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DOMAIN 8 PATHOGENESIS

Animal Enterotoxigenic *Escherichia coli*

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ABSTRACT Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of *E. coli* diarrhea in farm animals. ETEC are characterized by the ability to produce two types of virulence factors: adhesins that promote binding to specific enterocyte receptors for intestinal colonization and enterotoxins responsible for fluid secretion. The best-characterized adhesins are expressed in the context of fimbriae, such as the F4 (also designated K88), F5 (K99), F6 (987P), F17, and F18 fimbriae. Once established in the animal small intestine, ETEC produce enterotoxin(s) that lead to diarrhea. The enterotoxins belong to two major classes: heat-labile toxins that consist of one active and five binding subunits (LT), and heat-stable toxins that are small polypeptides (STa, STb, and EAST1). This review describes the disease and pathogenesis of animal ETEC, the corresponding virulence genes and protein products of these bacteria, their regulation and targets in animal hosts, as well as mechanisms of action. Furthermore, vaccines, inhibitors, probiotics, and the identification of potential new targets by genomics are presented in the context of animal ETEC.

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INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of *E. coli* diarrhea in farm animals (1). ETEC are characterized by the ability to produce two types of virulence factors: adhesins that promote binding and colonization of the intestinal epithelium and enterotoxins responsible for fluid secretion (2). The best-characterized adhesins are expressed in the context of fimbriae, such as the F4 (also designated K88), F5 (K99), F6 (987P), F17, and F18 fimbriae. Once established in the animal small intestine, ETEC produce enterotoxin(s) leading to diarrhea. Two major classes of enterotoxins are produced by ETEC: high-molecular-weight heat-labile toxin (LT) inactivated by heating at 60°C for 15 min and low-molecular-weight heat-stable toxins (ST) that are stable to 100°C for 15 min (3). ETEC strains produce LT, STa, STb, and/or enteroaggregative heat-stable toxin 1 (EAST1).

PATHOGENESIS

ETEC in the environment enter animals via the oral route. The stomach, duodenum, and jejunum of animals generally do not contain coliform

bacteria. The presence of ETEC in the environment of pigs, for example, is an important factor in the transmission of the pathogens, because these bacteria are able to survive for at least 6 months when protected by manure (4, 5). In humans, a dose of 10^8 to 10^{10} microorganisms is required for disease (6), but a lower infectious dose may be sufficient for transmission in animals. Bacteria transit and colonize the small intestine. Inside the animal, they can attach to the intestinal epithelium through fimbrial or nonfimbrial adhesins (also called colonization factor [CF] antigens) by recognition of specific receptors on the small intestine and then colonize this habitat (2). There, they can multiply rapidly and reach up to 10^9 bacteria per gram of intestine. The degree of colonization determines whether or not disease will result from infection. Once established, ETEC can synthesize and secrete one or more types of enterotoxins. Concomitant production of several enterotoxins was confirmed in piglets (7). ETEC are commonly associated with some animal species such as newborn (suckling) and weaned pigs and in newborn calves (8–10). ETEC infections are very rare or almost nonexistent in other important farm animals such as rabbits, poultry, or horses, but do occur in sheep. For now, no clear explanation of this species tropism can be given because these animals do possess the receptors for the enterotoxins. We can only speculate that the gut environment of these animals is probably not appropriate for ETEC establishment and/or colonization. Close adhesion of ETEC to the intestinal epithelium permits efficient toxin delivery (11). Secretion of water and electrolytes in the intestinal lumen results from toxin activity. ETEC are known to cause rapid onset of secretory diarrhea leading to dehydration. Diarrhea is defined as soft to watery feces containing less than 10% dry matter. Lethal ETEC infections occur as a result of severe dehydration and electrolyte imbalance.

ETEC affect various animal species, causing profuse neonatal diarrhea in piglets, calves, sheep, and dogs and postweaning diarrhea (PWD) in piglets. In these animals, ETEC provoke diarrhea through a set of different enterotoxins. Colonization factors F5, F6, F17, and F41 are associated with ETEC strains causing neonatal diarrhea, whereas F18 is generally associated with strains causing PWD, and F4 with both types of strains (12–19). ETEC-producing STa as the only toxin are associated with disease in neonatal pigs, calves, lambs, and dogs (5, 20).

Pigs

Diarrhea, one of the most common diseases in piglets worldwide, is transmitted from asymptomatic carrier piglets, or sows and piglets with diarrhea to naïve animals (20). Animals can be infected at early age (neonatal diarrhea) and after weaning (PWD). At birth, the pH of the piglet stomach and duodenum is relatively alkaline, and production of digestive enzymes is poor, favoring the establishment of infection. Diarrhea of newborn piglets is observed during the first 3 to 5 days of life. ETEC are frequently the primary and sole infectious agent (5). ETEC that have specific F antigens tend to be associated with a limited array of somatic O serogroups (Table 1).

PWD, on the other hand, is common in piglets 3 to 10 days after weaning. It is an important cause of death in weaned pigs and occurs worldwide. The trend towards early weaning (at 3 to 4 weeks) may have been responsible for a concomitant increase in the occurrence of PWD. *E. coli* isolates from PWD are mostly ETEC, and one or more strains can be found in the gastrointestinal tract of sick animals. Pigs with PWD typically have watery diarrhea that lasts from 1 to 5 days. This condition is

Table 1 Fimbriae of animal ETEC

Fimbriae	Enterotoxins[a]	Host	Associated O-serotypes	References
F4ab, ac, ad (K88ab, ac, ad)[b,c]	LT, STa, STb	Neonatal and weaned piglets	8, 45, 138, 141, 147, 149, 157	77, 584–586
F41	STa	Calves, lambs and goat kids	9, 20, 64, 101	72, 586, 587
F5 (K99)	STa, STb	Calves, lambs and goat kids, piglets	8, 9, 20, 64, 101	77, 584, 586, 587
F6 (987P)	STa, STb	Neonatal piglets	8, 9, 20, 46, 101, 138, 141, 147, 149	77, 586–588
F18ac (2134P, 8813)[2]	LT, STa, STb	Weaned piglets	8, 25, 45, 108, 138, 141, 147, 149, 157	203, 589
F17a (F[Y], Att25)	STa, LT-IIa	Calves	19, 101	590–592

[a]By expressing additional toxins (e.g., a Shiga-like toxin or a cytotoxic necrotizing factor) ETEC strains can share the pathogenic properties of non-ETEC strains.

[b]The F4 and F18 fimbriae each have their antigenic variants (the designation "a" describing the common antigenic determinants, and the second letter, the variant-specific determinants) (133, 203, 593). Shiga-like toxin-producing *E. coli* responsible for edema disease in weaned pigs express usually F18ab (F107) or F4 fimbriae.

[c]Fimbriae were classified and renamed according to their antigenic properties (594).

a major cause of economic loss to the industry because of reduced growth rates and mortality. F18 and F4 are the fimbrial types commonly associated with PWD. F18 are typically associated with diarrhea of weaned pigs and F4 are associated with diarrhea in neonatal as well as weaned pigs. Adhesion involved in diffuse adherence (AIDA), a nonfimbrial CF, has also been reported in *E. coli* from weaned pigs with PWD (21, 22). Some STb-positive strains or STb:EAST1 virulotypes of neonatal or weaned pigs may also be AIDA positive and do induce diarrhea, at least in experimental infections of neonatal pigs (22).

Calves and Sheep

Typically, ETEC in calves and lambs produce only STa and fimbrial adhesins F5 and F41 (1). Binding of F5 is age dependent and gradually decreases from 12 h to 2 weeks of age. ETEC were implicated as the major cause of neonatal diarrhea in calves. These ETEC induce diarrhea in calves in the first 4 days of life, older calves or adult cattle being more resistant (10). Diarrhea accounts for more than half of all calf mortality on dairy farms.

Dogs

Most ETEC isolated from dogs with diarrhea are STa positive, but a small proportion of these are also STb positive (23, 24). No LT-positive ETEC has been associated with diarrhea in this species. Many canine ETEC carry uncharacterized species-specific fimbriae.

EPIDEMIOLOGY

According to National Swine Surveys in the United States during the nineties, diarrhea was a major cause of mortality and morbidity (25–27). In recent years, a general decrease in microbial porcine diarrhea in developed countries can likely be related to significant improvements in pig housing, management, sanitation, vaccination, and biosecurity. Nevertheless, ETEC remain frequent agents of porcine diarrhea and continue to be diagnosed in neonatal and postweaning piglets that die of diarrhea in various countries, including the United States (28–36), even though intestinal viruses became major topics of investigations. Notably, the presence of ETEC alone is not always sufficient for the disease to develop, indicating the influence of other factors such as feeding, weaning age, other infectious agents, and season (37). The prevalence of the three major fimbriae (F4, F5, or F6) expressed by ETEC strains that colonize the intestines of neonatal piglets shows both temporal and geographic variations (Table 2). Although F4 became the major fimbria of newborn piglet ETEC in the United States and Europe, F5 and F6 fimbriated ETEC remain a problem in some other countries. In the past 30 years, fimbriated isolates from sick pigs presented a less diverse F4- and O-serotype profile than 30 to 50 years ago. For example, the F4ac variant became more predominant than the other F4 variants and O149 became a major O-serotype of ETEC in America, Europe, and Australia (13, 28, 29, 38–42). It has been suggested that the observed variations of these serotypes over time are the result of vaccination pressures. Alternatively, successful selection of certain serotypes might relate to the changes the pig-farming industry has undergone over the years. However, the adaptation of animal ETEC is not exclusively clonal (43).

FIMBRIAE, A HISTORICAL PERSPECTIVE

Fimbriae-mediated colonization of bacterial pathogens was first demonstrated in pigs before similar studies were undertaken in other mammals or humans. The first described adhesive antigen of *E. coli* strains isolated from animals was the F4 antigen (44), originally named K88 because it was thought to be a capsular antigen (K for "Kapsel" or capsule in German). Its proteinaceous nature and dependence on the presence of a plasmid was demonstrated later (45). This antigen was visible by electron microscopy as a surface-exposed filament that was thin and flexible and had hemagglutinating properties (46). Like other nonflagellar hair-like appendages on the bacterial surface, these fimbriae were also called pili. However, the designation of fimbriae is preferred by some because of historical precedence (47) or to distinguish adhesive (fimbriae) from conjugative (pili) organelles. That bacteria (48), and particularly *E. coli* (49), can agglutinate erythrocytes, and that this property can be mediated by fimbriae (50), had been described previously. However, the fact that intestinal adhesion and colonization of *E. coli* in diarrheic animals (51) is mediated by fimbriae was first shown with F4 fimbriae (52). More importantly, the role of fimbriae as virulence factors was first demonstrated with these fimbriae (53). In a seminal paper, Smith and Linggood infected pigs with an enterotoxigenic *E. coli* (ETEC) that had either its enterotoxin- or its F4 antigen-determining plasmid missing to demonstrate that both the enterotoxin and F4 were needed to elicit severe diarrhea or death by dehydration. Moreover, in the absence of F4, significantly less *E. coli* could be isolated from the intestines, the difference being most impressive in the proximal portions of the small

Table 2 Numbers of F4-, F5-, and F6-fimbriated ETEC isolated from piglets in various studies since 1979. Whenever possible (most studies), data including only neonatal piglets are shown

F4	F5	F6	Country	Year[a]	References
52	7	6	Netherlands	1979	595
54	9	55	USA	1980	77
13	1	5	Hungary	1982	596
48	13	30	USA	1986	586
27	44	25	USA	1986	597
50	9	16	Norway	1986	598
20	4	13	USA	1987	599
71	19	9	Sweden	1988	600
0[b]	32	23	Canada	1988	587
20	16	5	Canada	1989	91
31	6	25	Japan	1990	601
55	6	16	England	1990	90
29	96	484	Indonesia	1991	602
790	23	157	Poland	1992	603
766	54	47	England	1993	604
10	5	5	USA	1994	605
59	8	16	Denmark	1994	606
0	6	13	Spain	1997	607
13	8	13	Spain	1997	608
18	9	26	Korea	1999	609
280	2	6	USA	1999	18
64	3	11	Japan	2001	610
13	7	1	Canada	2003	497
14	1	1	Australia	2005	611
95	33	0	Vietnam	2006	612
40	6	1	Canada	2008	441
8	31	4	Korea	2008	613
19	15	1	Zimbabwe	2009	30

[a]Year of publication.
[b]Only nonclassical serotypes were studied.

intestines. In this extensive investigation, the authors applied all the basic approaches for the study of bacterial virulence factors that were later reformulated as the molecular Koch's postulates (54). For example, intestinal colonization and the induction of diarrhea in neonatal or weaned pigs were studied with a variety of strains, including plasmid-cured porcine ETEC strains into which the same or similar plasmids were reintroduced. Alternatively, nonpathogenic isolates were rendered pathogenic by introduction of the corresponding plasmids. To support their results, the authors even undertook competition experiments between the same strain carrying or missing either one or both plasmids. Additional studies by Jones and Rutter confirmed the role of F4 in ETEC adhesion and colonization of the small intestines of piglets (55). The latter study yielded data on the antiadhesive properties of F4-specific antibodies. This result led the authors to the ingenious experiment demonstrating that neonatal piglets could be protected against ETEC by passive immunity acquired by colostral uptake from an immunized mother sow (56).

Finally, it should be noted that the F4 gene cluster was the first fimbrial system shown to be expressed and functional when cloned into a K-12 strain of *E. coli* (57). This work and further studies on both the F4ac (58) and F4ab

(59) genes and their products spurred the cloning (60) and genetic study of other fimbriae, such as the common type 1 and the P fimbriae (61, 62).

THE ROLE OF FIMBRIAE IN ETEC PATHOGENESIS

Even though the role of fimbriae in animal ETEC pathogenesis has been demonstrated with clinical strains cured of plasmids encoding the fimbrial genes, confirming experiments with defined isogenic mutants were never undertaken (54). Thus, it could be argued that the absence of other plasmid-encoded genes could have affected the results. Nevertheless, there is extensive additional evidence supporting the importance of the F4, F5, and F6 fimbriae as essential adhesive virulence factors of animal ETEC (32, 63–78). Evidence supporting the importance of the other fimbriae in Table 1 as adhesive virulence factors of ETEC is mainly epidemiological and indirect (e.g., passive protection with anti-F18 or F17 antibodies) (79–81). All the fimbriae in Table 1 have been found associated with enterotoxin (genes) on clinical isolates of animals with diarrhea. However, F17 fimbriae are also expressed by diarrheagenic non-ETEC strains or by extraintestinal *E. coli* (82, 83–85). Clinical isolates of ETEC have clonal properties, since there is a preferential association of fimbriae with certain enterotoxins and O-serotypes (Table 1) that have led to their classification into major pathotypes (86). Frequently, the same isolate carries the genes to express two or more fimbriae. Each fimbrial type carries at least one adhesive moiety that is specific for a certain host receptor, determining host species, age, and tissue specificities (Table 1).

Some fimbriae were not included in Table 1, because their involvement in the pathogenesis of ETEC remains unclear. The CS1541 fimbriae are expressed *in vivo* by porcine ETEC strains (87, 88), but they do not bind to porcine enterocytes *in vitro*. Other fimbriae such as the F165 (1) (similar to Prs or F11), F165 (2) (same as F1C), and CS31A (protein capsule) fimbriae have been reported to be associated with animal ETEC strains (producing ST). However, these fimbriae are mainly expressed on non-ETEC strains of porcine or bovine origin (89–92). Most isolates expressing these fimbriae do not coexpress ETEC enterotoxins, but more typically express a cytotoxic necrotizing factor (CNF), as found in necrotoxic *E. coli* (NTEC) or the enteroaggregative *E. coli* (EAEC) heat-stable toxin (EAST-1, a diarrheagenic toxin), in the absence of an ETEC enterotoxin (93). The CS31A fimbriae do not mediate bacterial adhesion to bovine or porcine enterocytes or their brush borders (94). Whether non-ETEC binding to enterocytes can be mediated by CS31A variants is not clear (95). No enteroadhesive property in relation to farm animals has been reported for both F165 fimbriae. Thus, both F165 and CS31A lack the typical properties of ETEC fimbriae. Some Dr-like fimbriae (Afa-7 and Afa-8) are expressed by diarrheagenic *E. coli* that are non-ETEC or extraintestinal *E. coli* (83, 96).

FIMBRIAL GENE CLUSTERS

Like most studied bacterial fimbriae, the production of animal ETEC fimbriae requires sets of genes that are organized in clusters that include one and possibly more operons (Fig. 1). They all belong to various phylogenic clades of the chaperone-usher class of fimbriae (97). Most animal ETEC gene clusters for fimbriae are located on plasmids. These plasmids are quite large (40 to 100 kb) and usually also encode enterotoxin genes (98–104) (Table 1). In some cases, the fimbrial genes are adjacent to an enterotoxin gene, creating a pathogenicity islet that includes all the genetic determinants responsible for the symptoms of diarrhea (105, 106). However, ETEC strains typically carry several large plasmids encoding additional enterotoxins and fimbrial and nonfimbrial proteins, suggesting that the resulting redundancy of colonization and diarrheagenic factors must be beneficial to the survival or the transmission of ETEC. These same plasmids can also carry antibiotic resistance genes (107–109).

The gene clusters for the fimbriae of animal ETEC (Fig. 1) encode proteins that have one of three essential functions for the production of fimbriae (110). First, two or more genes encode the structural components of the fimbriae. One protein, the major fimbrial subunit, forms most of the polymeric structure of the fimbriae, whereas the other components are incorporated as minor subunits. One (or sometimes more) subunit(s) of each fimbria carries at least one binding site for a specific mammalian host receptor. Second, the fimbrial biogenesis machineries all consist of two types of molecules, one or more periplasmic chaperones and one outer membrane protein, or usher (111). Finally, gene clusters for animal ETEC fimbriae encode typically regulatory proteins that are fimbriae specific. Models depicting the subcellular locations of fimbrial subunits, chaperones, and usher proteins involved in the biogenesis of the major fimbriae in animal ETEC are shown in Fig. 2.

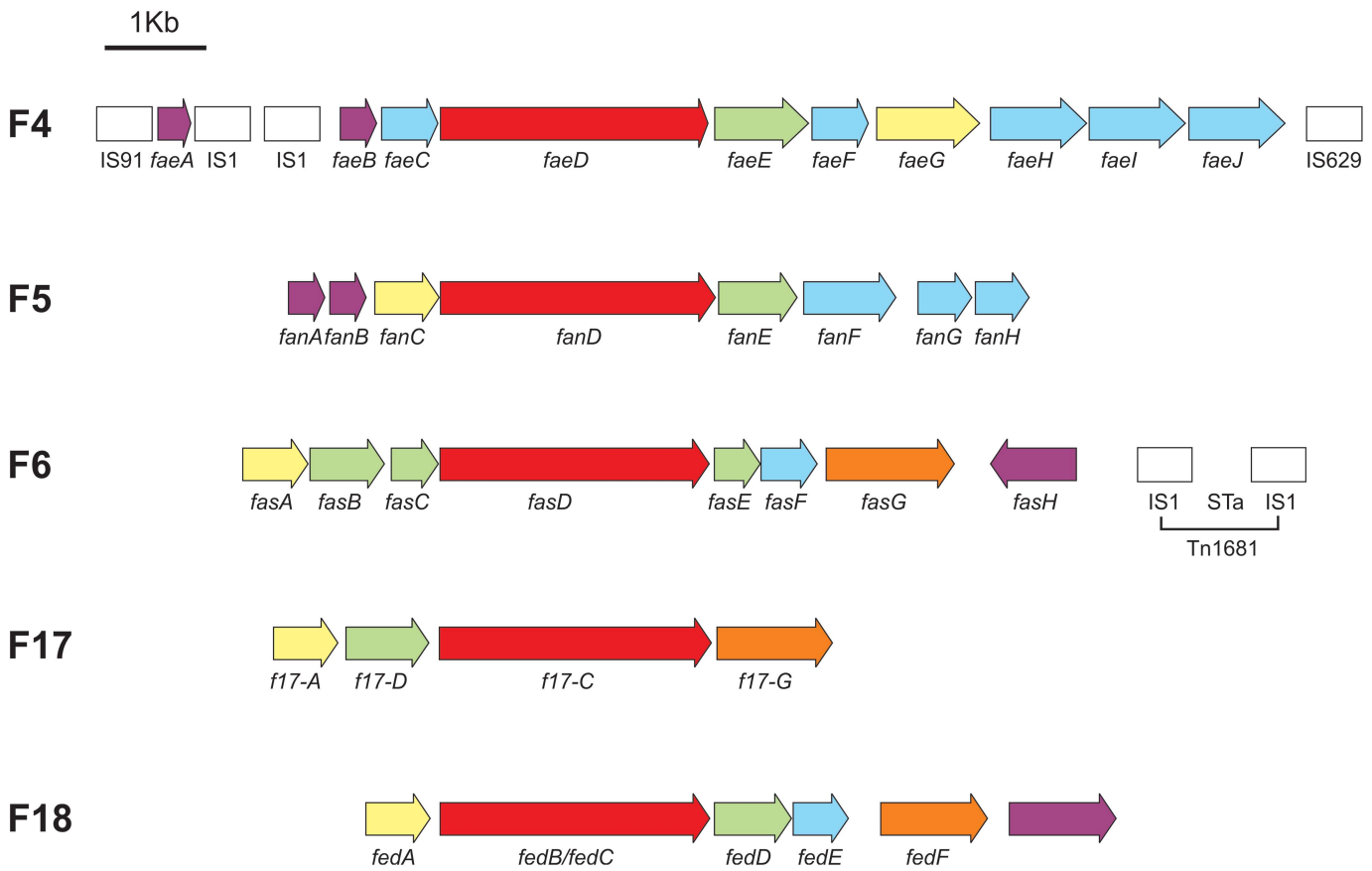


Figure 1 Genetic organization of animal ETEC fimbrial gene clusters. Genes encoding similar products or products with similar functions were labeled with the same pattern; genes for the major fimbrial subunit (yellow), minor fimbrial subunits (blue), minor adhesive subunit (orange), chaperones (green), usher (red), regulators (purple), and mobile or conjugation elements (white).

FIMBRIAL STRUCTURES

The hair-like appearance of fimbriae is best observed by negative staining electron microscopy. Fimbriae, which can reach 2 µm in length, have been typically classified by their thickness (diameter), which varies from 2 to 7 nm (Table 3). Diameter values cited in the literature seem to vary according to the bacterial growth conditions and the staining techniques used by each investigator (112–115). One fimbrial thread or fimbria consists of the spiral arrangement of hundreds of protein subunits along a filamentous axis (116). The less the subunits are compacted along the axis, the less they share surfaces of interactions and the thinner and more flexible the fimbria appears. The structure of thick or thin fimbriae is best illustrated by a helix or spring that is compressed or stretched apart, every helical turn touching the next turn only in the former situation, and the bending of the whole filamentous axis encountering the least amount of resistance in the latter situation. The model is also consistent with an axial hole (~ 2 nm), which is only visible on the electron micrograph of thicker fimbriae. Thick helical fimbriae can be stretched under certain *in vitro* conditions (117) or by using force to unwind the helix (118), accompanied by conformational changes in fimbrial subunits. It has been suggested that fimbrial stretching occurs *in vivo* to adjust and coordinate the lengths of the few hundred fimbrial threads anchoring the colonizing bacteria that are submitted to the intestinal peristalsis and its resulting shear force (119). Fimbriae-mediated bacterial adhesion to a target cell is enhanced by shear force, as described with F41 fimbriae (120). In addition, studies with adhesive subunits of a variety of fimbriae described a catch bond mechanism whereby tensile force induces stronger binding through the extension of the adhesin interdomain linker chain and an allosteric conformational change of the binding pocket that closes around the receptor moiety like a Chinese finger trap (121). The components of animal ETEC fimbriae are listed in Table 3. Immune electron microscopy of animal ETEC fimbriae typically shows that minor subunits are located at the

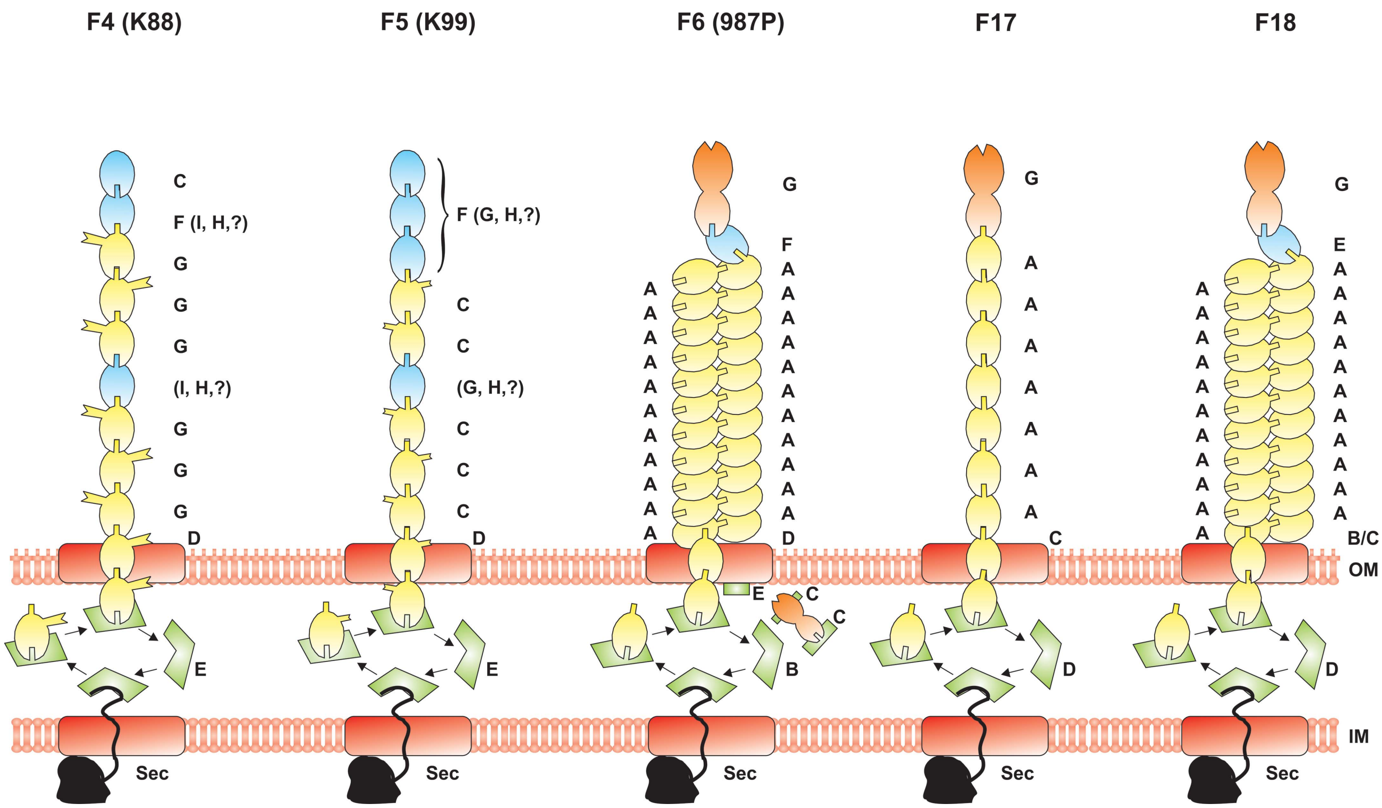


Figure 2 Fimbrial biogenesis models. The fimbriae all consist of the polymeric assembly of a major subunit (yellow) and of one or more minor subunits (blue), one of them being a tip adhesin (orange) for some fimbriae. For the K88 and K99 fimbriae, the major subunit is the adhesin. Usher proteins (red) locate in the outer membrane and channel the fimbrial subunits to the bacterial surface. All the fimbrial export systems use one periplasmic chaperone (green) for all the subunits, with the exception of the F6 fimbriae that have three chaperones, two being dedicated to two different fimbrial subunits. All the fimbrial proteins cross the inner membrane by using the general secretion (Sec) pathway (black), with the exception of fimbriae-specific regulators that remain in the cytoplasm (not shown).

fimbrial tips as well as at discrete sites along the fimbrial threads (122, 123). Mechanical fragmentation of isolated type 1 fimbriae of *E. coli* resulted in increased adhesin-mediated binding, suggesting the uncovering of hidden or incorporated adhesive minor subunits (124). However, that minor subunits can effectively be incorporated into a fimbrial body remains controversial, since broken off fimbrial tips could increase binding by sticking along the sides of fimbrial threads. Moreover, as discussed later, the current biogenesis model of affinity-determined ordered delivery of fimbrial subunits to the periplasmic domains of the usher speaks against it (123, 125, 126). Bacterial mutants of minor subunits are typically poorly fimbriated or lack fimbriae (122, 123), in support of the involvement of minor subunits in the initiation steps of fimbrial elongation and tip localization.

Table 3 The structural proteins of animal ETEC

Fimbriae	Fimbrial diameter	Major subunit	Minor subunits	References
F4	2–4 nm	FaeG	FaeC, FaeF, FaeH, FaeI, (FaeJ)	46, 58, 59, 113, 122, 246
F41	3.2 nm	Like F4	(No genes or protein names)	181, 182, 271
F5	~ 3 nm[a]	FanC	FanF, FanG, FanH	112, 251–254, 614, 615
F6	7 nm	FasA	FasF, FasG	106, 123, 195, 616, 617
F18	6.7 nm	FedA	FedE, FedF	115, 199, 258
F17	3–4 nm	F17-A	F17-G	215, 590, 618

[a]Reported wider values were likely due to strand bundling.

ADHESINS AND HOST RECEPTORS

It is generally assumed that the fimbriae of ETEC strains act only as anchoring devices to serve bacterial colonization of the intestinal surface. Fimbriae-mediated ETEC colonization of the intestines in pigs also induces innate immune responses (127–129), but it is not clear what bacterial molecules are responsible, whether lipopolysaccharides (LPS), flagellin, or other effectors. The pathogenesis of ETEC strains is specifically linked with the colonization of the small intestine and not of the large intestine. The distribution of the receptor(s) and the differential environmental signals regulating fimbrial expression in each intestinal segment (130, 131) determine the bacterial colonization sites.

Many fimbriae mediate hemagglutination. Since fimbriae most frequently act like lectins by binding to carbohydrate moieties of glycoproteins or glycolipids, fimbrial receptors have frequently been studied with red blood cells of various animal species. Although hemagglutination remains a convenient way to study and classify fimbriae (132–134), some fimbriae of animal ETEC such as F6 do not agglutinate red blood cells, or do so only after chemical treatment (135). Another caveat is that the *O*- and *N*-glycosylation profiles and the glycosylated host molecules on red blood cells might be quite different from the ones found in the intestinal mucus and on enterocyte brush borders of the relevant animal species. The presence, modification, or absence of some of these receptors in the mucus or on the brush borders varies with age, and these changes have been proposed to explain age-dependent intestinal colonization and ETEC-mediated diarrhea. For example, only newborn and weaned piglets had F4 receptors in their mucus, the latter more than the former (136), while these receptors were hardly detectable in the mucus of 6-month-old pigs (137). Similarly, intestinal cells from older pigs or calves were resistant to F5-mediated adhesion (138). This correlated to the age-dependent disappearance of the *N*-glycolyl group in intestinal glycolipids required for the F5 receptor activity (139, 140). Studies on the F6 receptors suggested that intestinal brush border receptors, particularly sulfatide, are released in the mucus of postneonatal pigs inhibiting fimbriae-mediated adhesion and colonization (141–143). Lactotetraosylceramide, which was recently detected as a carbohydrate receptor moiety, was suggested to be inactivated by fucosylation in older pigs (144). In contrast, the adhesion and colonization by F18-fimbriated ETEC isolates was proposed to be dependent on receptors that develop progressively with age during the first 3 weeks after birth (145). F17-mediated bacterial binding to ileal mucus of older calves was decreased in comparison with the binding to mucus of younger animals (146). The age-specific presentation and anatomical location in the intestines of the various receptor molecules for one fimbrial type do not determine alone the susceptibility of neonatal or weaned animals to fimbriae-mediated colonization. Also important is the genetic makeup of breeds and individual animals determining whether a receptor is expressed or not, and the adaptive immune responses eliciting passive (colostral) or active protection against colonizing ETEC. The major intestinal receptors for animal ETEC fimbriae and their cognate fimbrial adhesins are listed in Table 4.

F4 Fimbriae and Receptors

Biochemical studies identified several different receptors for the F4 fimbriae and its serological variants (Table 4). That the antigenic classification of the F4 variants also determines their binding particularities (147, 148) is consistent with the identification of the major fimbrial subunit FaeG as the F4 adhesin (149, 150). By definition, the F4 fimbriae are polyadhesins (110), since FaeG accumulates two roles by constituting most of the fimbrial structure and by mediating bacterial adhesion, unlike the better studied model that has a separate major subunit and an adhesive minor subunit that locates only at the fimbrial tip. FaeG combines its structural requirements with its adhesive role by grafting an additional ligand domain on its Ig-like core (151). Recombinant FaeG inhibits bacterial F4-mediated binding to enterocytes (152), and substituting the phenylalanine at position 150 of FaeG for a serine drastically reduces the hemagglutinating property of the F4ab fimbriae, suggesting that this residue is important for intestinal binding (150). Binding studies with engineered chimeric F4ac/ad indicated that amino acids 125 to 163 of FaeG are essential for F4 variant-specific binding (153).

The F4 receptor list in Table 4 is not exhaustive; other receptors of various molecular weights have been reported for the F4 fimbriae, as discussed in several reviews (154–156). Some of the additional mucin receptors might represent released degradation products of larger brush border receptors. Depending on the presence or absence of the different F4 receptors, up to 8 groups of receptor phenotypes have been described (157), with six having been studied in more detail (158) (Table 5). As shown with different glycoconjugate receptors, the three F4

Table 4 The fimbrial adhesins of ETEC and their receptors

Fimbriae	Adhesins	Intestinal receptor molecules	References
F4ab	FaeG(ab)	b: Transferrin *N*-glycan (74 kDa)[a]	159, 619
		Galactosylceramide, sulfatide, sulf-lactosylceramide, globotriaosylceramide	162
		bc: IMPTGP (210–240 kDa)[a,b]	620, 621
		bcd: Glycoproteins (45–70 kDa)[c]	137
		Aminopeptidase N	160, 161
F4ac	FaeG(ac)	bc: IMPTGP (210–240 kDa)[a]	620, 621
		Galactosylceramide	162
		bcd: Glycoproteins (45–70 kDa)[c]	137
		Aminopeptidase N	160, 161
F4ad	FaeG(ad)	d: Neutral glycosphyngolipids*a*	622
		bcd: Glycoproteins (45–70 kDa)[2]	137
		Aminopeptidase N	160, 161
F41	Major subunit	Unknown (erythrocytes: glycophorin A^M)	120, 183
F5	FanC	*N*-Glycolylsialoparagloboside	139, 140, 189,
		N-Glycolyl-GM3	190
F6	FasG	Sulfatide	142, 143, 165, 195
		Proteins (32–35 kDa)	
	FasA	Ceramide monohexoside (hydroxylated galactosylcerebroside)	
F18ac	FedF	Unknown [F18ab: α(1,2)-fucosyl-containing glycoconjugates]	205, 206
F17a	F17-G	Mucins, glycoproteins (170–200 kDa)	146

[a]As suggested by Billey et al. (155).
[b]Intestinal mucin-type sialoglycoproteins.
[c]As suggested by van den Broeck et al. (156).

variants demonstrate lectin activities specific for a minimal recognition sequence containing a β-linked HexNAc, a terminal β-linked galactose enhancing the binding (159). It is most likely that the context of this sequence in the different receptors is responsible for the binding specificities of the F4 variants. In contrast, the F4 aminopeptidase N receptor is glycosylated, and biochemical evidence has indicated that sialic acid is needed for binding (160), whereas transcription levels or single-nucleotide polymorphisms (SNPs) could not explain the various binding profiles of three F4 variants (161). Recent studies characterized some of the porcine intestinal carbohydrate receptor moieties interacting with the F4 fimbriae (162). Crystal structure comparisons of the FaeG variants and of the $FaeG_{ad}$-lactose complex suggested different variant-specific binding pockets with a potential involvement of conformational changes for the adhesion process (163). Noticeably, all the intestinal ceramides that act as receptors for the F4, F5, and F6 fimbriae need to be hydroxylated (140, 162, 164, 165), indicating the importance of the lipid moiety in the binding properties of gangliosides with short carbohydrate chains. Moreover, the membrane-embedded lipid portion of a glycolipid receptor determines the orientation of the carbohydrate target on the surface of host cells and thus plays an essential role in the recognition by a fimbrial lectin (166). Finally, although binding studies have focused on FaeG, whether some of the F4 minor subunits contribute to the adhesive properties of the fimbriae in animals remains an open question.

Table 5 Groups of F4/F4 receptor phenotypes, as originally classified (I to IV or A to E), with an updated nomenclature that distinguishes receptors with fully (R^{FA}) or partially (R^{PA}) adhesive phenotypes (155, 158, 623–626)

A (IV)	A1: F4abR^{FA}/F4acR^{+}/F4adR^{FA}
	A2: F4abR^{FA}/F4acR^{+}/F4adR^{PA}
B (III)	B: F4abR^{FA}/F4acR^{+}/F4adR^{-}
C	C1: F4abR^{PA}/F4acR^{-}/F4adR^{FA}
	C2: F4abR^{PA}/F4acR^{-}/F4adR^{PA}
D (II)	D1: F4abR^{-}/F4acR^{-}/F4adR^{FA}
	D2: F4abR^{-}/F4acR^{-}/F4adR^{PA}
E (I)	E: F4abR^{-}/F4acR^{-}/F4adR–

Genetic studies located the receptors of the F4ab/ac fimbriae on porcine chromosome 13 (167, 168), in the MUC4 region (169–171). MUC4-mediated susceptibility

was linked to the presence of high-molecular-weight glycoproteins (172). Based on linkage disequilibrium for MUC13 (173), and more specifically for six SNPs (two in MUC13) with the F4ab/ac receptor locus, this locus was located between the LMLN locus and microsatellite S0283 (174). Further studies confirmed a link between the F4ac receptor locus and MUC13, and pigs expressing at least one transcript predicted to encode a highly *O*-glycosylated MUC13 protein (MUC13B) were F4ac susceptible, whereas pigs homozygous for the nonglycosylated allele (MUC13A) were F4ac resistant (175). In differing studies, transcription of either MUC13 or MUC20, another gene associate to F4ac binding (176), did not relate to the adhesive phenotype (177). Moreover, biochemical studies were unable to detect an interaction between MUC13 and F4ac and genotyping assays suggested that a yet uncharacterized M13-adjacent orphan gene participates in glycosylation of the F4ac receptor (178). In another investigation, the SNPs or transcription of 12 genes involved in the assembly of glycosphingolipid carbohydrates could not be associated to a F4-binding phenotype (179). In contradiction to early studies, recent data suggested that pigs carrying F4ab/ac receptors had greater average daily weight gains than pigs lacking these receptors or having the F4ad receptor (180), possibly contributing to the prevalence of F4 variants in western countries.

F41 Fimbriae and Receptor

DNA hybridization and gene expression studies have indicated that the F41 fimbrial gene cluster is most similar to the F4 gene cluster, with the exception of the major subunit gene (181, 182). Thus, it is assumed that this fimbrial subunit acts as the F41 adhesin. An intestinal receptor for the F41 fimbriae remains to be identified, although it might include *N*-acetylglucosamine in a carbohydrate group that mimics one on glycophorin A^M, as determined by hemagglutination assays (120, 183). A quantitative trait locus (QTL) with a suggested candidate gene (ST3GAL1) was found on Chromosome 4 (SSC4) (184).

F5 Fimbriae and Receptors

Similar to the F4 fimbriae, the major F5 subunit FanC was shown to be responsible for the hemagglutinating properties of the fimbriae (185, 186). Site-directed mutagenesis of two positively charged residues, lysine 132 and arginine 136, affected the interaction with erythrocytes known to share some of the sialylated glycolipid receptors with piglet and calf intestines (139, 140, 187–191).

F6 Fimbriae and Receptors

In contrast to F4 and F5, F6 fimbriae do not agglutinate mammalian red blood cells, but only bind to intestinal cells of neonatal piglets (141, 143, 192–194). The F6 minor subunit FasG binds specifically to porcine histone H1 proteins (143, 195, 196). FasG also mediates F6 binding to a glycolipid receptor, porcine intestinal sulfatide (142, 165). Of twenty single arginine or lysine to alanine mutants, binding to sulfatide-containing liposomes was reduced in four cases (residues 17, 116, 118, 200) and abrogated for one mutant (lysine 117). All five mutants produced wild-type levels of F6 fimbriae. It was proposed that one or more of these residues communicate with the sulfate group of sulfatide by hydrogen bonding and/or salt bridges (197). All the allelic FasG proteins with reduced binding to sulfatide still interacted like wild-type FasG with the protein receptors of porcine brush borders. At least two segments of FasG that did not include lysine117 were involved in this interaction, suggesting that different residues, and thus different domains of FasG, are required for binding to the protein and the sulfatide receptors (198). In addition to the two enteroadhesive properties of FasG, a third type of F6 binding occurs between the major subunit FasA and piglet brush border hydroxylated ceramide monohexoside (165). More recently, F6 fimbriae were shown to bind to lactotriaosylceramide and lactotetraosylceramide isolated from the intestines of 6-week-old pigs (144). Whether these receptors are already expressed in younger neonatal piglets, which are the targets for F6-fimbriated ETEC, was not determined.

F18 Fimbriae and Receptors

For the F18 fimbriae, the minor subunit FedF of F18ab (199) was shown to act as an adhesin specific for porcine intestinal epithelial cells (200). The continuous FedF sequence from residue 60 to 109 was important for binding (201). Each of its charged residues was substituted for alanine, one at a time, and three mutations (replacing lysine 72, histidine 88, or histidine 89) significantly diminished bacterial binding to jejunal epithelial cells. Binding was abolished with a double mutant (lysine 72 and histidine 88) and the triple mutant. All these mutants produced wild-type levels of fimbriae. The *fedF* sequence of 15 clinical isolates indicated 97% identity, with 8 sequences showing an asparagine substitution to glycine or aspartic acid at position 73 (201). Both substituted and "wild-type" alleles were found in O139-serotyped strains, which typically express F18ab, and in O141-serotyped isolates, which usually express F18ac. Similar results were

obtained with a study of 37 strains of F18-expressing *E. coli* of various countries (202). Thus, it is likely that F18ab and F18ac bind to the same receptor(s), as suggested in earlier studies showing that either F18ab or F18ac fimbriae inhibit F18-mediated bacterial attachment to enterocytes (203). Whether the adhesive function of F18 fimbriae is modulated by the reported allelic sequence profiles of *fedF* in clinical isolates remains an open question. Genetic studies have shown that susceptibility to F18ab-mediated enteroadhesion was inherited in pigs as a dominant trait and that it was linked to the α(1, 2)-fucosyltransferase FUT1 gene on porcine chromosome 6 (204–206). These studies suggested that this glycosylase adds an essential fucose to one or more glycoconjugate receptors for the F18ab fimbriae. The role of FUT1 was consistent with the inhibition of F18-mediated receptor binding by a monoclonal antibody specific for the red blood group antigen H2, a trisaccharide that carries a terminal fucose (207), and confirmed biochemically by F18 binding to a variety of hexa- to nonaglycosylceramides from porcine intestinal epithelial cells, all carrying at least one terminal fucose (208). The crystal structure of FedF with its N terminus bound to the blood group A and B type 1 hexaoses revealed a shallow glycan-binding pocket with an adjacent polybasic loop proposed to stabilize F18 binding by interacting with intestinal cell membranes glycosphingolipids (209, 210). This polybasic loop was proposed to benefit particularly the intestinal binding of F18-ETEC to pig intestines, in the absence of inhibitory milk glycans that follows weaning (210). Several investigators in China detected that the homozygous resistance genotype occurred only in certain Western breeds and associated with genotypes for other forms of resistance (211–214).

F17 Fimbriae and Receptors

The F17G minor fimbrial subunit is the adhesin of the F17 fimbriae (215). F17G mutants produce normal fimbriae that do not bind to calf intestinal epithelial cells. Binding inhibition assays suggested that the carbohydrate specificity of the F17a fimbriae is a terminal or an internal *N*-acetylglucosamine on *O*-linked oligosaccharides of bovine mucins or intestinal glycoproteins (146, 210, 216). The crystal structures of the immunoglobulin-like lectin domains of two F17 adhesins (F17G and the related adhesin GafD) that bind *N*-acetylglucosamine were resolved (217, 218). Although an *f17G* gene encoding a different N-terminal sequence was recently described, it was not carried by an ETEC strain (85).

FIMBRIAL BIOGENESIS

Animal ETEC use the export and assembly apparatus of protein subunits known as the chaperone-usher pathway of fimbrial biogenesis (110, 219). Although the earliest models of fimbrial biogenesis were developed by studies on K88, much of the current knowledge was obtained by more detailed investigations on the type 1 and P fimbriae. To produce fimbriae on the bacterial surface, the structural subunits have to be transported directionally through two membranes (Fig. 2). With the exception of the fimbriae-specific regulatory proteins, both the fimbrial subunit and biogenesis proteins have typical signal sequences allowing them to cross the cytoplasmic membrane by using the general secretory pathway, also designated sec-translocase (220). Having reached the periplasm, an exported fimbriae-specific chaperone associate with a fimbrial subunit that appears on the periplasmic side of the inner membrane and lends a beta-strand to complete the immunoglobulin fold of the subunit by a mechanism termed donor strand complementation (221, 222). The chaperones stabilize and protect the fimbrial subunits against proteolytic degradation and premature assembly in the periplasm. Most importantly chaperones keep the subunits in an assembly- and export-competent conformation, the energy for subunit export and assembly being maintained by the conformational state of the chaperone-associated subunits (223). After their export to the periplasm, usher proteins reach the outer membrane, where they assemble in a beta-barrel structure with the help of the BAM assembly machinery (224, 225).

Fimbrial subunit release and delivery to the usher by the chaperone is coupled with the assembly of the subunit into a fimbrial fiber through a donor-strand complementation mechanism (110, 219, 226, 227). The usher molecule forms a gated channel that is required for the translocation of fimbrial subunits in a linear structure through the outer membrane (111, 221, 228). Although earlier findings suggested oligomeric and dimeric structures, recent work indicated that monomers are sufficient for fiber assembly and secretion, with a plug domain, a C-terminal domain required for filament assembly, and an N-terminal domain responsible for recruiting the chaperone-subunit complexes (229). Incorporation of subunits into the linear fimbriae with the help of the usher protein results in the extension of fimbriae on the bacterial surface, where they take their final helical conformation (230). Thus, the usher also acts as an anchor for the elongating fimbrial fiber (58). Minor fimbrial subunits are frequently observed on the tip of fimbriae by

immune electron microscopy. Since fimbriae grow from the base (231), tip-associated minor subunits have to be delivered to the usher before the major subunits. The role of these minor subunits can be essential in initiating fimbrial elongation, as described below for the individual fimbriae, when a mutation in a minor subunit gene results in the reduction or lack of fimbriation (122, 123).

F4 Biogenesis

DNA and protein sequence differences of the serologically differentiated F4ab, F4ac, and F4ad fimbriae are found between and within their major subunits FaeG (232). However, the sequences of the accessory genes of the F4ab and F4ac are identical (114, 233). The F4 chaperone FaeE (234–238) and the usher FaeD (239–241) are involved in the export of the adhesive major subunit FaeG and three to five minor subunits (FaeC, FaeF, FaeH, and possibly FaeI and FaeJ) (156, 221, 242–244). Genetic studies predict that the usher FaeD spans the outer membrane with 22 beta-strands, leaving relatively long N- and C-terminal ends in the periplasm (240, 241), which are likely used for subunit assembly and export as currently modeled (111, 229). FaeC, unlike FaeG, H, and F, interacts only weakly or indirectly with the chaperone FaeE in the periplasm, but binds well to the usher FaeD (245). The model of F4 biogenesis suggests that FaeC locates at the fimbrial tip and plays an essential role in initiating fimbrial export and assembly, since there is no fimbriation in its absence (246–248). FaeF is thought to act as an adapter that links FaeC to the fimbriae. In addition, FaeF locates also at distinct distances along the fimbrial length, as does FaeH, and both are involved in fimbrial biogenesis since 40 to 100 times fewer fimbriae are expressed in their absence (122). The FaeI and FaeJ proteins share sequence similarities with the other subunits. However, their role is not clear and the corresponding mutants have no detectable phenotypes. The F41 biogenesis apparatus has not been studied in great detail. However, it was shown that the F41 gene cluster contained a similar gene arrangement as the F4 gene cluster (181, 182). Moreover, F41 subunit-containing fimbriae were expressed by complementing its gene with the accessory genes from the F4 gene cluster, indicating that the F41 and F4 export and assembly system are closely related (249).

F5 Biogenesis

The export of the F5 subunits is coordinated by the monomeric chaperone FanE (234, 250) and the usher FanD (251). The minor subunit FanF is positioned both at the tip and along the fimbrial shaft (252). This is consistent with the phenotype of a *fanF* mutant that produces only 0.1% fimbriae, all being short. FanG and FanH are additional minor components of the F5 fimbriae (253, 254). They associate with FanF and participate in the initiation and elongation of the fimbriae, since *fanG* and *fanH* mutants are nonfimbriated or produce shorter and fewer fimbriae (1 to 2% wild-type levels).

F6 Biogenesis

FasG, an adhesive minor subunit, is the first exported subunit of the F6 fimbriae, followed by FasF, the second minor subunit proposed to act as a linker molecule, and FasA, the major structural subunit (123, 195). FasG and FasF can be visualized as well at the tip along the fimbrial shaft. In their absence, no fimbriae (or very rare short fibers for a *fasF* mutant) are expressed (106, 255). The outer membrane protein FasD is the F6 usher protein, and mutagenesis studies have suggested that its structure consists of a beta-barrel with 28 amphipathic beta–strands crossing the membrane (256). FasD was most accessible to proteases from the periplasmic side, implying the presence of a membrane-embedded usher with large periplasmic loops. In contrast to most other fimbrial systems, F6 fimbrial biogenesis involves three different chaperones (257). FasB is the chaperone associating with the major subunit FasA, whereas FasC acts independently of FasB as the FasG-specific chaperone. FasE, a chaperone-like protein, was also located in the periplasm. Although no FasE-associated Fas protein could be detected, FasE was shown to be required for optimal export of FasG.

F18 Biogenesis

The serologically differentiated F18ac and F18ab fimbriae consist mainly of a major subunit FedA that has different protein sequences both between and within the serovars (258–261). Accessory genes and products have only been investigated for the F18ab fimbriae. However, by analogy to the F4 fimbriae, it is assumed that the F18ab and F18ac accessory genes have the same or very similar sequences. Based on their sequences and their requirement for fimbriation, the *fedB* and *fedC* genes were proposed to encode the usher and periplasmic chaperone proteins of the F18ab fimbriae (200). Two minor fimbrial subunits, FedE and the adhesin FedF, are not essential for fimbriation, but corresponding deletion mutants formed longer fimbriae, indicating that their products control fimbrial elongation

(199). Interestingly, *fedE* mutants (like *fedF* mutants) do not bind to porcine intestinal villi, suggesting that FedE is involved in the export and assembly of FedF.

F17 Biogenesis

Analysis of the DNA sequence from the F17a gene cluster of a bovine ETEC strain revealed the presence of four genes (215). The chaperone F17a-D and the usher protein F17a-C are essential for the export and assembly of the major subunit F17a-A and the minor adhesive subunit F17a-G. An F17a-G mutant can make wild-type levels of unaltered fimbriae that do not bind, indicating the F17a-G adhesin is not required for fimbriation. A major subunit F17a-A mutant does not bind either. This suggests that the major subunit is needed for the export, the final conformation, or the presentation of F17a-G on the bacterial surface (215).

REGULATION OF FIMBRIAL EXPRESSION

Fimbrial gene clusters typically include one or two genes that specifically regulate the transcription of the genes in their cluster. It is generally assumed that fimbrial gene clusters have only one promoter per direction of transcription. However, when studied in more detail, some gene clusters were found to carry multiple promoters and operons for the different accessory proteins (262). In addition to the fimbriae-specific regulators, each fimbrial gene cluster typically belongs to specific regulons that can be activated or repressed by global regulators such as H-NS (histone-like nucleoid-structuring protein for temperature and osmolarity mediated signals) or CRP (cAMP receptor protein for catabolite repression). Some intriguing data highlighted the possibility that autoinducer molecules expressed by the intestinal microbiota or host catecholamines have an effect on intestinal colonization by ETEC (263). Recent data indicated increased gene expression and production of F4 fimbriae by an ETEC strain grown in the presence of conditioned media and epinephrine (264), but more work is needed to dissect the corresponding mechanisms of regulation.

Some fimbriae undergo phase variation. Transcriptional regulation ensures that all bacterial siblings synchronize their "on" or "off" switch for fimbrial expression. In contrast, a bacterial population regulated by phase variation always contains both "on" and "off" switched variants. The ratio of "on" and "off" variants depends on environmental growth conditions and one type of variant may be as scarce as mutants. It is thought that phase variation improves the survival rate of a bacterial population that is abruptly submitted to a new environment that selects for the scarcer phase variant. Fimbrial expression can be regulated posttranscriptionally. *E. coli* small RNA (sRNA) have been shown to regulate the transcription of some fimbriae (265, 266). However, it is not known whether sRNA also regulate ETEC fimbriae.

F4 Regulation

The F4 fimbrial gene cluster carries two genes, *faeA* and *faeB*, encoding proteins that are similar to regulatory proteins of the P and S fimbriae (267). FaeA negatively controls F4 expression, whereas FaeB does not appear to regulate F4. *Cis*-active regulation of fimbrial expression is mediated by the level of methylation of three GATC sites upstream of *faeB* (268, 269). Dam (deoxyadenosine methylase) methylation of the first GATC site prevents the coordinate binding of the global regulator LRP and FaeA at this site, a regulatory mechanism thought to inhibit a lethal overproduction of fimbriae. Methylation of the two other GATC sites destabilizes Lrp/FaeA binding and methylation of the third site activates transcription of *faeB* (and downstream *fae* genes). The population of F4-encoded plasmids in a culture consists of a mixture of replicons that are either methylated (20%, responsible for high-level expression) or nonmethylated (80%, responsible for low-level expression) at the third GATC site (the first site remaining methylated and the second nonmethylated). Thus, the methylation status of the third GATC site modulates the level of fimbrial expression. The presence of the two IS1 elements between *faeA* and *faeB* results in the lack of any detectable regulatory effects of FaeB on *faeA* or *faeB* transcription and in the constitutive expression and autoactivation of *faeA*. These effects were proposed to explain why the F4 fimbriae do not undergo phase variation as observed with the P fimbriae, despite the presence of similar regulatory proteins and GATC sites in *cis* (269).

No regulatory genes have been identified for the F41 fimbriae. However, F41 expression is repressed at low temperature and in the presence of alanine, indicating that the F4 and similar F41 fimbrial systems are regulated in a different manner (270, 271).

F5 Regulation

The production of F5 fimbriae or the transcription of the major subunit gene *fanC* is activated in the logarithmic

growth phase, by oxygen, by low pH, and by glycerol, whereas fewer fimbriae or *fan* transcripts are expressed in stationary phase, at high pH and in glucose-, pyruvate-, arabinose-, or lactose-containing media (272, 273). Studies on several compounds as carbon source for bacterial growth indicated that acetate has a strong inhibitory effect on fimbriation and that, together with glucose, it essentially suppresses F5 expression (274). Fimbrial subunit production is drastically reduced in a *cya* mutant, confirming that F5 is regulated by catabolite repression (275). Curiously, unlike many other fimbrial systems, H-NS is not involved in the noticeable downregulation of F5 expression at low temperature (270, 273). Moreover, two groups of F5-producing ETEC strains were distinguished based on the regulation of fimbriation by different growth conditions (276). At the 5′ end of the F5 gene cluster are two genes, *fanA* and *fanB*, that are transcribed in the same direction as the F5 genes and activate F5 expression (277). Studies on F5 gene transcription suggested that FanA and FanB act together as transcriptional antiterminators on two factor-dependent terminator sites after the *fanA* and *fanB* sequences (278). A third terminator after the *fanC* sequence includes a dyad symmetry for a potential stem-loop structure, suggesting that transcriptional termination at this site is factor independent. Lrp (leucine-responsive regulatory protein) acts as a F5 transcriptional activator by binding to the *fanA* promoter (279). It is likely that the inhibitory effects of alanine and leucine on *fan* gene transcription and F5 fimbrial expression (279, 280) are mediated through Lrp. Most interestingly, the F5 gene cluster seems to consist of three operons, one for the *fanA* to *fanD* genes, a second one for the *fanE* and *fanF* genes, and the third one for the *fanG* and *fanH* genes (262, 281). A stem-loop structure between *fanE* and *fanF* might act as an attenuator of *fanF* transcription. Putative promoters were mapped for the last two operons and the one upstream of *fanE* was adjacent to a CRP-binding consensus site, suggesting that the *fanEF* operon, like the *fanABCD* operon, is also regulated by catabolite repression.

F6 Regulation

Early studies recognized that F6 fimbriae are best expressed *in vivo* in piglet intestines or *in vitro* when bacteria are grown to stationary phase, forming pellicles at the air-medium interface (64, 65). F6 fimbrial expression undergoes phase variation. Specific environmental signals or growth conditions regulate the rate of phase variation (282). The mechanisms and the potential *cis*- or *trans*-active elements regulating F6 phase variation are different from those of the F6-similar CS18 fimbriae of human ETEC (130, 131, 283, 284). Unlike CS18, no F6 DNA segment is directly regulated by DNA inversion (283, 285). Moreover, dam methylation is not involved in F6 expression or phase variation (131, 286). The apparent stability of the duplicated F6 fimbrial genes in the same clinical strain on a plasmid and on the chromosome (70, 104, 131) may suggest that the merodiploid *fas* genes confer some advantage to the host strain. Alternatively, the two locations of the F6 genes could be due to separate bacterial populations in the same culture, some having integrated the plasmid or a mobile DNA element carrying the F6 genes in their chromosome. Whether potentially duplicated or mobile DNA is required for phase variation in not known. However, phase variation being recA independent, any mechanism of intrabacterial DNA exchange explaining phase variation would have to involve other recombinases (131). Expression of the major subunit FasA and of F6 is upregulated by FasH (FapR) (104, 287). FasH shares sequence similarity with the DNA-binding domain of the AraC transcriptional activator, and more specifically with the Rns subfamily of positive regulators of fimbriae and other virulence factors of *Enterobacteriaceae* (288). A portion of the proximal IS*1* sequence of the Tn*1681* transposon located upstream of *fasH* is involved in activating fimbrial expression (105). The expression of *fasH* and *fasA* are both regulated in response to the carbon source and the nitrogen source (131, 284). Since these nutritional signals are differentially modulated in the intestinal environment, they may provide a mechanism to allow preferential colonization of different segments of the intestine by various enteropathogens (130, 284).

F18 and F17 Regulation

No regulatory genes for F18 or F17 fimbriation have been described yet. Although special growth media seem to improve F18 fimbrial expression *in vitro* (203), not all the strains express F18 under these conditions (289). In contrast to F18ac, most F18ab fimbriae of clinical isolates are poorly expressed on commonly used media, suggesting a different mechanism of regulation for these two types of fimbriae. The F18 gene cluster is similar to the AF/R1 fimbrial gene cluster of rabbit-attaching and -effacing *E. coli* (290). Even though the F18 gene cluster lacks the upstream *cis*-active transcriptional regulators of AF/R1, it has an *araC*-like gene directly downstream of *fedF* (291), suggesting regulation by a potential F18-specific protein.

ENTEROTOXINS

Two classes of ETEC enterotoxins have been described: heat-labile (LT) and heat-stable (ST). An *E. coli* strain may produce one or both of these types of toxins (292). Both types of toxins are plasmid encoded. Nomenclature is based on toxin size, sequence, and biological activity. LT is structurally arranged as an AB_5 toxin, where A is the enzymatically active subunit and the B subunits correspond to the receptor-binding moiety. LT is related to *Vibrio cholerae* toxin (cholera toxin, CT) a highly immunogenic molecule. In fact, antibodies against CT cross-react with LT. STs are poorly immunogenic, and no immunological cross-reactivity has been observed between them. STs are single short polypeptides (less than 50 amino acids) and show no sequence similarity. Although the enteroaggregative *E. coli* (EAggEC or EAEC) enterotoxin (EAST1) was originally detected in human isolates of the corresponding *E. coli* pathotype (293), it was later shown to also be present in some porcine ETEC (294). Overall, all enterotoxins are associated with intestinal secretion of water and electrolytes in their normal hosts and/or in animal models. No significant pathological lesions or morphological changes in the intestinal mucosa result from the toxic activity of these enterotoxins (295). Many virulotypes of ETEC or classes of enterotoxin-producing *E. coli* that can be distinguished by their sets of virulence factors responsible for diarrheal diseases have been reported. Characteristics of ETEC responsible for enteric diseases in animals are listed in Table 6.

Table 6 ETEC implicated in diarrheal diseases of animals

Animal species	Type of diarrhea	Virulotypes
Pig	Neonatal	STa:F41
		STa:F6
		STa:F5:F41
		LT:STb:EAST1:F4
		LT:STb:STa:EAST1:F4
		STb:EAST1:AIDA
	Postweaning	LT:STb:EAST1:F4
		LT:STb:STa:EAST1:F4
		STa:STb
		STa:STb:F18
		STa:F18
Cattle	Neonatal	STa:F5:F41
		STa:F41
Sheep	Neonatal	STa:F5:F41
		STa:F41
Dog	Neonatal	STa:X[a]
		STb

[a]X: Unknown fimbriae.
Adapted from Gyles and Fairbrother (20).

HEAT-LABILE TOXIN

Generalities

In the 1950s it was observed that some *E. coli* strains could cause diarrheal disease similar to cholera (296). Although most *E. coli* strains lack the genes for toxin production, some strains secrete a heat-labile enterotoxin (LT) that is a homologue of CT produced by *V. cholerae*. These toxins share about 78% identity at the nucleotide level, and their structures and function are very similar. The genes encoding LT are located on a large plasmid called *pEnt* (297). This plasmid can be transferred to nonpathogenic *E. coli* bacteria, rendering them toxinogenic (298). LT enterotoxins are produced predominantly by human and porcine ETEC (1). In fact, the lion's share of the knowledge on the structure and function of LT was obtained from human ETEC strains. In humans, LT causes a cholera-like disease with watery diarrhea and stomach cramps. In animals, LT-positive ETEC typically produces F4 fimbriae and STb, suggesting a possible functional link between these virulence factors. However, STb and F4 are encoded on separate plasmids. Little heterogeneity among *Ent* genes found in porcine ETEC was observed (299). LT is part of an important group of toxins, the AB_5 toxin family. Two subtypes of LT, LTI and LTII, have been described. Differences between LTI and LTII are largely due to dissimilarity in their B subunit. LTI can be divided in LTIh and LTIp, produced, respectively, by human and porcine and human ETEC. These subtypes show slight differences in composition.

LTII consists of three antigenic variants, LTIIa, LTIIb, and LTIIc (300–302), that are related to LTI in their A subunit but differ in their B subunits. In contrast to the more similar A subunit sequences, the amino acid sequences of the B subunit of LTI and LTII are highly divergent (303–305). LTII is antigenically distinct from LTI, with only 41% sequence identity with LTI, but it possesses similar biological activities. LTII genes have been isolated from *E. coli* strains from humans, cows, buffaloes, pigs, and ostriches. LTII toxins have mainly been observed causing disease in humans and calves (1, 306). Purified LTIIa and LTIIb caused severe diarrhea in

neonatal pigs (302). LTII antigenic variants bind to various gangliosides, whereas LTI binds preferentially to ganglioside GM1 (Table 7). ETEC must be in close contact with the host cell to exert its effect, because a semipermeable filter could prevent toxicity expression (307). Efficient LT delivery to host cells most probably occurs via vesicles containing LT. Strains expressing LT have also been shown to have an advantage in colonization. In fact, Berberov et al. have shown that elimination of the genes for LT was associated with a concomitant reduction of toxicity and reduction in colonization of the intestine of gnotobiotic piglets, demonstrating that this enterotoxin plays a role in adhesion (7). In the same way, Johnson et al. have shown that LT promotes adherence of ETEC, the mechanism appearing to require the ADP-ribosyltransferase activity (308). Glucose, at an optimal concentration for LT expression, enhances bacterial adherence through the promotion of LT production (309).

Production and Regulation

ETEC strains do not produce similar amounts of LT (310). In fact, they can vary quite substantially in their production. However, we now know that ETEC strains that are kept frozen for long time periods just after their isolation show little change in toxin production (311). In general, conditions mimicking the human small intestine are optimal for production of LT. Growth condition influenced the amount of LT produced because no detectable toxin was detected at temperatures lower than 26°C, with the production increasing with temperature to reach a maximum at 37°C (312–314). Microaerophilic conditions as well as increased salt concentrations (optimum at 0.2 M) promoted LT production (315). Alkaline pH is a signal for production and secretion of LT and growth medium with a pH of 8.6 resulted in optimal LT production (316). Glucose, which is found in the small intestine in appreciable concentration, increased the release of LT with maximum production at 2.5 g/liter (312, 314, 317). In contrast, short-chain fatty acids (in particular, those with carbon chains between three and eight carbons) produced in large quantities in the colon impair production of LT (318). These conditions are believed to serve to indicate when LT production should be turned off or turned on.

Table 7 Receptors for ETEC toxins

Toxin	Subtypes	Receptor(s)
STa	STaH	Guanylate cyclase C
	STaP	Guanylate cyclase C
EAST1		Guanylate cyclase C
STb		Sulfatide
LTI	LTIh	[a]GM1, GD1b, GM2 asialo GM1, galactoproteins, galactose-containing glycolipids
	LTIp	
LTII	LTIIa	[a]GD1b, GD1a, GT1b, GQ1b, GD2
	LTIIb	[a]GD1a, GT1b, GM3
	LTIIc	[a]GM1, GM2, GM3, GD1a

[a]In order of decreasing binding strength.

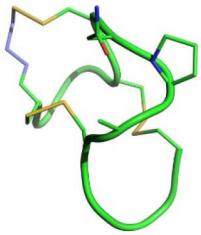
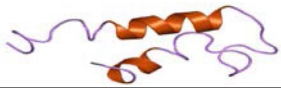
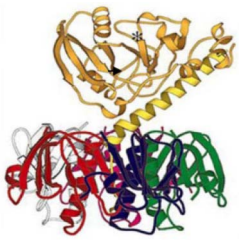
Structure

LTI is a high-molecular-weight molecule (approximately 85,000) of which activity is abolished after 15 min at 60°C (Table 8). It consists of a bioactive A subunit and five B subunits assembled in a doughnut-shaped ring (319) that binds to GM_1 ganglioside (Galβ1-3 GalNacβ1-4 [NeuAc2-3] Galβ1-4Glcβ1-1 Ceramide) receptors found on the intestinal epithelial surface. Binding of LTI to GM1 located in lipid rafts on host cells is critical, leading to expression of the toxic effect. Each B subunit binds cooperatively to one GM_1 ganglioside molecule. In addition, LT binds but more weakly to GD_1b, asialo-GM_1, GM_2, and a number of galactoproteins and galactose-containing glycolipids. In fact, this galactose-binding property was exploited for purification of LT (320).

Recently, the B subunit has been shown to bind to blood group determinants (321–323). LT was observed to bind best to pig brush borders with type A blood (324) and to human erythrocytes with A and B glycolipids (325). In fact, a cohort study in Bangladesh found a high prevalence of ETEC-based diarrhea among children with blood type A or AB (326). However, it has to be proven if blood group antigens are functional receptors *in vivo*.

In addition to its toxic activity, LT was shown to bind to the surface of *E. coli* cells (327, 328). LT localized to the cell surface by binding to LPS found on the bacteria. LPS is present in the outer membrane of Gram-negative bacteria and consists of a characteristic lipid moiety called lipid A, linked to a series of sugar residues (329). This LT-LPS association is independent of the A subunit (327). Even though it is bound to LPS, the B subunits remain able to bind to its mammalian cell surface receptor (330, 331). Free soluble LPS can significantly inhibit the binding of LT to the surface of ETEC strains (327). LPS lacking the O-antigen was effective in blocking binding, suggesting that the core sugars were responsible for

Table 8 Structural characteristics of toxins produced by ETEC

Toxin	# aa	*M*	Sequence/arrangement[a]	Structure
STa				
Subtype STaH	19	2,000	N S S N Y C C E L C C ***N P A C*** T G C Y	
Subtype STaP	18	2,000	N T F Y C C E L C C ***N P A C*** A G C Y	
EAST1	38	4,100		Not determined
17-2 strain			MPSTQYIRRPASSYASCIWC**T**T***AC***ASCHGRTTKPSLAT	
0-42 strain			MPSTQYIRRPASSYASCIWC**A**T***AC***ASCHGRTTKPSLAT	
STb	48	5,200	STQSNKKDLCEHYRQIAKESCKKGFLGVRDGTAGACFGAQIMVA-AKGC	
LTI		85,000	AB_5	
B-subunit	103	11,600		
A-subunit	240	28,000		

[a]Letters in bold and italics indicate the region involved in binding to the receptor. Underlined letters indicate the change observed for EAST1 variants.

binding. For full binding activity, the core sugars of LPS are required, although some weaker binding activity can be observed for highly truncated LPS as 3-deoxy-D-manno-octulosonic acid (KDO) is the minimal requirement for binding (332). LT binds specifically to unphosphorylated *E. coli* KDO residues, as was shown using *V. cholerae* phosphorylated KDO. LT may or may not associate with outer membrane vesicles (OMV) (333, 334), but the majority of LT activity in the extracellular environment is associated with OMVs (327, 332). OMVs are globular structures composed of lipid, approximately 50 to 200 nm in diameter, that are released from all Gram-negative bacteria studied so far (334, 335). Active LT molecules are found inside OMVs as well as associated with their surface (308, 328). LT within OMVs contributes to toxicity because LT can mediate internalization of entire vesicles (331). The LT-LPS association is robust, and OMVs could play a role in protecting LT from proteolysis (328). Overall, more than 95% of LT is attached to the OMVs via LPS.

Synthesis

Individual LT toxin subunits are produced in the cytosol under the control of a joint promoter. The operon encoding the A- and B-LT subunits (*eltAB*) is flanked by highly conserved regions followed by variable sequences that mainly consist of partial insertion elements (336). Sequence analysis indicates that genes encoding LT were acquired by horizontal transfer from *V. cholerae* approximately 130 million years ago (337). The global regulator H-NS is involved in regulation by repressing LT expression at lower temperatures (338). In high glucose conditions, such as found in the duodenum, LT genes are transcribed and toxin is produced as a result of the inactivation of bacterial CRP. The CRP system ensures a low level of cAMP, but, in the presence of glucose, this system is inactivated. In a low-glucose milieu, significant amounts of cAMP are produced and released by host cells in response to LT activity. This signal represses *eltAB* through CRP. Glucose limits LT production to where the toxin exerts its function (i.e., in the small intestine) and corresponds to an effective targeting strategy. On the other hand, short-chain fatty acids found in the colon negatively affect LT production (339).

The A (240 amino acids [aa]) and B (103 aa) subunits of LTI are synthesized with an N-terminal signal sequence in the cytoplasm. This sequence permits transport to the inner membrane (IM) and assembly as a holotoxin in the periplasm after the signal sequence is cleaved off.

Disulfide-bond-A oxidoreductase (DsbA) aids in disulfide bond formation, and peptidyl *cis-trans*-isomerase ensures the formation of a *cis*-proline; together these steps facilitate folding (340). LT-A and -B subunits spontaneously assemble into holotoxins. Once formed, these complexes are remarkably stable, remaining assembled from pH 2.0 to 11.0 (341, 342). Although strong acid conditions can dissociate the B pentamer, the free monomers reoligomerize readily following neutralization. The A subunit (M_r 28,000) consists of an A1 fragment (M_r 22,000; aa 1-194) containing the active enzymatic site and an A2 fragment (M_r 5,500; aa 195-240) that links A1 to the B (M_r 11,600) subunits (Table 8). Before activation, the A1 and A2 fragments are connected through a short linker where nicking takes place. In contrast to CT, this cleavage event is not required for LT toxicity to be expressed. Nevertheless, mutants unable to be nicked show a delayed toxic effect in cells in culture (343). These subunits upon nicking remain connected through a disulfide bond.

In a human *E. coli* strain, LTI was shown to be transported across the outer membrane (OM) by a type II secretion pathway (344). There has been no report of such a pathway in porcine ETEC. A type II secretion system consisting of a complex of 12 to 15 proteins spanning the IM and OM is found in numerous Gram-negative species (345). The genes encoding type II secretion apparatus are also regulated by H-NS (346). Thus, these genes are turned on by conditions that favor LT production. LT secretion relies on the B subunit, but the A subunit is not involved in the process (347). Some studies have shown that secretion of LT was dependent on a protein called LeoA, found to be a GTPase (348, 349). More recently, LeoA, B, and C were shown to be dynamin-like proteins responsible for potentiating ETEC virulence through membrane vesicle-associated secretion (350). Because few ETEC strains carry *LeoA*, a ubiquitous role in the secretion of LT cannot be imparted to this protein.

Internalization and Mechanism of Action

Human LT enterotoxin was assimilated to a prototype and used to decipher the mechanism of action of this significant toxin. Thus, it was observed that LT must enter the host cell's cytosol to exert its toxic effect (Fig. 3). LT binding results in the clustering of GM1 gangliosides targeting more GM1. The pentamer is required for entry into cells of the intestinal epithelium and disruption of the holotoxin prevents intoxication of host cells (351). After binding of the B subunit to their specific cell receptor, internalization of LTI is mediated by receptor-mediated endocytosis. Upon endocytosis, the GM1-associated LT toxin relocates to early and recycling endosomes (352). It is then transported in a retrograde manner to the *trans*-Golgi network independent of the late endosome pathway (353) and the endoplasmic reticulum (ER). At the extremity of the A_2 fragment an RDEL sequence aids in the transport of the toxin to the ER. Inside this structure, the proteolytic cleavage and reduction of a disulfide bond within the A subunit provoke the release the A1 fragment in the cell cytosol (354). This fragment possesses an adenosine diphosphate (ADP)-ribosyltransferase activity that acts on the Gs heterotrimeric protein complexes found concentrated in the lipid rafts. In the cytosol, a host ADP-ribosylation factor (ARF), which is a 20-kDa regulatory GTPase, binds to the A1 subunit, allowing it to bind to NAD in its active site (355). The Gs protein complex consists of a hormone stimulatory receptor, a regulatory Gs protein, and adenylate cyclase (AC) as the effector present in the basolateral membrane. Activation of Gs occurs when GTP is bound to Gsα. This component dissociates from Gsβ and Gsγ subunits and, in turn, activates its target, adenylate cyclase. Conversion of GTP to GDP by the intrinsic GTPase activity of Gsα acts as a turn-off switch inactivating the complex. The transfer of ADP-ribose from NAD by LT to the Gsα subunit results in inhibition of intrinsic GTPase activity. As stated previously, ADP-ribosylation is enhanced by an ADP-ribosylation factor (ARF6) that activates the A1 catalytic subunit. This modification results in activation of Gsα that constitutively turns on AC. In turn, AC produces the second messenger cyclic adenosine monophosphate (cAMP) from ATP. This second messenger targets protein kinase A (PKA), an activator of the membrane chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) located in the apical membrane of epithelial cells. Activation of CFTR provokes the opening of this anion channel and results in the secretion of chloride (Cl^-) and bicarbonate (HCO_3^-) ions from the cells into the intestinal lumen (356). The ions' electrolytic balance across the epithelium is affected, and the net effect is an increase in salt concentration in the intestine, making it hypertonic. The osmotic pressure forces large amounts of water out of the intestinal cells. Activation of CFTR is the major way by which LT toxin provokes water efflux from the cells to induce diarrhea. Nevertheless, PKA also phosphorylates and opens a basolateral potassium channel (357). In the basolateral membrane, PKA also

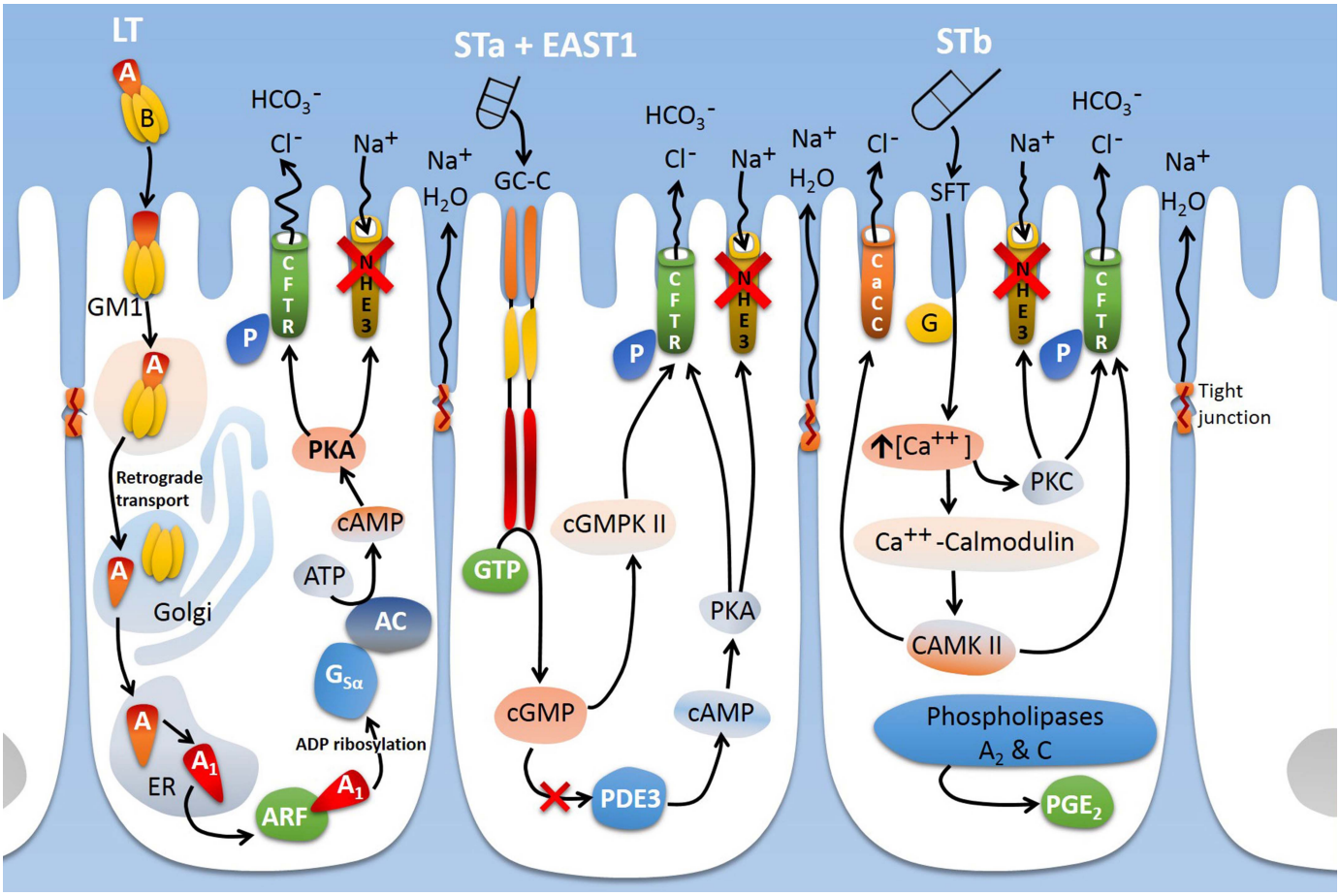


Figure 3 Mechanism of action of ETEC toxins on intestinal epithelial cells. Signaling leading to water and electrolyte loss through activation of ion channels and loosening of tight junctions by the various toxins is described. CFTR, cystic fibrosis transmembrane regulator; AC, adenylate cyclase; ARF, ADP-ribosylation factor; PKA, protein kinase C; PKC, protein kinase C; GM1, ganglioside GM1; GC-C, guanylate cycles C; SFT, sulfatide; ER, endoplasmic reticulum; Gsα, α component of an heterotrimeric G protein; NHE3, Na^+/H^+-exchanger 3; PDE3, phosphodiesterase 3; cGMPKII, cGMP-dependent protein kinase II; cAMPKII, calmodulin-dependent protein kinase II; CaCC, calcium-activated chloride channel; P, phosphorylation.

indirectly increases the activity of $Na^+/K^+/2Cl^-$ cotransporter (NKCC). This aids in the transcellular movement of chloride ions from the basolateral side of the intestinal lumen (356). Increased cAMP levels further contribute to enhance chloride secretion. In fact, cAMP inhibits the electroneutral absorption of Na^+ from the intestinal lumen. The A1 subunit has also been implicated in stimulating arachidonic acid metabolism, leading to the production of the secretagogue PGE2, in turn, stimulating intestinal secretion (358). In contrast to CT, LTI does not stimulate the production of 5-hydroxytryptamine (5-HT or serotonin) (359). The osmotic gradient formed is responsible for the water flow outside of the cells and the observed diarrhea. Loosening of tight junctions (TJs) as a result of LT activity can also contribute to fluid loss into the intestinal lumen (360–362). In summary, LT takes advantage of the host cell's machinery to exert its toxic effect, and the effect of LT once initiated is irreversible.

HEAT-STABLE ENTEROTOXINS

Generalities

E. coli heat-stable enterotoxins came to attention in the 1970s after it was observed that heat inactivation of bacterial cultures from patients and animals suffering from diarrhea failed to eliminate enterotoxigenic activity (363, 364). Heat-stable toxins are peptidic molecules of fewer than 50 amino acids. Their small size and 3D structure are responsible for resistance to boiling. Based on their sequences and their biochemical characteristics, we have recognized in this group STa (also known as STI), STb (or STII), and EAST1, all associated with ETEC (295).

STa

STa peptide

STa represents a family of toxins composed of a single peptide chain of approximately 2,000 Da. Toxins produced by human (STaH; 19 aa) and porcine (STaP; 18 aa) strains differ slightly in length and their amino acid sequence (Table 8). To date, STaP polypeptide has been observed in isolates from animal species including pigs, calves, lambs, chickens, and horses and also from humans (365). In contrast, STaH is produced solely by human isolates. A 13-amino-acid peptide in the carboxy terminus, which includes three disulfide bonds, corresponds to a highly conserved sequence, and this sequence represents a common antigenic determinant. The amino-terminal sequence up to the first cysteine is not involved in toxicity (366). STa is particularly associated with ETEC that cause disease in neonatal animals. This toxin is also produced by ETEC implicated in PWD in pigs but rarely as the sole enterotoxin.

STa toxins are synthesized as larger precursors (72 aa) that are later cleaved into the active mature toxin (367, 368). Six cysteine residues involved in disulfide bond formation are present at the same position in STaH and STaP (Table 8). The tertiary structure formed by the disulfide bonds is critical and required for full biological activity (369). Native STa toxins are poorly immunogenic. However, both homologous and heterologous antisera can neutralize toxicity (370). STa is an acidic peptide with a pI of 3.98 and a molecular weight of approximately 2000 (371). It is soluble in water and organic solvents including methanol and resists several proteases. The molecule is resistant to acidic but not to basic pH. Disruption of the disulfide bonds inactivates the toxin.

Genetics

Genes encoding STa (*estA*) are found on plasmids of varying molecular sizes (372). In animal ETEC isolates, it is common to find gene coding for STa, colonization factor, drug resistance, and production of a colicin on the same plasmid. Genetic studies demonstrated the existence of two types of *estA*. This gene has an AT content of 70% and is associated with transposons that are carried on plasmids of a wide range of molecular weights. The gene cloned from a bovine isolate (STaP) was shown to be part of a transposon (Tn*1681*) which is flanked by inverted repeats of IS1 (373). STaP genes from ETEC isolated from other animal species (including humans) are part of the same transposon (374). Genes encoding STaH and STaP may be carried by a single human ETEC strain. Sequencing of the STa gene revealed it was identical in 52 ETEC strains of porcine origin (299). Synthesis of STa by *E. coli* is subject to catabolite repression, and optimal yields of toxin are obtained in glucose-free media (375).

Secretion and disulfide bonds formation

STa is produced as a 72-amino-acid precursor molecule referred to as pre-pro-STa (376, 377). This polypeptide consists of a 19-amino-acid signal peptide (pre-STa), a 35-amino-acid pro sequence, and an 18- or 19-amino-acid mature STa. The pro-STa is translocated across the cytoplasmic membrane and requires secA-dependent transport. The signal sequence is cleaved by signal peptidase 1 (378). STa pro-region guides it into the periplasmic space (379), but this region does not seem to be involved in the extracellular transport of the peptide (380). Three intramolecular disulfide bonds are formed in the periplasm by DsbA protein prior to secretion (381–383). Proteolysis is then required to obtain biologically active 18 and 19 amino acids (378). STa toxin is secreted from the cell as it is synthesized. Mature STa molecules use TolC to cross the OM (384, 385) Formation of the three intramolecular disulfide bonds is not required for the mature toxin to pass through the OM (386). The three intramolecular disulfide bonds in STaH link cysteine 6 and 11, 7 and 15, and 10 and 18; in STaP disulfide bonds link cysteine 5 and 10, 6 and 14, and 9 and 17 (Table 8). These bonds stabilize the spatial structure (387). By using STa analogues, it was demonstrated that the second disulfide bond is essential for toxicity, whereas analogues without the first or the third disulfide bond showed only reduced toxicity (388).

Structure and toxic domain

STa tertiary structure consists of a folded peptide backbone assembled as a right-handed spiral from the first cysteine at the NH_2 terminus to the last cysteine residue at the COOH terminus (389) (Table 8). Three β-turns, located along the spiral, are stabilized by the three intramolecular disulfide bonds (390, 391). Overall, a 13-amino-acid sequence from the amino-terminal cysteine to the carboxyl-terminal cysteine is essential for toxicity (392–394). This segment was defined as the toxic domain of STaH and STaP. Four amino acids (N-P-A-C) are conserved in STaP and STaH enterotoxins (Table 8).

Receptor

STa expresses its toxicity by elevation of cyclic GMP (cGMP) in intestinal epithelial cells (367, 395). Cloning the receptor from cDNA libraries of rat, pig, and human intestine led to the identification of the STa receptor (396). The deduced amino acid sequence and functional expression in mammalian cells indicated that the STa receptor is guanylate cyclase C (GC-C) belonging to the atrial natriuretic peptide receptor family (367). GC-C is a glycoprotein that is expressed primarily on intestinal epithelial cells. It is present on the brush border of villous and crypt intestinal cells. It consists of an extracellular receptor domain, a transmembrane domain, and a cytoplasmic domain including a kinase homology domain and a guanylate cyclase catalytic domain at the COOH terminus (397). The endogenous agonist for GC-C was found to be a 15-amino-acid hormone called guanylin (367). This hormone appears to play a role in fluid and electrolyte homeostasis in the gut. Guanylin is 50% homologous to STa, containing 4 cysteine residues involved in disulfide bond formation essential for biological activity. This hormone is less potent than STa in activating GC-C and in stimulating chloride secretion (Cl^-) (398, 399). STa is mimicking the hormone guanylin and the basal gut fluid homeostasis is altered through activation of GC-C. STa receptors are present throughout the human intestine and colon. Their number is decreasing along the longitudinal axis of the gut, and binding of STa was noted in both crypts and villi of the small intestine and in crypts and surface epithelium of the colon (400). Binding is maximal on the villus and decreased along the villus-to-crypt axis (401). Based on the concentration of receptors, the posterior jejunum appears to be the major site responsible for STa hypersecretion of fluid. There is also good evidence that STa toxin binds to other receptors (295, 402).

Mechanism of action

STa is a potent toxin with rapid action, but short duration. For example, 6 ng of STa results in a positive fluid response in mouse intestine compared with 200 ng of STb or cholera toxin (CT) in the same model (403). STa exerts its toxic activity through activation of an intracellular signaling cascade, leading ultimately to watery diarrhea. STa receptor is present on villus of the jejunum and ileum brush border of intestinal epithelial cells (400) (Fig. 3). The binding of STa to the extracellular domain of GC-C and activation of the intracellular catalytic domain of GC-C results in hydrolysis of GTP and cellular accumulation of cGMP (404). Elevated cGMP level activates cGMP-dependent protein kinase II (cGMPKII), resulting in the phosphorylation of CFTR (395). Activation of CFTR induces secretion of Cl^- and HCO_3^- and a net fluid secretion in the lumen of the intestine (356). Osmotically driven water secretion results thereafter. Elevated cGMP also inhibits phosphodiesterase 3 (PDE3), resulting in an increased level of cAMP that activates protein kinase A (PKA). This enzyme phosphorylates CFTR as well as it inhibits Na^+ reabsorption following phosphorylation of the Na^+/H^+-exchanger 3 (NHE3) (367). The effect of STa is reversible.

STa and tight junctions

TJ, a structure responsible for sealing the epithelium, could be held responsible, at least in part, for the electrolytes and fluid loss due to STa. TJs are highly organized structures where numerous proteins, including claudins and occludins, are involved in keeping closely associated areas of two neighboring cells whose membranes join together, forming a virtually impermeable barrier to fluid and ions (405). Altering TJs could provoke loss of water and electrolytes within the intestinal lumen (406). Nakashima et al. observed that treating T84 polarized cell monolayers with STa elicited a reduction in transepithelial resistance (TER), indicating a loss of TJ integrity (407). However, no increase in paracellular permeability to a high-molecular-weight marker (fluorescein isothiocyanate-dextran) was noted. In contrast to STa, guanylin did not affect TER. Although both STa and guanylin induced cellular cGMP production, only STa reduced barrier integrity, suggesting that STa not only causes an induction of water secretion, through channel activation, but also induces intestinal barrier dysfunction. The effect of STa on epithelial TJs contributes to the enterotoxic activity and most likely plays a role in the pathogenesis of STa-producing ETEC.

EAST1

Generalities

EAST1 is a peptidic toxin originally recognized in an enteroaggregative *E. coli* (EAEC) strain isolated from the stools of a Chilean child suffering from diarrhea (293, 408). The gene coding for this toxin was also observed in other diarrhea-causing *E. coli*, including ETEC, and in some other human enteric pathogens such as *Salmonella* (294, 409, 410). EAST1 is often compared with *E. coli* STa enterotoxin because it shares some physical and biological similarities. However, it does not hybridize with

STa-specific DNA probes or react with anti-STa antibodies (293). EAST1 is widespread among porcine ETEC in various countries (411–416). This toxin is not well characterized in terms of both function and contribution to ETEC-mediated disease. Diarrhea and sometimes death in a gnotobiotic piglet model resulted from infection with the prototype EAEC strain 17-2 (408, 417). EAST1 is associated with *E. coli* strains isolated from cattle and pigs with diarrhea (418–421). Alone, EAST1 does not seem capable of producing disease (22) but, together with LT, it is efficient in producing diarrhea (7). The role of EAST1 in mediating diarrhea in animals remains controversial to this day (416).

EAST1 peptide

EAST1 is a heat-resistant 38-amino-acid peptide with a molecular weight of 4,100 and a calculated pI of 9.25 (Table 8) (422). Four cysteines at positions 17, 20, 24, and 27, are involved in the formation of two disulfide bridges required for toxicity expression. Unlike STa and STb, a classic signal peptide was not observed in the NH_2 terminus of the predicted EAST1 sequence (423). EAST1 is immunologically distinct from STa, and a polyclonal anti-STa antibody does not neutralize the biological activity of EAST1 (293). The toxic domain comprises a peptide spanning residues 8 to 29 (293, 423).

Genetics

The *astA* gene was detected in EAEC strains 17-2 and O-42 (424, 425). Variants differ by only one base at codon 21 (ACA→GCA), resulting in a change in the amino acid threonine to alanine (Table 8). Heterogeneity in virulence of EAEC strains 17-2 and O-42 (424) was reported. Because strain O-42 was able to provoke diarrhea in volunteers, whereas strain 17-2 did not, it has been proposed that variant O-42 could contribute to the virulence of EAEC in a more significant way (425). Moreover, the toxin produced by strain O-42 can be observed more frequently in epidemiological studies (425, 426). Numerous other variants of EAST1 have been observed (294, 427). These molecules have usually been reported only once and probably represent less frequently distributed EAST1 variants. The toxicity of these variants has not been evaluated in animal models.

The *astA* gene, a 117-bp-long DNA sequence, encodes EAST1. There is no homology between *astA* and *estA*, the structural gene for STa. The G+C content of *astA* is 53%, which is similar to the mean value for *E. coli* (50.8%), while for *estA* (coding for STa) it is 30.6% (410). In EAEC strains *astA* is associated with a 60-MDa plasmid mediating aggregative adherence (410). *astA* was observed in one or more copy on plasmids of variable size but was also found on the chromosome of various bacteria, including ETEC (428). It has been detected in human, bovine, and porcine ETEC, and it is commonly found on plasmids in F4-positive ETEC strains from pigs with diarrhea (421). The gene found in animals has more than 98% homology with the human isolates (7). Very little is known about the genetic regulation of EAST1 expression. Nucleotide sequences upstream of *astA* from porcine and bovine ETEC strains are identical, but are divergent from human ETEC. Even though results obtained by Yamamoto and Nakazawa point toward heterogeneity of DNA sequences between *E. coli* affecting humans and animals, there seems to be a certain consensus among *astA* flanking sequences (429). This gene was also reported to be on transposon-like sequences, near insertion elements or inverted repeats, which could represent means by which the EAST1 gene is spread.

Mechanism of action

The small size and relative heat stability of EAST1 and the finding that cGMP is the molecule acting as a second messenger led to the comparison of EAST1 with STa toxin. Also, EAST1 protein shares 50% identity with the enterotoxic domain of STa (amino acid residues 6 to 18) (423). EAST1 is also structurally and functionally similar to guanylin, both having four cysteines and activating the production of cGMP (293). Interaction with GC-C could occur through the N-P-A-C motif common to STa and partially conserved for EAST1 (i.e., X-X-A-C) (Table 6). Hence, the mechanism of action of EAST1 is proposed to be identical to that of STa (Fig. 3).

Toxicity of EAST1 has been evaluated in Ussing chamber and in the suckling mouse assay (293, 428). No concordance between the presence of *astA* and toxicity of live bacterial strains or culture filtrates could be established for several strains tested (410). EAST1 did not stimulate an increase of intracellular cAMP or cGMP levels in human T84 or in porcine IPEC-J2 cell lines (430). In addition, 5-day-old gnotobiotic pigs challenged with *E. coli* strains, expressing EAST1 as the only toxin, did not develop diarrhea or clinical signs 72 h postinoculation. EAST1 alone seems not sufficient to cause diarrhea in 5-day-old gnotobiotic pigs, suggesting that EAST1 is likely not a virulence determinant in ETEC-associated diarrhea.

An experimental infection with *E. coli* strains positive for EAST1 and AIDA and a F4/EAST1-positive strains, in gnotobiotic piglets, did not produce diarrhea either (431). Although the EAST1/AIDA strain used in the study was present in fecal shedding of challenged animals, it was not markedly associated with intestinal epithelial surface. Overall, these studies showed that EAST1 toxin alone does not induce diarrhea in the animals tested.

STb

Generalities

ETEC producing STb are associated almost exclusively with pigs, and the majority of porcine ETEC produce STb enterotoxin. This toxin has also been detected in ETEC of human origin (432, 433). STb is recognized as a potent enterotoxin in weaned pigs (292, 434–436).

STb peptide

STb-positive *E. coli* strains have been isolated principally from pigs, but also sporadically from cattle (including water buffaloes), chickens, dogs, cats, ferrets, and humans (437). STb comprises 48 amino acids with four cysteine residues involved in disulfide bridge formation (Table 8). The enterotoxin has a M_r of 5,200 and bears no homology to STa or EAST1 enterotoxins. STb peptide is synthesized as a 71-amino-acid precursor comprising a 23-amino-acid signal sequence (438, 439). The first seven amino acids at the NH_2 terminus of the mature toxin are not involved in either the structure or toxicity (440). The peptide spanning from Cys10 to Cys48 has full biological activity. STb isolated from various animal species have the same nucleotide and amino acid sequences. Nevertheless, a STb variant with a His to Asn substitution at position 12 was identified in *E. coli* isolates from pigs suffering from diarrhea (441). This variant shares structural and mechanistic properties with wild-type STb (442). No differences in biological activity of the variant have been reported. STb is a highly basic protein with an isoelectric point of 9.6 (443). It is soluble in water and some organic solvents but is insoluble in methanol and the toxin loses biological activity following β-mercaptoethanol or trypsin treatment (363, 444, 445). It resists acid (pH 2), alkaline (pH 12), and 8 M urea treatments (444). STb is very susceptible to protease degradation (446, 447). STb is poorly immunogenic. A serological response can be obtained following immunization with either fusion proteins or proteins chemically coupled to STb (448–451). The anti-STb antibodies can neutralize STb toxicity but are unable to neutralize STa or CT toxins (452).

Genetics

The *estB* gene encodes STb. This gene is found on heterogeneous plasmids that often code for other properties, including other enterotoxins, colonization factors, drug resistance, colicin production, and transfer functions (372, 453). It is part of a transposon of approximately 9 kb designated Tn4521 (454–456). This transposon is flanked by defective IS2 elements, but it is functional because *estB* can transpose from one plasmid to another. The structural gene for STb from different clinical isolates is uniform in size, but the flanking sequences are heterogeneous, suggesting that *estB* could be found on different transposons. Transposition of *estB* is probably one of the mechanisms by which this virulence factor is disseminated among ETEC. The promoter for *estB* expression is weak (457), capable of binding RNA polymerase, but seems to be a poor transcription initiator, and, hence, very little STb is produced. Production is under the control of environmental conditions. STb synthesis by wild-type *E. coli* strains varies with the composition of the culture medium (444, 458), and a repressive effect of glucose on STb production was reported (459).

Secretion and disulfide bond formation

Intramolecular disulfide bonds must be correctly formed to produce an active STb toxin. The STb polypeptide is synthesized as a 71-amino-acid precursor (438, 439). The NH_2 terminus of pre-STb, residues 1 to 23, has characteristics of a signal sequence that is cleaved by a signal peptidase during export to the periplasm by using the Sec export system (460). Thus, an 8.1-kDa precursor (pre-STb) is converted to a transiently cell-associated 5.2-kDa form consisting of 48 amino acids. Conversion of pre-STb to cellular STb depends on the secA gene product. Translocation of the precursor to the periplasm requires energy. The export of STb relies on the general export pathway of *E. coli*. STb is detected as a cell-associated molecule and an indistinguishable extracellular form becomes apparent, indicating that no proteolytic processing occurs during mobilization of STb from the periplasm to the culture supernatant. Mature STb is found preferentially in the culture supernatant (460). STb was absent from the culture supernatant of *dsbA* and *tolC* defective mutants, indicating that these genes are required for secretion (461). Two intramolecular disulfide bonds must be formed for the efficient secretion of STb (462). Elimination of either one of the bonds renders the toxin susceptible to periplasmic proteolysis. STb is exported

across the OM through TolC involving accessory proteins. MacAB, an ABC transporter, interacts with TolC and participates in secretion (463, 464). MacAB probably binds the toxin in the periplasm and transports it through the pore formed by TolC (465, 466). MacA is a subunit of the MacAB transporter stimulating the MacB ATPase activity (467). The central region of STb from amino acid 19 to 36 is involved in the secretory process (468), and DsbA is necessary for STb to adopt a structure that can then cross the OM.

Toxic domain and structure

A nuclear magnetic resonance study established that STb is helical between residues 10 and 22 and residues 38 and 44 (Table 8) (440). The helical structure in the region 10 to 22 is amphipathic, exposing several polar residues to the solvent. The loop region between residues 21 and 36 contains a cluster of hydrophobic residues. The integrity of the disulfide bonds is crucial for the structure and function of the toxin, as already discussed. Oligomerization was observed for STb, resulting in the formation of hexamers and heptamers (469). The region responsible for this process comprises hydrophobic residues M, I, and F, found close to or in the hydrophobic α-helix (aa 37 to 42).

STb receptor

A glycosphingolipid present in high number in the plasma membrane was shown to be the STb receptor (470, 471). Binding of STb to commercially available glycosphingolipids was evaluated (472). STb binding varied greatly depending on the molecule tested. Sulfatide ($SO_4$3-galactosylceramide) was the molecule to which STb bound with greatest affinity. The reaction was dose dependent and saturable. Total lipid extraction of pig jejunum epithelium and thin-layer chromatography indicated the presence of sulfatide on this tissue. A mass spectrometer analysis on the lipids isolated following high-performance thin-layer chromatography of pig jejunum epithelium confirmed the nature of the receptor (473). A dissociation constant of 2.4 ± 0.61 nM for the STb-sulfatide interaction was observed (474). The functionality of sulfatide, a widely distributed acidic glycosphingolipid on the intestinal epithelium, was finally proven in the rat ligated loop assay (472). The binding site of STb with sulfatide comprises between aa 18 and aa 30. The region responsible is partly within the amphipathic α-helix and the flexible loop rich in glycine (475, 476).

Mechanism of action

STb is a toxin with rapid action, but with moderate potency (477). In mouse intestinal loops, purified toxin elicits a response in 30 min and fluid accumulation reach a maximum after about 3 h (403). STb stimulated a cyclic nucleotide-independent secretion. This enterotoxin is thus a cytotonic toxin with properties and a mechanism of action different from STa. *In vivo* significant accumulation of Na^+ and Cl^- occur intraluminally following STb intoxication. As well, STb stimulates bicarbonate (HCO_3^-) secretion (478, 479).

The level of prostaglandin E2 (PGE2) in the intestinal intraluminal fluid increases as a result of STb action (480). The quantity of PGE2 produced by intestinal cells is directly related to the dose of STb administered and the quantity of PGE2 correlated with the volumes of fluid released into the intestinal lumen. Levels of arachidonic acid are also elevated following STb intoxication, indicating that arachidonic acid metabolism is stimulated possibly through phospholipase A2 activity. The mode of action of STb may be somewhat similar to that of CT stimulating the release of both PGE2 and 5-HT and suggesting a potential effect on the enteric nervous system (3). STb could also act directly on the muscle cells of the ileal serosa, increasing the spontaneous motility of the intestine and resulting in contractions (403). Atropine could not abolish toxicity, indicating that it was not the result of the excitation of cholinergic nerves. Papaverine, which causes relaxation of smooth muscles, can inhibit STb, implying that STb could act directly on muscle cells. Internalization of STb toxin within rat intestinal jejunum epithelium was observed (481), and the process was also confirmed by using a confocal microscope and NIH-3T3 cells (482).

When cells are intoxicated, STb binds through its galactose sulfate moiety to an acidic glycosphingolipid, sulfatide, a molecule widely distributed on intestinal epithelial cells (470) (Fig. 3). STb stimulates a GTP-binding regulatory protein, resulting in a Ca^{2+} level increase inside the cell-activating calcium/calmodulin-dependent protein kinase II (483). Activation of protein kinase C (PKC) is induced following the Ca^{2+} increase, and phosphorylation of the CFTR ensues (483, 484). PKC also inhibits Na^+ uptake by acting on an unidentified Na^+ channel. Calcium/calmodulin-dependent protein kinase II opens a calcium-activated chloride channel (CaCC) and could be involved in phosphorylation of CFTR, as well. The increased Ca^{2+} levels also influence the activities of phospholipases A2

and C, leading to the release of arachidonic acid from membrane phospholipids and formation of prostaglandin E2 (PGE2) and 5-HT (or serotonin), two secretagogues from enterochromaffin cells. Both compounds mediate the transport of H_2O and electrolytes out of the intestinal cells by a yet-unknown mechanism (452, 485–487).

Using brush border membrane vesicles (BBMV) isolated from piglet jejunum and a membrane-potential-sensitive probe, STb was shown to permeabilize the intestinal tissue. It can do so by forming nonspecific pores confirming previous trypan blue and planar lipid bilayer (PLB) studies (473, 488). An electrophysiological study using PLBs where the receptor for STb was reconstituted into large unilamellar vesicles made of phosphatidylethanolamine osmotically fused to phosphatidylethanolamine: phosphatidylcholine:cholesterol (7:2:1) showed resolved channels currents (489). Thus, STb appears to be involved in the opening of a voltage-dependent channel. The previous observation that STb forms oligomers (469) can indicate that STb may allow formation of pores that could alter the cellular membrane. Permeabilization of intestinal cells *in vitro* was observed without cell death. The formation of pores/channels within the plasma membrane may constitute a signaling event triggering fluid secretion associated with diarrhea.

In human (human colon tumor, HRT-18) and animal (rat ileum epithelium, IEC-18) cell lines, caspase-9, the initiator of mitochondrion-mediated apoptosis and caspase-3, an effector of caspase-9, were both activated following STb intoxication (490). DNA fragmentation was observed as well as condensation and fragmentation of nuclei. Overall, the data indicated that STb toxin could induce, at least in these cell lines, a mitochondrion-mediated caspase-dependent apoptotic pathway.

STb and tight junctions

A significant reduction of TER parallel to an increase in paracellular permeability to BSA-FITC for STb-treated cells was noted in T84 human colon cells compared with untreated cells or cells treated with a nontoxic STb mutant (491). The increase in paracellular permeability was associated with a marked alteration of F-actin stress fibers. F-actin filament dissolution and condensation observed in the presence of STb were accompanied by redistribution and/or fragmentation of ZO-1, claudin-1, and occludin. An 8-amino-acid peptide (GFLGVRDG) present in STb sequence and corresponding to a consensus sequence of *V. cholerae* zonula occludens toxin (Zot) affected T84 cells in the same way as STb (492, 493). A scrambled octapeptide (STb24-31) showed no effect compared with untreated cells. Further studies showed that STb provoked a redistribution of claudin-1, a protein playing a major role in TJ permeability. Claudin-1 was displaced from TJs and found in the cytoplasm. The loss of this protein from TJs was accompanied by its dephosphorylation (494). Thus, STb induces epithelial barrier dysfunction through changes in TJ proteins that could contribute to diarrhea. More studies are required to understand the pathways involved in STb-mediated alteration of TER and TJ proteins modulation.

OTHER VIRULENCE FACTORS

Although fimbriae with their adhesins and enterotoxins remain the characteristic virulence factors of enterotoxigenic *E. coli*, additional encoded proteins can play various roles in the pathogenesis of these bacteria (495). For example, and as mentioned earlier, the autotransporter protein AIDA is a nonfimbrial adhesin that is expressed by certain strains causing PWD in pigs (31, 496, 497). Iha is another adhesin usually associated with shigatoxin-producing *E. coli* that can be detected in some animal ETEC (495, 498). In addition to AIDA and Iha, it is likely that animal ETEC express other virulence factors, including some nonfimbrial adhesins and proteases described for certain human ETEC strains, such as the adhesins and invasins Tia and autotransporter protein TibA (499–502), the autotransporter adhesin TleA (503), the host-activated adhesin EaeH (504, 505), the two-partner secreted EtpA adhesin that binds to the flagellar tip and acts as an adhesive bridge (506), the mucin-degrading proteases EatA, an autotransporter serine protease, and YghJ, a secreted metalloprotease (507, 508). Genomic studies and molecular epidemiology will help to evaluate the presence, distribution, and frequencies of these or similar virulence factors in animal ETEC strains (509–511), whereas expression and functional investigations will be needed to identify new animal-specific factors and potential host-adapted activation dependency (504).

VACCINES AND COLONIZATION INHIBITORS

Neonatal Diarrhea Vaccines

Studies on ETEC fimbriae have helped to better understand the biology and role of these organelles in

pathogenesis; they have also opened the door to new diagnostic, prophylactic, and therapeutic tools. Following on the seminal studies of Rutter and Jones (55, 56, 512, 513), demonstrating that colostral antibodies induced by maternal immunization protected neonatal piglets, many additional *in vitro* and *in vivo* studies confirmed that fimbriae are highly immunogenic proteins and that the induced antibodies protect by inhibiting adhesion to enterocytes and intestinal colonization (67–69, 73, 76, 81, 514–517). Studies with ETEC strains of veterinary relevance have led to the development of effective parenteral antiadhesive vaccines based on four types of fimbriae, F4 (K88), F5 (K99), F6 (987P), and F41. In general, these vaccines have been quite successful in the prevention of neonatal diarrhea in piglets and calves. Vaccination of dams is a cost-effective health-management strategy to prevent ETEC diarrhea in neonates (32, 518). However, immunity generated by the current generation of vaccines that are based on lacteal immunity is not effective in the prevention of PWD (discussed further below).

Fimbriae are thought be very good immunogens because they are proteinaceous and contain a set of epitopes that are repeated 10^2 to 10^3 times on each fimbrial thread. Because bacteria have multiple copies of fimbriae on their surfaces, each bacterium can contain as many as 10^5 to 10^6 epitopes on each bacterial surface. However, recent data have shown that the use of a single fimbrial subunit from F4, the fimbrial adhesin subunit FaeG, does not provide good protection unless it is found in the polymerized fimbrial structure (232). Likewise, it has been shown that F5 needs to be assembled to induce immunity (519).Thus, it is likely that vaccines for food animals produced using technologies that focus only on fimbrial subunits as immunogens will not result in good protection compared with polymerized, mature fimbrial strands. Notably, it has been shown that when foreign epitopes are genetically engineered into fimbrial subunits and displayed in a polymeric form on attenuated live bacteria, they can be used to increase their immunogenicity (520–525).

Because there are antigenic variants of some fimbriae, such as F4 (F4ab, F4ac, and F4ad), an important question is whether all three types are required in a vaccine for efficacy against all three types. Fortunately, there is strong immunological cross-reactivity between the three variants, presumably based on the common "a" antigen. Thus, cross-reactivities of the major and minor structural subunits of F4 result in good protection regardless of the origin of F4 in the vaccine, if the vaccine contains polymerized F4. However, since there is no cross-reactivity between F4, F5, F6, and F41, each of these types of fimbriae needs to be in the vaccine for the broadest level of protection. Among various advantages of using assembled ETEC fimbriae in vaccines are their relative resistance to enteric proteases and their ability to induce mucosal immunity; fimbriae are also fairly easy to extract intact from whole bacterial cells.

Other approaches for vaccines for ETEC include the use of formalin-treated fimbriated ETEC, the use of live fimbriated but nontoxigenic ETEC, or the use of attenuated *Salmonella enterica* mutants expressing cloned fimbriae. These vaccines have been used to immunize animals by the oral route and to successfully induce antiadhesive antibodies (526–530). Oral vaccinations, combined with parenteral applications, can increase and prolong the duration of lacteal immunity (529). A potential advantage of fimbriae of enteric pathogens is that they possess enteroadhesive properties, which they share with other mucosal immunogens such as the enterotoxins. The binding of fimbriae to their complementary intestinal receptors in the appropriate host species is important for the activation of mucosal immunity after oral immunization, as shown with the F4 fimbriae (531, 532). Other carrier bacteria have also shown some level of usefulness inducing anti-F5 antibodies, including *Lactobacillus acidophilus* (533). The use of plant-based vaccines also has been the subject of investigations. The gene encoding the main subunit of F4 and its adhesin (*faeG*) has been cloned into tobacco, barley, and alfalfa genomes, and these plants have been shown to express *faeG* (152, 534, 535). With the use of an *in vitro* binding assay that employed pig villi, the FaeG produced in tobacco and barley was shown to inhibit binding of F4-positive *E. coli* to the villi. Using the alfalfa-based vaccine, these investigators also showed that the vaccine could reduce shedding of ETEC in challenged pigs. The reduction in shedding was equal to vaccination with purified F4 fimbriae.

Fimbriae-specific monoclonal antibodies also have been used in protection against neonatal ETEC diarrhea. This approach has been most successfully applied for use in calves (536, 537) where monoclonal antibodies against F5 were administered to neonatal calves that also were challenged with an F5-expressing ETEC. F5-producing ETEC are the most common ETEC found in cattle. Calves receiving the F5-specific monoclonal antibody had reduced levels of diarrhea and mortality than nonvaccinated controls. However, passive vaccination with

anti-fimbriae monoclonal antibodies is expensive and labor intensive (32).

Postweaning Diarrhea Vaccines

While the current generation of commercial vaccines confers excellent protection of neonatal piglets and calves against ETEC, these vaccines have not been shown to be effective against postweaning ETEC infections in pigs. PWD due to ETEC remains an important disease of young pigs and causes significant morbidity and mortality immediately after weaning. The reasons why the current vaccines are not effective for PWD are probably related to a loss of protective antibodies in piglets receiving colostrum and milk and the subsequent loss from circulation. Titers of antifimbrial antibodies in milk fade by the time pigs are weaned. Since it is believed that the mechanism of protection against ETEC is by blocking adhesion to enterocytes and by agglutination of the ETEC in the lumen of the intestines, the lack of antibodies in the intestinal lumen postweaning leads to a lack of protection against the intestinal colonization by the ETEC. Consistent with this is the observation that the feeding of anti-F18 antibodies to weaned pigs can be protective (80, 538). Spray-dried serum-containing anti-F4 and anti-LT also have been shown to reduce postweaning diarrhea and shedding of ETEC (539). This could be a useful approach in the reduction of the within-herd spread of ETEC. The recent construction and use of nanobodies to inhibit the attachment of F18- and F4-fimbriated *E. coli* to pig enterocytes is an interesting approach that needs to be investigated in pigs (540, 541).

Various active immunization studies have focused on the relevant F18 and F4 fimbriae to protect piglets against postweaning ETEC infections. Administration of non-attenuated live ETEC strains expressing F18ab or F18ac fimbriae to pigs shortly before or after weaning had some protective effects after challenge with ETEC, but most of the vaccinated pigs suffered mild to severe diarrhea (538, 542). Slower colonization of F18- fimbriated ETEC versus F4-fimbriated ETEC paralleled a slower induction of the humoral immune response (543). The oral administration of enteric-coated F4 or microencapsulated F18 fimbriae to newborn piglets at best marginally reduced intestinal colonization upon challenge after weaning (544, 545). All things considered, an efficient vaccine protecting against postweaning ETEC infections awaits further developments, including the design and evaluation of attenuated live bacteria and/or fimbrial protein vaccines that include adjuvants (546, 547). An impediment to creating better vaccines for weaning diarrhea is that, unlike neonatal diarrhea, good and reproducible pig disease models are not available. A newer approach that has been developed is based on the creation of vaccines that incorporate the expected protective epitopes of fimbriae and enterotoxins in a single protein molecule (548–551). The incorporation of epitopes from F4, F18, and heat-stable and heat-labile enterotoxins in a single protein molecule has been shown to elicit antibodies that can neutralize attachment of ETEC to mucosal surfaces and neutralize the enterotoxins.

Oral immunization of weaned pigs with F4 and F18 was shown to be better at priming a mucosal response than intramuscular administration. Induction of a primary immune response occurred only in pigs expressing the corresponding intestinal F4 receptor, suggesting that receptor binding may facilitate antigen uptake (532). Pigs with the F4 receptor were protected against a challenge with F4-fimbriated ETEC. However, parenteral priming with F4-induced suppression of a mucosal F4 recall response upon oral infection with $F4^+$ fimbriated bacteria (552). In contrast, orally administered F4 was able to prime an immune response in both F4-susceptible and -resistant pigs, indicating that F4 given by the mucosal route does not induce oral tolerance (553).

Since pigs are weaned when they are approximately 3 to 4 weeks of age, active protection against ETEC must be developed during the pig's first month of life. However, one difficulty in eliciting protection during this timeframe is that most pigs also are receiving antibodies specific for the ETEC antigens in vaccines via colostrum and milk. Since protection is likely to depend on local mucosal immunity and thus depend on oral immunization, any vaccine antigens orally administered to baby pigs must escape luminal antibodies (from the dam) specific to the vaccine antigens. Thus, strategies to elicit an active mucosal immune response in the presence of passive antibodies from the dam need to be developed. This could possibly include both oral and parenteral vaccinations. A recent review discusses immunization strategies and problems for ETEC-mediated PWD in pigs (554). An alternate approach that could be considered is the search for new, highly antigenic molecules that are not related to fimbriae but that are required for ETEC colonization and persistence in the small intestines of pigs. Because these antigens would not be part of the current generation of fimbrial-specific vaccines for neonates,

they could be administered directly to suckling pigs and would not be eliminated due to the maternal antibodies in milk and colostrum being received by piglets. The discovery of such antigens would need to be broadly expressed by ETEC-causing PWD. While these antigens have not yet been identified, they could be discovered using extensive genomic data as a reverse genetics discovery tool. Phage display also has been used to screen for new potential antigens for vaccines (555).

Inhibitors of Colonization

Identification and characterization of the binding moieties of ETEC fimbrial adhesins should be useful for the design of new prophylactic or therapeutic strategies. Studies have described potential receptor or adhesin analogues that interfere with fimbria-mediated colonization (556–560). However, more studies including efficient inhibition of the relevant panoply of ETEC fimbriae are needed for this approach to be applied in agriculture. Although fat globule membranes of sow's and cow's milk were reported to contain receptors for ETEC fimbriae (556, 561–564), the postulated protective role of these receptors in the intestines of young animals remains unknown. Oral administrations of proteases that degrade intestinal receptors have been investigated with some success (565, 566).

The use of probiotics is also being considered for the prevention of ETEC-induced diarrhea in livestock and humans. Certain probiotics such as lactobacilli can bind to enterocytes without interfering with the attachment of F4-fimbriated ETEC (567). It was suggested that the coaggregation of certain *Lactobacillus* isolates with the F4 ETEC decreases ETEC colonization. Probiotics were the most efficient in controlling diarrhea in calves when used in conjunction with fimbria-based vaccines (568). The *in vivo* relevance of F4-mediated adhesion inhibitors found in certain *Lactobacillus* culture supernatants remains to be determined (569, 570). Commensal bacteria such as *Lactobacillus* that express F5 have shown some degree of protectiveness against F5+ ETEC (533). Some commensal bacteria are being considered as directly blocking ETEC colonization of the intestines and might be useful in prevention or treatment of disease. Certain strains of *Lactobacillus* have shown the most promise to date (567, 569, 571, 572) along with strains of *Bifidobacterium*, *E. coli*, *Bacillus, Enterococcus, Pediococcus*, and *Saccharomyces* (573–577). In recent years, the pathogenesis of ETEC has been linked to epithelial inflammatory responses in the form of cytokine expression (578). However, whether these responses are due to known or new ETEC virulence factors or to indirect effects on the microbiome and/or its metabolites remains to be determined. Probiotics, albeit frequently with dietary supplements, modulate detected host factors of inflammation (574, 579–581), possibly through their metabolites (582). More *in vivo* studies are needed to determine whether probiotics can be sufficiently protective and cost-effective with regard to ETEC diarrhea in farm animals.

Genomics for Future Vaccines

Several completed genomes of animal-source ETEC are publicly available. Shepard et al. first described the genomes of F4+ and F18+ porcine ETEC and performed phylogenetic comparisons of a large collection of porcine ETEC (291). This study demonstrated complex genomes with remarkable plasmid complements encoding a variety of previously identified virulence factors including the F4 and F18 fimbrial operons. It was also evident that porcine ETEC comprise strains from multiple phylogenetic lineages that have acquired these plasmids, including strains within the *E. coli* phylogenetic groups A, B1, and D. However, it was also apparent from this study that the number of lineages containing porcine ETEC is somewhat limited in comparison with human ETEC, suggesting that a specific chromosomal background is required to harbor porcine ETEC plasmids enabling virulence in the host.

Specific lineages may also have enhanced virulence potential. For example, the genome of an O157 porcine ETEC strain involved in an outbreak in pigs was analyzed, and it was LT+, STa+, and STb+, yet lacked common porcine ETEC fimbriae such as F4, F5, or F18 (510). The phylogenetic background of this strain was distinct from classical O157:H7 human clinical isolates and was actually most similar to O78 strains of avian pathogenic *E. coli* (583). This again supports the concept that combinations of ETEC virulence factors in the appropriate phylogenetic background are required for enhanced virulence. Despite the apparent diversity of porcine ETEC, scanning of porcine ETEC genomes for antigenic candidates has revealed several candidates that are differentially present in porcine ETEC compared with porcine commensal *E. coli* found across porcine ETEC lineages and predicted to be surface exposed and accessible to the host (509). Therefore, reverse vaccinology exploiting

available animal-source ETEC genomes could be an effective approach toward the development of subunit vaccines.

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