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Surface Structures of *Escherichia coli* That Produce Diarrhea by a Variety of Enteropathic Mechanisms

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Strains of *Escherichia coli,* mostly of human origin, were obtained from several different investigators who had isolated them from patients with diarrhea from many different parts of the world. The mechanisms by which these *E. coli* were thought to have caused diarrhea included: (i) synthesis of labile, stable, or *Shigella dysenteriae*-like enterotoxins; (ii) invasion of the intestinal mucosa; and (iii) unknown. Each strain was carefully examined for pili or flagella to correlate the presence or absence of such surface structures with a particular mechanism of diarrhea. The presence of pili was determined by colony morphology on minimal media, pellicle formation in static broth culture, and transmission electron microscopy. The pili were categorized as type 1 if the bacteria fermented rhamnose and if pellicle formation was inhibited by α-methyl-D-mannoside. The presence of flagella was confirmed in motility media and by transmission electron microscopy. Six invasive *E. coli* strains lacked pili and flagella. Ten *E. coli* strains that synthesized enterotoxins or produced diarrhea by an unknown mechanism were piliated (seven with type 1 pili), and all but one had flagella. Pili and flagella seem to be associated with strains of *E. coli* that produce diarrhea by enterotoxin synthesis or unknown mechanisms. Strains that produce diarrhea by mucosal invasion lack both types of surface structures.

Surface structures, including pili and flagella, are thought to be important to the ability of certain bacteria to produce diarrhea (1, 5, 15, 17, 21, 23). The purpose of our study was to determine the presence of pili and flagella among *Escherichia coli* isolated from patients with diarrhea from several different areas of the world. All such strains had been exhaustively examined by the investigators who originally isolated them for the presence or absence of one of several mechanisms by which *E. coli* are known to cause diarrhea. It was hoped that the possession of pili and/or flagella could be associated with a particular enteropathic mechanism (hereafter, a strain of bacteria that causes diarrhea or a mechanism by which a bacteria produces diarrhea will be referred to as enteropathic).

MATERIALS AND METHODS

Bacterial strains. The strains studied are described and referenced in Table 1. The mechanisms by which these various strains produce diarrhea include the ability to invade or to synthesize labile and/or stable enterotoxin. We ascertained that the invasive *E. coli* (InvEC) strains were invasive by the Serény test (22). We confirmed that the toxin-synthesizing strains were toxigenic by the rabbit ileal loop assay (18). All loops were read at 6 h because we found 6-h experiments to be more reproducible than 18-h experiments. In collaboration with O'Brien et al. (A. D. O'Brien, M. R. Thompson, J. R. Cantey, and S. B. Formal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B103, p. 32) at the Walter Reed Army Institute of Research, we have found that RDEC-1, a strain of *E. coli* that produces diarrhea in rabbits (3), synthesizes small amounts of a *Shigella dysenteriae*-like enterotoxin. The four strains of enteropathic *E. coli* (EEC) obtained from M. J. Gurwith were felt by him to be the cause of diarrhea in patients from whom they were isolated, and it was determined that the strains did not possess any of the known mechanisms for producing diarrhea (16). We ascertained previously that the non-enteropathic *E. coli* (NEC)-VA lacks the ability to invade or synthesize enterotoxin. The other NEC strains, NEC-PH, -JC, -WL, and -CH, were isolated from four healthy adult males in our laboratory and were assumed to be non-enteropathic. NEC-K12, which is a lactose-negative strain, was found to lack enteropathic mechanisms by Stanley Falkow of the University of Washington, Seattle, Wash. NEC-CDC was checked in a similar manner by Eugene Gangarosa of the Center for Disease Control, Atlanta, Ga., and found to be non-enteropathic.

Biochemical assays and determination of the presence of pili and flagella. All strains were identified as *E. coli* at the time of receipt by using the API-20 system (Analytab Products, Plainview, N.Y.).

TABLE 1. *Characteristics of E. coli strains*

Strain[a]	Serotype	Mechanism of diarrhea[b]	Isolation locale	Reference[c]
InvEC-SF-1	0143:k?:H-	Inv	Vietnam	10
ToxEC-SF-1	078:H11	LT	Bangladesh	13
InvEC-BS-1	0144:H-	Inv	Vietnam	10
InvEC-CDC-1	Untypeable	Inv	Mexico	19
InvEC-CDC-2	Untypeable	Inv	United States	A
InvEC-CDC-3	028	Inv	United States	A
InvEC-CDC-4	028	Inv	United States	A
ToxEC-CDC-1	Untypeable	LT	Mexico	19
ToxEC-CDC-2	Untypeable	ST	Mexico	19
ToxEC-CDC-3	06:H16	LT-ST	Mexico	19
NEC-CDC	Not done	None	Mexico	19
ToxEC-MG-1	0159	LT-ST	Canada	16
EEC-MG-1	055	Unknown	Canada	16
EEC-MG-2	0119	Unknown	Canada	16
EEC-MG-4	0111	Unknown	Canada	16
EEC-MG-6	0126	Unknown	Canada	16
RDEC-1	015	SDET	United States	3
NEC-VA	Not done	None	United States	3
NEC-PH	Not done	None	United States	B
NEC-JC	Not done	None	United States	B
NEC-WL	Not done	None	United States	B
NEC-CH	Not done	None	United States	B
NEC-K12	?:K12	None	United States	3

[a] SF, Obtained from S. B. Formal, Walter Reed Army Institute of Research, Washington, D.C.; BS, obtained from R. B. Sack, University of Maryland, Baltimore, Md.; CDC, obtained from E. J. Gangarosa, Center for Disease Control, Atlanta, Ga.; MG, obtained from M. J. Gurwith, University of Manitoba, Winnepeg, Manitoba, Canada; Inv, invasive; Tox, toxigenic; N, non-enteropathic; E, enteropathic (diarrhea associated, mechanism unknown); RDEC-1, rabbit strain (see text for other details).

[b] Inv, Invasion; LT, heat-labile enterotoxin; ST, heat-stable enterotoxin; SDET, *S. dysenteriae* type 1-like enterotoxin (see text for other details).

[c] A, Personal communication from E. J. Gangarosa, Enteric Disease Laboratory, Center for Disease Control, Atlanta, Ga.; B, isolated from the feces of four healthy adult males in our laboratory.

The original cultures were inoculated into Trypticase soy broth (Difco Laboratories, Detroit, Mich.) and incubated under static conditions at 37°C for 18 to 24 h. Samples were taken and stored in vials in 10% glycerol at −90°C until used.

The ability of each strain to ferment rhamnose was assessed by the red to yellow color change of tryptic agar base (Difco Laboratories) fitted with a rhamnose sugar disk (Difco Laboratories).

The ability of a strain to form a pellicle was assessed by the serial static broth culture technique (20). A standard volume (0.1 to 0.2 ml) was inoculated into Trypticase soy broth or Mueller-Hinton broth (Difco Laboratories), incubated under aerobic or anaerobic static conditions at 37°C, and inspected after 72 h for the presence or absence of a pellicle on the surface of the broth. The formation of a pellicle that is not easily dispersed indicates that the culture contains piliated bacteria (8). The inhibition of pellicle formation by α-methyl-D-mannoside (grade III; Sigma Chemical Co., St. Louis, Mo.) was assessed by culturing the bacteria in Trypticase soy broth containing 0.5% α-methyl-D-mannoside. Piliated (pellicle-forming) strains were designated as type 1 if the strain was capable of fermenting rhamnose and if pellicle formation in broth was inhibited in the presence of α-methyl-D-mannoside (6, 20).

Charles Brinton of the University of Pittsburgh, Pittsburgh, Pa., recommended that we test our strains with a technique recently developed in his laboratory (C. C. Brinton, Jr., et al., *Proceedings of the 13th Joint Conference on Cholera*, in press). The technique consists of growing the bacteria on minimal media agar for 16 h at 37°C and examining the colonies with a dissecting stereomicroscope equipped for transillumination with a diffuse light source. A piliated colony has a continuous dark halo along its perimeter, whereas a nonpiliated colony lacks a continuous halo.

Motility of each strain was assessed by inoculating tubes containing either motility-indole-ornithine media (Difco Laboratories) or semisolid motility media (Difco Laboratories).

Electron microscopy. The negative staining technique for transmission electron microscopy (24) was used to identify the surface structures of *E. coli* isolates. The isolates from the surface broth of 72-h

cultures, as well as clones grown for 16, 24, and 48 h on blood agar base (Difco Laboratories) or MacConkey agar (Difco Laboratories), were studied. The bacteria were mounted on plastic-coated grids, negatively stained with 1% phosphotungstic acid and/or 2% aqueous uranyl acetate, and examined on an Hitachi HS-12A electron microscope. A minimum of 100 bacteria were examined per grid.

Controls. The rhamnose fermentation, piliation, and motility tests, as well as electron microscopy, were performed on each strain in duplicate on at least two different occasions. The purity of the cultures was rigorously documented by biochemical and bacteriological means and in some cases by the slide agglutination technique, using specific antisera.

RESULTS

The results are summarized in Table 2. All six strains that synthesized enterotoxin fermented rhamnose, as did two of six InvEC strains, three of four of EEC strains, and five of seven NEC strains. Thus, rhamnose fermentation was not reliably associated with enteropathogenicity among the strains of *E. coli* that we assayed.

All of the enterotoxin-synthesizing and EEC strains and five of seven NEC strains had pili, mostly type 1 pili. None of the six InvEC strains possessed pili. Strains of bacteria that had been maintained in the laboratory for a prolonged period (2 years or more) had to be passed as many as six times before pellicle formation in static broth culture occurred.

Flagella were found on all but one of the strains that synthesized enterotoxins, as well as on all of the EEC and NEC strains. None of the six strains of InvEC had flagella.

The observation of serial static broth cultures for pellicle formation was a simple, specific, and sensitive assay for detecting piliated bacteria and was used extensively early in the study. However, the minimal media screening assay of Brinton et al. offers several advantages over observation for pellicle formation. Piliated clones can be detected on the transilluminated minimal media agar without previous serial passage, thus obviating the difficulty in keeping cultures pure during serial transfer. The results

TABLE 2. *Surface structures and biochemical characteristics of EEC and NEC*

Strain	Rhamnose fermentation	Presence of pili as determined by:			Presence of type 1 pili[a]	Presence of flagella
		Minimal media screening assay	Pellicle formation	Electron microscopy		
Invasive strains						
InvEC-SF-1	−	−	−	−	NA[b]	−
InvEC-BS-1	+	−	−	−	NA	−
InvEC-CDC-1	−	−	−	−	NA	−
InvEC-CDC-2	+	−	−	−	NA	−
InvEC-CDC-3	−	−	−	−	NA	−
InvEC-CDC-4	−	−	−	−	NA	−
Toxin-producing strains						
ToxEC-SF-1	+	+	+	+	+	+
ToxEC-CDC-1	+	+	+	+	+	+
ToxEC-CDC-2	+	+	+	+	+	+
ToxEC-CDC-3	+	+	+	+	+	+
ToxEC-MG-1	+	+	+	+	+	+
RDEC-1	+	+	+	+	+	−
Enteropathic strains						
EEC-MG-1	+	+	+	+	−	+
EEC-MG-2	+	+	+	+	−	+
EEC-MG-4	−	+	+	+	−	+
EEC-MG-6	+	+	+	+	+	+
Non-enteropathic strains						
NEC-VA	−	−	−	−	NA	+
NEC-CDC	−	−	−	−	NA	+
NEC-K12	+	+	+	+	+	+
NEC-PH	+	+	+	+	+	+
NEC-JC	+	+	+	+	+	+
NEC-WL	+	+	+	+	+	+
NEC-CH	+	+	+	+	+	+

[a] A piliated strain which can ferment rhamnose and whose pellicle formation is inhibited by α-methyl-D-mannoside.

[b] NA, Not applicable.

from the minimal media assay are available in 1 day, whereas the pellicle formation method may require several weeks. The presence of pili and flagella (2, 9) was confirmed by electron microscopy. Bacteria grown on enriched agar media (blood agar base) had fewer numbers of piliated organisms and fewer pili per bacteria than did those grown in Trypticase soy broth.

DISCUSSION

Our finding that six InvEC strains, originally isolated in widely separated locales, lacked surface appendages of any sort is very good evidence that InvEC strains, and perhaps invasive bacteria in general, do not require pili and flagella to be enteropathic. Duguid and colleagues have reported previously that some strains of other invasive enteropathogens, including salmonella and shigella (5–8), may lack pili as well as flagella. In previously reported studies in the rabbit model of InvEC diarrhea, we found no evidence of adherence of the bacteria to the microvillous border of the ileum and proximal colon (4). Perhaps pili and flagella interfere with the ability of the invasive bacteria to penetrate mucosal epithelial cells.

All of the toxin-synthesizing and EEC strains, which were obtained from many different parts of the world, possessed pili, mostly of the type 1 classification. Human strains of toxin-synthesizing *E. coli* have previously been examined for and reported to have pili (11, 12; Brinton et al., in press). The present study adds to that list and provides further evidence that toxin-synthesizing strains of *E. coli,* with the exception of one strain which produces only heat-stable enterotoxin (11), possess type 1 pili. Evans et al. have reported that many such strains also possess their colonization factor antigen (11). Porcine and bovine strains frequently possess the K88 and K99 pili, respectively (17, 23), and our rabbit enteropathogen, strain RDEC-1, has type 1 pili. Only one of the EEC strains had type 1 pili, a fact which is of some interest because other types of pili are not felt to be virulence factors for EEC (5).

Our data and those of others thus reveal an association between pili and toxin-synthesizing *E. coli.* It would be difficult to draw any firm conclusions from such a finding in view of our evidence and that of others that the majority of strains of *E. coli* obtained from urine, wounds, or feces of healthy and ill individuals are piliated and most have pili of the type 1 classification (5, 9, 14). The very high frequency of the occurrence of pili among other types of *E. coli* does make the finding of a complete absence of pili among InvEC strains more remarkable.

Pili are thought to mediate adherence to gut mucosal epithelium of bacteria that cause diarrhea (5, 11, 17, 21, 23). Flagellum-mediated motility may be helpful in chemotaxis towards gut mucosal epithelium (1) and penetration of the mucous barrier that overlies mucosal epithelium (15). The present study is further evidence for an association between *E. coli* that synthesize enterotoxins and pili and flagella. It is quite clear from our studies of invasive *E. coli* and the studies on others of salmonella and shigella (5–8) that neither surface structure is indispensable to invasive gut pathogens.

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