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THE ROLE OF INTESTINAL BACTERIA IN ACUTE DIARRHEAL DISEASE

Annual Report
September 1978

by

Sherwood L. Gorbach, M.D.

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A large body of evidence exists which implicates enterotoxin-producing Escherichia coli as the cause of a high percentage of undiagnosed diarrheas in man.

Enterotoxigenic E. coli from animal sources have been found to produce an additional virulence factor -- species-specific, surface-associated antigens (K88,K99) which confer the ability to colonize the small bowel of

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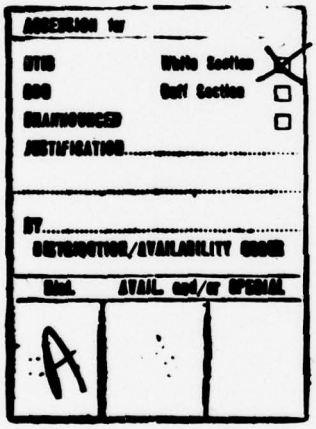
LT(−) ST(−) HA(−) LT(+) ST(+) HA(+)

20.

certain animals. These K antigens also have mannose-resistant hemagglutinin activity (MR-HA).

Using a pair of isogenic strains 334 (LT^+/ST^+, $MR\text{-}HA^+$ buccal adherent) and 334 (LT^- ST^-, HA^- buccal non-adherent) we have been able to isolate the specific MR-HA receptor which we term specific 334 MR-HA pili. Examination by electron microscopy revealed the presence of an apparently homogenous paracrystalline material. This preparation was used to produce antisera against the specific MR-HA pili. Using the buccal assay, MR-HA of various blood cells are reactivity with specific pili antisera, we have detected adherent strains which have differing adherence antigens. Presumably, these strains attach to buccal cells by different pili or non-pili components. ETEC strains from man will be compared in their ability to adhere to human mucosal epithelial cells, human fetal intestinal cells in tissue culture and to the small bowel of infant rabbits in order to develop specific adherence assays for toxigenic E. coli. The physiologic and genetic control of adherence antigens was investigated along with a study of their biological properties.

Greater understanding of the adherence antigens present on toxigenic E. coli from humans will then allow the development of specific "blocking" or competing substance(s) designed to interfere with bacterial colonization.



The investigations during the year of this Contract covered five major areas:

1. Testing of *E. coli* strains isolated from humans with diarrheal disease for enterotoxin production and presence of colonization-specific surface antigens.
2. Methodology for isolation of specific pili (i.e. surface antigens which function in binding to small bowel tissue).
   a. pili isolation procedure
   b. chemical characterization
   c. preparation of pili specific antisera
   d. electron microscopic study of pili morphology
3. *In vitro* adhesion assay specific for recognition of *E. coli* strains which have the potential to adhere to human small bowel tissue.
4. Conditions for attachment of ETEC strain 334 to human buccal mucosal cells
   a. pH
   b. ionic effects
   c. temperature
5. Genetic control of adherence antigens present on ETEC strains from man.

Copies of the following abstracts and publications are included:

Abstracts:

Thorne, G. M., Deneke, C. F., and S. L. Gorbach. Hemagglutination reactions and human buccal cell adherence of human toxigenic E. coli. A.S.M. Abstracts, 1978.

Deneke, C. F., Thorne, G. M., and S. L. Gorbach. Purification of mannose-resistant hemagglutination pili of human toxigenic Escherichia coli. A.S.M. Abstracts, 1978.

Deneke, C. F., Thorne, G. M., and S. L. Gorbach. Attachment of E. coli pathogenic for humans. International Conf. Antimicrob. Ag. and Chemother., 1978.

Publications:

Thorne, G. M., Deneke, C. F., and S. L. Gorbach. Hemagglutination and adhesiveness of toxigenic Escherichia coli isolated from humans. Infect. Immun., accepted for publication.

Deneke, C. F., Thorne, G. M., and S. L. Gorbach. Attachment of enterotoxigenic Escherichia coli of human origin to human buccal mucosal cells. Manuscript in preparation.

Deneke, C. F., Thorne, G. M., and S. L. Gorbach. Attachment pili from enterotoxigenic E. coli pathogenic for man. Manuscript in preparation.

SECTION 1.

ENTEROTOXIN PRODUCTION AND MANNOSE RESISTANT HA TESTING OF E. COLI STRAINS OF HUMAN ORIGIN:

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 enterotoxin and for the presence of a surface antigen(s) (analogous to the K88 antigen of porcine Enteropathogenic E. coli). The K88 protein antigen can cause a specific hemagglutination (HA) of guinea pig red blood cells at 4^{o} C in the presence of mannose. We have screened our test strains for mannose resistant HA (MR-HA) of guinea pig and human type A and B red blood cells. A description of the strains examined is given in Table 1. The various patterns of MR-HA of the three types of blood cells used are presented in Table 2. Two strains, (334, 193-4) were found to cause MR-HA of human A, B and guinea pig rbc's. Twelve strains gave strong MR-HA reactions with both human A and B rbc's, but not with guinea pig cells. Three test strains were MR-HA reactive only with guinea pig rbc's.

The results of tests for enterotoxin production by these strains are also indicated on Table 2. The Y1 mouse adrenal cell assay and/or 18 hr. rabbit ideal loop tests were used to detect LT enterotoxin, and the suckling-mouse assay was used to detect ST enterotoxin. Several of the K-series and TD-series of strains were found to be nontoxigenic when tested in our laboratory - although when originally isolated by Dr. R. B. Sack, the strains produced enterotoxins.

SECTION 2.

PILI AND COLONIZATION FACTOR:

A wide range of E. coli strains show mannose-sensitive binding to host cells and to erythrocytes (causing hemagglutination). The bacterial surface component responsible for mannose-sensitive binding has been termed the type 1 pilus. Table 3 outlines various physical properties exhibited by type 1 pili compared with those of the two K protein antigens K88 and K99 found to be associated with strains of enterotoxigenic E. coli pathogenic for animals. The table also contains information concerning the CF antigen of a human ETEC strain (H10407) and the MR-HA specific 334 pili which are currently under study in our laboratory. There are a number of obvious differences between type 1 pili and those felt to function in intestinal adherence. Chief among these is the inhibitory effect of the sugar, mannose, or mannosides, on binding by type 1 pili. Binding is also known to be inhibited by a mannose-seeking plant lectin (concanavalin A) which presumably competes with the bacterial surface component (type 1 pili) for the host cell-surface mannose receptor. These two types of pili also have different pH optima for attachment. The type 1 pili bind at acid pH, typically pH 5.5, while the mannose-resistant attachment pili reported here have a binding optimum near neutrality, which would be more typical of physiological conditions in the small intestine.

Thus, the K protein antigens (K88, K99, etc.) can be distinguished from the lectin-like substance (type 1 pili) by their

binding to mucosal cells in the presence of mannose. Like type 1 pili, their production always correlates with the appearance of fine filamentous projections covering the surface of the cell.

a. Purification of specific pili: Type 1 pili and K antigens are surface appendages which may be removed and isolated by purely physical means, i.e. ultracentrifugation, precipitation and chromatography. However, to make antisera specific for the adherence antigens (K-like protein antigens) it is important to isolate the specific pili involved. We have isolated the E. coli 334 receptor which is responsible for the agglutination of guinea pig red blood cells in the presence of mannose at 0 C. Thus, we have used a specific absorption reaction based on the following observations: the MR-HA of guinea pig red blood cells by E. coli 334 could be reversed by increasing the temperature of the reaction from 0 C (ice bath) to 37 C. Upon recooling, the MR-HA reaction was again observed, suggesting a temperature-dependent reversible equilibrium, rather than an irreversible process.

For the preparation of specific K-like pili, E. coli 334 was grown overnight on peptone agar and was harvested and washed twice in saline. The washed cells were treated in a blender for 3 minutes using short bursts and cooling with ice to prevent heat denaturation. The intact cells and cellular debris were removed by centrifugation (10,000 RPM for 10 min.) and the supernatant containing pili which had been sheared off was mixed with washed guinea pig erythrocytes in PBS containing 1% mannose and incubated 15 minutes in an

ice bath . The red cells which should have attached K-like pili, but not type 1 pili (which exhibit mannose sensitive HA reactions), were washed with PBS+ mannose. The supernatants were termed PBS-mannose 1 through 3 (Figure 1). The MR-HA mediating pili were then eluted from the red cell surface by raising the temperature. The first two PBS washes were each incubated for 5 minutes at 37 C; then the temperature of the 5 minute incubation was increased to 45 C for PBS fraction 3 and 55 C for PBS fraction 4. Following ultracentrifugation which removes red cell debris these supernatants are found to contain specific pili protein. This procedure is summarized in Figure 1.

b. Characterization of K-like pili: The availability of purified E. coli pili, the MR-HA receptor, has allowed us to begin physio-chemical characterization. The molecular weights of the components in the isolated pili preparations were determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

As isolated by the methods described above, the purified pili preparation of strain 334 contains two polypeptide chains, with molecular weights of 13,100 and 12,500 (Figures 2,3). Other material staining with Coomassie Blue was not found.

Protein bands with equivalent molecular weights as those of strain 334 were detected in pili preparations from a number of other toxigenic E. coli strains (Figure 2). These strains all strongly adhere to human buccal mucosal cells. The buccal binding characteristics of the strains will be discussed in Section III

of this report.

These K-like pili, which can be selectively isolated by taking advantage of their specific mannose-resistant binding to red cells, also bind to human buccal mucosal cells. Evidence of direct attachment of these components was obtained using SDS-PAGE. Use of this technique was possible since buccal cells solubilized in SDS do not appear to have significant Coomassie Brilliant Blue staining material in the molecular weight range of bands found in the bacterial pili preparations (Figure 4). When buccal cells were incubated with pili material and then extensively washed, pili were removed in the first wash (Figure 4, column 5) but not in subsequent washes (Columns 6-8). Pili which firmly attached to the buccal cells were found in the washed cell pellet (Column10).

Thus, we feel that these specific pili components are responsible for both the MR-HA reaction and for buccal cell binding.

c. Electron microscopic study of pili morphology: Electron microscopic examinations of the pili preparations of strains 334 and 193-4 have revealed the presence of fiber-like paracrystalline structures (5 x 300 nm) (Figure 5). At present we have examined a number of Group I strains for the presence of surface pili and found structures of identical size and morphology to those found in pili preparations of strains 334 and 193-4. As an example we have included electron micrographs of strain 334, which demonstrate the surface pili (Figure 6).

Table 4 outlines the data collected concerning detection of K-like surface pili on strains of ETEC isolated from man using SDS-PAGE and electron microscopy. The control nontoxigenic strains H10405, 334LL, CD-1 do not appear to produce these components.

All ETEC strains in the three classes described in Section III of this report will undergo examination by electron microscopy and SDS-PAGE. Surface structures found on these ETEC strains will undergo immunologic studies to determine the prevalence of cross-reacting antigens and for different components involved in binding to eukaryotic cells. This will be accomplished using immunodiffusion and rocket immuno-electrophoretic techniques.

d. Antisera: We have used the pili preparation from E. coli 334, as well as the intact organism, to prepare specific rabbit antisera. This specific antisera has then been used to examine the antigenic surface structures of various toxigenic E. coli strains and their derivatives. These data are summarized in Table 2. As can be seen, strain 193-4 which shows binding to the buccal cells also gives a positive reaction (agglutination) with either antisera against 334 or 334 pili. This strain also agglutinates human A, B and guinea pig red blood cells in the cold and in the presence of mannose, which suggests a common antigenic site is present in both these strains and is responsible for the mannose-resistant HA reaction. Results with the buccal cell adhesion assay indicate that these strains also share the characteristic of binding to

human epithelial cells. These results will be discussed in Section III of this report "Buccal Adherence Assay".

We are still in the process of preparing a set of pili specific antisera and antisera against whole bacteria (Table 5). This material will also allow us to examine the serologic cross-reactivity of these surface adherence antigens.

Recently we have prepared the F_{ab} portion of IgG immunoglobulin directed against the surface pili of strain 334. The pili specific F_{ab} was found to effectively block the binding of strain 334 to human buccal cells (Table 6). We are now in the process of preparing 334 pili labelled with ^{125}I to obtain more precise data concerning the binding to buccal cells of this purified bacterial protein. Such studies should also lead to the determination of whether bacterial binding occurs to specific receptor sites on eukaryotic membranes.

Evidence to date appears to indicate that the surface components of the eukaryotic cells (buccal cells and red blood cells) which are the substrates for the bacterial binding reactions are different. The MR-HA^+ ETEC strains will bind to buccal cells from a blood type O donor but will only hemagglutinate red blood cells of blood type A or B. As a type O individual does not synthesize either type A or B blood group substances, the buccal binding reaction cannot involve these particular oligosaccharides. Also, the bacterial MR-HA reaction is freely reversible at 37 C, while the

attachment of either isolated pili or whole bacteria to buccal cells occurs avidly at 37 C. The attachment sites on the two eukaryotic cells differ as well in their ability to bind the pili. The buccal cells apparently having a higher affinity since the temperature range of this binding reaction is broader and the pili remain bound during washing. This suggests that the buccal cell receptor more nearly "fits" the pili binding site(s) and is, therefore, closer to the "natural" substrate.

SECTION 3.

HUMAN BUCCAL CELL ADHERENCE ASSAY:

We have developed an adherence assay employing human buccal epithelial cells. (A manuscript has been accepted for publication in Infection and Immunity and is included as part of our contract renewal application.) This system is attractive since it used human cells against human pathogens - the homologous system. Binding to human buccal epithelial cells was first 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. ETEC strains have been cultured from the throats of infants with diarrhea - presumably an indication of colonization of the oral cavity. Also, the buccal cells are easy to collect from the same group of human volunteers, and these cells share certain similarities with the gastrointestinal mucosa. The

buccal cells are obtained from a secreting tissue,contiguous with the GI tract, and ABO and Lewis blood group substances are known to be present in secretions and cells lining the entire GI tract, including the oral cavity.

We have employed two different systems to monitor the adherence of human toxigenic E. coli to the human buccal cell suspensions: (1) staining the cell mixtures with an indirect fluorescent antibody technique; (2) radioactively labeled bacteria and a membrane filtration technique. These two binding assays have shown good agreement. These studies have allowed for the detection of strains with special surface properties which permit binding to the buccal epithelial cells in the presence of mannose, a sugar which is known to inhibit cellular binding and HA reactions due to common type 1 pili.

The buccal adherence data of 31 toxigenic E. coli strains tested to data are presented on Table 7. Eighteen of the 31 toxigenic test strains were found to adhere at a level similar to that of strain 334. The mean net number of attached cells per buccal cell of adherent strains ranged from 101 to 470. Toxigenic non-binders averaged from 17-86 attached bacterial cells per buccal cell.

E. coli strains pathogenic for animals that produce enterotoxins and special surface receptors appear to be separable by this assay. The animal E. coli pathogens were P307, a porcine pathogen; RDEC-1, the cause of diarrhea in rabbits; and the plasmid-containing strains K12 K99 and K12 K88. These strains show only

background level adherence in the buccal assays.

There was no direct correlation between ability to adhere to human buccal cells and ability to cause mannose-resistant HA in any of the three blood cell types employed (i.e. guinea pig, human A and B). Therefore, strains are described as being MR-HA$^+$ in Table 8 if they react with any of the rbc's used.

To date, strains examined in this study can be placed into three groups (Table 8). The first group is composed of strains which are MR-HA$^+$ and adhere to buccal cells; eleven of the twelve strains also react with 334 pili antisera (described above). These strains probably share a similar pilus type. EM and serologic studies of these strains are in progress. Strain K324$_{cl}$ of Group I probably makes a different pilus type. Further study of this strain is in progress. The second group of strains adhere to the epithelial cells but do not HA. We plan to study these strains for the presence of different pili or non-pili adherence components. There are four MR-HA$^+$ strains which did not bind to buccal cells (Group III). These strains will also be examined by EM and serologic means in order to gain some insight into the cause of their differing binding capacities in relation to Group I strains.

SECTION 4.

CONDITIONS FOR ATTACHMENT OF ETEC STRAIN 334 TO HUMAN BUCCAL MUCOSAL CELLS:

A manuscript entitled "Attachment of Enterotoxigenic Escherichia coli of Human Origin to Human Buccal Mucosal Cells" is being submitted to the Journal of Infection and Immunity. The following has been exerpted from the manuscript.

a. pH Effects

The binding of E. coli 334 to buccal cells in the presence of mannose was found to be a pH dependent process (Figure 7). The buccal binding reaction had a pH optimum near neutrality and it decreased under either acid or alkaline conditions. There was no binding at pH 5; increasing the pH to 8.0, caused a reduction in binding to approximately 25% that of neutrality.

b. Ionic Effects

Table 9 shows the effects of Ca^{++} and Mg^{++} on the binding reaction. The presence or absence of divalent cations did not appreciably change the numbers of bacteria retained by the filters either with or without buccal cells. Equivalent buccal attachment occurred in absence of divalent cations; bacterial self-aggregation was also not changed. Thus, divalent cations are not required for the bacterial attachment reaction.

c. Effect of Temperature

There are 2 possible attachment reactions involving pili which are under consideration here: bacterial self association and bacterial binding to buccal cells. In contrast with the other type of aggregate , the specific binding of bacteria to buccal when corrected for the bacterial self-aggregation does not vary with temperature, over the range 0-37 C (Table10). Bacterial self-aggregation (the value in the absence of buccal cells) decreased from 10,500 to 8,000 (mean CPM) when the temperature is raised from 0 to 37 C. Further evidence that this represents bacterial self-association is shown in Figure 8. An increase in temperature from 0 to 37 C decreased the amount of radioactive E. coli 334 retained by the filter for all pore sizes, suggesting a decrease in the size of the bacterial aggregate. This is not removal of individual bacterial cells as all the pore sizes used were 5 to 10 times larger than the diameter of the individual bacterial cells. Non-specific adsorption of the bacteria to the polycarbonate filter material is likely to have remained constant with varying pore size. Supporting evidence that strong bacterial self-aggregation occurs is seen with E. coli 193-4, in which case the bacterial aggregates were visible (ca 1-2mm). These large aggregates could also be dispersed by elevating the temperature.

A temperature effect similar to that of the bacterial self-aggregation is seen with the mannose-resistant hemagglutination (MR-HA) reaction, where increasing the temperature from 0 C to 37 C will reverse the MR-HA reaction. This temperature reversal is the basis of our pili isolation procedure.

SECTION 5.

GENETIC CONTROL OF ADHERENCE ANTIGENS PRESENT ON ETEC STRAINS FROM MAN:

Since the structural genes for the K88 and K99 antigens involved in adherence are located on transmissible plasmids like those that code for the E. coli enterotoxins (LT,ST), a number of plasmid "curing" procedures were used in attempts to isolate MR-HA negative derivatives of strain 334 (Table11). The usual curing agents, ethidium bromide and SDS, as well as growth at elevated temperatures, were not effective. Rifampicin and nalidixic acid treatment did yield MR-HA derivatives. Rifampicin specifically interacts with RNA polymerase and has been shown to eliminate the F plasmid from E. coli. This antibiotic is also known to interfere with synthesis of plasmid-mediated surface components. The phenotypic change exhibited by strain 334-27 (HA negative under both test conditions) suggests that rifampicin is affecting synthesis of both the K-like antigen responsible for mannose resistant HA and type 1 pili responsible for mannose sensitive HA. Derivative $334P^{+}15$ is also missing plasmid band #4, yet is MR-HA positive. The nalidixic acid resistant mutant was selected in order to perform plasmid superinfection matings. The absence of plasmids was noted after the strain was screened by the agarose electrophoresis technique of Meyers et. al., J. Bact. 127: 1529-1537, 1976. The data given in Table 12summarized the information

concerning toxin production and HA ability of strain 334 and its derivatives. From the phenotypic characteristics of derivatives 334 Nx^{R-1}, $334P^{+}15$, K-like antigen and toxin production by strain 334 are not related to presence of plasmid bands #2, #3, or #4. It seems more likely that plasmid #1 (60×10^6 daltons) will be found to determine both toxin and adherence antigen production. Plasmids mediating LT/ST production studied to date have been found to belong to a highly related group with an approximate molecular weight of 60×10^6 daltons which is similar to plasmid band #1.

Initial attempts have been made to genetically label the "cryptic" plasmids in strain 334 by insertion of transposable antibiotic resistance genes.

To date, we have only been able to detect transfer of plasmid band #3 into a polA recipient which, due to its small size, was probably co-transferred by one of the large plasmids which was then lost. It does not confer the ability to cause MR-HA which supports the curing data described above. These studies will be continued in the proposed work.

In testing ETEC for the presence of K-like antigens (i.e. mediating MR-HA), several strains were found to undergo loss of this phenotype. The buccal binding exhibited by three MR-HA positive strains and their spontaneous MR-HA negative derivatives is shown in Table 13. The $MR\text{-}HA^{+}$ strains bind avidly in the presence

of mannose. The average number of bacteria per buccal cell ranged from 172-246. Low level binding occurred with the MR-HA$^-$ derivatives (range of the mean number of bacteria per buccal cell was 13-38). The MR-HA negative derivatives of strain 334 and $M403_{c3}$ no longer were agglutinated by 334 pili antisera. (Strain $K324_{c1}$ does not react in the 334 pili antisera.) The plasmid bands found when cleared lysates of two of these strains 334 and 334LL were studied using the agarose gel electrophoretic technique is shown in Figure 9, Channels C and F. Strain 334 has 6 plasmid bands, while only the chromosomal fragment is apparent when strain 334LL is analyzed by this method. Additional experiments are planned to investigate the role of plasmids in mediating production of surface antigens involved in binding to eukaryotic membranes.

SUMMARY

In summary, the buccal adherence and strain reactivity with 334-pili antisera appear to indicate that multiple surface sticky antigens (adherence factors) are present on these toxigenic strains of E. coli isolated from humans. The occurrence of Group II strains (Table 8) cautions against complete reliance on the MR-HA phenotype to help detect E. coli with the potential to adhere to human mucosal

tissues. Preliminary genetic study suggests that the K-like antigen present on strain 334 may be plasmid mediated.

The 334 pili-specific antisera has allowed for detection of cross-reactivity between strains of differing serotype, O78:K80:H12, O78:H11, O15:H11, and O25:H42 (Table 8), which also exhibit buccal binding ability and mannose resistant HA of red blood cells. Using the H10407 (O78:H11) CFA antisera of the Evans group, the Ørskovs (Med. Microbiol. Immunol. 163: 99, 1977) recently reported the detection of CFA surface antigen in O78:H$^-$, O78:H11, and O78:H12 strains but not in any strains of the other serotypes commonly isolated from cases of human diarrhea.

The reaction of Group I strains with erythrocytes raises the possibility that these organisms are adhering to ABO and Lewis blood group receptors or similar structures. These blood group substances are known to be present in sections and on other tissues including those of the entire intestinal tract. This *in vitro* model system allows for increased understanding of the complex and presumably species-specific interactions between bacterial attachment components and receptors on eukaryotic cell membranes.

The buccal cell adhesion assays allow us to measure the affinity of various toxigenic *E. coli* for human epithelial tissue. This should permit recognition of colonization ability in a particular strain and, therefore, should separate *E. coli* truly pathogenic in humans from non-colonizing strains, both of which might still produce enterotoxin.

TABLE 1

ESCHERICHIA COLI STRAINS EXAMINED

Human Strains	Enterotoxins Status	Serotype	Source
334	LT/ST	015:H11	AD India
334LL	-	015:H11	LP
334 P+15	LT/St	015:H11	LP
193-4	LT/ST	N.T.	AD India
052005-74	LT/ST		AD Mexico
408-3	LT/ST		AD India
H10407	LT/ST	078:H11	AD India
H10407P	LT/ST	078:H11	LP
Tx-1	ST	078:K80:H12	ID Texas
Tx-85	ST	078:K80:H12	ID Texas
B2C	LT/ST		AD Viet Nam
B7A	LT/ST		AD Viet Nam
214-4	ST		AD MD
H10405	-		adult feces
HS	-		adult feces
K108c3	LT/ST		AD Kenya
K324c1	LT/ST	08:060:H9	AD Kenya
K344c2	LT		AD Kenya
K130c1	LT/ST		AD Kenya
K135c2	-		AD Kenya
K325c3	-		AD Kenya
K326c5	LT/ST	025:H42	AD Kenya
K328c4	-		AD Kenya
K325c1	LT		AD Kenya

Human Strains	Enteropathogenic Status	Serotype	Source
TD462c1	LT/ST	06:H16	AD Mexico
TD260c1	LT	06:H16	AD Mexico
TD514c1	-		AD Mexico
TD412c1	LT/ST		AD Mexico
TD514c2	-		AD Mexico
TD427c2	LT		AD Mexico
TD213c2	ST	0128	AD Mexico
TD234c4	LT		AD Mexico
TD219c1	ST	06:H16	AD Mexico
TD451c2	LT/ST		AD Mexico
TD327c2	-	05	AD Mexico
M403c3	ST		AD Morocco
Animal Pathogens and Pladmid Containing Strains			
RDEC-1	Shiga entero-toxin		ovine diarrhea
152	LT/ST	K88ac	porcine diarrhea
341	ST	K99	bovine diarrhea
P307		K88	porcine diarrhea
K12	-		LP
K12K88ab	-	K88ab	LD
K12K99	-	K99	LD

AD Adult diarrhea

ID Infant diarrhea

LP Laboratory passage

LD Laboratory derived

TABLE 2

TOXIN PRODUCTION AND AGGLUTINATION REACTIONS

BY HUMAN STRAINS OF ESCHERICHIA COLI

STRAIN	TOXIN PRODUCED	HEMAGGLUTINATION[1] HUMAN		GUINEA PIG	AGGLUTINATION[2] PILI-ANTISERA
		A	B		
334	LT/ST	+	+	+	+
334LL	-	-	-	-	-
193-4	LT/ST	+	+	+	+
Tx-1	ST	+	+	-	+
Tx-85	ST	+	+	-	+
214-4	ST	-	-	-	-
B2C	LT/ST	-	-	-	-
B7A	LT/ST	-	-	-	-
$M403_{c3}$	ST	-	-	-	-
$K108_{c3}$	LT/ST	-	-	-	-
$K324_{c1}$	LT/ST	-	-	+	-
$K344_{c2}$	LT	-	-	-	-
$K130_{c1}$	LT/ST	-	-	-	-
$K135_{c2}$	-	-	-	-	-
$K325_{c3}$	-	-	-	-	-
$K326_{c5}$	LT/ST	+	+	-	+
$K328_{c4}$	-	-	-	-	-
$K325_{c1}$	LT	-	-	-	-
$TD462_{c1}$	LT/ST	-	-	-	-
$TD260_{c1}$	LT	-	-	-	-
$TD514_{c1}$	-	-	-	-	-
$TD412_{c1}$	LT/ST	-	-	-	-
$TD514_{c2}$	-	-	-	-	-
$TD427_{c2}$	LT	-	-	-	-
$TD213_{c2}$	ST	+	+	-	-
$TD235_{c4}$	LT	-	-	-	-
$TD219_{c1}$	ST	+	+	-	-
$TD451_{c2}$	LT/ST	-	-	-	-
$TD327_{c2}$	-	-	-	+	-

TABLE 2 continued

STRAIN	TOXIN PRODUCED	HEMAGGLUTINATION[1] HUMAN A	HUMAN B	GUINEA PIG	AGGLUTINATION[2] PILI-ANTISERA
H10407	LT/ST	+	+	-	+
H10407P	LT/ST	-	-	-	+
D370855	LT/ST	+	+	-	-
D444	LT/ST	-	-	-	-
D563	LT/ST	+	+	-	+
D513	LT/ST	-	-	-	-
D542	LT/ST	+	+	-	+
D481	LT/ST	+	+	-	+
D280551	LT/ST	+	+	-	+
D370844	LT/ST	+	+	-	-
D524	LT/ST	-	-	-	-
D280561	LT/ST	-	-	+	-
CONTROL STRAINS					
H10405		-	-	-	-
CD-1		-	-	-	-
HS		-	-	-	-
RDEC-1		-	-	-	-
P307		-	-	+	-
K12		-	-	-	-
K12K88		-	-	+	-
K12K99		-	-	-	-

1. mannose-resistant hemagglutination (HA) of washed human group A,B or guinea pig erythrocytes at 0°C.

2. agglutination by rabbit antisera prepared against surface structures present on strain 334.

TABLE 3

TYPES OF PILI FOUND ON E. COLI

	Type I Pili	Intestinal Attachment Pili
Examples:	Common fimbriae	K88,K99, CFA, 334-Like
Heat	stable 60^{o} unstable 100^{o} 1 hr	unstable 65^{o}C 1 hr.
Growth at 18^{o}C	+	-
HA	Mannose-sensitive	Mannose-resistant
Blood types	guinea pig fowl horse not human	human guinea pig
Antigenic	+	+
Glucose	+	represses synthesis of CFA, 334-like
Genetic locus	chromosome	plasmid?
Size	10nm	K99 8.4 nm x 130nm CFA 8-9 nm 334 ∿2 nm K88 8-13 n
pH optimum for cell adherence	4.5 (vero cells)	7.0 (mucosal cells)

TABLE 4

DETECTION OF K-LIKE PILI ON STRAINS OF TOXIGENIC *E. COLI* ISOLATED FROM MAN

Group I Strains[1]	Pili Detected by: SDS-PAGE[2]	Electron Microscopy[3]
334	+	+
193-4	NT[4]	+
TX-1	+	+
D542	+	NT
D481	+	+
D563	NT	+
Control Nontoxigenic Strains		
H10405	-	-
334LL	NT	-
CD-1	NT	-

1. Strains in Group I are MR-HA$^+$ bind to human buccal mucosal cells and all but strain K 324_{Cl} are agglutinated by 334 pili-specific antisera (See Table 2).
2. Representative gels are shown in Figures 2,3 .
3. Representative electron micrographs are shown in Figures 4 and 5.
4. NT = not tested.

Table 5 Whole Cell and Pili Antisera Available 3-28-78

WHOLE CELL ANTISERA	PILI ANTISERA
334	334
334LL	193-4
H10407	TX-1
H10407P	TX-85
$K324c_1$	$TD219c_1$
$K326c_5$	$M403c_3$
$TD327c_1$	$TD219c_2$
TX-1	214-4
TX-85	K12K99
$TD213c_2$	
214-4	
193-4	
RDEC-1	
HS	
K12K88	
K12K99	
1111A	
H10405	

WHOLE CELL AND PILI ANTISERA BEING PREPARED

$K325c_3$	D370844
$K135c_5$	D513
$TD235c_4$	D280561
$TD462c_1$	D280551
$M403c_3$	D563
$TD219c_1$	D481
	D542
	D370855

Table 6

INHIBITION OF BUCCAL BINDING BY ANTI 334 HA PILI F_{ab}

Strain	F_{ab} (µl)	Mean Net No. Bacteria / Buccal Cell
334	-	100
334	200	60
334	400	10

1. Bacteria alone were pretreated for 15 minutes with F_{ab} preparation.

TABLE 7.

BUCCAL CELL ADHERENCE

BY TOXIGENIC AND NON-TOXIGENIC STRAINS OF E. COLI

Toxigenic Human Strains[1]	Mean Net No. Attached Bacteria / Buccal Cell	± SEM[2]	Number Assays
334	246	± 48	11
214-4	108	± 14	5
H10407	253	± 73	4
H10407P	255	± 104	7
193-4	171	± 30	3
TX-1	147	± 28	4
TX-85	101	± 10	4
B2C	183	± 40	4
D280551	128	± 20	4
D563	102	± 37	3
D481	135	± 37	3
D444	112	± 21	4
D542	115	± 11	3
$K324_{c1}$	172	± 66	3
$K325_{c3}$	132	± 55	4
$K135_{c2}$	470	± 56	3
$TD235_{c4}$	144	± 39	5
$TD462_{c1}$	275	± 86	3
$M403_{c3}$	28	± 11	7
$TD219_{c1}$	50	± 28	3
$TD213_{c2}$	46	± 6	7
$M409_{c1}$	25	± 5	4
$K344_{c2}$	84	± 42	5
$TD451_{c2}$	76	± 15	4
$TD327_{c2}$	30	± 20	2
D280561	86	± 14	4
D370855	50	± 10	6
D370844	39	± 5	4
D513	22	± 15	4
D524	17	± 9	3
B7A	75	± 58	4

TABLE 7.

continued

Non-Toxigenic Human Strains	Mean Net No. Attached Bacteria Buccal Cell	± SEM	Number Assays
10405	259	± 93	4
HS	61	± 17	5
CD-1	37	± 9	5
334LL	13	± 3	10
Animal & Lab Strains			
RDEC-1	43	± 14	4
K12	31	± 15	4
K12 K88ab	81	± 38	4
K12 K99	48	± 19	4
P307	38	± 29	3

[1] Stain characteristics are listed in Table 1.

[2] SEM, standard error of the mean

TABLE 8.

COMPARISON OF AGGLUTINATION REACTION AND/OR BUCCAL CELL ADHERENCE OF HUMAN TOXIGENIC *E. COLI* STRAINS

STRAIN	SEROTYPE	MR-HA[2]	BUCCAL ADHERENCE	334-PILI ANTISERA[1]
Group I				
334	015:H11	+	+ (246)	+
193-4	NT	+	+ (171)	+
TX-85	078:K80:H12	+	+ (101)	+
TX-1	078:K80:H12	+	+ (147)	+
$K324c_1$	08:060:H9	+	+ (172)	-
H10407	078:H11	+	+ (253)	+
$K326c_5$	025:H42	+	+ (136)	+
D542		+	+ (115)	+
D563		+	+ (102)	+
D481		+	+ (135)	+
D444		+	+ (112)	+
D280551		+	+ (128)	+
Group II				
H10407P	078:H11	-	+ (253)	+
$K325c_3$		-	+ (132)	-
$K135c_2$		-	+ (470)	-
$TD235c_4$		-	+ (144)	-
$TD462c_1$		-	+ (275)	-
214-4		-	+ (108)	-
B2C		-	+ (183)	-
Group III				
$TD213c_2$		+	- (46)	-
TD219		+	- (50)	-
D370855		+	- (50)	-
D370844		+	- (39)	-

[1] agglutination by rabbit antisera prepared against surface structure present on strain 334

[2] mannose-resistant hemagglutination (MR-HA) of washed human group A, B or guinea pig erythrocytes at $0^{o}C$

TABLE 9

EFFECTS OF DIVALENT CATIONS ON ATTACHMENT OF
E. COLI 334 TO BUCCAL CELLS

BUFFER USED	BACTERIA RETAINED BY FILTER		
	WITHOUT BUCCAL CELLS	WITH BUCCAL CELLS	BACTERIA/ BUCCAL CELL
Hanks Balanced Salts	2.37×10^3	2.64×10^8	809.
Hanks Balanced Salts Without Calcium or Magnesium	2.39×10^8	2.72×10^8	862.

Bacteria were mixed with either buccal cells or buffer in the presence of either Hanks balanced salts solution containing Ca^{++} and Mg^{++} or in Hanks balanced salts solution without these divalent cations. Following a 5 minute incubation at 37 C, aliquots were filtered through 5 μ Nucleopore filters, washed and radioactivity determined using a liquid scintillation counter. The specific activity of the bacterial suspension was determined from the optical density and radioactivity retained by a 0.2 μ filter.

TABLE 10

ATTACHMENT OF RADIOACTIVELY LABELED E. COLI 334

	CPM RETAINED BY FILTER	
	37 C	0 C
Bacteria + Buccal Cells	9930. ± 410.	12790. ± 650.
Bacteria Alone	7940. ± 280.	10680. ± 310.
Bacteria Bound by Buccal Cells	1990.	2020.

Data is expressed as counts per minute retained by 5 μ Nucleopore filters. Bacteria alone or bacteria and buccal cells together were incubated at 37 C or 0 C for 5 minutes. Aliquots were then filtered and washed with 5 ml of PBS at the appropriate temperature. Data is given as mean ± standard error for 12 determinations.

TABLE 11. SUMMARY OF CURING EXPERIMENTS

USING *E. COLI* 334 (LT/ST, HA+)

"CURING" TREATMENT	NO. COLONIES TESTED	NO. COLONIES HA-
1. GROWTH AT 42°C	62	0
2. GROWTH AT 44°C	105	0
3. GROWTH AT 48°C	56	0
4. GROWTH AT 49°C	14	0
5. ETHIDIUM BROMIDE		
30 µg/ml	110	0
60 µg/ml	57	0
90 µg/ml	3	0
120 µg/ml	1	0
240 µg/ml	2	0
6. LAURYL SULFATE, SODIUM SALT		
1%	62	0
2%	100	0
3%	58	0
4%	55	0
5%	50	0
7. RIFAMPIN		
40 µg/ml	43	0
20 µg/ml	27	0
2 µg/ml	60	18
0.5 µg/ml	26	1
8. ACRIDINE ORANGE		
5 µg/ml	10	0
9. NEOMYCIN SULFATE		
5 µg/ml	9	0
10. SUPERINFECTION WITH Tc R-FACTOR		
D1-76	4	2
11. NALIDIXIC ACID		
50 µg/ml	1	1

TABLE 12 BIOLOGICAL PROPERTIES AND PLASMID COMPONENTS OF E. COLI 334 AND ITS DERIVATIVE STRAINS

STRAIN	METHOD OF ISOLATION	HA TESTS[1]		ST[2] TOXIN	LT[3] TOXIN	PLASMIDS[4] LARGE		SMALL				GEL REFERENCE
		0°C (+) mannose (K-like antigen)	24°C (-) mannose (type 1 pili)			#1, #2		#3, #4, #5, #6				
334		+	+	+	+	+	+	+	+	+	+	Figure 9
334 NX^R-1	nalidixic acid	+	+	+	+	+	-	-	+	+	+	
334-27	rifampin	-	-	+	+	+	+	+	-	+	+	
334P+15	rifampin	+	-	+	+	+	-	+	-	+	+	Figure 9
334LL	storage	-	+	-	-	-	-	-	-	-	-	Figure 9

Guinea pig erythrocytes were used for the hemaglutination (HA) tests

1. HA test (+) mannose, at 0°C is specific for K88-like sticky antigen.
   HA test (-) mannose, at 24°C is an indication of the presence of type 1 pili.
2. ST toxin was assayed in the suckling mice.
3. LT toxin was assayed in YI adrenal cell cultures.
4. Plasmid DNA components were observed following agarose gel electrophoresis as described.
   - #1 is the largest plasmid seen at the top of the gel, #6 is the smallest and fastest moving species seen at bottom of the gel.

TABLE 13

BUCCAL ADHERENCE
OF HA^+ AND HA^- ISOGENIC STRAINS

STRAIN	MEAN NO. BACTERIA ± SEM / BUCCAL CELL	
	HA^+	HA^-
334	246 ± 48	13 ± 3
$M403_{C3}$	192 ± 98	19 ± 10
$K324_{C1}$	172 ± 66	38 ± 4

Figure 1

Isolation of K-like Pili from E. coli 334

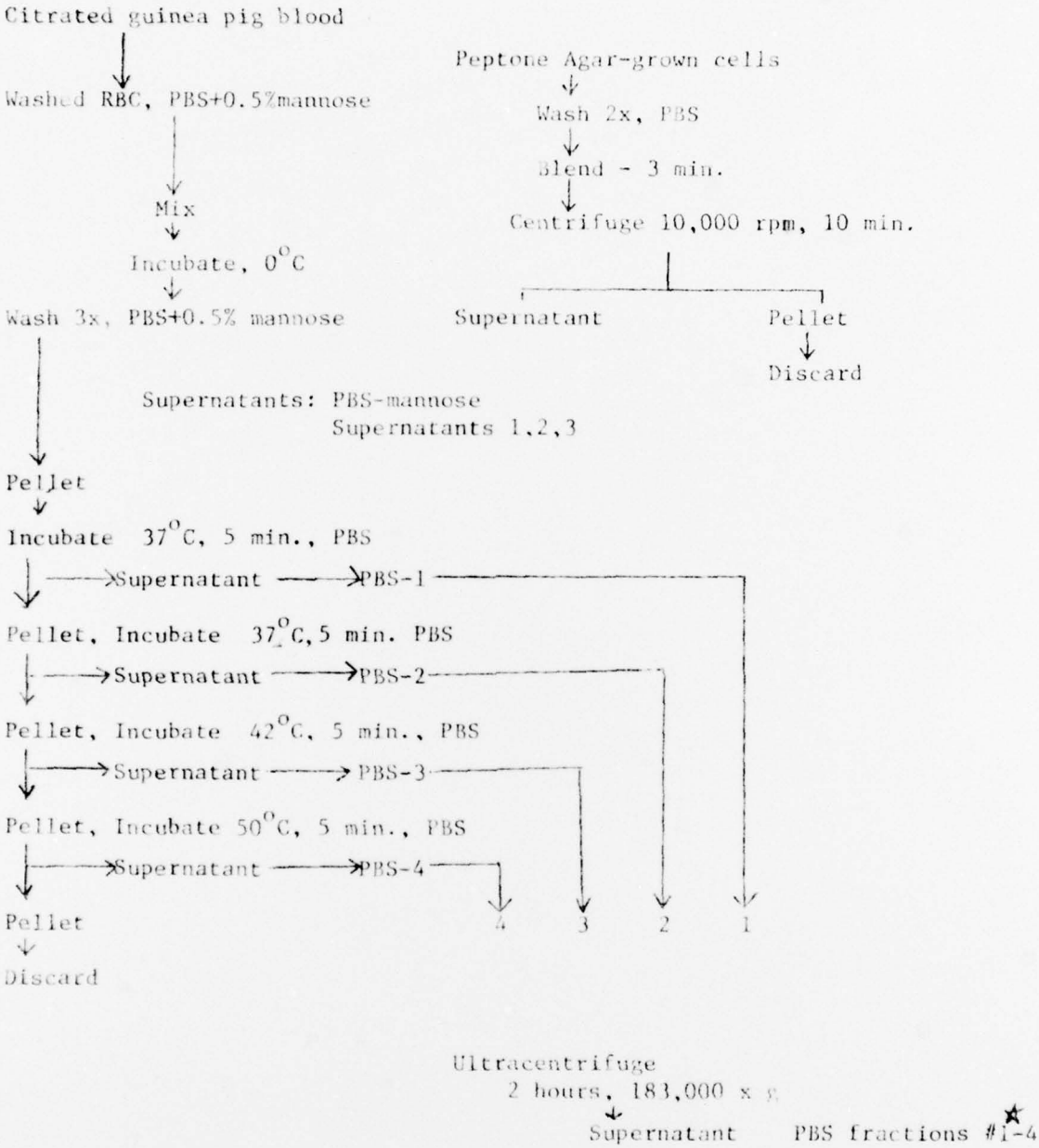
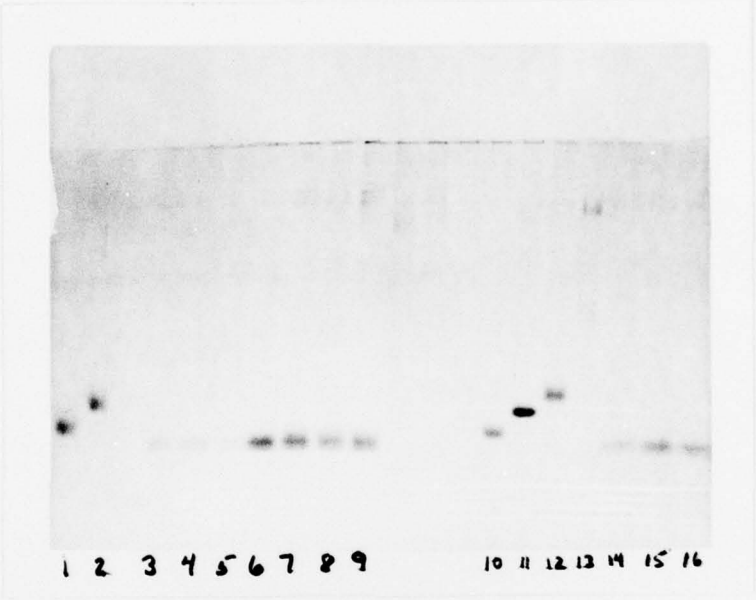


Figure 2

SDS Polyacrylamide gel electrophoresis of pili and molecular weight standards



Sample No.	Sample	Sample No.	Sample
1	lysozyme, m.w. 14,300	10	cytochrome C, m.w. 11,700
2	ribonuclease A, m.w. 13,700	11	myoglobin, m.w. 17,200
3	334 pili PBS-mannose wash, OC, 1st wash*	12	-lactoglobin, m.w. 18,400
4	334 pili PBS-mannose wash, OC, 2nd wash*	13	bovine gamma globulin, m.w. 50,000 and 23,500
5	334 pili PBS-mannose wash, OC, 3rd wash*	14	pili from E. coli TX-1*
6	334 pili PBS wash, 37C*	15	pili from E. coli D542*
7	334 pili PBS wash, 37C*	16	pili from E. coli D481*
8	334 pili PBS wash, 42C*		
9	334 pili PBS wash, 50C*		

* two bands are visible on close inspection (see Figure 3).

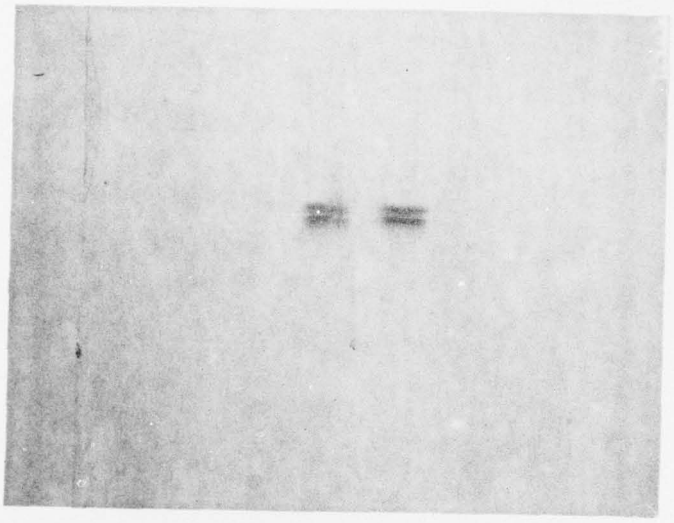


Figure 3.

SDS Polyacrylamide gel electrophoresis of 334 pili

Electrophoresis was carried out for 6 hours to increase separation of the two bands.

Figure 4 SDS Polyacrylamide Gel Electrophoresis of Pili and Human Buccal Cells

1. myoglobin
2. β-lactoglobin
3. lysozyme
4. 334 pili
5. 1st PBS wash of 334 pili and buccal cell mix
6. 2nd PBS wash
7. 3rd PBS wash of 334 pili and buccal cell mix
8. 4th wash
9. initial mixture of 334 pili and buccal cell mix (1X)
10. final washed buccal cell pellet (1X)
11. buccal cell preparation (concentrated 25X)

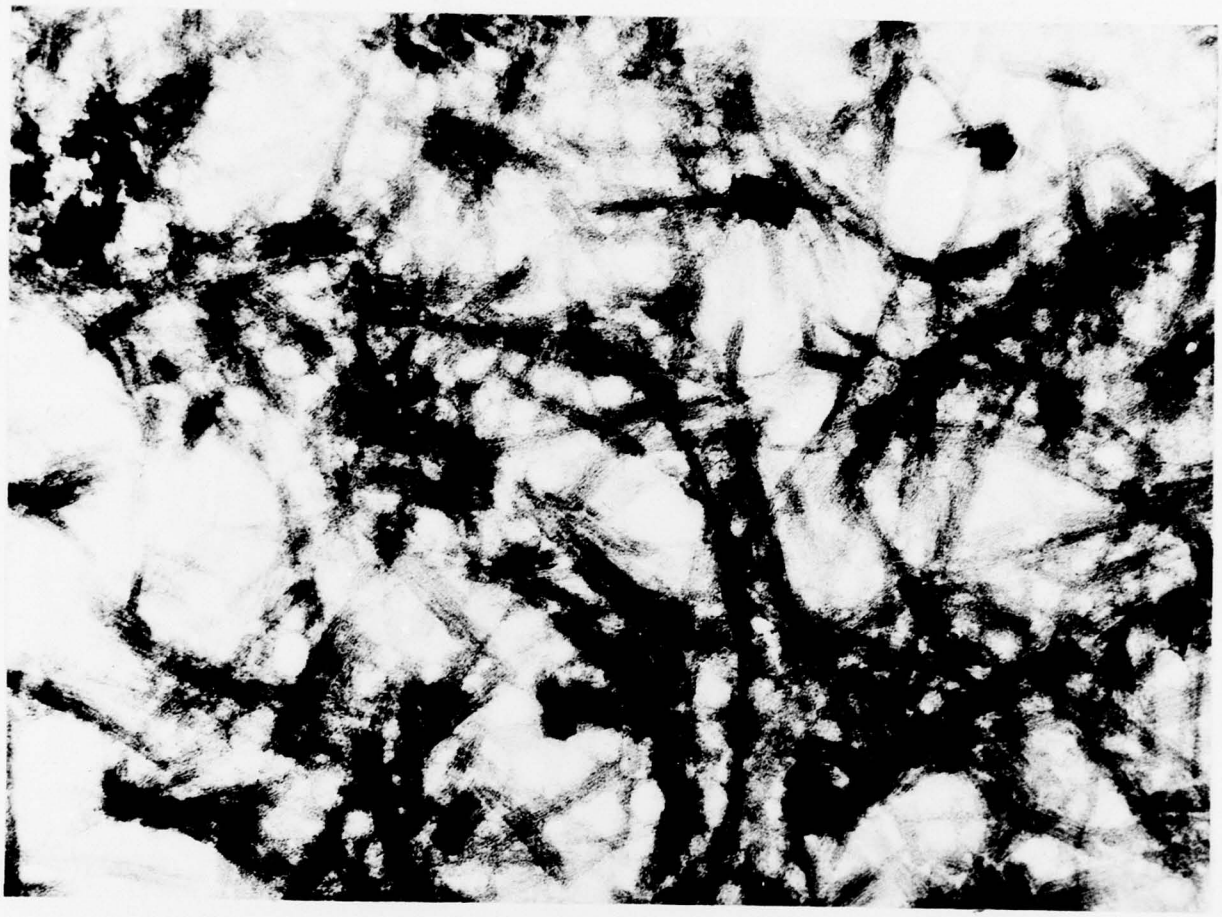


Figure 5. Specific pili preparation from strain 193-4 LT+ST+ HA^+.
Negatively stained with 2% uranyl acetate (X 240,000).

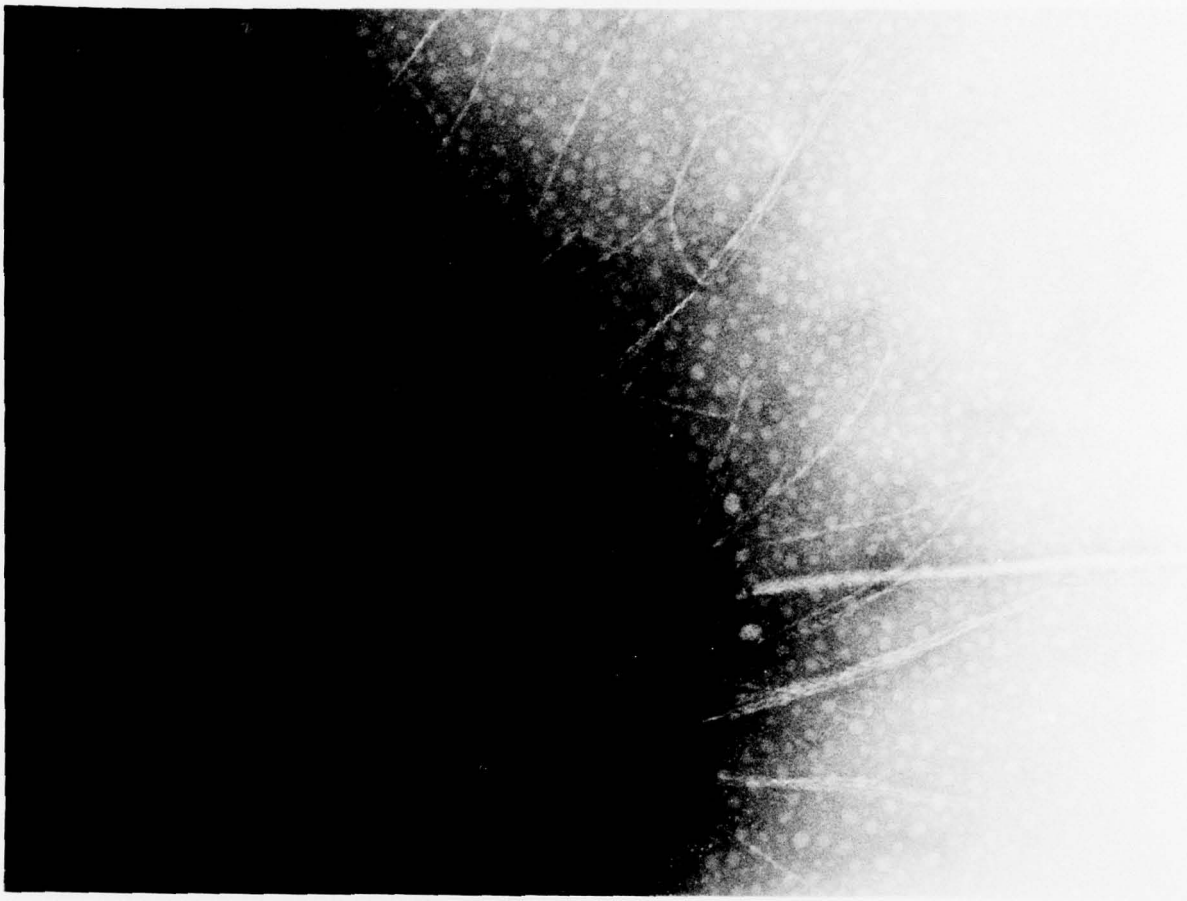


Figure 6. Pili on surface of whole cell 334 $LT^{+}ST^{+}$ MR-HA^{+}.

Negatively stained with 2% potassium phosphotungstate (X 100,000).

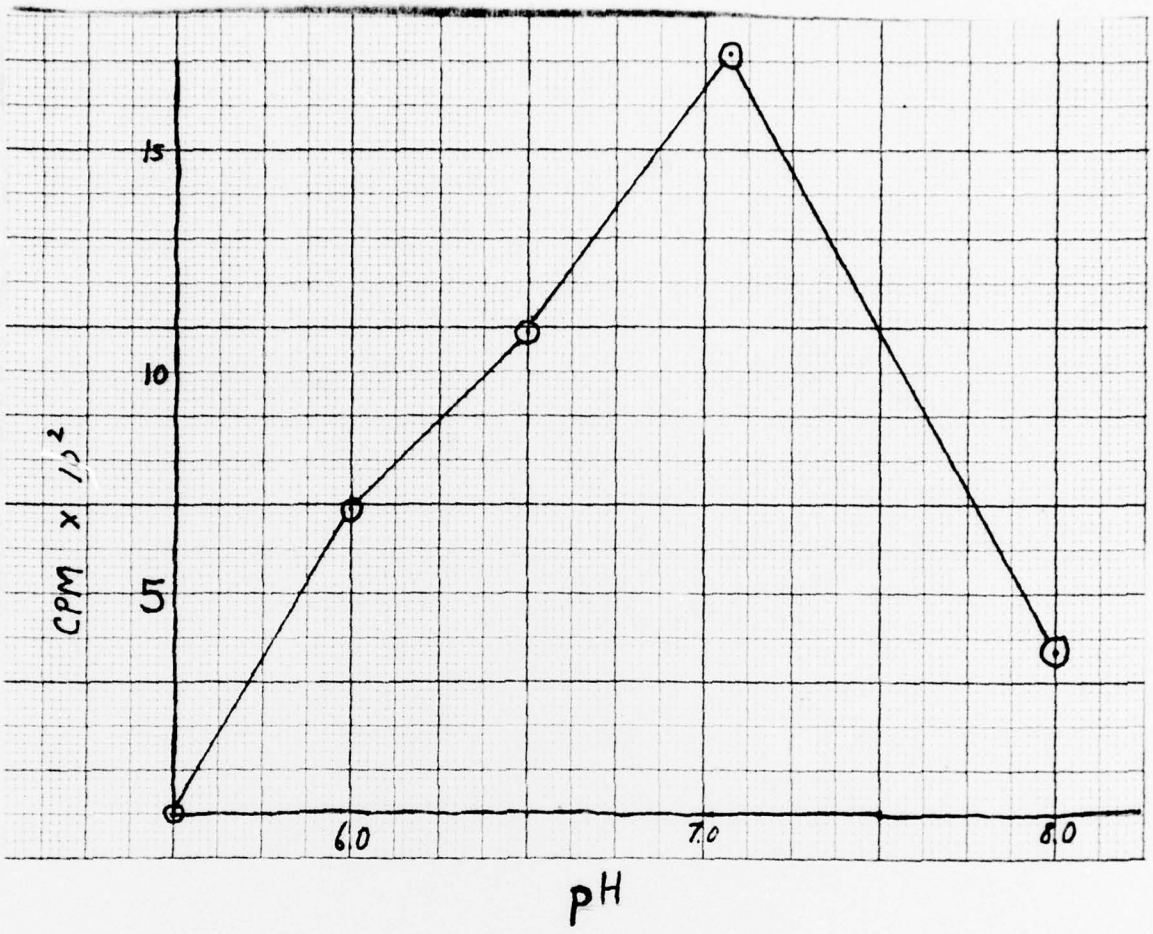


FIGURE 7. EFFECT OF pH ON BUCCAL BINDING BY E. COLI 334. E. coli 334 were grown for 6 hours on peptone agar containing 25 μCi each ^{3}H-alanine and ^{3}H-leucine. The bacteria were resuspended in PBS at the appropriate pH and mixed with either saline or buccal in saline. Values have been corrected for bacterial self-aggregation and represent only bacterial-buccal association.

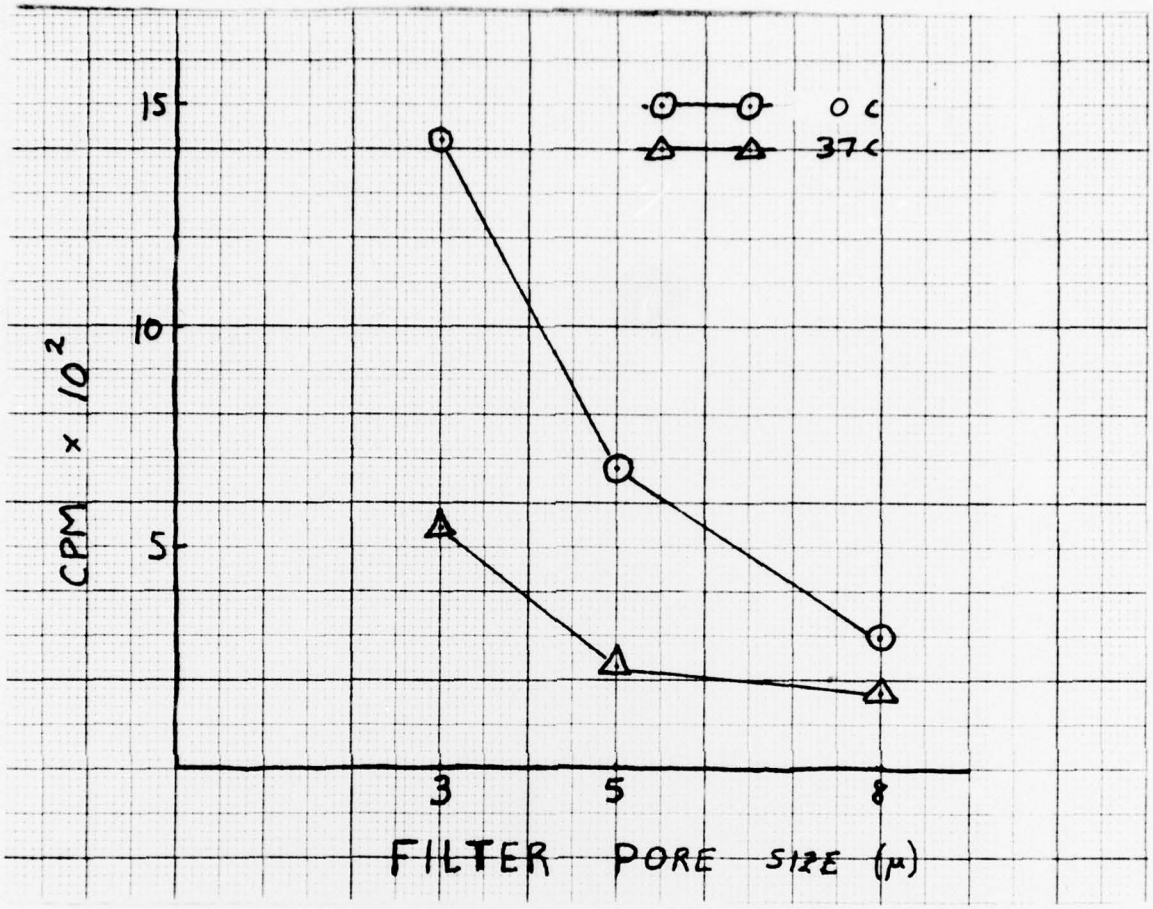


FIGURE 8. BACTERIAL SELF AGGREGATION OF E. COLI 334.
E. coli 334 was grown for 6 hours on peptone agar containing 25 μCi each ^{3}H-alanine and ^{3}H-leucine. The bacteria were resuspended in PBS containing 0.5% mannose. Following incubation at either 0 or 37 C, 0.5 ml aliquots were filtered and radioactivity determined by liquid scintillation.

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