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THE ROLE OF INTESTINAL BACTERIA IN ACUTE DIARRHEAL DISEASES

Annual Report

Sherwood L. Gorbach, M.D.

January 1981

Supported by

US Army Medical Research and Development Command
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Our studies provide evidence for at least three serologically distinct groups of surface antigens present on ETEC isolated from man. E. coli strains in each of the three serologic groups produce surface pili of "identical" size, morphology, and molecular weight. The presence of these pili shows no apparent correlation with the ability to cause MR-HA of a particular rbc type(s). In the year 1979, our ETEC culture collection doubled in size; E. coli strains belonging to various O-groups have been found to react with our three serologic types of pili-specific antisera.

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Publications during the past contract year.

Abstracts:

C.F. Deneke, G.M. Thorne, and S.L. Gorbach. Adhesive pili for enterotoxigenic Escherichia coli (ETEC) pathogenic for humans. International Conference Antimicrobial Agents and Chemotherapy. 1979.

C.F. Deneke, G.M. Thorne, and S.L. Gorbach. Adhesive pili for enterotoxigenic Escherichia coli pathogenic for humans. Fifteenth Joint Conference on Cholera. U.S. Japan Cooperatifve Medical Science Program. Washington, D.C., 1979.

Publications:

Thorne, G.M., Deneke, C.F., and S.L. Gorbach. Hemagglutination and adhesiveness of toxigenic Escherichia coli isolated from humans. Infect. Immun., 23:690-699, 1979.

Deneke, C.F., Thorne, G.M., and S.L. Gorbach. Attachment pili from enterotoxigenic E. coli pathogenic for man. Infect. Immun., 26:362-368, 1979.

Deneke,C.F., Thorne, G.M., and S.L. Gorbach. Serogroups of attachment pili found on enterotoxigenic Escherichia coli of humans. and Immunity. In Press.

Thorne, G.M., and S.L. Gorbach. General Characteristics: Nomenclature of Microbial Toxins. In International Encyclopedia of Pharmacology and Therapeutics (ed) Friedrich Dorner. Pergamon Press, Oxford, New York submitted for publication.

SECTION 1

Testing of E. coli isolated from humans with diarrheal disease for production of enterotoxins

During the Contract period, we have continued to screen Escherichia coli isolated from humans with diarrhea for their ability to produce LT and/or ST enterotoxins and for the presence of surface antigen(s) analogous to the K88 antigen of porcine enteropathic E. coli. An outline of the characteristics of known adherence surface antigens (pili) found on ETEC strains of human origin is given in Table 1A.

The Evans group have described antigens CFA/I and CFA/II. Production of these two antigens always correlates with the appearance of fine filamentous projections covering the surface of the bacterial cell (pili) and mannose-resistant hemagglutination activity for specific rbc types. These two antigens have been found on ETEC of a limited number of O-groups, mostly in the 078 serogroup in the case of CFA/I. (These investigators have used an infant rabbit intestinal colonization assay as their adherence model.)

Our studies which are detailed below provide evidence for at least three serologically distinct groups of surface antigens present on ETEC isolated from man. E. coli strains in each of the three serologic groups produce surface pili of "identical" size, morphology, and molecular weight. The presence of these pili shows no apparent correlation with the ability to cause MR-HA of a particular rbc type(s). In the year 1979, our ETEC culture collection doubled in size; E. coli strains belonging to various O-groups have been found to react with our three serologic types of pili-specific antisera (Tables 1-6). These data are described in detail in Section 2e.

SECTION 2. Isolation and characterization of pili found on ETEC

The studies described in the following Sections a-d have been published in Infection and Immunity, 26: 362-368, 1979 (Deneke, C.F., G.M. Thorne, and S.L. Gorbach).

a. Purification: Purification of adherence pili from ETEC by temperature-dependent red cell absorption and elution was described previously (Annual Progress Report 1978). Pili isolated by this protocol have undergone further characterization.

b. Electron Microscopic (EM) Studies: Short, needle-like pili (5-10 nm x 300 nm) were found to be present on the surface of a number of different ETEC strains but not on non-enterotoxigenic control strains of E. coli (Fig. 1A to F). Pili preparations have also been examined by EM and appear to be homogenous. The isolated pili had the same diameter as the small pili on the intact bacterial cells (Figure 2).

c. Molecular Weight Determination: SDS-PAGE analysis of purified pili of various ETEC strains detected protein subunits of 12,500 and 13,100 molecular weight.

d. Biological Reactivity: The isolated pili have also been shown to bind to human buccal cells under temperature conditions (37°C) which prevent binding of these pili to erythocytes.

The F_{ab} fragment of anti-pili immunoglobulin G antibody has been shown to block buccal cell binding by intact bacterial cells.

e. Serological Characterization: An important aim of this study has been to develop rabbit antisera against purified adherence pili for use in screening ETEC isolates from man. The results obtained with 84 ETEC are summarized in Table 7. We find that 53 of 84 ETEC strains (63%) react with one or more of the three pili-specific antisera. The ETEC were also examined for possible

correlations between pili serogroup and O somatic antigens, enterotoxin profile, geographic origin, and/or MR-HA ability. The O somatic antigens of the ETEC strains are detailed in Table 8. In our study there is no apparent correlation of pili antigen type with specific somatic antigens. Thus, our findings are dissimilar to those of the Evans' group as indicated in Table 1A. Likewise, there is no apparent correlation of pili serogroup with toxin profile (Table 9). ETEC strains which produce both toxins (LT/ST), LT-only, or ST-only have been found to synthesize each of the three pili types recognized by our antisera. In adition, there was no correlation of pili serogroup with MR-HA ability or geographic location of the strains (Tables 10, 11).

An additional 18 ETEC strains were received from Dr. Robin Ryder from human cases of diarrheal disease in Panama. Production of type 1 or type 2 pili was detected in 5/18 of these isolates (Table 6).

We are currently preparting antisera against the surface pili present on ETEC strains 2016-10, H326$_c$I, CDC 5206-70, C921d, 54e-14 (Tables 1-5). These strains are MR-HA positive yet do not react with the three pili antisera. Unfortunately, we have encountered repeated loss of our rabbits due to two mini-epidemics of P. multocida in the rabbit facility. Consequently, our production of "novel" pili antisera is off schedule and we have not been able to build sufficient antisera to serologically characterize the five piliated strains mentioned above. One or more new pili serogroups may be recognized when these strains are studied further.

Section 3. Studies with adherence antigens

a. Serologic relationship of our three pili serogroups to the CFA/I and CFA/II antigens: The precise immunological relationship between our three adherence pili and the Evans' CFA/I and CFA/II antigens is still unclear. Published descriptions indicate that CFA I has a molecular weight of 23,800 following precipitation with ammonium sulfate andelution from DEAE Sephadex. (Purification and characterization of CFA/II has has not been reported). Strains possessing CFA/I

and II are described as being associated with the ability to cause mannose-resistant hemagglutination (MR-HA) of specific red blood cells: CFA/I-human A, bovine, chicken; CFA/II-bovine). In our studies the MR-HA patterns have proven to be unreliable markers for the presence of certain pili types. The MR-HA patterns of ETEC strains in each of our three serologic groups are variable. Our type 1 strain 334(O15:H11) causes MR-HA of human A,B, bovine and guinea pig RBC's. The type 2 prototype M9800-5 (O6:K15:H16) only causes MR-HA of bovine red cells. The type 3 prototype D542 (not typable) causes MR-HA of both human A and bovine red cells. Following isolation by our red-cell absorption-elution techniques (Deneke et al., Infection Immunity 26:362-368, 1979), all three pili types were found to have two subunits with molecular wieghts of 12, 12,500 and 13,100 by SDS PAGE. Thus, according to published data, the pili differ; alternatively, we may be reporting the monomer size while the Evans' report a dimer configuration. Recently, Wevers et al., (Infection and Immunity 29:685-691, 1980) reported the molecular weights of subunits of CFA/I and CFA/II of 12,000 and 13,000, respectively, when using SDS-PAGE analysis.

During serologic studies of our three pili types, we have found no cross-reactivity between heterologous sera and whole bacterial cells of each of the three prototype strains. Ouchterlony immunodiffusion analysis of isolated pili has also supported the serologic differences of our three pili types. Figure 3

The fact that the CFA/I strain H10407 reacts with both our type 1 and 3 antisera has raised concern about the specificity of the sera. Recently, we prepared CFA/I by the Evans' method and tested the antigen preparation in Ouchterlony plates with our type 1 and 3 sera. The enclosed photograph, Figure 4 shows the single precipitin line resulting from reaction with type 1 and type 3 pili antisera. The resulting precipitin lines were crossed which is a reaction of non-identity. Thus, both our antigens are present in the CFA/I pili preparation, and they are not identical.

We have also obtained CFA/I antiserum from Dr. M. Levine which was prepared by injection of whole cells of H10407 followed by cross-absorption by H10407P, the pili-negative derivative. Ouchterlony immunodiffusion analysis of purified CFA/I antigen (by the Evans method Infect Immun 25:738-748, 1979.) with the Levine antiserum and our antisera from serogroups 1 and 3 is shown in Figure 5. Both Levine's CFA/I serum and our serogroup 1 antiserum gave single lines with CFA/1 antigen, but these lines cross, indicating non-identity. Thus, it would appear that the antigen (334-like pili) recognized by serogroup 1 antiserum is not identical with CFA/I antigen. The single broad immunoprecipitin line seen with Levine's CFA/I antiserum splits into multiple lines with the serogroup 3 antisera (D542 pili antisera) (well 3). We interpret this reaction as one of partial antigenic relatedness between CFA/I and our D542-like pili (serogroup 3).

We cannot explain the variations described above but these immunologic reactions may be affected by the particular H10407 strain used and by the method of antigen preparation (i.e. whole bacteria vs. isolated pili).

Dr. D.E. Evans, Jr., sent us the H10407 strain used to prepare the purified CFA/I antigen in Figures 4 and 5, and it clearly has both serogroup I and 3 pili antigens. The CFA/I antiserum provided by M. Levine was prepared against intact H 10407 and absorbed with H10407P and does not recognize both pili antigens.

The isolated pili used to prepare our three types of antisera have been subjected to electron microscopic examination and piliate structures are seen in these preparations. Of course, one would need monoclonal antibody preparations to assure that the sera are reacting with adherence antigens only. However, a reasonable level of confidence in the sera can be reached, since:

1). the antisera are prepared against isolated red cell absorbed-eluted pili.

2). the antigens give two subunit bands in SDS-PAGE without foreign proteins.

3). EM examination detects pili structures

4). Ouchterlony immunodiffusion testing with isolated pili and pili specific

antisera gives single precipitin bands.

In our screening study of 106 ETEC strains from around the world, other strains (like H10407) but of various O:H serotypes were also found, that is, ones which react with one or more of our three pili antisera. Presumably, such strains are producing more than one serologic type of pilus.

In summary, the differences between our three pili types and the Evans' CFA/I and II are evident in both the comparison of the published data and in our serologic testing to date. Thus a definitive answer is impossible, and only points out the necessity of more studies in order to clarify the relationships of these important bacterial antigens. As outlined in our contract renewal application, complete biochemical and serologic characterization of these antigens is planned.

b. Pili Cross-Linking PDM: We attempted to clarify the antigenic interrelationships of the pili mediating MR-HA reactions and buccal cell adherence by Ouchterlony immunodiffusion analysis. Isolated pili are unstable, however, tending to disassociate into their monomeric units, which apparently have a single antigenic combining site. Behaving like a hapten, monovalent pili subunits proteins absorb anti-pili antibody and an immunoglobulin-mediated precipitation reaction does not occur. (The antibody titer against whole bacterial cells drops following preincubation with purified pili.) Therefore, we have cross-linked the pili protein subunits with N, N'-p-phenylenedimaleimide (PDM). Using highly purified agarose (Seakem HGT-P) and barbitol buffer, we have been successful in performing immunodiffusion studies of the three pili serogroups recognized to date. The Ouchterlony analysis with PDM-linked pili antigens is shown in Figure 3. The precipitation lines show a reaction of non-identity (i.e. the precipitate lines intersect since the samples contain no antigenic determinant in common).

c. ^{3}H-NEM labeling of isolated pili: The isolated, partly purified pili have been labeled with ^{3}H-N-ethylmalemide (NEM). The availability of isolated labeled pili will allow us to quantitatively study the attachment of these pili to various human cells, including buccal mucosal cells, red cell ghosts, and human small in-

testinal cells. Our preliminary experiments with ^{125}I labeling, using the lactoperoxidase glucose oxidase method, were unsuccessful due to very low incorporation of the label into the pili fraction and loss of buccal binding ability. Subsequently, we discovered that oxidants such as H_2O_2 denatured or inactivated the pili, thereby making them unable to attach to buccal cells. We now have evidence from N-ethylmaleimide titrations that a single -SH group is apparently not involved with the binding reaction, since pili-mediated functions such as mannose-resistant hemagglutination occurs in the presence of Hg++ ions. The availability of these intact pili with the high specific activity of ^{3}H-NEM allows the study of the attachment of these pili to various cells.

d. Binding of ^{3}H-NEM labeled pili to buccal mucosal cells: Using the ^{3}H-NEM pili we have carried out an experiment to show that the labeled pili can be used in cell attachment studies. The time course of the attachment of ^{3}H-NEM pili to human buccal cells is given in Figure 6. The rate of attachment is not perfectly linear, decreasing slightly with time, and it is markedly slower than the attachment of the intact bacteria to buccal cells. This is a result of the large number of simultaneous attachment reactions occurring when the whole bacteria attach to buccal cells; that is, buccal binding by intact bacteria is the sum of multiple binding events, in contrast to the single binding event occurring with the labeled pili system. This reaction has not reached completion by 30 min. The supernatant after 5 min. incubation when added to fresh buccal cells shows the same level of binding. This finding suggests that after 5 min. of incubation the amount of functional ^{3}H-NEM-labeled pili has not significantly decreased.

As demonstrated in figures 7 and 8, this binding can be interpreted as an $A+B \rightleftharpoons AB$ equilibrium as a change in either the concentration of pili or of buccal cells results in a shift in final equilibrium position. That is, if the concentration of either component is altered, the final equilibrium will also change in the same direction. If pili concentration increased more binding results (Figure 5).

Similarly, if the concentration of buccal cells increases more binding results (Figure 6). Therefore, neither the number of receptor sites, nor the number of attaching pili is limiting. The final amount of binding reflects an equilibrium between bound and free pili rather than a binding reaction that goes to completion. If the binding reaction went to completion, then the extent of binding would be limited only by the availability of either receptor (buccal cell) or ligand (pili), and either the addition of more pili or more buccal cells would not cause greater binding because one reagent should be in excess over the range of concentrations chosen. The pili preparations used here were "aged" and follow second order equilibrium compatible with the binding of pili-pili dimeric subunit. Using other pili preparations, not in the monomeric state similar results are obtained but these preparations give linear binding vs. concentration. These differences probably reflect differences in the quaternary structure of the pili. Binding of the labeled pili does not occur in the absence of buccal cells under our experimental conditions. The low absolute activity of the pili preparation has precluded accurate determination of the binding affinity using Scatchard plot analysis but preliminary studies indicate values on the order of 10^{10} -10^{11}.

In the future, this system will allow us to examine the pH and temperature effects on the binding of isolated pili to buccal cells and to compare the binding conditions for isolated pili and intact bacteria. We should be able to determine whether pili binding is the exclusive mechanism involved in the buccal cell attachment reaction. Kinetic studies, similar to those applied to enzymes, may allow us to further describe the interactions between the pili and eukaryotic receptors.

SECTION 4. Adherence of ETEC to Eukaryotic Cells

a. Human Buccal Cell Adherence Assay: We have developed an adherence assay employing human buccal epithelial cells. This system is attractive since it is used human cells against human pathogens - the homologous system. Binding to human buccal epithelial cells was explored by us since it was known that another enteric pathogen, Vibrio cholerae, is present in high number in the oral cavity during acute and convalescent periods. Also, the buccal cells are easy to obtain, and they share certain similarities with the gastrointestinal mucosa. The buccal cells are part of a mucosal, secreting tissue contiguous with the gut, and ABO and Lewis blood group substances are known to be present in secretions and cells lining the entire GI tract. This study has been published (Thorne, G.M., Deneke, D.F. and Gorbach, S.L. Infection and Immunity 23:690-699, 1979).

b. Scanning Electron Microscopy: The adherence of ETEC to human buccal cells was examined by scanning electron microscopy. Buccal cells alone, or following incubation with ETEC strains 334 or 334LL were applied to glass slides and fixed in 5% glutaraldehyde in PBS. The specimens were dehydrated in alcohol, critical point dreied, fixed, mounted, and coated with gold. The scanning electron micrographs are shown in Figures 9-11. Individual bacilli can be seen attached to the buccal cells in the preparation containing ETEC strain 334 (Figure 9). Buccal cells alone were free of attached organisms as shown in Figure 10. Specimens prepared from mixtures of buccal cells and E.coli 334LL (enterotoxin negative, non-adherent MR-HA, plasmid free, derivative of ETEC strain 334) were found to be free of any attached organisms. In Figure 11, bacterial strands can be seen to interconnect the bacterial cells (ETEC 334) and to make contact with the buccal cell surface.

c. Development of Adherence Assays of Human ETEC Strains to Human Ileal Cells: Because of our ongoing interest in the interactions of human bacterial pathogens with the small bowel of humans, we continue to search for an optimal eukaryotic intestinal cell or tissue. We believe that it is important to use human cells to study the attachment of bacterial strains pathogenic for man. Buccal cells or red blood cells are not the most appropriate target cell. Therefore, we have made an effort to prepare human small intestinal cells in order to have a better model for adherence studies. We have established a protocol for the use of intestinal cells from ileostomy patients. Our volunteers have included a 52 year old woman with a history of Crohn's Disease. She has a mature ileostomy (six years) and takes no medication. Our other volunteer is a 34 year old woman who has had her ileostomy for four years. Briefly, the ileostomy is perfused with 250-300 ml of saline. The return perfusate contains approximately 10^5 columnar cells/ml. Radiolabeled ETEC test strains are used to test for adherence with the intestinal cells. The "buccal cell" filter assay protocol is followed.

i. Preservation of Human Ileal Cells.

Our initial efforts involved the preservation of ileal cell morphology and

functional binding characteristics. These studies used hematoxylin-eosin and hematoxylin-periodate-Schiff staining of the isolated ileal cells as a function of storage time in various buffers. We determined the binding ability of human ETEC strains to the ileal cell preparations, and the data are summarized in Table 12. Briefly, neither Dulbecco's, Hank's salts solution, nor tissue culture medium elevated initial binding or protected the cells during centrifugation. The addition of extra NaCl (Providing a hypertonic support medium) did not significantly change the bacterial attachment. The best binding was observed in the buffer containing gelatin, glucose, HEPES and NaCl described in Table 13. The addition of fetal calf serum decreased the initial binding but seems to have resulted in less cell loss during centrifugation. The presence of fetal calf serum during the initial isolation resulted in less degradation morphologically, possibly because of proteolytic enzyme inhibitors present in serum, and more initial binding although there is still presumably some fetal calf serum interference with binding. Our present procedure is to lavage the small intestine through the ileostomy stoma with phosphate buffered saline (PBS). The PBS-containing ileal cells is then removed and added immediately to chilled fetal calf serum and concentrated (10X) buffer. Morphological features of the isolated ileal cells are shown in Figure 12.

ii. Ileal Cell Preparation

The ileal cell suspension at this stage represents a heterogenous cell population with at least two major cell populations. The physically largest population represents masses of cells embedded in sheets of mucus, and they can be removed by centrifugation at very low speed (45 RPM, ea 3xg) for 15 minutes. The ileal cells remaining in the supernatant are then removed using a 600 xg centrifugation. However, if the mucus entrapped material is not removed first, the isolated cells and mucus sheets will be forced together, resulting in a solid mucus mass which can not be resuspended for use in the binding assay. When separated, both of these preparations are active in the binding assay (Table 14).

iii. Binding of ETEC and Control Strains to Human Ileal Cells.

The various ETEC strains tested can be grouped by their ileal cell binding (Table 15). Strains isolated from humans such as B2C and 334 generally bind well while control srrains such as 334LL (a non-toxigenic, pili-free derivation of 334) does not bind. Other human ETEC strains such as TX1, M9800-5, and D542, all of which are recognized by one of our antisera, also bind but show greater variability from day to day and a generally lower absolute level of attachment. Strains which are not recognized by our antisera including H410C1, recent isolate from Honduras, also bind. Generally H410C1 exhibits high level binding (equivalent to B2C) reproducibly. Curiously, the human strains H10407 and its derivative H10407P have not exhibited reproducible binding, although they have maintained their MR-HA characteristics. The rabbit pathogen, RDEC-1, does not bind, nor do K99-producing strains, including B44 and 1129R. However, two K88 strains, P307 and G205, do exhibit adhesion in our assay. Additional K88 strains are being investigated, and preliminary data indicate that ileal cell attachment is not observed with other K88 strains.

SECTION 5. Genetic Studies of Plasmids Controlling Production of Adherence Antigens

a. Cloning of DNA Controlling Pili Production

During the past year, we have attempted to clone DNA controlling pili production onto plasmid pBR322 using restriction-ligation techniques. This relaxed plasmid, 2.6 x 10^6 daltons, contains a unique PstI site within an ampicillin-resistance gene and four other unique restriction sites (EcoR1, Hind III, Bam HI, and Sal I) in the tetracycline-resistance gene (Figure 13).

The plasmid is sensitive to col E1, and the antibiotic resistance genes on pBR322 are not transposable. Briefly, preparations of vector plasmid DNA (pBR322) and plasmid DNA from a pili-producing ETEC strain are treated with a single restriction enzyme. At the present time, we are employing Bam HI, although with this vector four other restriction endonucleases can be used. Bam HI was chosen because it is know to cleave outside the K99 region (S. Falkow, personal communication). Restriction enzyme cleavage products are then incubated with T_4DNA ligase, and used to transform E.coli recipient strain JE2571 (pil, fla, str, leu, thr), a

strain which does not produce flagella or pili, is streptomycin resistant, and requires leucine and threonine for growth.

In experiments, transformed colonies were selected on ampicillin (25 µg/ml) containing medium and screened for tetracycline sensitive clones which would result from insertion of cloned DAN into the Bam H1 site. Very recently, a piliated transformant was isolated from an experiment using ligation products of Ban H1 treated pBR322 the vector plasmid. This transformant called JE(D542:p3R322) has the phenotypic markers outlined in Table 16. This strain was found to cause a weak MR-HA reaction with human type A blood. Upon electron microscopic study, it produced fine piliate structures identical in size to those of the parental ETEC strain D542. Electron micrographs of these two strains and of JE2571 the fla pil recipient strain are given in Figures 14, 15, 16.

Unfortunately, preliminary restriction endonuclease characterization of this hybrid plasmid have provided confusing results. The agarose gel shown in Figure 17 contains the plasmid vector pBR322 before and after BAMH1 treatment (lanes 3 and 4). Treatment of the "hybrid" plasmid did not release a fragment of the molecular size anticipated by a "normal" insertion into the BAMH1 site of pBR322. In fact, this fragment produced by BAMH1 treatment of the hybrid is closer in molecular size to BAMH1 cleaved pMB8:AP3 (lane 10), which is 5 x 10^6 daltons.

Further restriction endonuclease analysis is given in Figure 18. The vector pBR322 contains single sites for five restricton endonucleases; its single PST 1 fragment is shown in Figure 18, lane 10. The "hybrid" plasmid was not affected by Sal I or Hind III treatment. EcoR1 treatment reveals a single fragment; following Pst1 treatment, four fragments were released. Proof that this plasmid resulted from a hybridization of DNA fragments from ETEC strain D543 + pBC400 (pBR322) awaits further hybridization analysis using the Southern "blot" technique.

Should this hybrid prove to share sequences with parental plasmids, sub-cloning experiments will be performed (the PstI fragment pattern Figure 18, lane 5 would be an appropriate step in sub-cloning). If the correct homologies are proven, the piliated hybrid plasmid containing strain will be tested for production of LT and ST enterotoxins, adherence to human buccal cells, and human ileal cells, and undergo serologic classification of its pili. At present, the JE2571 strain agglutinates in normal rabbit sera. The three pili specific sera are currently being absorbed with JE2571 so that the "hybrid" plasmid containing transformant can be tested. Long range plans include cloning the pili genes from prototype ETEC strains producing serologically different pili and comparing these pili genes and plasmids for areas of DNA homology.

b. Attempts to Insert Tn3 onto Cryptic Plasmids Found in ETEC Strains

Efforts have continued over the past year to insert transposable antibiotic resistance onto the cryptic plasmid(s) suspected of involvement in production of adherence pili. As described in methods, plasmid pMB8:Tn3 has a strict dependence upon the polymerase I function of E. coli. This property can be exploited in a technique of "marking" cryptic plasmids. Transposition of Tn3 from pMB8:Tn3 normally occurs at frequencies of about 6×10^{-4} per F+ transconjugant.

In our laboratory, various piliated ETEC strains (334,193-4, M9800-5, D543, were transformed with plasmid pMB8:Tn3. The resulting ampicillin-resistant ETEC strain was then (1) checked for presence of previous plasmid-band pattern + pMB8:AP3 band and (2) ability to cause MR-HA reactions. Plasmids were then isolated from these strains and used to transform E. coli PolA strain, or conjugation experiments were performed in order to detect transposition of Tn3

from pMB8:AP3 to a cryptic plasmid. To date, we have failed to detect a transposition event using the pMB8:AP3-PolA system. In the future we will use the phage λb221 rex::Tn5cI857 system described by Dr. D. Berg. This λ phage contains a transposable kanamycin element Tn5. We are hopeful that this technique will be more fruitful. Approximately 10^{-2} of cells infected with λTn5 phage are transduced to Kan^R. Hopefully with this high rate, cryptic plasmids in piliated ETEC strains will pick up the kanamycin element.

TABLE 1A

ETEC ADHERENCE ANTIGENS

TYPE:	ADHERENCE PILI	CFA/I	CFA/II
MR-HA	Serogroup 1,2,3,		
RBC Type	Human A, B $^{\pm}$	Human A^{+}	Bovine $^{+}$
	Bovine $^{\pm}$	Bovine $^{+}$	
	Guinea pig $^{\pm}$	Chicken $^{+}$	
MOLECULAR Weight:	13,00	23,800	?
	12,500		
SIZE:	5-10nm	7nm	
TISSUE:	Human buccal	Rabbit	Rabbit
	Mucosa	Intestine	Intestine
ENTEROTOXIN(s)	LT/ST	LT/ST	LT/ST
	ST^{+}	ST^{+}	
O-Group:	06, 08, 015	06, 08, 015	06, 09
	025, 027, 078	025, 078	085
	0128, 0148, 0149		

TABLE 1

ESCHERICHIA COLI STRAINS EXAMINED

Human Strains	Origin	Serotype	Enterotoxins Status	MR-HA[2] AB	GP	BOV	Pili[3] Serogroups
334	AD India	015:H11	LT/ST	+	+	+	1
334LL	LP	015:H11	-	-	-	-	NR
193-4	AD India	N.T.	LT/ST	+	+	+	1
H10407	AD India	078:H11	LT/ST	+	-	-	1,3
H10407P	LP	078:H11	LT^+	-	-	-	NR
Tx-1	ID Texas	078:K80:H12	ST	+	-	+	2,3
Tx-85	ID Texas	078:k80:H12	ST	+	-	+	2,3
B2C	AD Viet Nam		LT/ST	-	-	-	2
B7A	Ad Viet Nam		LT/ST	-	-	-	NR
214-4	AD MD		ST	-	-	-	NR
K108c3	AD Kenya	-	LT/ST	-	-	-	
K324c1	AD Kenya	08:060:H9	LT/ST	-	+	-	2
K344c2	AD Kenya	-	LT	-	-	-	NR
K130c1	AD Kenya	-	LT/ST	-	-	-	NT
K135c2	AD Kenya	-	-	-	-	-	2
K325c3	AD Kenya	-	-	-	-	-	2
K326c5	AD Kenya	025:h42	LT/ST	+	-	-	2
K328c4	AD Kenya	-	-	-	-	-	NT
K325c1	AD Kenya	-	LT	-	-	-	NT
TD462c1	AD Mexico	06:H16	LT/ST	-	-	-	2,3
TD260c1	AD Mexico	06:H16	LT	-	-	-	2
TD514c1	AD Mexico		-	-	-	-	NT
TD412c1	AD Mexico		LT/ST	-	-	-	NT
TD514c2	AD Mexico		-	-	-	-	NT

TABLE 1 CONTINUED

Human Strains	Origin [1]	Serotype	Enterotoxins Status	MR-HA [2] AB	MR-HA [2] GP	MR-HA [2] BOV	Pili [3] Serogroups
TD427c2	AD Mexico		LT	-	-	-	NT
TD213c2	AD Mexico	0128	ST	+	-	-	?
TD234c4	AD Mexico		LT	-	-	-	NT
TD219c1	AD Mexico	06:H16	ST	+	-	-	3
TD451c2	AD Mexico		LT/ST	-	-	-	NT
TD327c2	AD Mexico	05	-	-	-	+	2
M9800-5	USA(Crater Lake)	06: K15:H16	ST	-	-	+	2
SS34560	USA(Crater Lake)	06:H16	ST	-	-	-	2
SS34561	USA(Crater Lake)	06:H16	ST	-	-	+	2
D370855	Dacca	?	LT/ST	+	-	+	2,3 neg
D444	Dacca	?	LT/ST	-	-	+	2
D563	Dacca	?	LT/ST	+	-	+	3
D513	Dacca	?	LT/ST	-	-	-	2
D542	Dacca	?	LT/ST	+	-	+	3
D481	Dacca	?	LT/ST	+	-	+	3
D280551	Dacca	?	LT/ST	+	-	-	2
D370844	Dacca	?	LT/ST	+	-	+	2,3 neg
D524	Dacca	?	LT/ST	+	-	+	2
D280561	Dacca	?	LT/ST	-	+	-	2,3 neg
TD235C4	AD Mexico	?	LT	-	-	-	NR
TD425C2	AD Mexico	?	LT	-	-	-	NR
M403C3	Morocco		ST	-	-	-	
M409C1	Morocco		ST	-	-	-	

TABLE 1 CONTINUED

1. AD - adult diarrhea

   LP - lab passage

   ID - infant diarrhea

2. Mannose-resistant hemagglutination of washed human A, B, guinea pig and bovine RBC's at $4^{o}C$.

3. NR - non-reactive in antisera

   NT - not typable due to auto-agglutination.

ETEC ISOLATES PROVIDED BY DR. I. KAYE WACHSMUTH

of THE CDC. ATLANTA, GA.

THE ORIGIN OF THESE STRAINS IS DETAILED IN TABLE

STRAINS	ORIGIN	SEROTYPE	TOXINS	MR-HA AB	MR-HA GP	MR-HA BOV	Pili SEROGROUPS
CDC-69-2707	Maryland	O15: H11	LT/ST	+	+	-	1
CDC-70-5206	New Mexico	O128: H21	ST	+	+	-	1
CDC-70-5610	Dacca	O15: H11	LT				
CDC-70-5726	Pakistan	O78: H11	LT	-	-	-	NR
CDC-70-5203	New Mexico	O128: H21	ST	+	+	-	1
CDC-70-5727	Pakistan	O78: H11	LT	-	-	-	1
CDC-70-5605	Dacca	O20: H -	LT/ST				
CDC-70-5729	Dacca	O15: H11	LT	-	-	-	1
CDC-72-5467	Washington, D.C.	O6: H16	ST	-	-	-	2
CDC-72-5460	Washington, D.C.	O6: H16	LT/ST	-	-	-	2
CDC-73-1119	California	O79: K80: H12	LT/ST	-	-	-	
CDC-73-0562	?	O27: H20	ST	-	-	-	NR
CDC-77-1781	?	O25: K98: H -	LT	-	-	-	NR
CDC-77-1780	Mexico	O25: K98: H -	LT	-	-	-	NR
CDC-69-5400	?	O15: H11	ST	+	+	+	1,3
CDC-70-0423	Vietnam	O148: H28	LT/ST	-	-	-	
CDC-71-1694	Foreign	O27: H20	ST	-	+	-	2
CDC-72-5448	?	O148: H28	LT/ST	-	-	-	2
CDC-72-1782	Foreign	O27: H20	ST	-	-	-	2
CDC-72-1943	?	O27: H20	ST^+LT?	-	-	-	
CDC-72-2513	Massachusetts	O6: H16	ST^+LT^+	-	-	-	2,1
CDC-73-0563	?	O27: H20	ST^+	-	-	-	2

STRAINS	ORIGIN	SEROTYPE	TOXINS	MR-HA AB	MR-HA GP	MR-HA BOV	Pili Serogroups
CDC-73-2694	?	O149: H7	ST^+	-	-	-	1?
CDC-76-1086	Arizona	O15: H -	LT/ST	-	-	-	-
CDC-76-0100	?	O78: K80: H12	(ST^+) *	-	-	-	
CDC-77-2520	Louisiana	O128: H7	(ST^+) *	-	-	-	1
CDC-77-2521	Louisiana	O15: H -	LT/ST	-	-	-	1
CDC-77-3502	?	O15: H11	LT/ST	+	+	+	1
CDC-77-2368	North Dakota	O128: H7	(ST^+) *	-	-	-	?
CDC-77-1782	Cruise Ship	O148: H28	ST/LT	-	-	-	2
CDC-77-2417	Massachusetts	O15: H11	ST/LT	-	-	-	1,3
CDC-79-1203	Cruise Ship	O25: H -	LT	-	-	-	

1. NR - non-reactive in antisera

* K.W. - reported these cultures ST negative upon retesting

TABLE 3.

ETEC ISOLATES FROM CASES OF HUMAN DIARRHEAL DISEASE

IN HONDURAS

(PROVIDED BY DR. R. B. SACK, BALTIMORE, M.D.)

STRAIN	-TOXINS-	MR-HA AB	MR-HA GP	MR-HA BOV	Pili Serogroup
H 326 $_{c1}$	LT/ST	+	-	+	
H 410 $_{c1}$	LT/ST	+	-	+	
H 415 $_{c1}$	LT	-	-	-	
H 150 C6B	LT	-	-	-	1,3
H 109 C6I	LT	-	-	-	
H 426 $_{c2}$	LT	-	-	-	
H 556 $_{c11}$	LT	-	-	-	
H 142 C5A	ST	-	-	-	
H 439 $_{c3}$	LT	-	-	-	
H 326 $_{c2}$	LT	-	-	-	3
H 111 C6A	LT	-	-	-	
H 341 $_{c5}$	LT	-	-	-	2
H 218 $_{c5}$	ST	-	-	-	2
H 326 $_{c3}$	ST	+	-	+	3
H 449 $_{c3}$	ST	-	-	-	

TABLE 4

ETEC ISOLATES FROM CASES OF HUMAN DIARRHEAL

IN ETHIOPIA AND SWEDEN

(STRAINS PROVIDED BY DR. TORKEL WADSTROM, UPPSALA SWEDEN)

TRAIN	ORIGIN	SEROTYPE	ENTEROTOXINS	HUMAN AB,	GUINEA PIG,	BOVINE	PILI SEROGROUP
922f	Sweden	O6: K15:M	LT/ST	-		-	2
921d	Sweden	O6: K15:M	LT/ST	-	-	-	2
5-8	Ethiopia	O78:KN:NM	LT/T	+		-	NT[b]
30	Ethiopia	O6:K15:M	LT/ST	+		+	2
016-10	Ethiopia	ND:M	ST^+	+	-	-	NT
016-16[a]	Ethiopia	ND:M	-	-	-	-	NT
528-15	Ethiopia	O78:KN:NM	LT/ST	+		+	3
271a	Ethiopia	O6:K15:H^-	LT/ST	-		-	2
4e-14	Ethiopia	O78:KN:NM	LTST	+		-	NT

[a] Strain 2016-16 is an enterotoxin negative, MR-HA^-, spontaneous derivative of strain 2016-10.

[b] NT = non-typable, strains spontaneously agglutinate.

TABLE 5

E. COLI CONTROL STRAINS

Animal Origin	Serotype	MR-HA[1] AB	GP	BOV	Pili Serogroups
RDEC-1 (Ovine diarrhea)	?	-	-	-	NR
P307 (Porcine diarrhea)	-K88[+]	-	+	-	NR
LAB DERIVED					
K 12					NT
K 12 K88	K88	-	+	-	NT
K 12 K99	K99	-	-	-	NT
ADULT FECES (Non-TOXIGENIC)					
H10405 (LT ? Klipstein)	?	-	-	-	NR
HS	?	-	-	-	NR
CD-T	?	-	-	-	NR
Ec #1	?	-	-	-	NR
#7	?	-	-	-	NR
#8	?	-	-	-	NR
#9	?	-	-	-	NR
#10	?	-	-	-	NR
#11	?	+			NR
#12	?	-	-		NR
#13	?	+			NR
#14	?	-	-		NR
#15	?	-	-		NR
#16	?	-	-		NR

TABLE 5 CONTINUED

Animal Origin	Serotype	MR-HA[1] AB	GP	BOV	Pili Serogroups[2]
ADULT FECES (cont'd)					
#17	?	+			NR
#18	?	-	-		NR
#19	?	-	-		NR
#20	?	+			NR
#21	?	-	-		NR

1. Mannose-resistant hemagglutination of washed human A, B, guinea pig and bovine RBC's at 4°C.

2. NR - non-reactive in antisera

   NT - not typable due to anto-agglutination.

TABLE 6

TESTING OF ETEC STRAINS FROM PANAMA FOR ADHERENCE ANTIGENS

STRAIN	MR-HA REACTION			Pili Serogroup Reactivity
	Human A	Bovine	Guinea Pig	
A-31-12	−	−	+ω	
A-33-12	−	−	−	
A-39-1b	−	−	−	1
A-40-1c#1	−	−	+ω	
Ac50b	−	+	+	1
B-14-1d	−	−	−	
BV-132	−	−	−	
BV-48b	−	−	−	
C-5-2b	−	−	−	
C-15-1f	−	−	−	
C-28-9	−	−	+ω	
C-39-1c	−	−	+ω	
Pc-48c hly	−	+	+	
PC-48c non-hly	−	−	−	
PC 60a	+	+	+	2
PC 61f	+	+	+	2
PC 61d hly	−	+	+	
PC 61d non-hly	−	+	+	

1. MR-HA reaction: Mannose resistant hemagglutination reactions were tested in PBS + 1% mannose at 0°C.

2. Pili Serogroup Reactivity: Bacterial suspensions were tested with serial dilutions of the three pili-specific antisera. Postive reactions were scored for titer ≥512. Negative reactions with heterologous sera or normal rabbit sera (control) were ≤ 128.

TABLE 7

REACTION OF 84 ETEC STRAINS WITH PILI SPECIFIC ANTISERA

PILI SEROGROUPS	NUMBER OF STRAINS	%
Group 1	12	14.3
Group 2	24	28.6
Group 3	7	8.3
Group 1,3	4	4.8
Group 2,3	6	7.1
Total Pili Reactive	53	63.1
Total Negative	31	36.9
Total	84	100.0

TABLE 8

O GROUPS OF ETEC STRAINS WITH ADHERENCE PILI

Pili Serogroup	Number of Strains	O Group		
		06,08	015	Other
1	12	0	7	078,0128,0149,NT
2	24	7	0	025,027,0128,0148,NT
3	7	1	0	0128,NT
1,3	4	1	1	078
2,3	6	1	0	078,NT
Negative	31	0	2	020,025,027,078,0128, 0148,NT

TABLE 9

ENTEROTOXIN PROFILES OF ETEC STRAINS

	ENTEROTOXINS		
PILI SEROGROUPS	LT/ST	LT	ST
Group 1	6	2	4
Group 2	12	4	8
Group 3	3	1	3
Group 1/3	2	1	1
Group 2/3	4	0	2
Negative	8	15	8
TOTALS	35 (41.7%)	23 (27.4%)	26 (30.9%)

TABLE 10

ETEC TESTED FOR MR-HA WITH RED CELLS

FROM: HUMAN A, B, BOVINE, GUINEA PIG

		MR-HA	
		Postive	Negative
PIli Serogroup	1	6	6
	2	8	16
	3	6	1
	1,3	2	2
	2,3	4	2
Unreactive		5	28
		31	53

TABLE - 11

Geographic Origin of ETEC Isolates Examined for Presence of 3 Serologic Pili Types

Country	Pili Serological Groups					(NT,NR)
	Type 1	Type 2	Type 3	Type 1,3	Type 2,3	negative
U.S.A.	5	2		1	2	4
"Cruise Ships"		1				1
Mexico		2	1		1	3
Honduras		2	2	1		8
Sweden		2				0
Morocco						2
Kenya		4				1
Ethiopia		2	1			4
India	2			1		
Pakistan	1					1
Bangladesh	2	4	3		3	
Vietnam		1				2
"Foreign"		2				
Unknown	2	2		1		5
TOTALS	12	24	7	4	6	31

TABLE 12 EFFECT OF VARIOUS BUFFERS ON ETEC BINDING TO HUMAN ILEAL CELLS

Buffer	CPM bound by ileal cells	
	before centrifugation	after centrifugation
DBSS	1095	1111
HBSS	1736	414
TC media	719	0
TC media + 1% NaCl	894	0
TC media + 2% NaCl	1148	59
1x Goop	5022	3089
10% FC 1x Goop	2468	2038
10% FC in 1x Goop[1]	6435	2502

CPM bound of ETEC strain B?C background (binding without ileal cells) has been subtracted. Buffers: DBSS = Dulbecco's balanced salts solution without Ca^{++} and Mg^{++}; HBSS = Hank's balanced salts solution containing both Ca^{++} and Mg^{++}; TC media = MEM with Earle's salts: 1x Goop buffer described in Table 2; 10%FC - 1x Goop buffer described in Table 1 containing 10% heat inactivated fetal calf serum.

[1]ileal cells collected in fetal calf serum

TABLE 13 ILEAL CELL ISOLATION BUFFER pH 7.2

gelatin	0.01%	0.1 g/l
HEPES	25 mM	
glucose	1%	10 g/l
NaCl	0.9%	9 g/l

TABLE 14 BINDING BY ILEAL CELLS PREPARED BY TWO DIFFERENT PROCEDURES

Strain [1]	Centrifugation Speed: 45 RPM, 15 min (∿3xg) CPM	45 RPM, 15 min (∿3xg) # bacteria bound	600 RPM, 10 min (∿600xg) CPM	600 RPM, 10 min (∿600xg) bacteria bound
B2C	17,009	5.01×10^6	33,348	9.83×10^6
334	10,327	1.49×10^7	18,829	2.69×10^7
334LL	898	4.72×10^5	3,289	1.73×10^6
P307	171	6.81×10^5	1,243	4.93×10^6
G205	1,758	1.12×10^7	3,405	2.17×10^7
RDEC-1	0	0	8	4.09×10^4

[1]. Strains

B2C	human ETEC with serogroup 2 adherence pili
334	human ETEC with serogroup 1 adherence pili
334LL	plasmid-free laboratory derivative, lacks serogroup 1 pili
P307	pig pathogen K88 ab+
G205	pig pathogen K88 ac+
RDEC-1	rabbit pathogen

TABLE 15 Binding of ETEC and Control Strains to Human Ileal cells[1]

ETEC Strain	Adherence Antigens	Experiment 1 CPM bound	Experiment 1 bacteria bound x10^5	Experiment 2 CPM bound	Experiment 2 bacteria bound x10^5
B2c	2	7637	876	17,009	501
334	1	3345	390	10,328	149
334LL	none	27	3	899	5
P307	K88	4399	336	172	7
G205	K88	0	0	1758	112
TX-1	2+3	0	0	321	96
H410c1	uncharacterized pili	1866	147	28,617	339
H10407	1+3, CFA/I	3002	217	8,808	96
H10407P	none	0	0	6,104	64
1129R	K99	187	18	73	8
B44	K99	0	0	690	8
M9800-5	2	7211	53	6986	977
RDEC-1	uncharacterized pili	417	28	0	0
D542	3	_	_	1677	17

1. Ileal cells were prepared by the 45 RPM, 15 min. method
2. Adherence antigens: 1, 2 and 3 are serogroups of attachment pili we have described, CFA/I is the pili described by Evans et al (51). K88 and K99 are animal pathogens. Uncharacterized pili includes strains where pili have been visualized by electron microscopy, but which have not been fully characterized.

TABLE 16

Phenotypic traits of transformant

JE2571 (D542: pBR322) and recipient JE 2571

Phenotype	Strains	
	JE2571	JE 2571 (D542: pBR322)
Amp	S	R
TC	S	S
Sm	R	R
API TESTS		
ONPG	+	+
ADH	-	-
LDC	+	+
ODC	-	-
CIT	-	-
H_2S	-	-
URE	-	-
TDA	-	-
IND	+	+
VP	-	-
GEL	-	-
GLU	+	+
MAN	-	-
INO	-	-
SOR	+	+
RHA	+	+
SAC	-	-
MEL	-	-
AMY	-	-
ARA	+	+

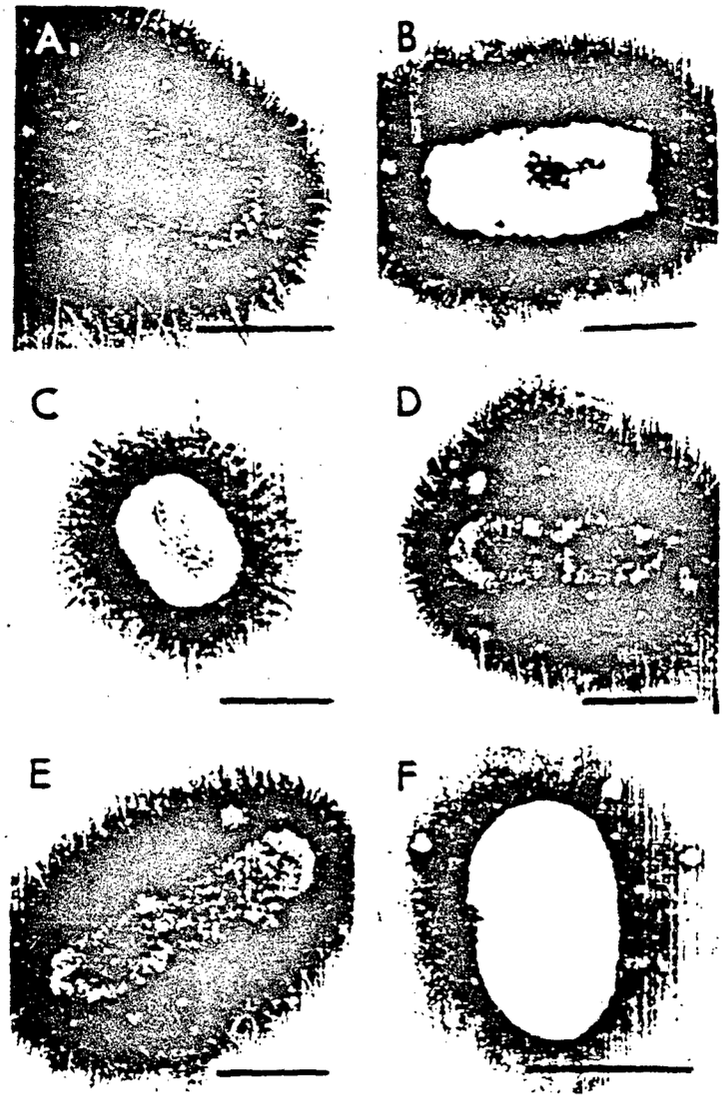


Figure 1. Five strains of toxigenic E. coli and one non-enterotoxigenic strain negatively stained with 2% phosphotungstic acid. A. 334; B. 193-4; C. D563; D. D481; E. TX-1; F. 334LL (negative control). Attachment pili are small,5-10 nm, needle-like structures. Bar = 1.0μ.

Figure 2. Pili preparation of ETEC strain 193-4 stained with 4% uranyl acetate. Bar = 1.0μ.

pili preparations were cross-linked with N, N^1-p phenylenedimaleimide.

A. Well 1, anti334 pili; Well 2, anti M9800-5 pili; well 3, M9800-5 pili; well 4, 334 pili

B. Well 1, anti D542 pili; well 2, anti M9800-5 pili; well 3, M9800-5 pili; well 4 D542 pili.

C. Well 1, anti 334 pili; well 2, anti D542 pili; well 3, D542 pili; well 4, 334 pili.

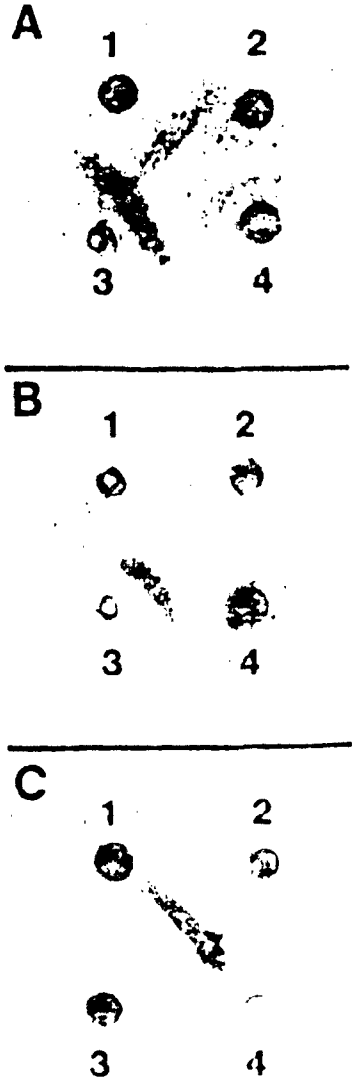


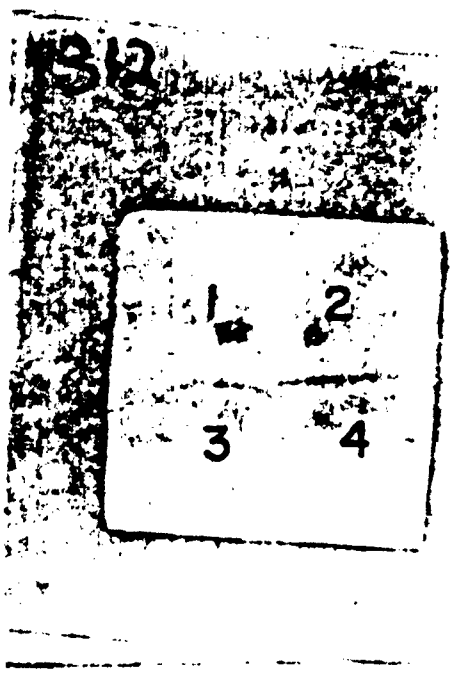
FIGURE 4.

Ouchterlony immunodiffusion analysis of an H10407 pili preparation, and antisera raised against isolated pili of serogroups one and three.

Well 1 and 2. H10407 pili prepared by ammonium sulfate precipitation

Well 3 Antiserum to 334 pili (334 pili, serogroup 1)

Well 4 Antiserum to D542 pili (D542 pili, serogroup 3)



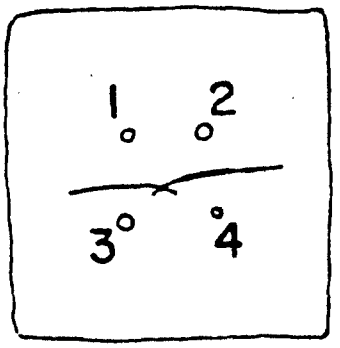


FIGURE 5.

Ouchterlony immunoprecipitation reactions of various pili antisera with purified CFA/I pili.

Center well. Purified CFA/I pili*

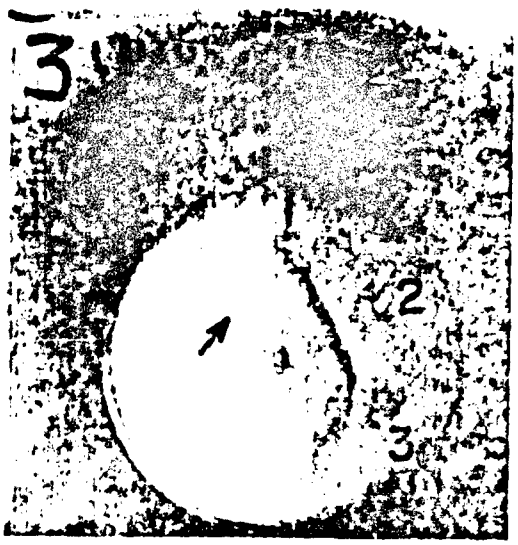
Well 1. Antiserum to 334 pili (334 pili, serogroup 1)

Well 2. Absorbed antiserum against ETEC, H10407 (donated by Dr. M. Levine)

Well 3. Antiserum to D542 pili (D542 pili, serogroup 3)

*Prepared from ETEC strain H10407 by ammonium sulfate precipitation and DEAE-Sephadex chromatography.

Arrow points to faint precipitation line between CFA/I and antiserum to 334 pili



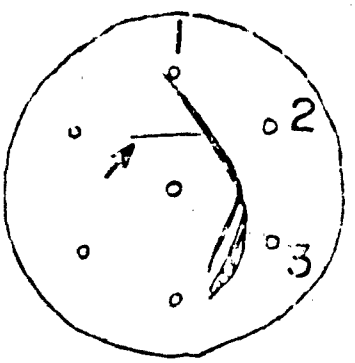
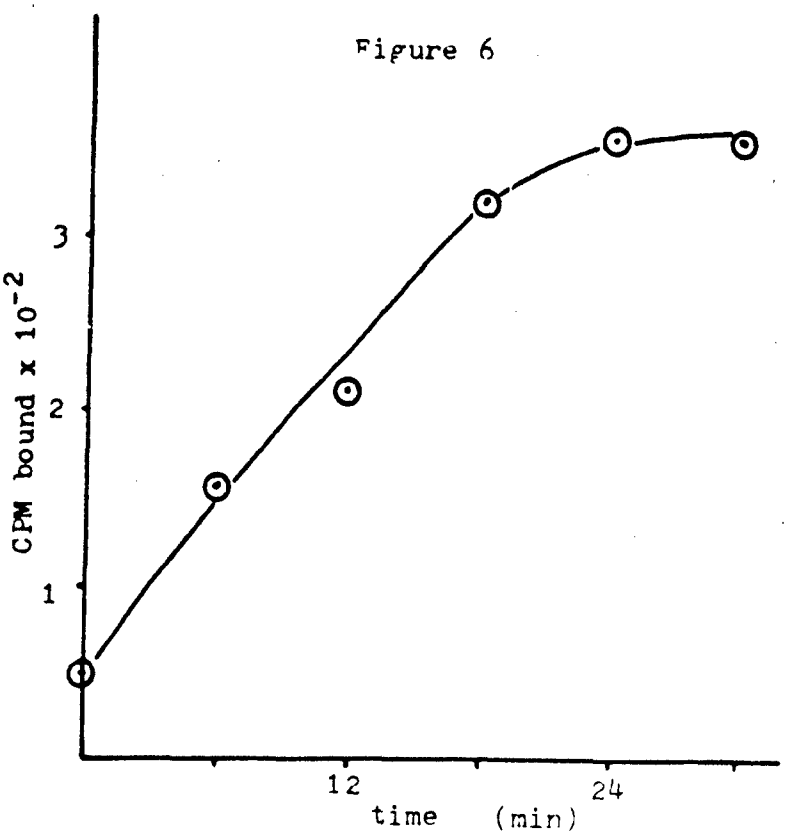


Figure 6. Time course of attachment of labeled pili to human buccal cells. Isolated pili were labeled with ^{3}H-N-ethyl maleimide, and free label removed by gel filtration with BioGel P-6. After incubation for various periods of time at 37C, the bound and free pili were separated by centrifugation.



FIGURES 7 AND 8. Attachment of labeled pili to human buccal cells. Buccal cells and labeled pili were mixed for 30 minutes at 37 . Then unbound pili were removed by centrifugation (microfuge, 30 sec.) and washing twice. The final buccal cell precipitate was resuspended in water, transferred to scintillation vials and radioactivity determined by liquid scintillation counting. Microfuge tubes were prewashed with 0.01% bovine serum albumin to decrease loss of pili onto the wall of the tube.

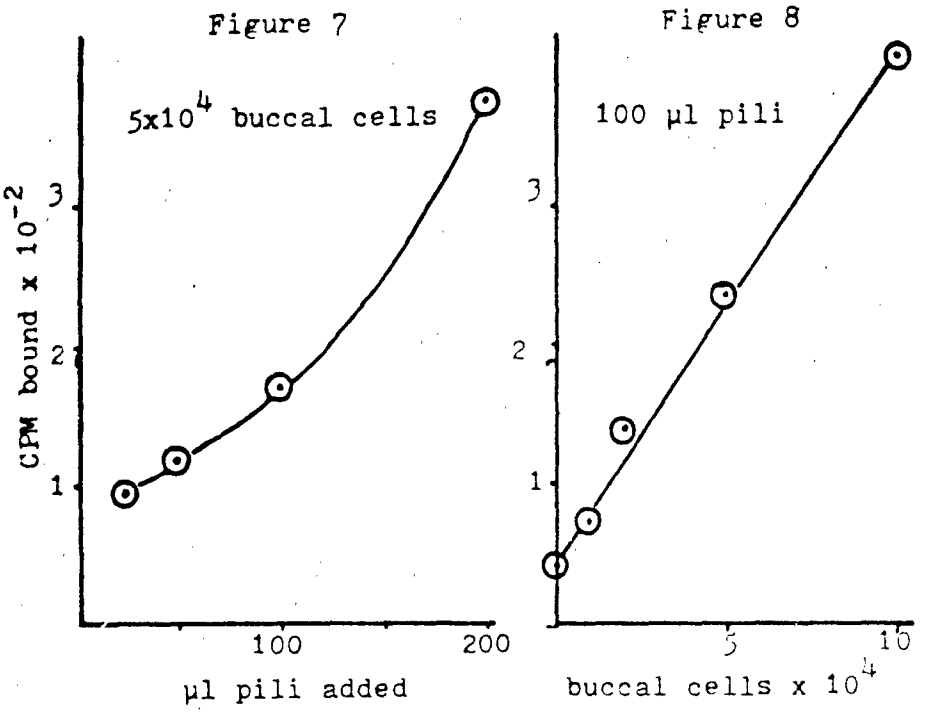


Figure 9 Scanning electron micrograph of human buccal cells with attached ETEC strain 334 (x 1500)

Figure 10 Scanning electron micrograph of human buccal cells with 334LL (toxin$^-$, MR-HA$^-$ derivative of ETEC strain 334). (x 1500)

Figure 11 Scanning electron micrograph of human buccal cells with attached ETEC 334 (x 20,000)

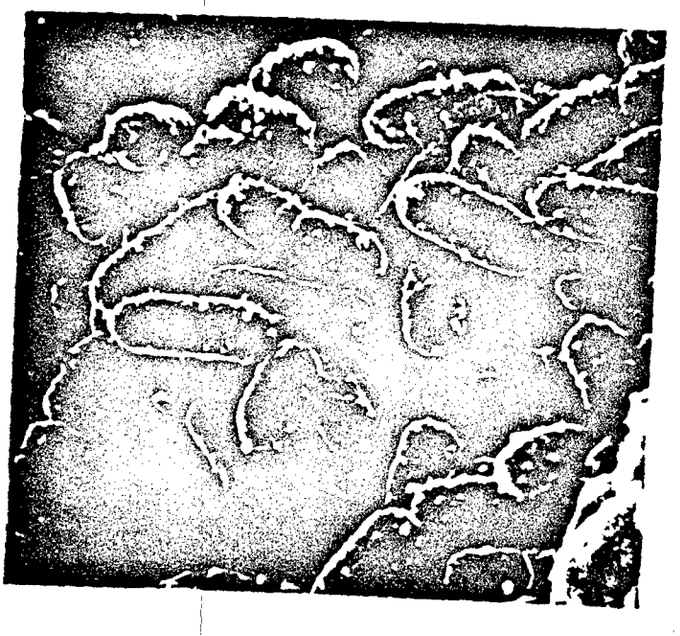
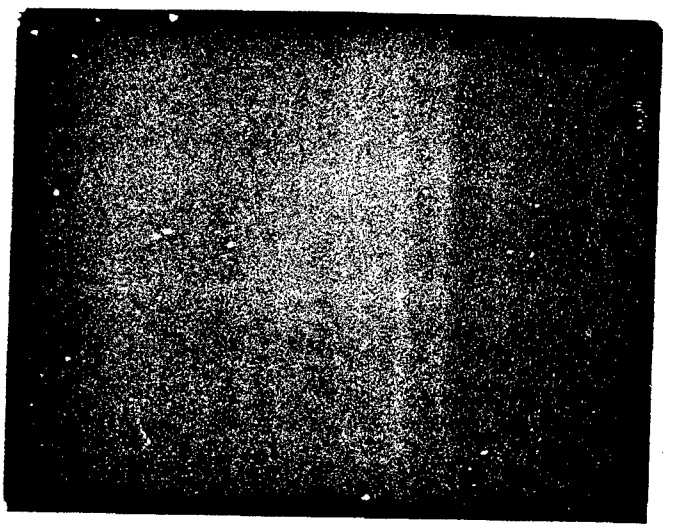


FIGURE 12

Light Microscopy of isolated human ileal cells fixed in phosphate buffered formaldehyde, collected on millipore filters, and stained with hematoxalin-periodate Schiff.



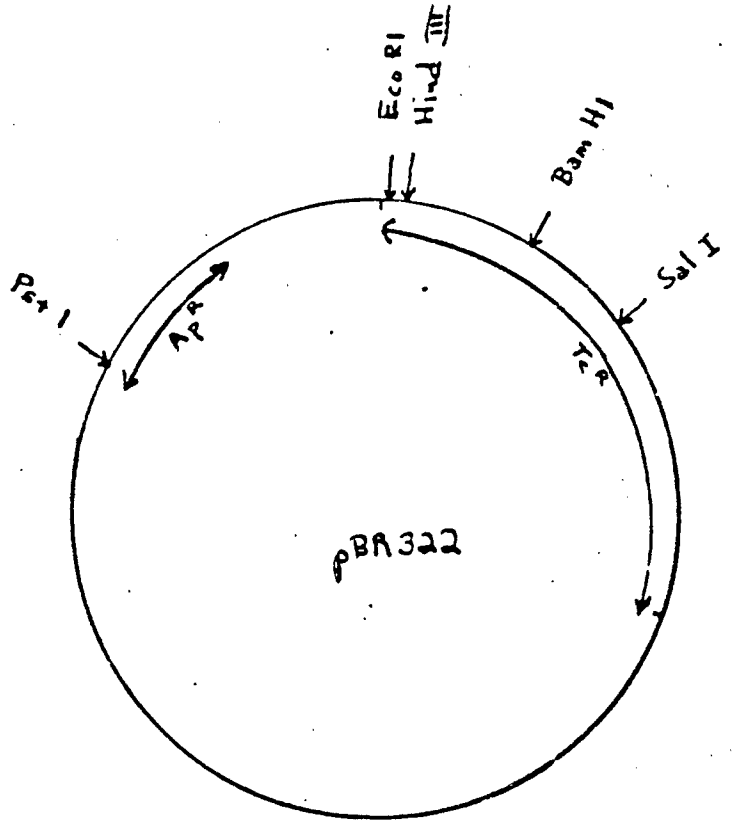


Figure 13 The circular restriction map of pBR322.

The ampicillin and tetracycline resistance genes are indicated by Ap^R and Tc^R respectively. The relative positions of the restriction sites for the 5 unique enzymes are shown.

The circular Restriction map of pBR322 p. 687. In: DNA, Insertion elements, plasmids and episomes. (ed) AI Bukhari, JA Shapiro, SL Adhya. Cold Spring Harbor Laboratory, 1977.

Figure 14. Electron micrograph of *E. coli* strain JE 2571 (D542: pBr322) negatively stained with 2% phosphotungstic acid (40,000x)

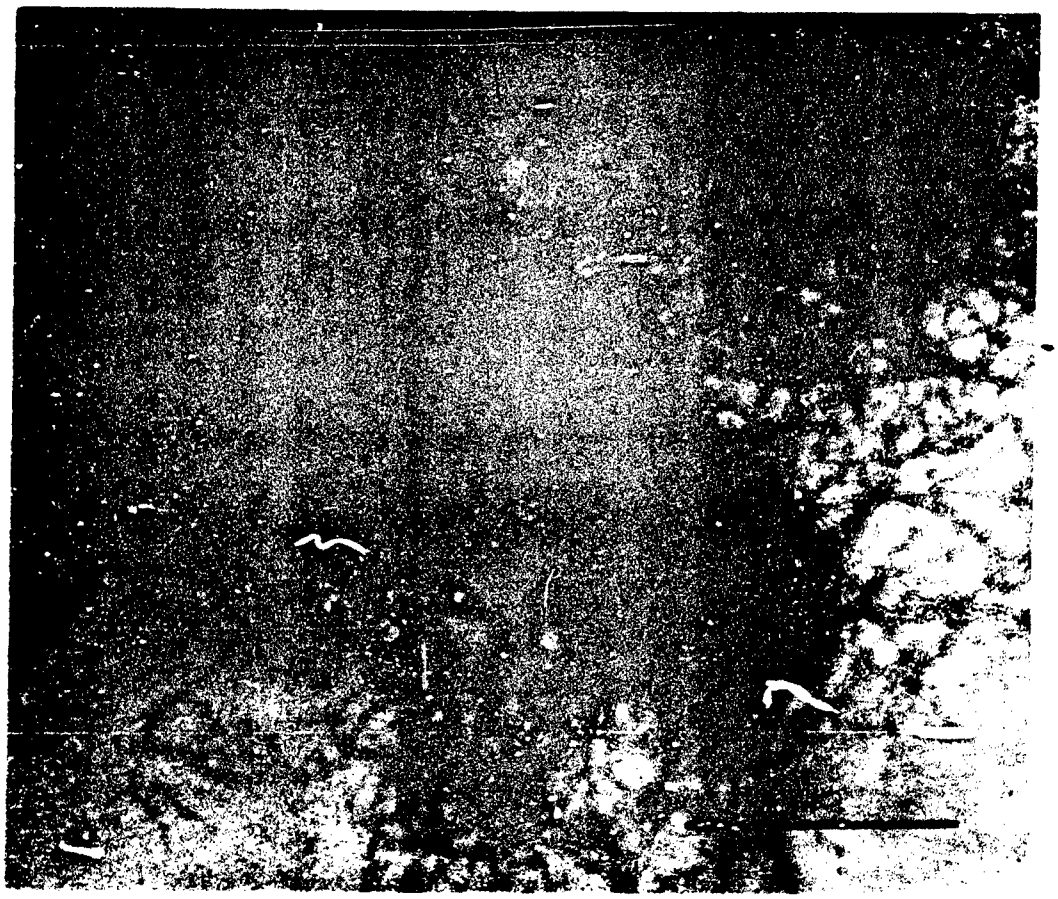


FIGURE 15. Electron micrograph of ETEC strain D542 negatively stained with 2% phosphotungstic acid (125,000x)

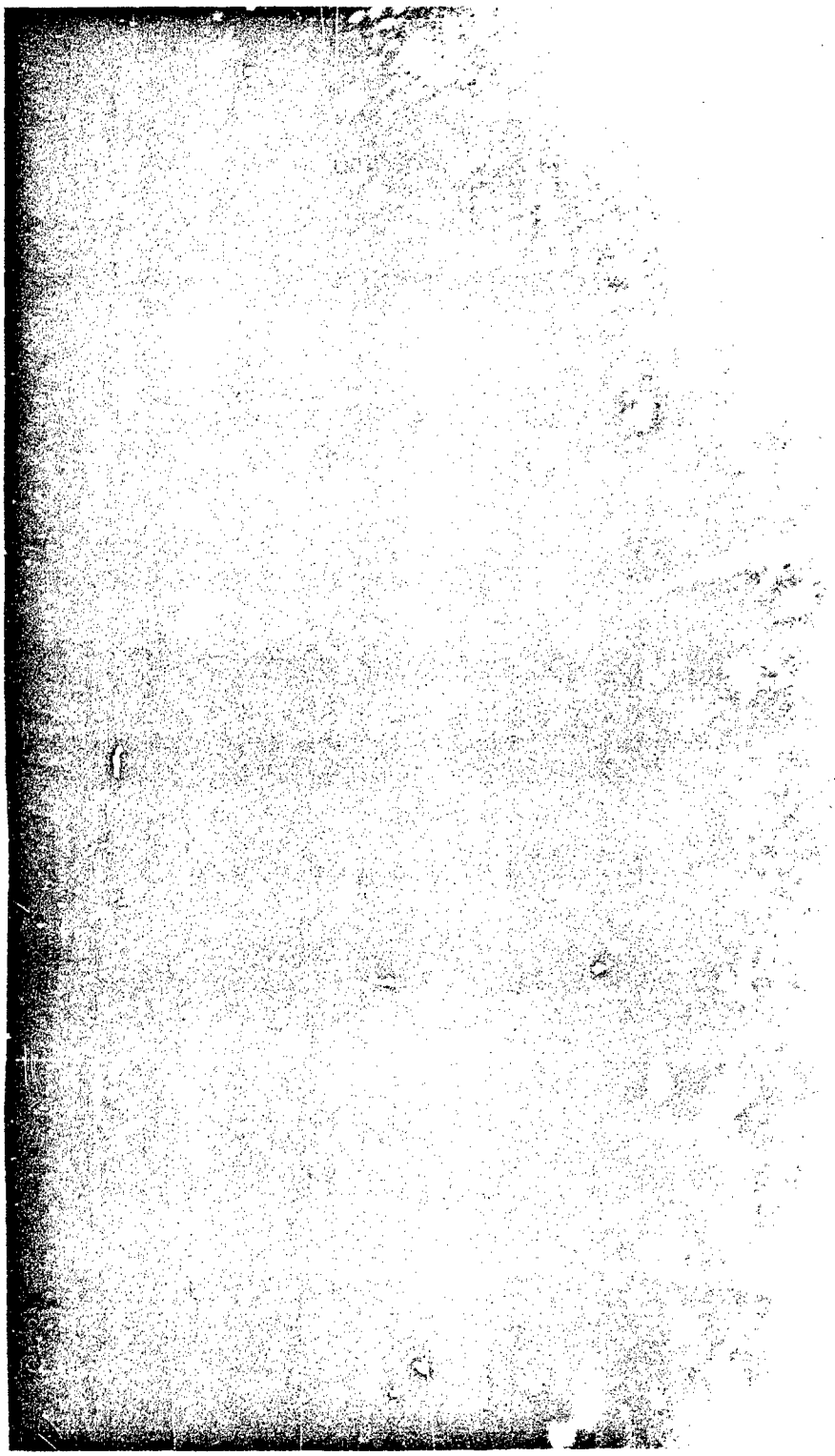


FIGURE 16 Electron micrograph of *E. coli* strain JE 2571 (*pil fla*) negatively stained with 2% phosphotungstic acid (40,000x)

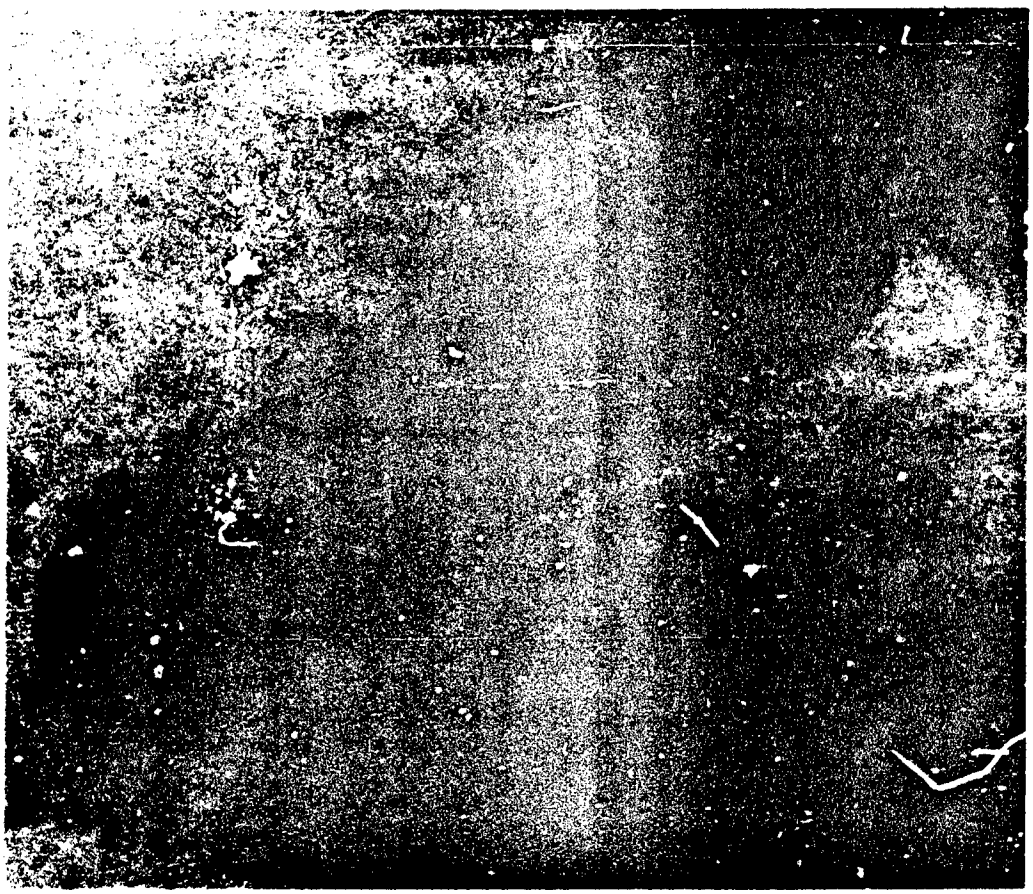


FIGURE 17. Agarose gel of plasmid found in strain Je 2571 (D542: pBR322)

1) λ

2) λ BAMH1 fragments 17.4, 7.3, 6.76, 6.47, 5.54, 5.54

3) pBR322 (2.6 x 10^6 dalton)

4) pBR322 BAMH1

5) JE (D542:pBR322)

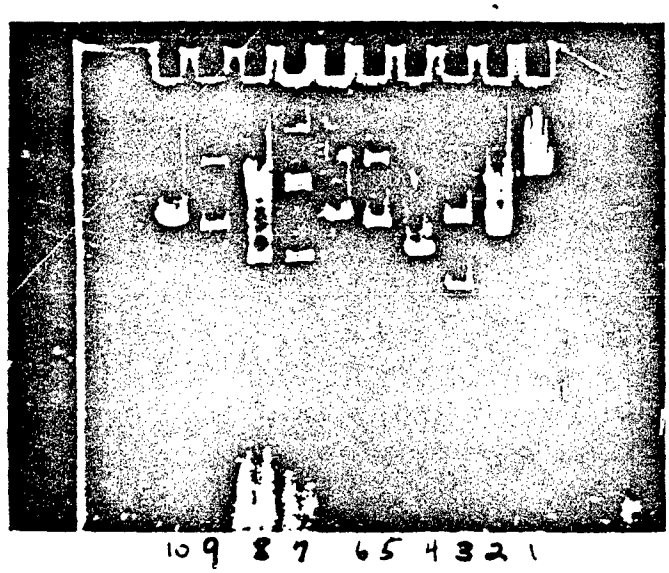
6) JE (D542:pBR322) BAMH1

7) D542

8) D542 BAMH1

9) pMB8:AP3 5.0 x 10^6 dalton

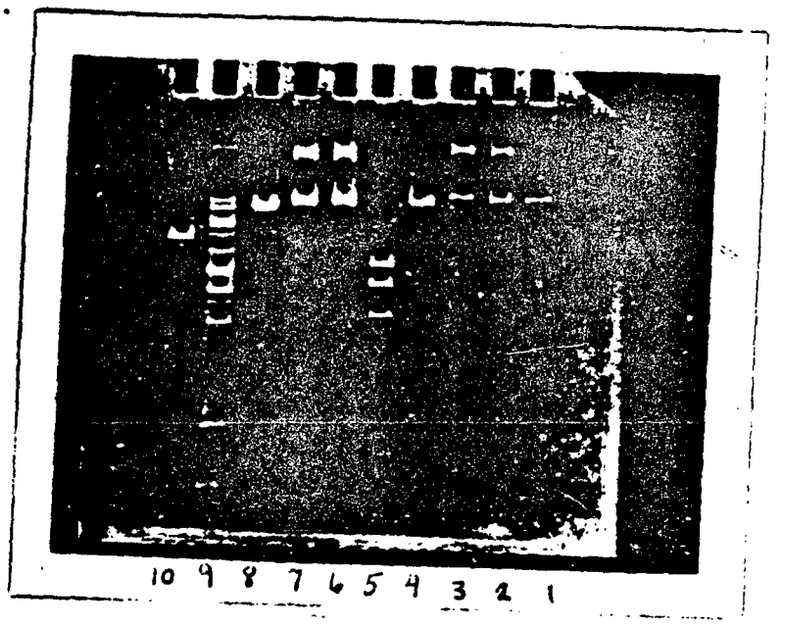
10) pMB8:A03 BAMH1



Endonuclease characterization of hybrid plasmid

JE 2571 (D542: pBR322).

1) JE 2571 (D542: pBR322)
2) JE 2571 (D542: pBR322) Sal I
3) JE 2571 (D542: pBR322) Hind III
4) JE 2571 (D542: pBR322) EcoR1
5) JE 2571 (D542: pBR322) PST I
6. pMB8:AP3 Sal I
7. pMB8:AP3 Hind III
8. pMB8:AP3 Eco R1
9. pMB8:AP3 PST I
10. pBR 332 PST I



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