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Host-Specific Fimbrial Adhesins of Noninvasive Enterotoxigenic *Escherichia coli* Strains

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INTRODUCTION

The ability of certain bacteria to adhere to eucaryotic cells is recognized as a fundamental feature for the colonization of host tissues in vivo. For many pathogenic organisms, adherence is of paramount importance since they have to compete with the commensal microorganisms for a successful colonization of the host epithelial cell surfaces or the skin. Specific adhesive mechanisms have been found to play an important role in the attachment of *Escherichia coli*, *Neisseria*, *Streptococcus*, and *Mycoplasma* species to various epithelial tissues.

Enteric diseases caused by enteropathogenic *E. coli* strains can be classified into at least two different forms. Some strains are invasive and cause a dysentery-like syndrome (enterocolitis); others isolated from diarrheal illnesses are noninvasive enterotoxigenic strains. The latter strains are characterized (i) by their ability to proliferate in the small intestine and (ii) by the production of one or both of two types of enterotoxins. Colonization of the mucosal epithelium is mediated by specific adhesins with which the cells can resist the flushing action of the peristalsis of the gut. The enterotoxins stimulate the epithelial cells to secrete fluid into the lumen of the gut, thereby causing the actual diarrhea. Two types of enterotoxin can be distinguished on the basis of their thermostability.

The surface of *E. coli* cells is covered with substances capable of provoking immunological reactions which are commonly used for the serological classification of pathogenic and nonpathogenic strains. These surface antigens include substances referred to as adhesins which are responsible for the attachment of the cells to the epithelial mucosa. The most common antigens of *E. coli* are the O, K, and H antigens. The terminology O (from the German: Ohne Hauch) and H (from the German: Hauch), introduced by Weil and Felix (190, 191) is used for the somatic (O) and flagellar (H) antigens of *E. coli*. The somatic O antigens are lipopolysaccharide complexes and constitute part of the outer membrane. They are thermostable and not inactivated by temperatures of 100 or 121°C. The flagellar H antigens are protein in nature and are inactivated at 100°C. The term K antigen (from the German word Kapsel) was introduced by Kauffmann and Vahlne (86). They are usually acidic polysaccharides which form an envelope or capsule around the cell wall.

A new type of K antigen, numbered K88, was first described in 1961 in a paper about the

serological analysis of a number of *E. coli* strains frequently isolated from edema disease and enteritis in swine (133). Since the discovery of the K88 antigen, the surface antigen characteristics of porcine enterotoxigenic *E. coli* strains have attracted considerable attention.

It was recognized that porcine neonatal diarrhea is characterized by proliferation of certain serotypes of *E. coli* in the small intestine, many of which bear the K88 antigen on their surface (176). The ability of these *E. coli* strains to proliferate and cause disease has been attributed to their adherent properties for the piglets' intestinal epithelium (4, 8, 34, 195). More recently, Nagy et al. (123, 124) reported the colonization of the porcine small intestine by enterotoxigenic *E. coli* strains that lack the K88 antigen. These strains were found to possess another adhesin, designated as 987P.

In 1972, Smith and Linggood (166) reported a discovery made by Sojka that many calf and lamb enteropathogenic strains, although having different O antigens, possess a closely related or common K antigen. This antigen was referred to as the "common K antigen," KCo, and was later designated as K99 (132). K99-positive strains proliferate in the small intestine whereas K99-negative strains do not. For this reason it was conceivable that the K99 antigen functions in the same manner as the K88 antigen by facilitating adhesion of the bacteria to the intestinal epithelium (17).

A surface antigen, with characteristics similar to the K88 antigen, on enterotoxigenic *E. coli* of human origin was first described by Evans et al. (51). This antigen was found on an *E. coli* strain (H-10407) isolated from the liquid stool of a patient with severe cholera-like diarrhea in Dacca, Bangladesh, and was termed colonization factor antigen (CFA). This surface antigen was called CFA/I when a second, immunologically distinct, surface antigen from human enterotoxigenic *E. coli* was discovered. The second surface antigen was consequently termed CFA/II (42).

All of these surface antigens which confer adhesive properties on the enterotoxigenic strains have been identified as nonflagellar, filamentous, proteinaceous appendages on the bacterial cell surface. The existence of this type of surface appendage on the surface of different species of bacteria was already reported in 1949 by Anderson (3) and by Houwink and van Iterson in 1950 (70). The most common terminologies for these structures are "fimbriae," introduced by Duguid et al. (38), and "pili," introduced by Brinton (13). Following the recommendation of Ottow (136) and Jones (80), we used the term fimbriae. Fimbriae are thinner and more numerous than flagella and confer on bacteria adhesive properties including hemagglutinating activity. Both Brinton (13) and Duguid and co-workers (36) distinguished several types of fimbriae among *Enterobacteriaceae* mainly based on their morphology and hemagglutinating properties. The most common type of fimbriae are named as type 1 fimbriae.

The majority of both pathogenic and nonpathogenic *E. coli* strains produce type 1 fimbriae. These fimbriae enable *E. coli* to adhere to a wide variety of eucaryotic cells, i.e., erythrocytes of several animal species (150), leukocytes (10), epithelial cells (127), and most other animal, plant, and fungal cells as far as tested (37). The adhesive properties of type 1 fimbriae are inhibited by D-mannose. It is clear that type 1 fimbriae are the most universal adhesin of *E. coli*. Their adhesive properties can help to colonize epithelial surfaces and therefore contribute to the pathogenicity of enteropathogenic *E. coli* strains. Several reviews describe the characteristics of type 1 fimbriae (13, 14, 37, 136). This review is restricted to the description of another class of *E. coli* fimbriae characterized as host-specific adhesins. In contrast to type 1 fimbriae, these adhesins seem to occur exclusively on the enterotoxigenic *E. coli* strains. They adhere to the intestinal epithelia of a very limited number of animal species and specifically agglutinate only certain species of erythrocytes. In contrast to type 1 fimbriae, the hemagglutinating activity of these adhesins is not inhibitable by mannose.

OCCURRENCE

Association of Adhesins and O Serogroups

In contrast to type 1 fimbriae which are present on the majority of both commensal and pathogenic *E. coli* strains, the presence of host-specific adhesins on enterotoxigenic strains seems to be associated with a relatively small number of O serogroups (Table 1). Frequently occurring (classical) serotypes of *E. coli* associated with diarrhea in newborn piglets are O8:K87, O45, O138:K81, O141:K85, O147:K89, O149:K91, and O157 (175). Incidentally, K88-positive *E. coli* strains belonging to other O serogroups have been isolated from diseased pigs (60). Furthermore, the occurrence of K88-positive enterotoxigenic *E. coli* strains of various O groups is known to fluctuate with the geographical area and time, as well as with the vaccination policy of a particular country. Söderlind and Möllby (172) examined *E. coli* strains isolated from 200 piglets with neonatal diarrhea in Sweden and found that one-third of the strains were enterotoxigenic. O group 149 comprised 24% of all strains, and all but one of the strains belonging to O group 149 possessed the K88 antigen. In another comparative study

TABLE 1. Occurrence of adhesins in relation to O serogroups

Adhesin	Origin	O serogroups[a]
K88	Piglet	O8, O45, O138, O141, O147, O149, O157
987P	Piglet	O9, O20, O141
K99	Calf, lamb	O8, O9, O20, O101
K99	Piglet	O64, O101
F41	Calf	O9, O101
CFA/I	Human	O15, O25, O63, O78
CFA/II	Human	O6, O8

[a] Strains belonging to O serogroups which have only occasionally been found to possess one of the adhesins are not included.

on piglets with diarrhea and healthy piglets of the same age, Söderlind and Möllby (173) isolated 810 intestinal *E. coli* strains from 81 piglets. A clear difference was found between *E. coli* strains isolated from both groups of piglets with regard to the distribution of O groups, K88 antigen production, and the frequency of enterotoxigenicity. The K88 antigen was found exclusively in enterotoxigenic strains of O groups 149 and 8. Guinée and Jansen (62) studied 101 enterotoxigenic *E. coli* strains of porcine origin isolated in the Netherlands. The K88 antigen was found on 52 of these strains. Six strains had another porcine-specific adhesin (987P), and seven strains possessed the K99 antigen. Of the K88-positive strains, 92% belonged to the so-called classical serogroups, and the others belonged to serogroups O9 and O20.

Nagy et al. (124) investigated the prevalence of the 987P antigen among porcine enterotoxigenic *E. coli* strains that lack the K88 antigen. Of 119 strains tested, all belonging to O groups 9, 20, and 101, 50% of the O9 and 14% of the O20 serogroup strains produced colonies that were agglutinated by anti-987P serum, whereas none of the strains of serogroup O101 reacted with the antiserum. In another study, Moon et al. (109) tested 111 enterotoxigenic *E. coli* isolates from the intestines of neonatal pigs with diarrhea that did not react in preliminary tests with K88 antiserum. Fifty-five of these strains were 987P positive, 9 isolates produced K99, and 4 produced the K88 antigen. The 987P adhesin was found to be most common in strains of serogroups O9, O20, and O141, and occasionally in O groups 101 and 149 (Table 1). Apparently, the majority of enterotoxigenic strains isolated from neonatal pigs belong to a restricted number of O serogroups and produce either the K88, 987P, or K99 adhesin. Porcine enterotoxigenic strains which lack these antigens may produce another hitherto unrecognized type of adhesin which mediates their adherence to the intestinal epithelial cells. Recently, Awad-Masalmeh et al. (5) studied three of such strains, designated as $3P^-$, in more detail. The strains adhered to and colonized the pig intestine and produced diarrhea. Furthermore, they exhibited mannose-resistant hemagglutinating activity. The authors could not demonstrate that these activities were associated with a particular type of fimbriae, but they were able to exclude a role for the type 1 fimbriae produced by these strains in mediating adhesion to the intestinal epithelial cells of pigs.

The occurrence of the K88 as well as the 987P adhesin appears to be restricted to porcine enterotoxigenic *E. coli* strains. K88- or 987P-positive strains have not been reported among enterotoxigenic strains isolated from other animals or humans.

E. coli strains isolated from calves or lambs in epidemiologically unrelated cases of diarrhea were examined by Ørskov et al. (132) for the presence of the K99 antigen. All K99-positive strains belonged to serogroups O8, O9, and O101. Myers and Guinée (121) reported the serotyping of 35 enterotoxigenic *E. coli* strains isolated from calves in the United States. The following serotypes were detected: O8:K25, O8:K85, O9:K35, O20:K?, O101:K28, and O101:K30. In another study, Guinée and Jansen (62) described that 74 unselected field isolates of bovine enterotoxigenic *E. coli* strains from the Netherlands and the United States belonged to serogroups O8 (16%), O9 (27%), O20 (3%), and O101 (54%). Several other reports on the occurrence of the K99 antigen among bovine enterotoxigenic strains (11, 112, 158) confirmed the frequent association of K99 production with O groups 8, 9, 20, and 101 (Table 1).

Moon et al. (110) reported the isolation of K99-positive enterotoxigenic *E. coli* strains from pigs. Several enterotoxigenic *E. coli* strains of human origin were also tested for the presence of the K99 antigen, but all were negative. Furthermore, K99-positive enterotoxigenic *E. coli* strains of both calf and pig origin produced K99 in the pig ileum in vivo, adhered to the pig epithelium, and caused profuse diarrhea in newborn pigs. The K99 antigen appears to occur rather commonly among pig strains of O groups 64 and 101 and occasionally also on strains of O group 9. These data indicate that although K99 also facilitates adhesion to and colonization of the pig intestine, not all K99-positive enterotoxigenic *E. coli* are able to do so.

The occurrence of the K99 antigen seems to be restricted to bovine, ovine, and porcine enterotoxigenic strains.

The adhesins of enterotoxigenic *E. coli* strains associated with diarrhea in adults and children were discovered more recently and designated as CFA/I and CFA/II (42, 51). The enterotoxigenic strains possessing these adhesins are con-

sidered a major cause of traveller's diarrhea and diarrhea among young children in developing countries. The occurrence of CFA/I and CFA/II on strains of human origin also appeared to be associated with a very restricted number of serotypes. CFA/I is found predominantly on strains of serogroup O78, and CFA/II is found predominantly on strains of serogroups O6 and O8 (Table 1). Less frequently, both adhesins were observed on strains of other serogroups. Table 2 summarizes some reports on the occurrence of the CFA/I adhesin among human enterotoxigenic *E. coli* strains of various serotypes isolated at different locations all over the world. Although the CFA/I adhesin was frequently encountered among the human enterotoxigenic *E. coli* strains, an important percentage of these strains does not possess the CFA/I or the CFA/II adhesin (23, 42). CFA/I and CFA/II, therefore, are not prerequisites of virulence for all *E. coli* strains that cause diarrhea in humans (149). In general, however, it should be stressed that underestimation of the frequency of occurrence of a particular adhesin may easily occur if one does not take into account that the production of these adhesins is influenced by growth conditions.

Association of Adhesin and Enterotoxin Production

Enterotoxigenic *E. coli* strains associated with acute diarrhea in man and domestic animals all produce a heat-stable (ST) and/or a heat-labile (LT) enterotoxin (148). The production of both types of enterotoxins is controlled by transmissible plasmids (66, 163). The correlation between enterotoxigenicity and the presence of adhesins

TABLE 2. Occurrence of CFA/I on enterotoxigenic *E. coli* strains of human origin[a]

No. of strains tested	No. of CFA/I-positive strains	O serogroup[b]	Reference
26	5	O78 (5)	54
50	22	O78 (22)	6
60	30	O15 (3), O25 (11), O63 (6), O78 (10)	42
76	9	O62 (1), O63 (4), O128ac (3)	141
77	16	O78 (16)	130
89	6	O63 (1), O78 (4), O153 (1)	59
178	38	O78 (34)	170
187	31	O25 (2), O63 (9), O78 (18), O128 (1), O153 (1)	22

[a] Strains isolated in various geographic areas.

[b] Numbers in parentheses represent the number of CFA/I-positive strains belonging to that particular O serogroup.

TABLE 3. Association of adhesin and enterotoxin production

Adhesin	Enterotoxin		
	ST	LT	ST + LT
K88	+	+	+
987P	+	−	−
K99	+	−	−
F41	+	−	−
CFA/I	+	+	+
CFA/II	−	−	+

has been the subject of many studies to establish the enteropathogenicity of *E. coli* strains isolated from stools.

Classical pig enterotoxigenic *E. coli* strains were originally thought to fall into two categories: strains producing only ST and strains producing both ST and LT. The K88 antigen was found to be associated with strains producing both enterotoxins (65–67, 161). Guinée and Jansen (62), however, reported that 37 out of 52 K88-positive strains produced only LT whereas the other 15 strains produced ST + LT. Söderlind and Möllby (173) reported an almost complete correlation between K88 production in strains belonging to serogroup O149 and the production of both ST and LT. Remarkably, however, they observed that the K88-positive strains belonging to serogroup O8 produced only ST. Obviously, the presence of the K88 antigen may be associated with ST, LT, or both enterotoxins (Table 3).

The second adhesin detected on pig enterotoxigenic strains, 987P, is always found to be associated with the production of ST (62, 109; Table 3). Also, for the K99 antigen, a positive correlation has been reported with ST production (62, 77, 112; Table 3). With bovine strains, Guinée and Jansen (62) detected a 100% positive correlation between ST production and the presence of the K99 antigen on 74 strains. None of the 43 non-enterotoxigenic strains of bovine origin possessed the K99 antigen. Kaeckenbeek et al. (84) reported that 99% of the calf enterotoxigenic strains they studied possessed the K99 antigen, but in addition the K99 antigen was detected on 21% of the 441 non-enteropathogenic strains. Moon et al. (112) as well as Isaacson et al. (77) reported that only 77% and 87%, respectively, of the enterotoxigenic strains possessed the K99 antigen. Differences in the frequency of association of the K99 antigen with enterotoxigenic strains are probably due to difficulties in the detection of the K99 antigen (63, 132). However, the presence of unknown adhesins cannot be excluded. In most porcine and bovine enterotoxigenic *E. coli* strains, the production of adhesins and of enterotoxins ap-

peared to be encoded on separate plasmids. In some K99-positive strains, however, the ability to produce K99 and ST appears to be encoded by a single plasmid (C. L. Gyles, personal communication).

A close association exists between the presence of CFA/I and the production of ST + LT or ST only (42, 45, 59, 95, 141, 160). CFA/I-positive strains producing only LT seem to be relatively rare (95). The production of CFA/I and ST has been shown to be controlled by a single nonconjugative plasmid (23, 51, 99, 140, 160) of about 60×10^6 daltons. The size of this plasmid isolated from various sources is somewhat variable. Occasionally a plasmid encoding the production of all three properties (CFA/I, LT, and ST) has been observed (99). However, in most cases the production of LT is encoded by a separate plasmid (100, 186). Compared with *E. coli* strains which produce only ST, the diarrhea caused by *E. coli* producing both enterotoxins is more severe and of longer duration (103). Analysis of CFA/II-positive enterotoxigenic *E. coli* strains from different geographic locations indicated that the majority of these strains produce both ST and LT (23, 42). The genes encoding the three properties were found to be located on a single plasmid of about 60×10^6 daltons (138). The data indicate that, in contrast to enterotoxigenic *E. coli* strains associated with diarrhea in domestic animals, human enterotoxigenic strains harbor plasmids which encode for both adhesin and enterotoxin production. Consequently, the occurrence of CFA/I and CFA/II in a very restricted number of *E. coli* serotypes may be explained by the close association of enterotoxigenicity with certain serotypes (6, 102, 131). The reason for this association between chromosomally encoded cell wall antigens and plasmid-determined enterotoxin and/or adhesin production is not yet understood. It has been suggested that one of the factors that might contribute to this nonrandom association might be the ability of certain strains to accept and to harbor the virulence plasmids more stably than others (47). Enterotoxigenic strains of human origin can be divided into different groups depending on the tendency of strains with respective O antigens to lose the ability to produce LT and/or ST (6, 47).

HEMAGGLUTINATION

Hemagglutination was the first observed manifestation of the adhesive properties of fimbriated enteric bacteria (38). The majority of *E. coli* strains exhibit a similar pattern of hemagglutinating activity with erythrocytes of various animal species. This hemagglutination is mannose sensitive, subject to phase variation, and correlated with the presence of type 1 fimbriae (12, 38). The host-specific adhesins described in this review differ from the adhesins which exhibit mannose-sensitive hemagglutination in several properties: their hemagglutinating activity is not inhibitable by D-mannose and its analogs, no adhesin production occurs on cells grown at 18°C, agglutinated bacteria can be eluted from the erythrocytes at 37°C, and the hemagglutinating activity is destroyed by heating the cells for 30 min at 65°C. Although the terminology "mannose-resistant hemagglutination" has little significance because the hemagglutination is also "resistant" to numerous other substances, we prefer to hold on to it in view of its common use in the literature (35). In Table 4, the hemagglutinating activity of strains carrying various adhesins is summarized.

Stirm et al. (179) showed that in two K88-positive strains investigated, the presence of the K88 antigen was associated with the ability to agglutinate guinea pig erythrocytes. The hemagglutination reaction was best performed in the cold. Unlike hemagglutination by common or type 1 fimbriae, the hemagglutination by K88 fimbriae was mannose resistant (13, 38, 150). Jones and Rutter (82) showed that all of the 108 K88-positive *E. coli* strains tested caused mannose-resistant hemagglutination of guinea pig erythrocytes at 0 to 3°C. Hemagglutination was negative in K88-positive strains grown at 18°C or in K88-negative mutants of a K88-positive strain. Extracts containing the K88 antigen possessed hemagglutinating activity which could not be separated from the K88 antigen by fractionation or serological procedures. The authors concluded that K88 was a hemagglutinin and suggested that the mechanism of hemagglutination and attachment to the mucosa of piglets may be similar. The observation, however, that hemagglutination was effective at 4°C but eluted at 37°C might indicate that the fit for the natural receptor of the K88 antigen on the intestinal epithelial cells is better than for guinea pig

TABLE 4. Hemagglutinating activity of strains carrying various adhesive antigens

Adhesin	Origin	Erythrocytes hemagglutinated
K88	Porcine	Guinea pig, chicken
987P	Porcine	—[a]
K99	Bovine, ovine, porcine	Horse, sheep
F41	Bovine, porcine	Human, guinea pig, horse, sheep
CFA/I	Human	Human, bovine, chicken
CFA/II	Human	Bovine, chicken

[a] The 987P adhesin fails to hemagglutinate erythrocytes from horse, guinea pig, rabbit, sheep, pig, or cattle.

erythrocytes. At higher temperatures, the binding of the K88 antigen to the erythrocyte receptor may be disrupted as a result of the increase of molecular agitation. Parry and Porter (137) tested a large number of different types of erythrocytes for their agglutination by cell-free preparations of two serological variants of the K88 antigen (K88ab and K88ac) as well as by whole cells carrying these antigens. They observed a powerful and consistent affinity of K88ab for chicken erythrocytes. The adhesion was stable at room temperature and mannose resistant. K88ac-positive cells failed to adhere to the chicken erythrocyte membrane, and cell-free K88ac preparations did not cause hemagglutination. Guinea pig erythrocytes were agglutinated by both K88ab- and K88ac-positive bacteria. They confirmed the previous observation that the reaction with guinea pig erythrocytes was unstable at room temperature. The number of cells adhering to erythrocytes was low compared to the adhesion observed on microvilli. Hemagglutination of chicken erythrocytes by cell-free K88ab and the adhesion of K88ab-positive bacteria to these erythrocytes was readily inhibited by using antisera to K88ab. Antisera against K88ac gave a very poor inhibition of these reactions. Furthermore, antisera to the K88b and K88c determinant were only capable of inhibiting the hemagglutinating activity of their homologous antigen. However, anti-K88ac and anti-K88a sera showed extremely low inhibition of K88ab agglutination of chicken erythrocytes compared to their corresponding inhibition of K88ac agglutination of guinea pig erythrocytes. Probably, the selectivity of K88ab for the chicken erythrocyte may involve adhesion via the K88b determinant.

The ability of the 987P antigen to hemagglutinate various types of erythrocytes was tested with purified 987P and whole cells carrying the 987P antigen (79). However, no agglutination reaction was observed either at 4°C or at room temperature. This observation does not exclude the possibility that 987P can agglutinate erythrocytes of types not yet included in the tests.

In a first report on the establishment of the K99 adhesin of calf and lamb enterotoxigenic *E. coli* strains, Ørskov et al. (132) described that some K99-positive strains were able to hemagglutinate guinea pig erythrocytes in the cold. The reaction was not inhibited by mannose. Tixier and Gouet (183) studied the hemagglutinating ability of various enterotoxigenic strains of bovine, porcine, and human origin with horse and snake erythrocytes at 4°C. The calf enterotoxigenic strains exhibited a strong mannose-resistant hemagglutination of horse erythrocytes and possessed fimbriae which could be the K99 adhesin. These strains as well as the other enterotoxigenic and non-enterotoxigenic strains were also able to hemagglutinate snake erythrocytes, but this reaction was mannose sensitive as indicative for the presence of type 1 fimbriae. Burrows et al. (17) demonstrated that enterotoxigenic strains of calf origin caused mannose-resistant hemagglutination of sheep erythrocytes and were able to demonstrate that the presence of the K99 antigen on the cell surface was responsible for this effect. When grown at 37°C, the K99-positive strains attached to brush borders prepared from the small intestine of calves. The hemagglutinating and adhesive properties were not exhibited by strains grown at 18°C, at which temperature the K99 antigen is not expressed (132). The paired nature of hemagglutination and adhesive properties for the K99 antigen were further demonstrated by transfer of the K99 plasmid to a K99-negative recipient strain. Transfer of the K99 plasmid conferred both properties on the exconjugants. Cell-free K99 antigen also possessed hemagglutinating activity and is able to inhibit the adhesion of K99-positive bacteria to brush borders. At 37°C, the cell-free K99 antigen showed no hemagglutinating activity.

Morris et al. (114, 116) confirmed the observation that cell-free K99 antigen is able to hemagglutinate sheep erythrocytes and also showed that guinea pig and human type O erythrocytes were agglutinated. Heating of the antigen for 15 min at 100°C destroyed the hemagglutinating activity. In contrast with these data, Isaacson (71) reported that purified K99 antigen did not react with guinea pig erythrocytes and suggested (72) that the K99 preparations used by Morris and co-workers contained an additional adhesin not identical with K99. The existence of another adhesin on certain calf enterotoxigenic strains was recently established by Morris et al. (117, 118). This adhesin, designated as F41, is produced by strains belonging to serogroups O9 and O101. The hemagglutinating activities of both adhesins were recently reported by De Graaf and Roorda (29). Purified K99 antigen showed a strong hemagglutination of horse and a weaker reaction with sheep erythrocytes, but no activity with guinea pig erythrocytes. On the other hand, purified F41 antigen showed a strong hemagglutination reaction with guinea pig erythrocytes and a weaker activity with sheep and horse erythrocytes.

Mannose-resistant hemagglutination of human group A erythrocytes by enterotoxigenic strains of several different serogroups and isolated from adults with diarrhea was found to be associated with the presence of CFA/I on those strains (48). Neither CFA/I nor hemagglutination were produced when the strains were grown at 18°C. Hemagglutination was completely inhibited by

pretreatment of CFA/I-positive cells with anti-CFA/I serum. Cell-free purified CFA/I fimbriae do not hemagglutinate erythrocytes (44). Thus the CFA/I released from bacterial cells is monovalent. Hemagglutination, however, can be obtained by sensitizing micro latex beads with purified CFA/I or by aggregates of the purified CFA/I which were induced at low pH. Purified CFA/I also retains its affinity for the epithelial cells of the rabbit small intestine and blocks the adhesion of CFA/I-positive bacteria (44). CFA/I released by heating cells at 60°C for 30 min does no longer agglutinate human erythrocytes (48, 54). A hemagglutination typing system for enterotoxigenic *E. coli* possessing the colonization factor antigens CFA/I or CFA/II has been proposed by Evans et al. (46). CFA/I-positive enterotoxigenic *E. coli* exhibit mannose-resistant hemagglutination with human, bovine, and chicken erythrocytes with decreasing intensity and not with guinea pig erythrocytes. CFA/II-positive *E. coli* strains exhibit only mannose-resistant hemagglutination with bovine and chicken erythrocytes at 4°C. The association of CFA/I and mannose-resistant hemagglutination of human and bovine erythrocytes and of CFA/II and mannose-resistant hemagglutination of bovine erythrocytes was also observed by a number of other investigators (6, 23, 33, 54, 59, 95, 130, 131, 170, 182). However, a correlation between mannose-resistant hemagglutination and the presence of colonization factor antigens was not always observed, i.e., enterotoxigenic *E. coli* strains producing hemagglutination of human and/or bovine erythrocytes but lacking CFA/I or CFA/II were frequently encountered. Furthermore, the use of hemagglutination typing systems as proposed by Evans et al. (46) may easily lead to erroneous results if one does not realize that a particular strain may be able to produce more than one type of adhesin.

PRODUCTION AND DETECTION

Production of adhesins is generally a function of the cultivation conditions, i.e., temperature and composition of the growth medium as well as of the actual strain. All adhesins of enterotoxigenic *E. coli* strains studied so far are not produced at 18°C except for type 1 fimbriae which appear to be produced at all temperatures. Commercially available nutrient agars, designed for the isolation of *Enterobacteriacae*, are often not suitable for the detection of adhesins on *E. coli* isolates. The influence of growth conditions on the detectability and production of adhesins has been studied extensively for type 1 fimbriae (12, 13). Only a limited number of studies, mainly concerning the K99 and the 987P antigen, have been published on the influence of growth conditions on the expression of host-specific adhesins.

As mentioned before, the initial detection of the K99 antigen was derived from the observation of Sojka that many calf and lamb enteropathogenic strains, although having different O antigens, reacted not only with homologous but also with heterologous OK antisera in slide agglutination tests, suggesting that these strains might share one or more common antigens. Detection of this common antigen appeared to be dependent on the presence of capsular K antigens and on the medium used for cultivation (132). In strains without capsular K antigen or in more transparent colonies of mucoid-growing strains, which have the least amount of capsular antigen, this common antigen could be detected more readily. Guinée et al. (63) undertook a study on the detection of the K99 antigen because of its possible significance for the identification of calf enterotoxigenic *E. coli* strains. Identification of these strains by means of serotyping was hampered by the fact that strains with the same serotype are often also found in healthy calves. They developed a buffered semisynthetic medium (Minca medium), which contains less amino acids and carbohydrate than common nutrient media (63, 64) to suppress the production of capsular K antigens. The diminished production of capsular antigen facilitated the detection of the K99 antigen by slide agglutination as well as by immunoelectrophoresis.

In an attempt to explain the difficulties in the detectability of the K99 antigen on commercially available nutrient agars, De Graaf et al. (30) determined the extent of K99 production by strains grown under different conditions. They observed that, instead of Minca medium, a minimal salt medium with glucose can be used to obtain a high K99 production. In complex media like nutrient broth the production of K99 was strongly reduced, and slide agglutination tests are not sensitive enough to detect the low amount of K99 produced under these conditions. Optimal amounts of K99 antigen were produced at 37°C. At 30°C a weak production of K99 antigen was detected by hydrophobic interaction chromatography or enzyme-linked immunosorbent assay (ELISA) (Fig. 1). The ELISA also appeared to be a reliable assay for the detection of the K99 antigen in calf feces (40). Furthermore, the production of the K99 antigen appeared to be related to the O antigen carried by the host strains, but seemed to be independent of the absence or the presence of various K polysaccharide antigens. Under all conditions tested, strains with antigen O101 produced more K99 antigen than did strains belonging to O antigen group 8, 9, or 20. De Graaf et al. (26) have been able to give an explanation for the

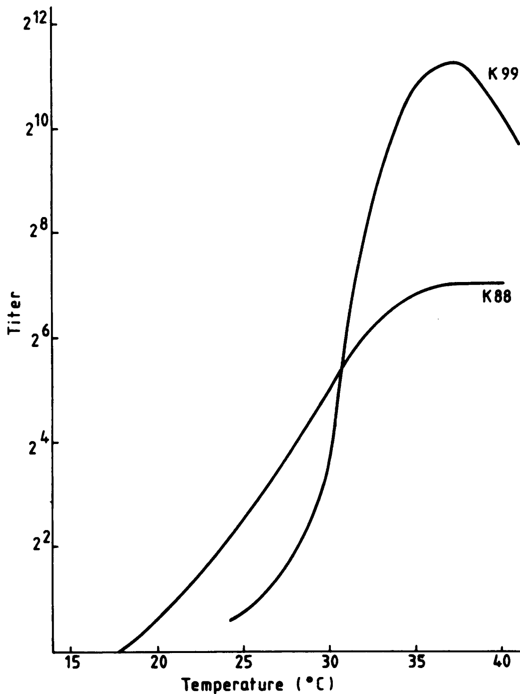

FIG. 1. Temperature-dependent production of the K88 and K99 adhesins. The adhesin production was determined in sonicated cell suspensions, using an ELISA. Titers are determined in standard ultrasonic extracts and expressed as the highest twofold dilution which still showed coloring.

observed differences in K99 production and detectability between complex and minimal media. By addition of various compounds to the minimal medium, it appeared that alanine specifically represses K99 biosynthesis. A 1 mM concentration of L-alanine was sufficient to reduce the K99 production by about 95%. These results were confirmed by Contrepois et al. (21). Experiments on the kinetics of alanine-induced repression indicated an immediate effect on K99 production without a significant lag period (Fig. 2). D-Alanine also inhibits K99 production when the bacteria are grown in a medium with D-alanine as sole carbon source, but the metabolic degradation product pyruvate showed no effect (26). Another factor that might affect K99 production is glucose, as indicated by Guinée et al. (64). In a recent paper, Isaacson (73) described that high concentrations of glucose (0.5%) repressed K99 production. This repression could be overcome by addition of 0.5 mM cyclic adenosine monophosphate, indicating that K99 synthesis is subject to cyclic adenosine monophosphate-dependent catabolite repression. On the other hand, Contrepois et al. (21) reported that two types of K99-producing strains can be distinguished. In one type of strain K99 production is not affected by glucose, whereas in the other type K99 production is glucose dependent. The nature of this phenomenon remains to be explained.

The ELISA appeared to be a reliable assay for the detection of the K99 antigen in calf feces (40).

The production of the F41 adhesion antigen is also dependent on the composition of the growth medium and comparable to the production of the K99 antigen (29, 30). Like the production of the K99 antigen, the production of F41 antigen is repressed by alanine, which indicates that the regulation of production of both antigens may have a similar molecular basis.

In contrast with the regulatory phenomena observed for K99 production, no such effects have been described for K88 production except for the influence of temperature (Fig. 1). No significant differences in K88 production were observed for an *E. coli* K-12 strain harboring the K88 plasmid and cultivated in a variety of media.

Relatively little is known about cultivation conditions that affect the detectability and production of CFA/I. Evans et al. (49) prepared a hyperimmune serum against purified CFA/I, and cell suspensions of different CFA/I-positive strains were tested for CFA/I production by titrations with this serum. The known CFA/I-positive strains were negative when grown on MacConkey agar. The most efficient medium for detecting CFA/I on *E. coli* isolates was a pep-

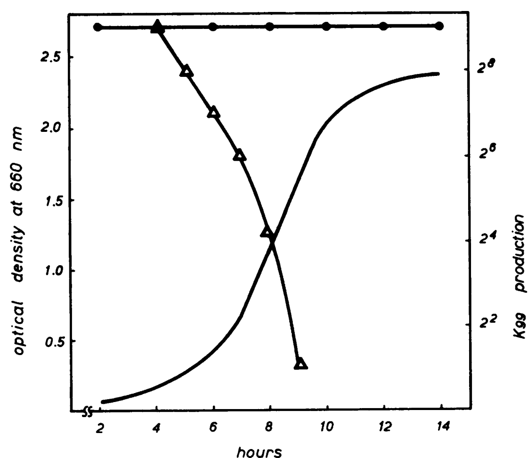

FIG. 2. Kinetics of the alanine-induced repression of K99 biosynthesis. L-Alanine (10 mM) was added to the culture at an optical density of 0.2. Symbols: ●, K99 production in the control culture without alanine; △, K99 production in the presence of L-alanine; ——, optical density. Titers are expressed as described in the legend of Fig. 1.

tone-agar medium composed of 2% peptone, 0.5% NaCl, and 2% agar. Tergitol-grown cells were either negative or gave a very low titer. Maximum CFA/I titers were obtained with an agar medium consisting of 1% Casamino Acids, 0.15% yeast extract, 0.005% $MgSO_4$, 0.0005% $MnCl_2$, and 2% agar (pH 7.4), designated as CFA agar (48). The ELISA technique appeared to be a useful tool for the diagnosis of diarrhea caused by CFA/I-positive strains (43).

Brinton (13, 15) has shown that the production of type 1 fimbriae is subject to a phenotypic control mechanism called phase variation. Within one bacterial strain, fimbriate and nonfimbriate cells were distinguishable from each other by electron microscopy and electrophoresis. The electrophoretic mobility of the fimbriated cells was about half that of the nonfimbriate cells (16). Colonies of fimbriated cells are smaller and smoother than colonies of nonfimbriated cells (12). Phase variation describes the all-or-none oscillation between the fimbriate and nonfimbriate state. The probability of switching is strongly influenced by environmental conditions. The change appeared to be spontaneous and takes place in either direction at a rate of about once per 1,000 bacteria per generation. Phase variation of type 1 fimbriae was found to be under transcriptional control (39). Independently of phase variation, an additional regulatory mechanism (181) appears to determine the number and/or length of fimbriae per cell. The mechanism of this kind of regulation, named quantitative regulation of fimbriation by Swaney et al. (181), is not understood at present.

With respect to the other adhesins of enterotoxigenic *E. coli* strains, the phenomenon of phase variation and associated changes in colony morphology has been described for the 987P antigen (55, 124). This adhesin, like type 1 fimbriae, is probably also encoded by chromosomal genes. Phase variation for CFA/I in *E. coli* H10407 has been demonstrated by Brinton (15). For the other adhesins this phenomenon has not been described so far.

The possibility of phase variation and the effect of environmental conditions thereon must be taken into account when selecting methods for the detection and production of a particular type of adhesin, especially when cells are capable of synthesizing more than one type of fimbriae.

CHARACTERIZATION

The K88 Adhesins

In a first report about the discovery of the K88 antigen, Ørskov et al. (133) described the adhesin as a thermolabile substance, numbered as K88 (L), the development of which was suppressed at 18°C. An isolation procedure for the K88 antigen was given by Stirm et al. (178, 180) who released the antigen from the cells by heating a cell suspension for 20 min at 60°C or by treating a suspension in a Waring blender. Purification was achieved by repeated isoelectric precipitation and preparative ultracentifugation. Chemical analysis revealed that the purified K88 antigen was a protein, in contrast to the polysaccharide nature of all other K antigens known at that time. The morphology of the K88 antigen was studied in the electron microscope (179, 188). It was found that K88-positive bacteria were covered with a material of filamentous appearance. The purified K88 antigen had the same fimbria-like structure and was visualized as thin flexible threads (Fig. 3). The diameter of the K88 fimbriae was estimated as 2.1 nm (188).

With the use of specific antisera, Ørskov et al. (134) could distinguish two variants of the K88 antigen, K88ab and K88ac. The nomenclature indicates that a common antigenic determinant is present on both variants. The K88ab variant was originally found on *E. coli* strains isolated from swine edema or pig enteritis in England. The K88ac variant was first described on *E. coli* strains isolated in Ireland and the German Democratic Republic (135). Additional minor serological variations of the K88ac antigen have been observed, but these have not been taken into the diagnostic scheme (134). Recently a new K88 variant, termed K88ad, was described on porcine enteropathogenic *E. coli* strains isolated in the Netherlands (61). Similar minor variations as found for the K88ac antigen were found for the K88ad antigen. The K88 variants could be distinguished on the basis of their behavior in double diffusion tests against their homologous antisera. All K88-positive strains had one determinant in common—K88a. Upon immunoelectrophoresis in Noble agar (Difco) K88ab was cathodic, K88ac was cathodic or anodic depending on the strain from which the antigen was isolated, and the K88ad was not mobile or anodic. This immunoelectrophoretic behavior of the K88 variants did not alter upon transfer of the corresponding plasmid to *E. coli* K-12, indicating that this property was not host dependent. Anodic and cathodic K88ac antigens could not be distinguished serologically. The serological variations of the K88 antigen may represent an effort of the bacterium to escape the immunological pressure imposed upon the K88-positive bacterial population by large-scale vaccinations with K88-containing vaccines. Another explanation for the observed serological K88 variants could be that the occurrence of pigs "resistant" to a particular type of K88 adhesin, due to the presence of altered "receptor sites" on the intestinal epithelial cells, has led to the selection

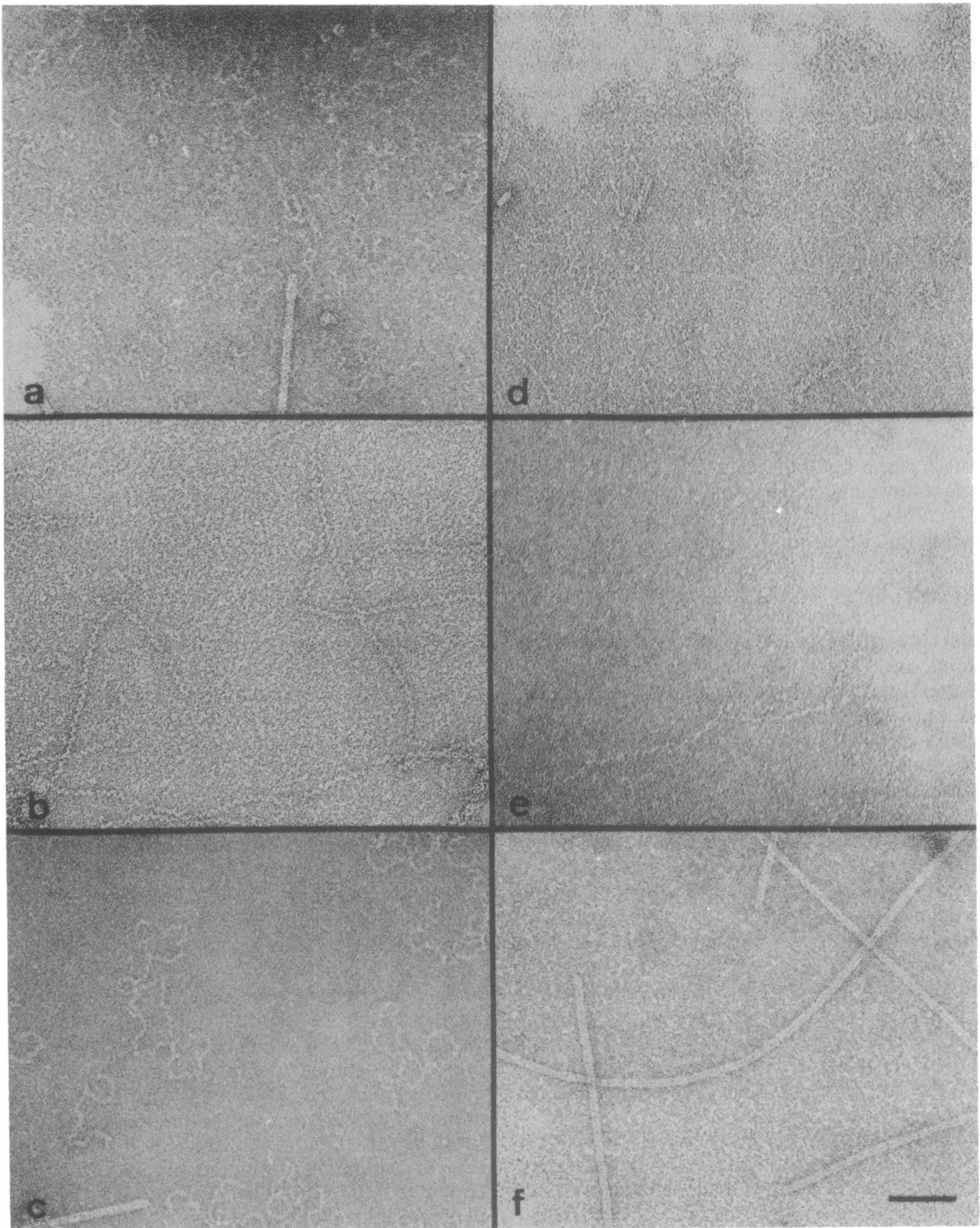

FIG. 3. Electron micrographs of purified adhesion preparations. (a) K88ab adhesin; (b) K99 adhesin; (c) F41 adhesin; (d) CFA/I adhesin; (e) CFA/II adhesin; (f) 987P adhesin. The bar represents 64 nm. Photo kindly provided by J. van Breemen. CFA/I and CFA/II were a gift from P. Klemm; 987P was a gift from L. K. Nagy.

of other naturally occurring K88 variants that are better adapted to these altered "receptors." This last possibility could very well explain the observations of Bijlsma et al. (9) who observed specific binding sites for the various serological K88 variants. The idea that the serological variants of the K88 antigen are adaptations to altered circumstances is supported by the fact that the adhesiveness was found to be associated with the K88c or K88b antigens and that this

ability is only neutralizable by the homologous antisera (195). It is interesting that most K88-positive *E. coli* strains isolated from infected piglets nowadays have the antigen of the K88ac or K88ad type (61, 195), and there seems to be a prevailing tendency towards the disappearance of the K88ab antigen.

Mooi and De Graaf (105) purified the K88 variants by gel filtration on a Sepharose CL-4B column after initial treatment of a cell suspension in a Waring blender and precipitation of the crude K88 preparation with ammonium sulfate. Analysis of purified K88 adhesins on sodium dodecyl sulfate-polyacrylamide gels showed a single protein band with an apparent molecular weight of 23,500 to 26,000 depending on the K88 variant isolated. It is clear, therefore, that the K88 fimbriae consist of hundreds of identical protein subunits. Very little variation was observed in the amino acid composition of the serologically different K88 proteins (Table 5).

The K88 preparations contain all the common amino acids, with the exception of cysteine. This implies that the subunits in the K88 fimbriae are not held together by disulfide bridges but by hydrophobic or electrostatic interactions between adjacent subunits. Carbohydrate analysis indicated the presence of trace amounts of sugars in the preparations of Mooi and De Graaf (105). Apparently, the K88 antigen is no glycoprotein. However, there are three regions in the amino acid sequence of the K88ab protein subunit where *N*-glycosidation could have occurred. The empirical rule of Eylar (52) that *N*-glycosidation only occurs at asparagine residues in Asn-X-Ser/Thr sequences is fulfilled at amino acid residue positions 7–9 (Asn-Gly-Ser), 127–129 (Asn-Ala-Ser), and 202–204 (Asn-Ile-Thr) in the primary structure of the K88ab subunit protein (Fig. 4). The N- and C-terminal amino acid sequence of the different serological K88 variants has been determined by Gaastra et al. (56), together with the N-terminal amino acid sequence of the cyanogen bromide fragments derived from the K88 antigens. No difference was found between the K88 variants in the first 23 N-terminal amino acids, nor in the 24 C-terminal amino acids. This probably indicates that there is an evolutionary constraint on these parts of the K88 protein and implies a role of these parts of the protein structure in either the function of the fimbriae or in the subunit-subunit interaction within the fimbriae. The N-terminal sequence of some of the cyanogen bromide fragments was different for the different K88 antigens. The nature of these differences and the position within the molecule are discussed together with the primary structure of the K88ab antigen.

The primary structure of the K88ab antigen was determined by two groups in different ways. Klemm (91) determined the amino acid sequence of the K88ab protein subunit. Gaastra et al. (57) determined the base sequence of the deoxyribonucleic acid (DNA) of the gene encoding the K88ab protein. The amino acid sequence of the protein is given in Fig. 4. It was found that the K88ab subunit is synthesized in a precursor form with an additional 21 amino acid residues at

TABLE 5. Amino acid composition of various adhesin subunits

Amino acid	No. of residues							
	K88ab	K88ac	K88ad	987P	K99	F41	CFA/I	CFA/II
Asx	30	34	30	34	23	27	12	14
Thr	29	25	25	28	19	18	15	9
Ser	18	16	19	23	15	33	17	6
Glx	17	17	21	16	10	24	11	20
Pro	5	8	9	8	5	11	7	3
Gly	36	35	35	26	19	39	10	11
Ala	28	25	26	26	18	16	19	8
Cys	0	0	0	2	4	ND[a]	0	0
Val	21	20	20	15	8	17	19	7
Met	3	3	4	1	3	3	3	2
Ile	13	13	13	12	9	10	5	4
Leu	20	22	20	17	9	14	12	7
Tyr	10	10	9	5	7	13	4	5
Phe	11	9	10	3	9	9	2	4
His	0	2	2	0	3	6	1	1
Lys	11	10	10	10	9	12	8	5
Arg	8	10	7	2	6	4	1	3
Trp	4	ND	ND	1	ND	ND	1	ND
HyLys[b]	0	0	0	0	0	10	0	0

[a] ND, Not determined.
[b] HyLys, Hydroxylysine.

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5' A A A C G G A G C C G C G G G A T G G T T T T A C G G T A A T T C C G G A A A A A T A A G G G T T A C C G A T T T C A G T T T A T

T A T T T G T G G A T A T C A A G G G G T T T A A T T T T A T G A A A A A G A C T C T G A T T G C A C T G G C A A T T G C T G C A
                                                             -20                       -15                   -10
K88ab                                                    Met Lys Lys Thr Leu Ile Ala Leu Ala Ile Ala Ala

T C T G C T G C A T C T G G T A T G G C A C A T G C C T G G A T G A C T G G T G A T T T C A A T G G T T C G G T C G A T A T C G G
                 -5              1           5                   10
K88ab Ser Ala Ala Ser Gly Met Ala His Ala Trp Met Thr Gly Asp Phe Asn Gly Ser Val Asp Ile Gly
K88ad                                     Trp Met Thr Gly Asp Phe Asn Gly Ser Val Asp Ile Gly

T G G T A G T A T C A C T G C A G A T G A T T A T C G T C A G A A A T G G G A A T G G A A A G T T G G T A C A G G T C T T A A T G
      15              20                  25                  30
K88ab Gly Ser Ile Thr Ala Asp Asp Tyr Arg Gln Lys Trp Glu Trp Lys Val Gly Thr Gly Leu Asn
K88ad Gly Ser Ile Thr Ala Asp Asp Tyr

G A T T T G G T A A T G T A T T G A A T G A C C T G A C C A A T G G T G G A A C C A A A C T G A C C A T T A C T G T T A C T G G T
35              40                  45                  50                  55
K88ab Gly Phe Gly Asn Val Leu Asn Asp Leu Thr Asn Gly Gly Thr Lys Leu Thr Ile Thr Val Thr Gly
K88ad

A A T A A G C C A A T T T T G T T G G G C C G A A C C A A A G A A G C A T T T G C T A C G C C A G T A A G T G G T G C T G T A G A
            60                  65                  70                  75
K88ab Asn Lys Pro Ile Leu Leu Gly Arg Thr Lys Glu Ala Phe Ala Thr Pro Val Ser Gly Gly Val Asp
K88ad

T G G A A T T C C T C A G A T T G C A T T T A C T G A C T A T G A A G G A G C T T C T G T A A A A C T C A G A A A C A C T G A T G
      80                  85                  90                  95
K88ab Gly Ile Pro Gln Ile Ala Phe Thr Asp Tyr Glu Gly Ala Ser Val Lys Leu Arg Asn Thr Asp
K88ad

G T G A A A C T A A T A A A G G T T T A G C A T A T T T T G T T C T G C C G A T G A A A A A T G C A G A G G G C A C T A A A G T T
100                 105                 110                 115                 120
K88ab Gly Glu Thr Asn Lys Gly Leu Ala Tyr Phe Val Leu Pro Met Lys Asn Ala Glu Gly Thr Lys Val
K88ad                                                 Met Lys Asn Ala Glu Gly Thr Lys

G G T T C A G T G A A A G T G A A T G C A T C T T A T G C C G G T G T G T T C G G G A A A G G T G G G G T T A C T T C T G C G G A
                125                 130                 135                 140
K88ab Gly Ser Val Lys Val Asn Ala Ser Tyr Ala Gly Val Phe Gly Lys Gly Gly Val Thr Ser Ala Asp
K88ad

C G G G G A G C T G T T T T C G C T T T T T G C G G A C G G G T T G C G C G C T A T C T T T T A T G G T G G T T T G A C G A C G A
      145                 150                 155                 160
K88ab Gly Glu Leu Phe Ser Leu Phe Ala Asp Gly Leu Arg Ala Ile Phe Tyr Gly Gly Leu Thr Thr
K88ad             Met Ala Leu Phe Ala Glu Gly

C T G T T T C G G G T G C T G C A C T C A C G A G T G G A G T G C C G C A G C G G C G C G C A C A G A G T T G T T T G G A A G T
165                 170                 175                 180                 185
K88ab Thr Val Ser Gly Ala Ala Leu Thr Ser Gly Ser Ala Ala Ala Ala Arg Thr Glu Leu Phe Gly Ser
K88ad                         Leu Pro Ala Gly Ser Ala Ala Ala Ala Arg Thr Glu Leu Phe Gly Ser

C T A T C A A G A A A T G A T A T T C T C G G A C A G A T T C A A A G A G T A A A C G C A A A T A T T A C T T C T C T T G T T G A
          190                 195                 200                 205
K88ab Leu Ser Arg Asn Asp Ile Leu Gly Gln Ile Gln Arg Val Asn Ala Asn Ile Thr Ser Leu Val Asp
K88ad Leu Ser Lys Asn Asp Ile Leu Gly Gln Ile Gln Arg Val Asn Ala Asn Ile Thr Ser Leu Val Asn

C G T C G C A G G T T C T T A C A G G G A A G A C A T G G A G T A C A C T G A T G G A A C T G T T T G T T T C T G C T G C C T A T G
      210                 215                 220                 225
K88ab Val Ala Gly Ser Tyr Arg Glu Asp Met Glu Tyr Thr Asp Gly Thr Val Val Ser Ala Ala Tyr
K88ad Val Pro Gly Ser Phe Asn Glu Lys Met Ala Tyr Thr Asp Gly Ser Val Val Ser Val Ala Tyr

C A C T G G G T A T T G C A A A C G G T C A G A C T A T T G A G G C A A C T T T T A A T C A G G C T G T A A C T A C C A G C A C T
230                 235                 240                 245                 250
K88ab Ala Leu Gly Ile Ala Asn Gly Gln Thr Ile Glu Ala Thr Phe Asn Gln Ala Val Thr Thr Ser Thr
K88ad Ala Leu Gly Ile Ala Asn Arg Gln Thr Ile Glu Ala Thr Phe Asn Gln Ala Val Thr Thr Ser Thr

C A G T G G A G C G C T C C G C T G A A C G T A G C A A T A A C T T A T T A C T G A A A G T T G C T G G A
            255                 260
K88ab Gln Trp Ser Ala Pro Leu Asn Val Ala Ile Thr Tyr Tyr
K88ad Gln Trp Ser Ala Pro Leu Asn Val Ala Ile Thr Tyr Tyr
```

FIG. 4. The nucleotide sequence of the gene encoding the K88ab protein subunit. The amino acid sequence of the K88ab subunit derived from the nucleotide sequence is also given, together with the available data on the amino acid sequence of the K88ad protein subunit. Symbols: ——→, The ATG start codon of the K88ab structural gene; —⊤—, the TGA stop codon of the K88ab structural gene. The nucleotides which are underlined encode ribonucleic acid bases which are complementary to the 3′ end of 16S ribosomal ribonucleic acid of *E. coli* and a possible Pribnow box. Differences between the amino acid sequences of the K88ab and K88ad protein subunits are enclosed in a box.

the N-terminal part of the molecule. This signal peptide is cleaved off during the transport of the subunit from the ribosome to its place of destination. It also became clear from the determination of the base sequence of the gene encoding the K88ab antigen that there is probably a promoter preceding this gene. The differences observed in the N-terminal amino acid sequences of the cyanogen bromide fragments of the various K88 antigens could be located around amino acid residues 150 and 220. As compared to the primary structure of the K88ab variant, 15 differences in the partial amino acid sequence of the K88ad variant have been observed at the moment (Fig. 4). These differences are spread along the polypeptide chain and are not confined to one region. Most differences involve charged amino acid residues, and therefore most likely occur at the surface of the protein. It is not yet known whether these differences in primary structure also reflect the observed differences in immunological behavior. No homology was observed between the primary structures of the K88 and CFA/I protein subunits and the partially known sequences of other adhesion antigens (27, 68, 90, 92; Fig. 5). As can be seen in Fig. 4, there is a specific charge distribution along the polypeptide chain of the K88 antigen. The C-terminal part of the molecule is rather hydrophobic, and among the last 45 amino acid residues, there is only one charged residue. The central part of the molecule, on the contrary, is rather hydrophilic. It is also this part of the molecule where most of the basic amino acid residues are found.

The 987P Adhesin

The isolation and purification of the 987P antigen by the method of Brinton (13) has been reported (55). The diameter of 987P fimbriae as observed in the electron microscope was 7 nm (Fig. 3), and the antigen appeared to be composed of protein subunits with an apparent molecular weight of 18,900 (Table 6). A more detailed report on the purification and partial characterization of the 987P antigen of pig enterotoxigenic *E. coli* strains was recently published by Isaacson and Richter (79). The 987P antigen was removed from the cells by homogenization and purified by repeated precipitation with $MgCl_2$. Chemical analysis showed that the 987P adhesin is composed primarily of protein but contains also an unidentified amino sugar. Electrophoresis of the purified antigen on sodium dodecyl sulfate-polyacrylamide gels showed a single protein band with an apparent molecular weight of about 20,000. In the electron microscope, the 987P antigen appeared as rather rigid fimbriae with a diameter of 7 nm and an apparent axial hole. Morphologically the 987P antigen could not be distinguished from type 1 fimbriae of *E. coli*. Both fimbriae have the same N-terminal amino acid, alanine (90). The isoelectric point of 987P protein is at pH 3.7.

TABLE 6. Some characteristics of adhesins of enterotoxigenic *E. coli* strains

Adhesin	Diameter of fimbriae (nm)	Mol wt of subunit	pI
K88	2.1	27,540	4.2
987P	7	20,000	3.7
K99	4.8	18,500	9.7
F41	3.2	29,500	4.6
CFA/I	3.2	15,058	4.8
CFA/II	3.2	13,000	

The K99 Adhesin

In 1977, Isaacson (71) reported a procedure for isolation and purification of the K99 antigen from an *E. coli* K-12 strain harboring the K99 plasmid. The antigen was removed from cells grown in Trypticase soy broth (BL Microbiology Systems) by salt extraction and homogenization of a concentrated cell suspension in a Sorvall Omnimixer. Subsequently the antigen was purified by ammonium sulfate precipitation and ion-exchange chromatography on diethylaminoethyl-Sephadex. The purified material was composed of two protein subunits: a major com-

```
        1           5              10             15             20
Type 1  Ala-Ala-Thr-Thr-Val-Asn-Gly-Gly-Thr-Val-His-Phe-Lys-Gly-Glu-Val-Val-Asn-Ala-Ala-

K88     Trp-Met-Thr-Gly-Asp-Phe-Asn-Gly-Ser-Val-Asp-Ile-Gly-Gly-Ser-Ile-Thr-Ala-Asp-Asp-Tyr-Arg-

K99     Asn-Thr-Gly-Thr-Ile-Asn-Phe-Asn-Gly-Lys-Ile-Thr-Ser-Ala-Thr-Cys-Thr-Ile-Glu-Pro-Glu-Val-

CFA/I   Val-Glu-Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-

F41     Ala-Asp-Trp-Thr-Glu-Gly-Gln-Pro-Gly-Asp-Ile-Leu-Ile-Gly-Gly-Glu-Ile-Thr- X -Pro-Ser-Val-
```

FIG. 5. The amino acid sequence of the N-terminal part of the K88 (56), K99 (27), CFA/I (90), and F41 (54) adhesin subunits. The amino acid sequence of the N-terminal part of the subunit of type 1 fimbriae (68) is given for comparison.

ponent (molecular weight, 22,500) and a minor component (molecular weight, 29,500). The ratio between both constituents was 5:1. When purified K99 was negatively stained and examined under the electron microscope, it appeared to possess a rod-like filamentous structure with a strong tendency to aggregate. Individual rods had an average diameter of 8.4 nm and mean length of 130 nm. The isoelectric point of the antigen was 10.1. In contrast to the K99-positive bacteria, purified K99 no longer hemagglutinated guinea pig erythrocytes in the presence of D-mannose. The hemagglutinating activity towards these erythrocytes was separated from the K99 antigen during purification.

Morris et al. (114) published another isolation procedure for the K99 antigen based on a method for isolation of the K88 antigen as described by Stirm et al. (180). *E. coli* B41 (O101:K99:H$^-$) was grown on blood agar, and the K99 was extracted from the cells by heating and shaking the bacterial suspension for 30 min at 60°C. Subsequently a partial purification of the antigen was obtained by repeated isoelectric precipitation at pH 4.9, gel filtration of the precipitated material on Sepharose 4B, and finally ion-exchange chromatography on quaternary aminoethyl Sephadex. During this procedure the ability to hemagglutinate sheep erythrocytes was not separated from the K99-positive material. Absorption of the antigen with monospecific antiserum to K99 abolished the hemagglutination, and the authors therefore identified the K99 antigen as a mannose-resistant hemagglutin. In another paper, Morris et al. (116) reported that the isoelectric point of the K99 antigen isolated from *E. coli* B41 was 4.2. Furthermore, the isolated K99 antigen showed hemagglutination of guinea pig as well as sheep erythrocytes. These results are in agreement with a study of Burrows et al. (17) who demonstrated that the determinants for K99 and hemagglutinin were located on the same plasmid and that culturing of K99-positive bacteria at 18°C suppresses both of these characters.

In an attempt to explain the anomalies in the characterization of the K99 antigen, Morris et al. (115) examined K99 antigen isolated from *E. coli* B41 and precipitated with ammonium sulfate by immunoelectrophoresis with OK sera prepared from a series of K99-positive strains belonging to O serogroups 9 and 101. They observed that the isolated material contained an anionic as well as a cationic component which could be separated by ion-exchange chromatography. The anionic component exhibited a strong hemagglutination reaction with sheep erythrocytes, whereas the cationic component showed only a weak hemagglutination reaction which was lost upon storage. These results suggested that the K99 isolated from *E. coli* B41 is composed of two antigenically distinct components. Isaacson (72) confirmed this conclusion but demonstrated that the discrepancy in the presence of only the cationic component or the presence of both the anionic and the cationic component was due to differences in the sera used to detect K99 as well as the strain from which the antigen was isolated. K99 isolated from *E. coli* K-12 (K99) and *E. coli* B41 (O101:K99:H$^-$) yielded a single precipitation line with antisera prepared against *E. coli* K-12 (K99) and *E. coli* (O64:K99). Using antisera prepared against *E. coli* of serotype O101:K30:K99, the same precipitation line was observed, but an additional line was produced with K99 isolated from *E. coli* B41. Apparently the K99 isolated from this strain contained an additional (anionic) component. K99 isolated from *E. coli* K-12 hemagglutinated sheep erythrocytes in the cold and horse erythrocytes, whereas K99 isolated from *E. coli* B41 hemagglutinated guinea pig, sheep, and horse erythrocytes with no temperature effect. Inclusion of standard absorbed anti-K99 serum in the hemagglutination reaction inhibits only the horse erythrocyte reaction. Recently Morris et al. (118) reported that antibodies to the cationic component of a K99 antigen extract prepared from *E. coli* B41 were present in antisera to all the K99-positive *E. coli* strains in serogroups 8, 9, 20, 64, and 101, but antibodies to the anionic component were only detected in antisera to *E. coli* strains from the O9 and O101 serogroups. The anionic component isolated from the K99 preparation hemagglutinated sheep, guinea pig, and horse erythrocytes, whereas the cationic components only showed hemagglutination of horse erythrocytes. In conclusion, the K99 antigen was definitively identified as a cationic hemagglutin. De Graaf et al. (27) recently purified the K99 antigen from different *E. coli* strains by salt extraction, gel filtration, and treatment with deoxycholate. The purified preparation was homogeneous upon sodium dodecyl sulfate-polyacrylamide electrophoresis and appeared to be composed of identical protein subunits with a molecular weight of 18,400. Amino acid analysis showed a preponderance of amino acids with apolar side chains as well as the presence of cysteine. The isoelectric point of the purified material was established as 9.75 (Table 6). Recently, Isaacson et al. (74) confirmed that purified K99 antigen is composed of only a single subunit. Analysis of the N-terminal amino acid sequence showed that the K99 antigen bears no resemblance to the N-terminal primary structure of the K88 and CFA/I adhesins (Fig. 5). When observed in the electron microscope, K99 appeared to be a fimbria with a helical structure and a diameter of 4.8 nm.

As mentioned before, when studying adhesins one should take into account the possibility that one strain possesses more than one type of adhesin. The conflicting data on the identification of the K99 antigen clearly demonstrate this issue.

The F41 Adhesin

The purification and characterization of the F41 antigen has been described by De Graaf and Roorda (29). F41 was characterized as a filamentous structure composed of protein subunits with a molecular weight of 29,500. The F41 adhesion antigen has a diameter of 3.2 nm, which is slightly thinner than the K99 adhesion antigen (Table 6). The N-terminal amino acid sequence of the F41 protein subunit showed a limited homology with the K99 protein subunit (Fig. 5).

The CFA/I Adhesin

Several isolation procedures for the CFA/I protein have been described. Initially the CFA/I was isolated by shearing the cells followed by differential ultrafiltration of the supernatant, isoelectric precipitation, and ultracentrifugation into sucrose (49). Later the isoelectric precipitation and ultracentrifugation steps were replaced by ammonium sulfate precipitation and ion-exchange chromatography (44). The purified antigen could be seen in the electron microscope as thin threads 7 nm in diameter and had an average molecular weight of 1.6×10^6 as determined by sedimentation equilibrium centrifugation. The apparent molecular weight of the CFA/I subunit was 23,800 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (44). Because of the work of other authors, however, these data have become questionable.

Klemm (90) isolated the CFA/I adhesin by heating the bacteria for 20 min at 60°C followed by shearing. This method has the disadvantage that prolonged heating of the CFA/I destroys its antigenicity, i.e., heat treatment of cells prevents agglutination of cells by anti-CFA serum. The CFA/I purified in the way described by Evans et al. (44) retained its morphology, antigenicity, and biological function. Purification of the CFA/I was obtained by Klemm (90) by gel filtration on Sepharose columns. Contrary to the data reported by Evans et al. (44), the molecular weight of the CFA/I subunits was found to be 14,500 (90), with valine as the N-terminal amino acid. Wevers et al. (192) reported molecular weights of 12,000 and 13,000 for the protein subunits of CFA/I and CFA/II, respectively. These authors suggested that the value for the molecular weight of the CFA/I protein subunit as reported by Evans et al. (44) is probably a dimer because a faint band corresponding to a molecular weight of 24,000 was detectable on their gels. The amino acid composition of the CFA/I protein subunit is given in Table 5. The complete amino acid sequence of the CFA/I protein subunit (Fig. 6) was recently determined by Klemm (92). It shows no homology with the amino acid sequence of the K88ab protein sub-

FIG. 6. The amino acid sequence of the CFA/I protein subunit (92).

unit nor with the partial amino acid sequences of other adhesin subunits (Fig. 5). The molecular weight of the subunit as calculated from the complete amino acid sequence is 15,058 (92). The CFA/I subunit is rather hydrophobic and like the K88 subunit contains no cysteine. The isoelectric point of the CFA/I antigen is at pH 4.8 (54). Isoelectric points at low pH values have also been determined for the type 1 fimbriae and K88 antigen of *E. coli* (13, 105). The pI values for these antigens are 3.92 and 4.2, respectively, in contrast to the K99 antigen which has a pI value of 9.75 (27). Electron micrographs of a CFA/I preparation isolated by shearing show flexible fimbria-like structures with a diameter of approximately 3 nm and resembling the K88 and K99 fimbriae (Fig. 3). A similar observation has been published by Brinton (15) who studied a clone of *E. coli* H10407 that produced a mannose-resistant but not the mannose-sensitive hemagglutinin. We have observed that, depending on the concentration, the CFA/I fimbriae tend to aggregate and form bundles with the diameter reported by Evans et al. (44). This concentration dependency was also observed by Brinton (personal communication). The presence of very thin fimbriae was also reported by Evans and Evans (42) on a CFA/II-positive strain.

Other Adhesins of Human Enterotoxigenic *E. coli*

In a survey of enterotoxigenic *E. coli* strains isolated from humans, Thorne et al. (182) studied the adhesive properties of these strains by using human buccal mucosal epithelial cells. Of 32 enterotoxigenic strains examined, 52% bound to the buccal cells. No correlation, however, was found between adhesion to buccal cells and mannose-resistant hemagglutination of human and guinea pig erythrocytes. One group of adherent strains were able to agglutinate human and/or guinea pig erythrocytes, but another group of adherent strains showed no reaction with these types of erythrocytes. In a further study, Deneke et al. (32) described the presence of fimbriae on enterotoxigenic *E. coli* strains that adhered to buccal cells and exhibited mannose-resistant hemagglutination of human group A and B and/or guinea pig erythrocytes. For purification, the fimbriae were detached from the cells by blending, absorbed onto guinea pig erythrocytes at 0°C, and subsequently eluted from the erythrocytes at 37, 42, and 50°C. The fimbriae of strain 334 appeared to be composed of two protein subunits with molecular weights of 13,100 and 12,500, respectively. Purified fimbriae bound to human buccal cells as did the whole cells. The binding could be inhibited by anti-fimbriae Fab fragments prepared from antiserum against the purified adhesin. The fimbriae exhibit a hemagglutination pattern that differed from the CFA/I and CFA/II adhesins. In a more recent paper, Deneke et al. (33) described serological studies of these fimbriae. Three distinct antigenic types were identified on the enterotoxigenic *E. coli* strains that adhered to buccal cells. Antisera against these three adhesin types reacted with 60 of 106 enterotoxigenic *E. coli* strains tested. These strains all produced LT and/or ST. Only a limited cross-reactivity was observed with the three types of fimbriae and their respective antisera, although each of the three types of fimbriae is composed of protein subunits with molecular weights of 13,100 and 12,500. There was no correlation between fimbrial serotype, enterotoxin production, O-antigen type, geographical source of isolation, and mannose-resistant hemagglutination of various types of erythrocytes.

It should be noted that *E. coli* strain H10407, which has been used for the isolation and purification of the CFA/I adhesin by Evans et al. (44) and by Klemm (90), and the CFA-negative H10407-P derivative strain both reacted with antiserum against the fimbriae isolated by Deneke et al. Furthermore, both strains adhered to buccal cells, but strain H10407-P had lost the ability to hemagglutinate human erythrocytes. Because it cannot be excluded that the antisera prepared by Deneke et al. contain antibodies against type 1 fimbriae, the observed cross-reactivity may be attributed to the presence of type 1 fimbriae on *E. coli* H10407 as reported by Brinton (15).

An in vitro binding assay for human enteropathogenic *E. coli* strains to human fetal small intestine was developed by McNeish and coworkers (101). With a strain of serotype O26:K60:H11 originally isolated from a baby with diarrhea, the adhesion to human intestinal tissue was shown to be mannose resistant, host specific, and plasmid mediated (193). The genetic determinant for this mucosal adherence is encoded by a transmissible plasmid, pLG101, 56 megadaltons (Md) in size, that also carried the genes encoding for the production of the bacteriocin, colicin Ib (194). The nature of this adherence factor is not known, but it appears to be distinct from the CFA/I or CFA/II adhesins.

A strain of *E. coli* serotype O18ac:H$^-$, isolated from the stool of a patient with diarrhea, was found to possess fimbriae that were able to agglutinate human group A erythrocytes but not bovine, chicken, or guinea pig erythrocytes (192). The fimbriae were not produced at 20°C. Assays for ST and LT were negative, but the strain produced hemolysin and colicin, which is characteristic of *E. coli* strains causing extraintestinal infections (104). The fimbriae were de-

tached from the cells by shearing and purified by isoelectric precipitation and gradient centrifugation in cesium chloride. The fimbriae had a diameter of 5 nm and variable length. Also these fimbriae were antigenically different from the CFA/I and CFA/II adhesins. The molecular weight of the protein subunits as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 21,000. Chemical analysis showed the presence of one phosphate group per subunit. The amino acid composition of this subunit differed from those reported for CFA/I, K88, and K99, as well as from type 1 fimbriae. The fimbriae contained two cysteine residues per subunit, but as in the case of the K99 antigen, no disulfide bridges between subunits were present. The isolated fimbriae were shown to retain their filamentous structure in the electron microscope. Isoelectric focusing revealed the presence of two protein bands having pI values of 5.1 and 5.6, respectively. It should be mentioned that Cravioto et al. (22) found that 23 out of 44 *E. coli* O18ac strains all exhibited mannose-resistant hemagglutination of human erythrocytes. All strains, however, were isolated from extraintestinal sources. In addition, Wevers et al. (192) reported that antiserum against purified fimbriae of their *E. coli* O18ac:H$^-$ strain also agglutinated *E. coli* strains of different serotypes obtained from urinary tract infections. This finding raises the question as to whether these fimbriae are also important for colonization of the intestine.

ADHESION

Adhesion to Intestinal Epithelial Cells

Although there is no doubt that the adhesins of enterotoxigenic *E. coli* strains confer adhesive properties to these bacteria, little is known about the nature of the mechanism of adhesion. General concepts and principles of the adhesion of bacteria to eucaryotic cells have been reviewed (80, 128). Bacterial cell surface characteristics such as charge and hydrophobic properties are supposed to be involved in attachment. Physicochemical aspects of the adhesion of microorganisms to surfaces have recently been reviewed by Rutter and Vincent (147). The surface of both procaryotic and eucaryotic cells is, in sum, negatively charged. The repulsive electrostatic force between these like-charged cell surfaces can be overcome by long-range and short-range attractive forces. Specific binding of fimbriae to receptors on the surface of the epithelial cells can overcome the repulsive forces between bacteria and epithelial cells. The role of short-range hydrophobic interactions in the adhesive properties of fimbriated enterotoxigenic strains has been demonstrated by hydrophobic interaction chromatography (169, 187, 188). Smyth et al. (169) suggested that reduction in the cell surface potential by the masking of the charge contribution of polysaccharide K antigens and lipopolysaccharide by adhesins with hydrophobic characteristics probably promotes adhesion and that hydrophobic bonds may be involved in the interaction of these adhesins with the intestinal mucosa. Fimbriae might have hydrophobic patches, as a consequence of a high content of apolar amino acids.

Adherence to and colonization of the epithelial cell surfaces of the small intestine, without actual tissue invasion, followed by proliferation to a large population is consistent with clinical and experimental observations of diarrhea in animals and humans, caused by enterotoxigenic *E. coli*. The role of the K88 adhesion antigen in promoting colonization of the intestine was studied by implanting the plasmid encoding the K88 antigen and a plasmid encoding the production of enterotoxin into nonpathogenic strains of *E. coli* or alternatively by removal of these plasmids from pathogenic strains and subsequently feeding such modified strains to the neonate (81, 164, 165). The implantation of the K88 and enterotoxin plasmids into a nonpathogenic *E. coli* strain resulted in the colonization of the small intestine and the development of diarrhea. K88-negative *E. coli* derived from K88-positive enteropathogenic *E. coli* failed to proliferate in the anterior small intestine and did not cause diarrhea in newborn pigs. Introduction of a K88 plasmid from another *E. coli* strain restored the ability to produce diarrhea. It should be noted that transfer of Ent and K88 plasmids to an *E. coli* K-12 strain did not convert this strain to enteropathogenicity, which emphasizes that possession of an adhesin and production of an enterotoxin is as such not sufficient to produce diarrhea (164).

The K88 antigen enables the organisms to proliferate in the anterior small intestine (Fig. 7), where normally very few bacteria are present, instead of being carried along with the normal movement of the chyme. It is this area of the intestine that is most sensitive to enterotoxic activity, and the gut becomes progressively less responsive after the first few feet (69, 162). Bertschinger et al. (8) compared two enterotoxigenic strains and a non-enterotoxigenic *E. coli* strain for their association with the porcine small intestinal epithelium. The enterotoxigenic strains were found from tip to base of the villi and contiguous to the brush border. These bacteria were not found in the crypts. In contrast, the non-enterotoxigenic strain characteristically remained in the gut lumen near the tips of the villi. Comparable results have been described by

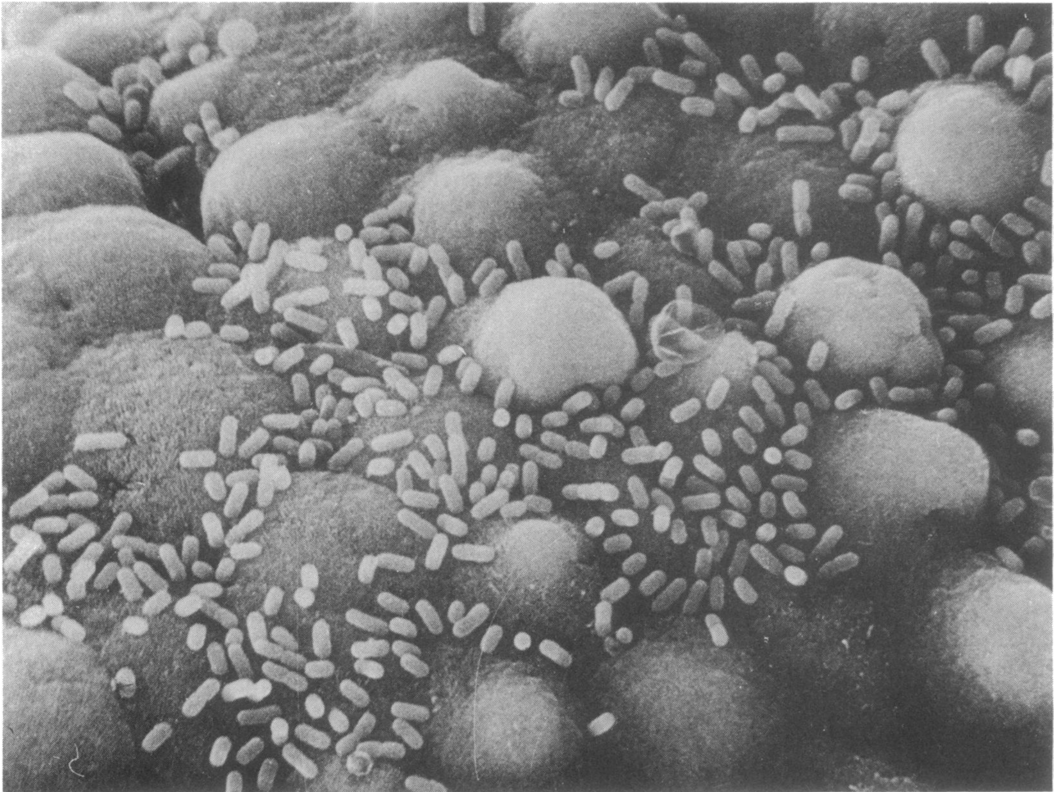

FIG. 7. Scanning electron micrograph of *E. coli* O141:K85ab:K88ab on villi of an ileum sample 18 h after infecting a newborn colostrum-deprived germfree piglet. Magnification: ×3,900. Photo generously provided by J. A. Morris.

Arbuckle (4). Adhesion of K88-positive bacteria to tissue from the small intestine in vitro could be inhibited by K88 antibodies. Adhesion of cell-free K88 antigen was also demonstrated. In several other reports the in vitro adhesion of K88-positive strains to either isolated intestinal epithelial cells (195) or epithelial cell brush borders (83) has been described. The importance of the K88 antigen and the role of adhesion in the diarrheal disease caused by enteropathogenic *E. coli* in piglets is shown clearly when the numbers of organisms present in the small intestinal contents and on the tissues in piglets from sows vaccinated with a K88-positive or a K88-negative vaccine are compared. The latter vaccine contains only O antigens and stimulates the production of O-specific antibodies, whereas the K88-positive vaccine stimulates the production of both O and K88 antibodies. Compared to nonvaccinated piglets challenged with 10^{10} K88-positive organisms, the K88-negative vaccine caused a fivefold drop in the amount of organisms present in the lumen of the small intestine and a threefold drop in the number of organisms present on the small intestinal wall. With the K88-positive vaccine, the drop in the number of organisms was 10- and 100-fold, respectively (189).

Moon and Whipp (111) reported the development of resistance with age by the swine intestine to some enteropathogenic *E. coli* strains, whereas the response to other enteropathogenic strains was positive at all ages. Walker and Nagy (189) challenged piglets with 10^{10} K88-positive cells at various time intervals after birth and observed a gradual decrease in the number of adherent organisms over a period of 5 weeks.

The adhesiveness of a pathogen for its particular host is highly specific. Enterotoxigenic *E. coli* strains isolated from pigs fail to produce diarrhea in calves and lambs by oral inoculation due to their inability to proliferate in the anterior small intestine of calves and lambs (162), whereas all enterotoxigenic strains isolated from diarrheic calves or lambs produced a severe diarrhea when administered orally to colostrum-fed neonatal calves and lambs younger than 20 h. The strains had no effect on 3-day-old calves or lambs whether or not fed with colostrum.

Several porcine enterotoxigenic *E. coli* strains

have been isolated that do not possess the K88 antigen. These strains colonize the pig intestine, and the presence of adhesive surface components was suggested (8, 69). Nagy et al. (123) reported that a number of K88-negative porcine enterotoxigenic *E. coli* strains all belonging to serogroups O9, O20, and O101 were able to cause severe diarrhea when inoculated into newborn pigs. The strains colonize the ileum and adhere to the ileal epithelium of the inoculated piglets. The presence of fimbriae on these strains was subsequently described (78). Strains of serogroup O101 possessed fimbriae with a diameter identical to the K99 antigen, but strains of serogroups O9 and O20, including strain 987, produced other fimbriae with a diameter of 7 nm. The number of fimbriated cells per culture varied from 1 to 100%. However, when inoculated into ligated intestinal loops, all of the cells became heavily fimbriated. Antiserum against the fimbriae of strain 987 agglutinated all of the strains of serogroups O9 and O20 that produced fimbriae morphologically identical to those produced by strain 987. However, in contrast to K88-positive strains, the 987P-positive stock cultures were unable to adhere to isolated intestinal epithelial cells in vitro (124). The differences between in vivo and in vitro adhesion could be explained by a difference in the production of fimbriae. Growth in the pig intestine in vivo appeared to select or promote the development of fimbriated cells. Fimbriated colonies isolated from the intestine can be distinguished from nonfimbriated colonies by colonial morphology on blood agar plates (124). Similar observations on colonial differences for fimbriated and nonfimbriated clones of *E. coli* possessing type 1 fimbriae were made by Brinton (12). With cells grown under conditions that promote adhesin production and selected fimbriated clones, Isaacson et al. (76) were able to demonstrate that 987P-positive cells adhere to porcine intestinal epithelial cells in vitro. Purified 987P fimbriae as well as Fab fragments specific for 987P fimbriae inhibited this adhesion. The attachment of fimbriated cells to the epithelial cells was a saturable process with a maximum of 30 to 40 bacteria per epithelial cell. Smith and Huggins (164) have confirmed the results of Moon and co-workers (78, 124) in the establishment of the 987P adhesin as a colonization factor in pigs. They observed that, in contrast to K88-positive bacteria which proliferated to a much greater degree in the anterior small intestine, 987P-positive strains are found in larger numbers in the posterior small intestine.

The importance of the K99 antigen in facilitating adhesion of K99-positive cells to calf and lamb intestinal epithelial cells in experimental infections was described by Smith and co-workers (162, 166). K99-positive strains of some serotypes are equally able to proliferate in the small intestine of pigs and cause a profuse diarrhea (164). Infection experiments with strains of different serotype indicated that the polysaccharide K antigen in a K99-positive strain and, perhaps to a lesser extent, the O antigen were important in determining whether or not a K99-positive strain would colonize the small intestine of pigs. Moon and co-workers (110) reported the isolation of several enterotoxigenic *E. coli* strains from pigs that were found to possess the K99 antigen. The highest number of K99-positive bacteria is always found in the posterior small intestine, and the concentration of bacteria decreased progressively towards the anterior small intestine (77, 164).

K99-positive bacteria adhere to porcine intestinal epithelial cells in vitro with a maximum of 30 to 40 bacteria per epithelial cell (76).

The ability of K99-positive enterotoxigenic *E. coli* strains to produce diarrhea appears to be age dependent. Calves and lambs become resistant to experimental challenge after about 2 days of age (162), and the isolation of K99-positive strains has not been reported from diarrhea in pigs after weaning. In contrast, K88-positive strains have been isolated from diseased pigs during both the neonatal and postweaning period (174). Suckling mice are susceptible to K88-positive and K99-positive enterotoxigenic *E. coli* strains, but because pathogenic strains rapidly lose their K88- and K99-encoding plasmids during growth in adult mice, the older mice are resistant (89). Runnels et al. (142) studied the adhesion of K99-positive strains to isolated intestinal epithelial cells taken from neonatal and older pigs, calves, and mice. It appeared that the epithelium in the small intestine develops resistance to K99-mediated adhesion with increasing host age. For the K88-positive cells, no such age-dependent adhesion could be detected.

Morris et al. (117) studied the adhesive properties of a calf enterotoxigenic *E. coli* strain carrying the recently discovered F41 adhesin. Germfree piglets infected with the *E. coli* B41M developed diarrhea within 16 h. The bacterial counts of scrapings from the washed ileal mucosa were greater than 10^8 bacteria per g, and scanning electron microscopy showed plaques of adherent bacteria in the mucosal folds of the villi. Indirect immunofluorescent staining demonstrated the presence of the F41 antigen in vivo. Isolated F41 antigen competitively inhibited the attachment of strain B41M to calf intestinal brush borders.

Volunteer studies and experiments in rabbits have confirmed the hypothesis that the CFA/I plays a significant role as a virulence factor in human diarrhea caused by enterotoxigenic *E.*

coli (31, 50). Volunteers were challenged orally with 10^8 organisms of a CFA/I-positive strain or its CFA/I-negative derivative. Diarrhea occurred only in those volunteers ingesting CFA/I-positive *E. coli* accompanied by the appearance of the organism in the stool for more than 7 days after the beginning of the experiment, whereas those ingesting the CFA/I-negative strain shed the organism in the stool for a maximum of 3 days (50).

A rather unusual type of virulent *E. coli* strain which causes diarrhea in young rabbits was initially described by Cantey and Blake (18). This strain, designated as RDEC-1, colonizes the ileum, cecum, and colon, is noninvasive, and does not synthesize either LT or ST. *E. coli* RDEC-1 binds to rabbit brush borders in vitro (19). This adherence was not influenced by the presence of mannose. In vivo infectivity of *E. coli* RDEC-1 was host specific. The strain heavily colonized the ileum and cecum of rabbits (10^9 cells per g of tissue), but minimal colonization was observed in guinea pigs and rats (20). The existence of fimbriae responsible for the mannose-resistant adherence to rabbit brush borders has not been described by Cantey or Cheney. The strain, however, does possess type 1-related fimbriae (55).

Nature of the Receptor Sites for Adhesins

Kearns and Gibbons (87) reported that plasma membranes prepared from brush borders susceptible to adhesion by K88-positive bacteria had lost 78% of their receptor activity. The K88 binding ability of the brush-border membranes was enhanced when the membranes were incubated with the supernatant fractions obtained during the preparation of these membranes. A comparable result was obtained when brush-border membranes isolated from a K88-resistant pig were incubated with the supernatant fraction of K88-susceptible brush borders. No increase in binding was observed on incubating brush-border membranes from either adhesive or nonadhesive brush borders with the supernatant fraction obtained from nonadhesive brush borders. Analysis of the supernatant fractions from adhesive and nonadhesive brush borders revealed a consistent difference in the glycolipid profiles of these fractions. Since this was the only chemical difference to be demonstrated between the different brush-border membranes and in view of the correspondence between chemical and genotypic differences, the difference in glycolipid profile was supposed to be an indication for the possible nature of the receptor for the K88 antigen. Attempts to inhibit the binding of radioactively labeled K88 antigen to brush borders with several mono-, di-, and oligosaccharides (152) suggested that in the binding of the adhesin to brush borders, complex interactions are involved and that galactosyl residues may be important (Table 7). Inhibition of the ability of the soluble K88 antigen to agglutinate guinea pig erythrocytes by some mucous glycoproteins suggested that a terminal β-D-galactosyl residue could be involved in the interaction of K88 with the erythrocytes and therefore that such a residue might be present in the receptor for the K88 antigen (58). A different specificity was reported by Anderson et al. (2) who used an in vitro binding assay employing differential filtration to show that purified ^{125}I-labeled K88 antigen formed complexes with isolated porcine intestinal brush-border membranes. The formation of these complexes was inhibited by glycoproteins with terminal *N*-acetylglucosamine and *N*-acetylgalactosamine residues and to a lesser extent by free *N*-acetylhexosamines. The difference in conclusion may result from the difference in the assay system. Gibbons et al. (58) studied the adhesion of K88 to guinea pig erythrocytes whereas Anderson et al. (2) used a direct binding assay with brush-border membranes. Sellwood (152) also tested several glycosidases to see whether they could destroy the receptor. None of them inhibited adhesion of iodated K88 preparations to adhesive brush borders. Triton X-100 extracts of adhesive brush borders were found to inhibit an ELISA developed for the K88 antigen (F. R. Mooi and A. Olthof, unpublished results). It was therefore concluded that extraction with Triton X-100 solubilized a substance that could be the receptor for the K88 antigen. The chemical nature of this substance, however, is not yet known.

A possible involvement of glycolipids in the binding of enterotoxigenic *E. coli* strains to the intestinal epithelium has become very likely since it has been demonstrated that glycolipids act as receptor for uropathogenic *E. coli* strains on human erythrocytes and uroepithelial cells (85, 93).

As indicated above, K88-positive bacteria do

TABLE 7. Carbohydrate chains in the receptors for the various adhesins

Adhesin	Carbohydrate[a]	Reference
K88	β-D-Gal or Glc, Gal, and Fuc or GM1?	58, 87, 152
K99	GalNacβ(1-4)Galβ(1-4)GlcCer | 2αNeuAc	53
CFA/I	GalNacβ(1-4)Galβ(1-4)GlcCer | 2αNeuAc	53

[a] Gal, Galactose; Glc, glucose; Fuc, fucose; GalNac, *N*-acetylgalactosamine; Cer, ceramide.

TABLE 8. Adhesion of K88ab-, K88ac-, or K88ad-positive *E. coli* strains to isolated pig intestinal brush borders

Brush border phenotype	Adhesion by K88-positive cells		
	K88ab	K88ac	K88ad
A	+	+	+
B	+	+	−
C	+	−	+
D	−	−	+
E	−	−	−

not adhere to the brush borders from all piglets. Two pig phenotypes were demonstrated and designated "adhesive" (signifying that K88-positive bacteria attached to their brush borders) or "non-adhesive" (K88-positive bacteria do not attach to their brush borders) (144, 154). The two phenotypes were found to be the products of two alleles at a single locus and are inherited in a simple Mendelian way (154). In a recent study that involved all three K88 variants known today, at least five different phenotypes could be distinguished in the brush-border adhesive test (9) (Table 8). One phenotype is susceptible to all three variants, three phenotypes are susceptible to only one or two variants, and one phenotype is entirely resistant to K88-mediated adhesion. Experiments in which the adhesion of cells carrying one of the three immunological K88 variants to the different brush-border phenotypes was inhibited with the various purified K88 adhesins indicate that the receptor sites for the K88ab and K88ac adhesins have a closer similarity than the receptor site for the K88ad adhesin. This observation fits very well with the fact that there is much more homology between the primary structures of the K88ab and the K88ac adhesins than there is between K88ab and K88ad. The chemical basis for the different receptor sites is not known.

The receptor for CFA/I has not yet been studied in human intestinal brush-border preparations. Faris et al. (53) studied the erythrocyte receptor that is responsible for the mannose-resistant hemagglutination of CFA/I- and K99-positive *E. coli* strains. They showed that the hemagglutination of these strains could be inhibited by mono- and disialogangliosides, especially the GM_2 at concentrations above the critical micellar concentration (Table 7). Pretreatment of erythrocytes with neuraminidase inhibited the hemagglutination of human erythrocytes by the CFA/I-positive strains but not the hemagglutination of sheep erythrocytes by K99-positive strains. No influence of treatment of erythrocytes with trypsin was observed, whereas pronase treatment resulted in negative hemagglutination. These results suggest that both glycolipids and glycoproteins may be involved in the erythrocyte receptor for these two adhesins.

IMMUNITY

As adhesion of enteropathogenic *E. coli* to the intestinal epithelium of the infected animal is an essential prerequisite for the development of enteric disease, the antibody-mediated inhibition of bacterial adhesion will be one of the potential defense mechanisms of the host against the pathogen. In a young animal, such antibody will be derived either from maternal sources through the colostrum and milk or later by local synthesis via the intestinal secretory immune system. Recently Sellwood (153) showed that colostrum from susceptible dams effected more efficient in vitro opsonic phagocytosis and killing of K88-positive *E. coli* than did colostrum from resistant dams. Furthermore, colostrum from susceptible sows inhibited the binding of purified K88 antigen to brush borders significantly better than the colostrum from resistant dams. Fractionation of colostrum revealed that fractions rich in immunoglobulin M had the highest opsonic activity.

Vaccination of a dam with a purified K88 preparation can confer passive immunity for a K88-positive pathogen on her offspring (145). Rutter et al. (146) used in vitro tests on serum and mammary secretions from vaccinated and nonvaccinated dams to investigate the nature of the protective factors and concluded that neutralization of the adhesive properties of the K88 antigen by K88 antibodies in colostrum and milk contribute significantly to the protection of piglets from vaccinated dams. The K88 antibodies appear to be predominantly in the immunoglobulin G fraction. Antibodies against the enterotoxin appeared not to be essential for protection, but a possible role of antibodies against the O antigen present in the colostrum could not be excluded.

Smith (159) showed that antiserum prepared against a K88-positive *E. coli* strain markedly delayed or prevented the proliferation of the strain in the small intestine of piglets to which it had been administered after challenge with the pathogenic strain. Comparable data were published by Rutter and Anderson (143). The effect of the antiserum was apparently due to anti-K88 antibodies (159). The antiserum was most effective if administrated orally. In vitro adhesion of K88-positive strains to isolated porcine intestinal epithelial cells was also almost completely inhibited by the addition of anti-K88 antiserum to the incubation mixture (81, 137, 154, 195). Antisera raised against K88ab and K88ac inhibited the adhesion of both K88ab- and K88ac-positive cells to the epithelial cells (137). The same result was obtained with antiserum specif-

ic for the K88a determinant, and antibodies against K88b and K88c determinants showed the appropriate selectivity for homologous strains. A protective effect of antiserum against the K88a determinant was not detected by Wilson and Hohmann (195). The effectiveness of the K88a antiserum prepared by these authors, however, is questionable since their serum may contain the intact K88 antigen.

Since transplacental immunity does not normally occur in pigs, the correlation between high titers of antibodies in maternal colostrum against the adhesin used for vaccination and protection of offspring leads to the conclusion that protection was a result of consumption of adhesin-specific antibodies in colostrum.

Another mechanism for countering the specific adhesion of K88-positive enterotoxigenic *E. coli* strains was described by Linggood and coworkers (96–98, 139). The passaging of K88-positive strains through media containing sow colostrum antibodies raised by vaccination of pigs with heat-stable antigens results in the loss of the K88 plasmid in vitro (97). Cured strains lose their ability to adhere to and agglutinate chicken erythrocytes. These observations imply that vaccination of dams may result in the presence of high concentrations of "curing antibodies" in the intestine of piglets fed with colostrum which subsequently may lead to a decrease in virulence of the enterotoxigenic bacteria. In other words, one of the main effects of immunization is thought to be the selection for adhesin-negative organisms in the intestine of neonatal animals. More recently, Linggood and Porter (98) observed examples of in vivo plasmid curing, giving rise to K88-negative cells which were unable to establish infection when administered to further animals. In this study sows were vaccinated by a combination of the oral and parenteral routes to provide an optimum level of serum and colostrum antibodies at the time of parturition and to convey passive immunity to the newborn piglets. A sow was challenged with large numbers of *E. coli* O149:K91:K88ac before parturition so that the piglets could receive the infection soon after birth. They observed a rapid loss of the K88 plasmid from the pathogen in the gut of the immunized sow. Shortly after parturition, 70% of the O149 cells present in the feces of the sow were K88 negative. In the feces of 10 piglets, a general trend was observed of initially higher numbers of K88-positive cells followed by an increasing number of K88-negative cells up to 100% of the O149 population. In another pig the reverse happened, and this piglet developed diarrhaea. Apparently, the curing phenomenon occurs both in the sow and in her progeny.

Davidson and Hirsh (24, 25) reported that inoculation of mice or pigs with a non-enterotoxin-producing K88-positive *E. coli* strain protects the animals against a subsequent challenge of an enterotoxin-producing K88-positive strain of the same serotype. They supposed that bacterial competition is responsible for protection.

Vaccination of sows with purified 987P adhesin confers protection to neonatal suckling pigs against diarrheal disease caused by enterotoxigenic *E. coli* strains possessing the same antigen (125). This protection did not extend to enterotoxigenic strains possessing other adhesins (75, 113). Protection was correlated to the level of 987P-specific antibodies in maternal serum and colostrum (75). Presumably, immunoglobulin G is the major protective antibody. Adhesion of 987P-positive strains to isolated porcine intestinal epithelial cells could be prevented by Fab fragments specific for the 987P antigen (76). In the same paper it was described that a laboratory strain of *E. coli* which possesses type 1 fimbriae also adhered to porcine intestinal epithelial cells in vitro. This adhesion was prevented by purified type 1 fimbriae and by Fab fragments specific for this antigen. The significance of this observation in relation to a possible pathogenicity for pigs of *E. coli* strains bearing only type 1 fimbriae remains to be clarified.

Several reports have been published about the passive immunization of newborn calves against experimentally induced enteric colibacillosis by vaccination of dams (120, 126). Vaccines used were either whole-cell vaccines (prepared from live or Formalin-killed bacteria) or concentrated crude toxin preparations. The first indication that immunity in neonates to enterotoxigenic *E. coli* strains might arise from the acquisition of anti-K99 immunoglobulins via ingested colostrum was reported by Morgan et al. (113) who were able to protect suckling pigs from enterotoxigenic colibacillosis after oral challenge with *E. coli* strain 431 (O101:K30,K99:NM) by vaccination of their dams with a purified K99 preparation. The incidence and duration of diarrhea and the degree of intestinal colonization (either duration or extent) were less than observed with other vaccines. In addition, Isaacson et al. (75) showed an increase of K99-specific antibodies in serum and colostrum of pregnant dams after parenteral vaccination with K99 antigen. Acres et al. (1) vaccinated pregnant cattle subcutaneously with (i) purified K99 adhesin, (ii) Formalin-killed whole cells of *E. coli* B44 (O9:K30,K99:H$^-$), or (iii) a bacterin containing six different strains of bovine enterotoxigenic *E. coli*. After birth, calves were allowed to nurse their dams and were challenged with cells of *E. coli* B44. Colostral antibody titers were determined against K99, K30, and O9 antigens of strain B44. In a nonvaccinated control group, 9 of 10 calves developed diarrhea and died within

24 to 72 h. Also, the calves vaccinated with the multiple-strain bacterin developed diarrhea and died. In contrast, calves nursing cows vaccinated with either purified K99 or the homologous whole-cell bacterin were protected against lethal diarrhea. The authors found a highly significant correlation ($P < 0.0005$) between protection against diarrhea and K99, but not K30 or O9, colostral antibody titers. Comparable results were reported by Nagy (122). All data are consistent with the hypothesis that K99 is a virulence factor and immunity against this adhesin prevents diarrhea, probably by blocking adhesion of the K99-positive bacteria to the intestinal mucosa. However, the K99 antibodies might also act by agglutinating or opsonizing the bacteria or both. A comparable study about the passive protection of lambs against enteropathogenic *E. coli* B44 (O9:K30,K99:NM) by vaccination of ewes with K99 adhesin isolated from *E. coli* B41 (O101:K99:NM) was published by Morris et al. (119, 177). After sucking their dams, the lambs were dosed orally within 4 to 21 h after birth. Their data suggested that the polysaccharide K antigen may contribute to the virulence of the enteropathogenic strains since with control lambs challenged with the nonmucoid form of strain B44 (O9:K99:NM), only loose feces were detected, whereas strains of the mucoid form produced a severe, watery diarrhea. None of the vaccinated dams developed diarrhea. Vaccination produced high levels of K99 antibodies in the serum and colostrum. The antibodies were predominantly immunoglobulin G and were shown to be associated with antiadhesive activity.

Unlike the adhesive antigens from *E. coli* strains producing diarrhea in animals, the colonization factor antigens of human enterotoxigenic *E. coli* have not been used to produce antisera that were used for vaccination. Immunity to enterotoxigenic *E. coli* was observed in human volunteers who were rechallenged 9 weeks after a first infection with an enterotoxigenic *E. coli* strain by Levine et al. (94). However, these investigators used CFA/I-negative strains. Their studies demonstrate that prior disease due to infection with an enterotoxigenic *E. coli* strain confers homologous immunity against a subsequent challenge.

GENETICS

Genetic Organization of the K88 Determinant

The genetic determinant of the K88 adhesin was found to be located on a plasmid that could be transferred to other bacteria by conjugation (129). Indications were obtained that two plasmids were involved in the transfer of the K88 determinant: (i) a transfer factor responsible for the transfer of the K88 genes and/or chromosomal markers to recipient cells, and (ii) another DNA segment on which the K88 determinant was located (129). Electron microscopic studies of Bak and co-workers (7) on isolated plasmid DNA of four *E. coli* strains carrying different varieties of the K88 determinant suggested that a plasmid carrying the K88 adhesin and a plasmid carrying transfer genes might associate to a composite plasmid that could dissociate again into a plasmid carrying the K88 determinant and the transfer plasmid. However, these different plasmids could not be observed as a single molecular species in separate cell lines, so definite phenotypes could not be assigned to the different plasmids observed. Smith and Parsell (167) found that the ability to utilize raffinose (Raf) as a sole carbon source is frequently cotransferred with the K88 determinant. Shipley et al. (157) determined that the K88 and Raf genes are located on a single nonconjugative plasmid, approximately 50 Md in size. In some strains larger conjugative plasmids were observed that were apparently recombinants between the Raf-K88 plasmid and a transfer factor. Mooi et al. (106) located the K88ab and Raf genetic determinants on a K88 plasmid. It appeared that the two determinants are not closely linked, but separated by a stretch of DNA of about 20 Md. Schmitt et al. (151) have found that K88 and Raf are flanked by direct repeats of IS1 and can be translocated by a *recA*-independent recombination. They suggested that Raf determinants endow enteropathogenic strains with a selective advantage, because they enable these strains to establish resistance against phagocytosis by cell wall production from raffinose.

The K88ab (88), K88ac (41, 155, 156), and K88ad (J. H. Meijerink, unpublished results) genes have been isolated by molecular cloning. In all three cases the K88 determinant appeared to be located on a *Hin*dIII fragment of approximately the same size (7 to 8 Md). The K88ac *Hin*dIII fragment, contained in the recombinant plasmid pPS002, expressed at least six polypeptides in minicells, ranging in size from 18,000 to 70,000 daltons. A 23,500-dalton polypeptide was identified as the K88ac subunit with anti-K88ac antibodies. This molecular weight is in good agreement with the size of purified K88 antigen subunit (105). Plasmid pPS002 was used to construct deletion mutants in vitro (88). One deletion derivate of pPS002 in which a K88ac DNA fragment of approximately 4.2 Md was retained was used to isolate Tn*5* insertion mutants. By analyzing the deletion and insertion mutants in minicells, four genes involved in the expression of the K88ac adhesin were identified and

mapped. The genes appeared to be organized into two operons. The first operon contains the genes for the 17,000-, 29,000-, and 70,000-dalton polypeptides. The second operon contains the gene of the fimbrial subunit. Mutants containing an insertion in the gene of the 17,000-dalton polypeptide do not express the fimbrial subunit, and it was suggested that this polypeptide acts as a positive regulator of the second operon. Mutants which contain an insertion in the gene of the 70,000-dalton polypeptide appear to shed the K88ac adhesin into the culture supernatant, suggesting a role for this polypeptide in the anchorage of the fimbriae. The deletion and insertion mutants were also analyzed for their ability to agglutinate in anti-K88ac sera, to cause mannose-resistant hemagglutination of guinea pig erythrocytes, and to adhere to isolated porcine intestinal epithelial cell brush borders. Strains harboring the 4.2-Md K88ac DNA fragment were positive for all three properties. Most insertion mutants were negative in these tests. One mutant, however, which contained an insertion in the gene of the 29,000-dalton polypeptide, was K88 positive, adhered to brush borders, but was unable to agglutinate guinea pig erythrocytes. A likely explanation for this observation could be that the hemagglutination test has a lower sensitivity than the adhesion test.

Mooi et al. (106) have cloned a 7.7-Md *Hin*dIII DNA fragment containing the K88ab determinant (Fig. 8). By excision of a 3.4-Md *Eco*RI fragment, a smaller plasmid, designated as pFM205, was constructed which still expressed the K88ab adhesin. The 4.3-Md K88ab DNA fragment contained in pFM205 expressed at least six polypeptides in minicells, with molecular weights of 17,000, 26,000, 27,000, 27,500, 30,000, and 81,000. The 26,000-dalton polypeptide was identified as the fimbrial subunit with anti-K88ab antibodies. The 30,000-dalton polypeptide appeared to be a precursor of the 27,000-dalton polypeptide (108).

The genes of the five polypeptides were located on the physical map of pFM205 by analysis of deletion derivatives of pFM205 in minicells. By inhibiting proteolytic processing in minicells, it could be determined that all five genes expressed by the K88ab DNA were translated into precursors that were approximately 2,000 daltons larger than the mature polypeptides. This indicates that all five polypeptides are transported across the cytoplasmic membrane by means of a signal sequence (107, 108). The presence of a signal sequence in the fimbrial subunit precursor was also derived from the base sequence of the gene encoding this fimbrial subunit (57) (Fig. 4).

To study the process of fimbria biosynthesis and to obtain information about the function of the gene products in this process, a set of deletion mutants of pFM205 was constructed, each containing a deletion in a different gene, and studied in minicells. Strains containing the deletion derivatives of pFM205 were also tested for their ability to adhere to porcine intestinal epithelial cell brush borders and to agglutinate guinea pig erythrocytes. An ELISA was used to determine whether the K88 antigen was properly transported to the outside of the cell (107, 108). A deletion in the gene of the 81,000-dalton polypeptide had no influence on the expression of the fimbrial subunit or the transport of subunits to the outside of the cell. The fimbrial

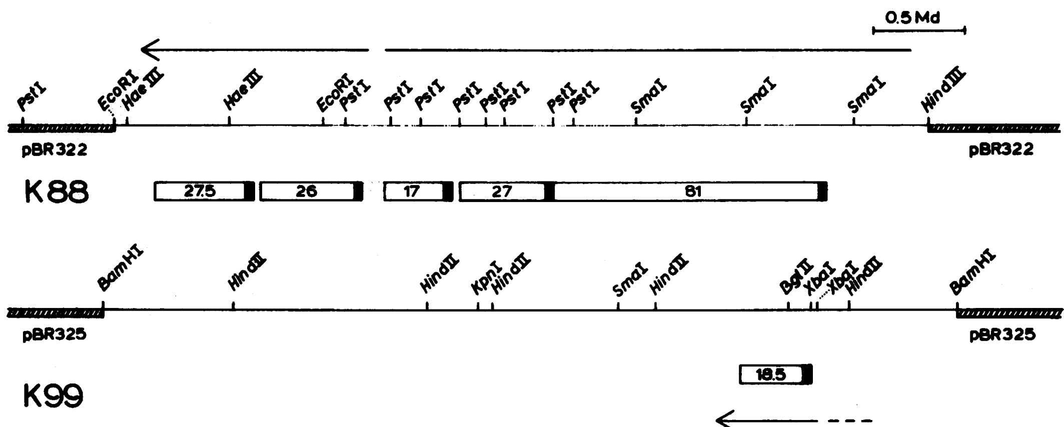

FIG. 8. Physical and genetic map of the K88 and K99 determinants. The numbers enclosed in the thick bars refer to the molecular weight (in kilodaltons) of the polypeptide encoded by that part of the DNA. The length of the thick bars corresponds to the length of DNA required to encode these polypeptides (a length of 0.5 is indicated). The black boxes indicate the presence of a signal sequence. The arrows show the direction of transcription.

subunits, however, were not assembled into normal fimbriae as indicated by the fact that they were more thermosensitive and could not be released from the cells at 65°C. Moreover, strains harboring this deletion plasmid mutant did not adhere to isolated brush borders or agglutinate guinea pig erythrocytes. Mutants with a deletion in the gene of the 27,000-dalton polypeptide also expressed the fimbrial subunit. However, in these mutants the subunit was rapidly degraded. Apparently, the fimbrial subunit is present in a form susceptible to proteolysis in the absence of the 27,000-dalton polypeptide. Although it is clear that there must be an interaction between the 27,000-dalton polypeptide and the fimbrial subunit, it is not clear at what stage of the biosynthesis of fimbriae this interaction occurs. Strains harboring a plasmid with a deletion in the gene of the 17,000-dalton polypeptide expressed the fimbrial subunit and exported it to the outside of the cell. These strains did not adhere to brush borders or agglutinate erythrocytes, indicating that the fimbrial subunits are not assembled into normal fimbriae. Possibly, the fimbrial subunit has to be modified by the 17,000-dalton polypeptide to be assembled in fimbriae. Strains harboring a plasmid with a deletion in the gene of the 27,500-dalton polypeptide produced very low amounts of the K88ab adhesin, but still adhered to brush borders and agglutinated guinea pig erythrocytes. All mutations except the deletion in the gene of the 27,500-dalton polypeptide could be complemented in trans. Therefore, it is not yet clear whether the 27,500-dalton polypeptide is involved in the biosynthesis of the K88ab fimbriae.

Although the overall picture is quite similar, there are some differences between the results obtained with the cloned K88ab and K88ac determinants. Kehoe et al. (88) suggest that the 17,000-dalton polypeptide is a positive regulator of the operon containing the gene of the fimbrial subunit. Mooi (108) has isolated several deletion derivatives of pFM205 which do not express the 17,000-dalton polypeptide but still express the fimbrial subunit. Moreover, the location of the 17,000-dalton polypeptide outside the cytoplasm (Table 9) excludes a positive regulatory role, at least at the level of transcription. Kehoe et al. (88) isolated an insertion mutant in the gene of the 29,000-dalton polypeptide which produced the K88ac adhesin, showed adhesion to epithelial cells, but did not agglutinate erythrocytes. This polypeptide is probably analogous to the 27,000-dalton polypeptide described by Mooi et al. (107), but strains harboring plasmids with a deletion in the gene of the 27,000-dalton polypeptide did not produce the K88ab adhesin (108).

TABLE 9. Cellular location and possible functions of the polypeptides encoded by the K88ab determinant

Mol wt of polypeptides	Cellular location	Possible function
81,000	Outer membrane	Assembly and/or anchorage of fimbrial subunits
27,500	Periplasm	
27,000	Periplasm	Necessary for integration of the fimbrial subunit into outer membrane
26,000	Outer membrane	Subunit of fimbriae
17,000	Periplasm	Involved in posttranslational modification of fimbrial subunits

Subcellular Location of Polypeptides Encoded by the K88ab Determinant

To obtain more information about a possible role of the polypeptides encoded by the K88ab determinant in the biosynthesis of the K88ab fimbriae, the subcellular location of these polypeptides was determined (184). Since all five polypeptides encoded by the K88 DNA (81, 27.5, 27, 26, and 17 kilodaltons) are synthesized as precursors, they were expected to be located outside the cytoplasm. Labeled minicells harboring the chimeric plasmid composed of pBR322 and the 4.3-Md fragment encoding the K88 antigen were fractionated by a passage through a French pressure cell into a membrane fraction and a soluble protein fraction. The membrane fraction was then separated into an inner membrane and an outer membrane fraction by sucrose gradient centrifugation. The periplasm and the cytoplasm fraction were isolated after conversion of minicells to spheroplasts and disruption of the spheroplasts by sonication. The 81,000-dalton polypeptide was found predominantly in the outer membrane, the 27,500- and 27,000-dalton polypeptides were mainly in the periplasm, and the 17,000-dalton polypeptide was found exclusively in the periplasm (Table 9). The K88ab protein subunit was found in varying amounts in all subcellular fractions, due to the compartmentation procedures used. Most K88ab protein was detected outside the cell.

Molecular Cloning of the K99 Determinant

In 1972 Smith and Linggood (166) reported that the K99 antigen is encoded by a conjugative plasmid. The size of this plasmid is about 52 Md (171). Recently, DNA fragments of the conjuga-

tive K99 plasmid, designated as pRI9901 and originating from *E. coli* B41, were cloned into the *Hin*dIII site of vector pBR322 and subcloned into the *Bam*H1 site of pBR325 (185). The smallest fragment obtained that still expressed the K99 antigen was 4.5 Md in size. With regard to the serological, adhesive, and morphological properties, no difference in the nature of the K99 antigen was observed between *E. coli* strains carrying the recombinant plasmid and the strain carrying pRI9901. One of the clones harboring the 4.5-Md fragment produced very little K99, although this fragment was indistinguishable from the fragments harbored by the K99-positive recombinants as analyzed by multiple-cutting restriction endonucleases. Spontaneous K99-negative mutants of *E. coli* B41 have been described (117) carrying plasmid DNA that showed no difference from their K99-positive parental strain. These observations might indicate that the expression of the K99 genes can be switched off without loss of the K99-encoding DNA as described for type 1 and 987P fimbriae. *E. coli* K-12 strains carrying K99 recombinant plasmids produced 16 to 32 times more K99 than did the *E. coli* K-12 strain harboring the parental plasmid pRI9901. The regulation of K99 expression by recombinant plasmid DNA was similar to that of natural K99-positive isolates. By deleting various regions in the K99 DNA, the position of the structural gene of the K99 subunit could be estimated. The precise location of this gene was determined by nucleotide sequencing (28). Like the K88 protein subunit, the K99 protein subunit is synthesized in a precursor form with a signal peptide of 22 amino acid residues (Fig. 8).

Using F41 antiserum, no F41 antigen could be detected on *E. coli* cells to which the K99-encoding plasmid was transferred by conjugation. This indicates that the genetic determinant for the production of F41 antigen is not located on the K99 plasmid (29). It is not known whether the F41 antigen determinant is located on the chromosome or on another plasmid carried by *E. coli* B41, i.e., the plasmid encoding for ST production.

Characterization of Plasmids Encoding CFA/I and CFA/II

E. coli strain H10407 (O78:K80:H11) contains three plasmids with molecular weights of 60, 42, and 3.7 Md, respectively (51). A laboratory-passed derivative strain H10407-P still possesses the 42- and 3.7-Md plasmids. The loss of the 60-Md plasmid in H10407-P is accompanied by the loss of CFA/I and ST production (51). Consistent with this observation, Smith and co-workers (160) have shown that the loss of expression of CFA/I and ST from five strains belonging to the O78 serogroup and isolated in different countries corresponds to the loss of a 56- to 61-Md plasmid. McConnell et al. (99) have proven that CFA/I and ST production are encoded by a single nonconjugative plasmid in strains belonging to serogroup O78. One strain of serotype O63:H^{-} contained a 65-Md plasmid coding for CFA/I and both ST and LT production (Table 10). Reis et al. (140) described a nonconjugative CFA/I-ST plasmid of 61 Md in strains of serotype O128ac:H12 isolated from children with diarrhea in Brazil. Plasmids coding for CFA/I and ST have also been identified in *E. coli* strains of serogroup O26, O63, O114, O128, and O153 (R. L. Shipley, D. G. Evans, and D. J. Evans, Jr., unpublished results; 23). The strains were isolated in different countries, and the majority also had a plasmid of approximately 60 Md in size. Cravioto et al. (23) compared the CFA/I-ST plasmids of several of these strains with the plasmids from the O78 strains by restriction enzyme analysis. Cleavage with the restriction endonucleases *Eco*RI or *Hin*dIII indicated that all plasmids shared several fragments. Generally there appeared to be more fragments in common between plasmids identified in strains isolated in the same geographical area.

One particular CFA/I-ST plasmid, NTP113, was characterized in more detail in order to identify the region(s) coding for CFA/I production. Different transposons were inserted into NTP113, and *E. coli* strains carrying the plasmid derivatives were tested for CFA/I and for ST production. CFA/I-negative, ST-positive mutants were obtained. These mutants were then used to construct deletions. It appeared that two regions of plasmid NTP113 are required for expression of CFA/I. The two regions are separated by a length of DNA corresponding to a molecular weight of at least 25 Md. A plasmid with a mutation mapping in one CFA/I region was tested for complementation with another plasmid derivative with a mutation in the other CFA/I region. Strains carrying these two plasmids produced CFA/I (23, 168).

Some of the CFA/II-positive strains examined by Cravioto et al. (23) lost the ability to produce the CFA/II simultaneously with the production of ST and LT. Other strains, however, lost these

TABLE 10. Genetics of adhesin production

Adhesin	Genetic location	Mol wt ($\times 10^6$)	Transmissibility
K88	Plasmid	51	Nonconjugative
		90	Conjugative
987P	Chromosome?		
K99	Plasmid	57	Conjugative
CFA/I	Plasmid	52–65	Nonconjugative
CFA/II	Plasmid	60	Nonconjugative

properties separately. Some of the strains that had lost the ability to produce the CFA/II, with or without enterotoxin production, had lost a plasmid. Other CFA/II-positive and -negative pairs, however, still had the same plasmid content. Penaranda et al. (138) described that CFA/II-positive strains from different geographic locations contain a 60-Md large nonconjugative plasmid coding for CFA/II, ST, and LT production.

CONCLUDING REMARKS

The etiological role of enterotoxigenic *E. coli* strains in diarrheal diseases of man and domestic animals has been firmly established during the last two decades. Besides the production of enterotoxins, at least one other important virulence factor, the ability to adhere to the intestinal epithelial mucosa by fimbria-like adhesins, has been recognized. However, other cell surface antigens (i.e., K antigens) also contribute to the capacity to provoke diarrhea in combination with adhesin and enterotoxin production. The majority of enterotoxigenic *E. coli* strains isolated from neonatal pigs, calves, or lambs possess one of the adhesive antigens—K88, K99, or 987P. A limited number of enterotoxigenic strains of human origin produce CFA/I or CFA/II. Other host-specific adhesins, however, have been observed on these strains.

In contrast to the presence of type 1 fimbriae which occur on the majority of *E. coli* strains, striking correlations have been observed between the presence of host-specific adhesins, the production of enterotoxins, and the serotype of the pathogen. Both the production of enterotoxins and these adhesins appear to be restricted to a relatively small number of serotypes. The reason for this restriction is not known. A possible explanation might be a difference in the ability of strains belonging to various serotypes to stably maintain the plasmids encoding these virulence factors. The location of CFA/I or CFA/II and enterotoxin production on the same plasmid obviously explains the close association of both properties. The close association of enterotoxin production and the presence of other adhesins like K88 or K99 cannot be explained in this way because their genetic determinants are mostly located on different plasmids. Unfortunately, the interpretation of the data presented in several studies on the occurrence and characteristics of the various adhesins is obscured by the fact that the authors insufficiently realized the possibility that *E. coli* strains produce more than one type of fimbriae at the same time. Cloning of cells producing only one type of fimbriae, as described by Brinton, can help to avoid this confusion.

Production of adhesins is affected by environmental conditions such as temperature and composition of the growth medium. None of the adhesins is synthesized below 20°C, indicating a regulatory mechanism that suppresses adhesin production at non-physiological temperatures. The effect of medium composition on adhesin production varies for the respective antigens. In general, the synthesis of adhesins seems to be optimal when the strains are grown in minimal media. Complex media can contain substances which inhibit adhesin production as shown for K99 and F41. At the present no data are available to indicate whether these factors regulate the number of adhesin-positive cells in a population, the number of fimbriae per cell, or the length of individual fimbriae. Another important mechanism that controls the production of fimbriae is the phase variation. This phenomenon has been extensively studied for the universal adhesin of *E. coli* strains, namely, type 1 fimbriae. Phase variation has also been described for some host-specific adhesins, such as the 987P antigen.

Most of the host-specific adhesins can be described as very thin and flexible filamentous proteinaceous appendages of the bacterial cell surface. Some of the adhesins however (i.e., 987P) cannot be distinguished from type 1 fimbriae as observed in the electron microscope.

Despite their similar function, no similarities in the primary structure of the adhesin subunits have been detected so far. This similar function, however, does not imply that adhesins are able to mediate the interaction between bacteria and any type of epithelial cell because each adhesin, except for type 1 fimbriae, exhibits a striking host and tissue specificity in vivo. This specificity resides in the chemical composition of a particular adhesin and the surface components of the host epithelial cells.

Relatively few studies have been done to resolve the identity of the intestinal epithelial cell surface components which function as "receptor sites" for adhesins. Several indications have been obtained that carbohydrate chains are essential components of these receptors. Furthermore, data obtained with K88- and K99-positive strains denote a nonrandom distribution of receptor sites along the intestinal mucosa. Hopefully, the study of receptor substances and the mechanism of fimbriae-mediated adhesion will receive more attention in the near future.

Immunity of neonates to infections has been obtained by immunization of dams with purified adhesin preparations. Several mechanisms have been proposed for the way in which this immunity is acquired, for instance antibody-mediated loss of virulence plasmids and prevention of adhesion.

Probably with the exception of 987P, the

genetic determinants encoding for the synthesis of host-specific adhesins are located on plasmids. The necessary genetic information appears to be organized in one or two operons encoding for several polypeptides. The possible role of these polypeptides in the biogenesis of adhesins will be a subject of future studies.

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