Subcloning of the fimbrial gene encoding for 987P for use as a diagnostic probe in swine diarrheal disease

by

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INTRODUCTION

Adherence of pathogenic bacteria to host tissues is often the first step in initiating the disease process. When adherence is absent, host defense mechanisms such as mucociliary action, peristalsis, and fluid flow across mucous membranes may remove invading pathogens. The ability of bacteria to adhere and maintain attachment in the face of host defenses is an important determinant of pathogenicity.

Although the ability to attach to and proliferate on epithelial surfaces is important in some diseases, there are other virulence factors of importance. Important factors contributing to virulence include the ability to produce enterotoxins or endotoxins, hemolysins, capsules and iron-sequestering proteins such as enterochelin and aerobactin. Attachment to host epithelial surfaces provides close contact allowing efficient and rapid action of toxins. Without close association of bacteria with the host cell, toxins could be washed away before exerting their maximal effects. Once attached to epithelial cells, bacteria are protected from the cleansing action of surface fluids. Toxins can then be released at their sites of action.

Different bacterial pathogens have evolved their own means of attaching to host cells. Cell surface components such as adhesive proteins (16), specialized attachment structures (40, 102), and sugar

polymers (55) are examples of non-fimbrial attachment factors. These types of factors allow organisms to adhere without specialized appendages to host cells. Research on pathogens possessing attachment organelles has been more extensive. Disease-producing organisms inhabiting the gut, urogenital tract, wounds, respiratory tract, and mucous membranes of the host generally express an adhesin on their surface. These adhesins are usually composed of protein although carbohydrates, glycoproteins, or glycolipids may be found in addition. Pathogenic bacteria found in human and veterinary diseases possessing adhesins include Klebsiella (22), Neisseria (104), Moraxella (3), Aeromonas (83), Pseudomonas (39), Shigella (25), Salmonella (23), Vibrio (99), Bordetella (2a), and Escherichia (6).

The functional properties of the adhesins of pathogenic bacteria provide useful information for their identification. In E. coli characteristics such as the ability to hemagglutinate certain species of erythrocytes (13) and adherence to host cells can be used to distinguish one adhesin type from another. Hemagglutinating activity of some E. coli strains was inhibited by the presence of 0.5 % D-mannose or methyl-alpha-D-mannoside (14, 24). Brinton (6) found these hemagglutinins, belonged to the Type 1 classification of fimbriae. However, Duguid et al. (24) found there were host-specific adhesins of various O serogroups that hemagglutinated erythrocytes in the presence of mannose. Therefore, fimbriae unable to hemagglutinate in the presence of mannose have been designated mannose-sensitive, while those

hemagglutinating regardless of the presence or absence of mannose are mannose-resistant. Although agglutination of erythrocytes is a possible indicator of fimbriae on the surface of a cell, the problem of whether the receptor on the red blood cell is the same as the receptor on host epithelial cells needs to be addressed. Jann et al. (47) studied E. coli strains bearing known fimbrial types and found no correlation between the ability to agglutinate red blood cells and adherence to mammalian cells.

The adherence of bacteria to mammalian host cells is a complex process. Host specificity of adhesins cannot be determined by hemagglutination testing with or without mannose. This requires a closer examination of adhesin-specific receptors on eukaryotic cells along with serological testing. For many pathogenic bacteria that possess adhesins, including E. coli, these tests offer a better understanding of the attachment mechanisms important in initiating a disease process.

Adherence is a primary area of study concerning diarrheal diseases affecting domestic animals and man. Among these diseases there are several caused by members of the family Enterobacteriaceae that are of major importance. Escherichia, Salmonella, and Shigella are genera belonging to this family known to be important causes of diarrheal disease. These bacteria adhere to epithelial surfaces of the intestine and / or invade epithelial and subepithelial cells. Upon invasion of these cells, enteritis and severe, profuse diarrhea often result.

Escherichia coli is probably a major contributor to diarrheal disease in young domestic animals.

There are four types of E. coli associated with diarrheal disease in humans and animals (58). These are: 1. Enteroinvasive (EIEC). This type is responsible for causing dysentery in humans only. 2. Enterohemorrhagic (EHEC). This is associated with hemorrhagic colitis and hemolytic uremic syndrome in humans, and hemorrhagic colitis in calves. 3. Enteropathogenic (EPEC). This is responsible for infant diarrhea in humans and diarrhea in calves. 4. Enterotoxigenic (ETEC). This type is associated with infant and travelers diarrhea in humans, and neonatal diarrhea in piglets, calves, and lambs, and post-weanling coli- bacillosis in pigs. Each type of diarrheagenic E. coli described above possesses a set of traits to distinguish it from the other. Enteroinvasive strains have a predilection for the colonic mucosa. They invade, proliferate and cause death of epithelial cells. These strains also secrete a cytotoxin although it had not been shown yet to have a role in the disease process. Enterohemorrhagic strains are often associated with O157:H7 serogroup. These bacteria may secrete one or both toxins, Verotoxin 1, which resemble Shigella dysenteriae type 1 cytotoxin, and Verotoxin 2. The sites of bacterial infection are the cecum and colon. Enteropathogenic bacteria characteristically attach to and destroy microvilli without invasion of the enterocyte. These strains usually contain a plasmid called EAF (enteropathogenic adherence factor) plasmid that encodes for a 94 kDa protein found on

the bacterial surface. This protein is thought to aid in attachment to the enterocyte surface. Enteropathogenic strains cause lesions primarily in the ileum and colon.

Enterotoxigenic E. coli are non-invasive and colonize the small intestine. They attach via fimbriae. These bacteria produce one or both of the following toxins: heat-stable (ST) or heat-labile (LT). Although their effects on intestinal fluid secretion are similiar, LT and ST toxins are different from each other structurally and functionally. Structurally, LT is similiar to cholera toxin. They are both composed of five receptor-binding units called B subunits and one active A subunit. The molecular weight of LT is approximately 86,500 daltons and there is only one type. ST is composed of two subsets STa (STI) and STb (STII). Both types are molecules containing no subunits and have a molecular weight of between 1,500 and 5,000 daltons. Genes encoding for LT, STa, and STb are located on plasmids that have inverted repeating sequences flanking the coding regions. The STa genes are found on transposon Tn1681 and are flanked inverted repeats of IS1. STb genes have a transposible element structure but there is no evidence of transposition. LT genes have not yet been found associated with transposons. Functionally the action of LT on epithelial cells, like cholera toxin, affects adenylate cyclase causing a prolonged fluid secretion by epithelial cells. The STa activates guanylate cyclase and is found in human, bovine, and porcine E. coli isolates. The STb is produced by certain strains of porcine ETEC and

the mechanism of action is not known. Both STa and STb cause a short-lived, rapid-onset fluid secretion from epithelial cells. The receptor for LT is GM_1 ganglioside and the STa receptor is thought to be either a protein or lipoprotein. The receptor for STb is not known.

Adherence of pathogenic and potentially pathogenic organisms to host cells is usually selective. For example, respiratory tract pathogens adhere to tracheal epithelial cells, while venereal disease pathogens adhere to epithelial cells of the urogenital tract. If an invading organism does not have a specific adhesin for a given cell type, host defense mechanisms or unavailability of attachment sites may make it difficult to replicate and elaborate other virulence factors.

LITERATURE REVIEW

Fimbriae on ETEC were first demonstrated in 1950 by Houwink and Van Iterson (39). Utilizing electron microscopy, they found a surface appendage different from previously described flagella. Flagella are long filamentous organelles on the bacterial surface (12, 54) responsible for locomotion of the organism (87). Houwink and Van Iterson observed filaments that were thinner and shorter than flagella. They were evenly distributed across the cell surface unlike flagella which are found in bundles. They suggested these appendages were responsible for attachment.

Duguid et al. (26) supported Houwink and Van Iterson's suggestion that these non-flagellar organelles function in attachment. Duguid suggested the name "fimbriae" (Latin meaning threads or fibers) be assigned to these appendages. They found that fimbriated E. coli would agglutinate red blood cells. Brinton (5) demonstrated the existence of fimbriated and non-fimbriated phases in E. coli. Fimbriae were present when bacteria were grown at 37°C but not at 18°C.

Members of the genus Escherichia important in veterinary diseases have adhesins on their surface. E. coli causing diarrheal diseases have adhesins that allow the organism to adhere and initiate the disease processes. Fimbrial types found on ETEC strains have tested mannose-resistant in hemagglutination and in in-vitro epithelial cell adhesion assays. Since many strains express more than one type of

fimbria on their surface, hemagglutination should not be used as an indicator of the presence or absence of a fimbrial type. Also, some ETEC strains produce fimbriae that fail to hemagglutinate any erythrocytes of any species tested so far. Along with hemagglutination, other criteria are necessary to identify specific fimbrial types. Studies have shown each fimbrial type recognizes a specific receptor on the gut epithelial surface. There appears to be no cross-reactivity between ETEC fimbrial types and their receptors (52b).

To date four major types of adhesins have been detected on E. coli: K88, K99, 987P, and F41.

K88

Fimbrial antigens on ETEC were first serologically described in 1961 by Orskov, et al. (81). They analyzed surface antigens present on E. coli isolated from swine with edema disease. One of these called L, was later found on several ETEC isolates from edema disease outbreaks and subsequently named K88 (80). Assays revealed K88 antigen agglutinated guinea pig erythrocytes (95) and chicken erythrocytes (84).

The incidence of K88 among swine diarrheal field isolates has been found to be approximately 45% (4, 103). The percentage may vary according to geographic location, herd health, and vaccination practices. Evans et al. (29) conducted a study in Michigan in which the

prevalence of all ETEC fimbrial types associated with swine diarrhea was determined and found K88 in only 27% of the field isolates tested.

Vaccination against K88 and other fimbrial antigens is a common practice in swine breeding operations. Usually the sow is injected several weeks before farrowing with a bacterin-toxoid or bacterin preparation. The vaccine consists of E. coli with the four important fimbrial types. The sow's immune system is stimulated to produce antibodies against these antigens. Antibodies are passed to the piglet through colostrum and provide protection against neonatal enteric disease.

Antigenic variation among K88 isolates has been described. Four variants now recognized are K88ab1, K88ab2, K88ac, and K88ad. There is a common factor "a" in these isolates, but each contains a distinct antigenic determinant in addition. The K88ab variant was originally found on edema disease isolates in England (30). The K88ac variant was isolated in Ireland (79). The K88ad variant was isolated in the Netherlands (35). Orskov et al. (80) detected K88ab and K88ac variants using specific antisera. The K88ab subtype was further divided into subgroups 1 and 2 based on sequencing of K88ab genes (27). Guinee and Jansen (35) examined the hemagglutination patterns and serological characteristics of the K88 variants. The location of divergent regions of the variant K88 fimbrial types was determined by amino acid (33, 53) and DNA sequencing (32, 34).

Incidence of K88 variants among field isolates has been studied by

Westerman et al. (101). They found K88ac was the predominant variant of K88 in 415 strains tested with monoclonal antibodies against each subtype. Gaastra et al. (33) suggested that K88 variants occur because of intensive immunization by swine producers. Vaccination against one variant was thought to apply selective pressure for those bacteria that could produce a modified subunit. Antibody specific for the K88 type present in the vaccine would not be protective against the variant.

Genetic analysis revealed the genes encoding K88 in E. coli are found on episomes or plasmids (78, 80, 96). Experiments were carried out using a donor possessing plasmid-encoded K88 genes and a host that did not produce the K88 adhesin. The plasmid encoding K88 was transferred from the donor strain and introduced into the $K88^-$ recipient. Daughter cells were serologically tested and found to produce K88. To confirm that K88 genes were present on a transmissible plasmid, K88-producing cultures were exposed to acridine orange. Acridine orange causes frameshift mutations in DNA and instability in replication mechanisms. Plasmids affected by this chemical become unstable and are lost during host cell division. Daughter cells treated with acridine orange showed no reactivity and proved the loss of K88 production.

Morphological analysis of K88 antigen (64, 96) showed this antigen to be a thin, flexible protein, with a diameter of 2.1 nanometers. Chemically, K88 antigen is soluble at pH 6.8 and has a subunit

molecular weight of 26,500 daltons. Subunits of fimbrial adhesins are proteins identical in structure and function that assemble on the outside surface of the bacterial cell. There is conservation of the amino acid sequence for necessary secondary and tertiary structure of the protein (53). The primary amino acid sequence of each fimbrial type is different since this is responsible for antigenic variation (27).

Smith and his colleagues (91, 92, 93) provided experimental evidence that K88-producing E. coli caused enteritis in neonatal swine but not in calves or lambs. These investigators found that piglets with diarrhea had colonization of the anterior small intestine by K88-producing E. coli. Smith and Halls (91) concluded that for E. coli to cause diarrhea it also must produce enterotoxin (Ent^+) along with the K88 adhesin. Smith and Linggood (94) found isolates lacking K88 ($K88^-$) and possessing the ability to secrete enterotoxin were fed to piglets. There was a lower incidence of diarrhea among those animals fed $K88^-$ Ent^+ cultures than those fed $K88^+$ Ent^+ cultures. This was confirmed later by a study that removed the K88 plasmid from E. coli and introduced the strain into weaned pigs. Subsequently, disease was not produced in the animal host. When K88 was reintroduced into the E. coli, the bacterium was able to produce diarrhea. Further investigation of ETEC possessing the K88 adhesin showed disease resulted from attachment and proliferation on the intestinal villus (93, 48).

K99

The K99 antigen was first described in 1972 by Smith and Linggood (94). This antigen was expressed on enteropathogenic Escherichia coli strains causing diarrhea. They reported Sojka had isolated calf and lamb enteropathogenic strains with different O antigen types, but possessing a closely related or common K antigen (Kco). Strains expressing this antigen were found to proliferate in the small intestine, unlike strains possessing K88 that were found in the anterior small intestine. Organisms possessing the Kco antigen were found to attach to calf intestinal villi in the same manner as K88 attaches to swine intestinal villi (9).

Serological analysis by Orskov et al. (82) revealed this antigen to be different from those previously known and proposed the Kco antigen be designated as K99. K99 was found to be a protein (42, 82).

Purification and characterization studies by Isaacson (42) using reference strain B41 which expressed K99, found the antigen to be composed of two subunits. These subunits were cationic with a molecular weight of 22,500 and 29,500 and a pI of 10. Isaacson also found that sheep and horse erythrocytes were hemagglutinated by strains expressing K99 and that K99-producing strains adhered to porcine intestinal epithelial cells in vitro (43). This finding was in contrast to earlier reports by Morris et al. (67, 68) that K99-producing strains agglutinated guinea pig erythrocytes in addition to

sheep and horse erythrocytes. Morris found the fimbrial subunits had a pI of 4.2. They also discovered an anionic component present on reference strain B41 in addition to the cationic component reported earlier. Subsequent study of strains producing K99 fimbriae was carried out by De Graaf et al. (19) and the subunit size of the cationic component was measured as 18,500 daltons, with a pI of 9.5. These data were in agreement with Isaacson's findings and 18,500 daltons is now the accepted size.

Morris et al. (70) explained the differences in published results concerning K99 by comparing isolation procedures. The K99 antigen that precipitated with ammonium acetate (42) was found to agglutinate horse and sheep erythrocytes in contrast to the acid precipitated antigen (9, 67) that agglutinated these and guinea pig erythrocytes. Both methods used B41 as the host strain for K99 isolation. Investigators were using different isolation methods to obtain the K99 antigen. Since one isolation procedure employed the use of a basic solution and the other procedure used an acidic solution, to compare which purification procedures were better was not possible. Since ammonium acetate favors K99 precipitation, the anionic protein remained in solution and only sheep and horse erythrocytes were agglutinated. Under acidic conditions the anionic component was preferentially precipitated (70). This anionic component was later named F41.

Like the K88 genes, K99 genes were found to be encoded on a transmissible plasmid (94). These experiments used E. coli K12

recipient strains ($K99^-$) and calf and lamb enteropathogenic strains expressing Kco. To test if expression of $K99^+$ antigen could be lost, cultures were treated with acridine orange as described previously for K88. To confirm transfer and lack of expression of genes, adhesion to ligated calf intestinal loops was used as a testing system. Bacteria lacking the plasmid adhered poorly to calf epithelial cells in contrast to bacteria expressing $K99^+$ antigen.

F41

The F41 antigenic type was characterized by De Graaf and Roorda (21). They described a spontaneous mutant of B41 (B41M) that did not react with K99 antiserum and produced only the anionic antigen (discussed above). The adhesin was named F41 (69) and found to be a protein like the other adhesins previously described. The F41 subunits were 29,500 daltons and had a pI of 4.6.

The B41M strain was not the only F41-producing organism investigated. Some atypical ETEC isolated from outbreaks of diarrheal disease in piglets failed to produce the three common fimbrial antigens- K88, K99, or 987P (1, 65, 98). These isolates were referred to as $3P^-$ strains (F41 in later studies) since they did not react with antiserum specific for the three common fimbrial antigens previously mentioned. Isolates with F41 adhered to the villi in in the lower small intestine of neonatal pigs (65). In calves and lambs F41 isolates appeared to colonize the lower small intestine, similar to

K99.

Moseley et al. (72) isolated the genes encoding F41. Using DNA probes derived from K88, K99 and F41 subunit genes they screened calf and swine diarrheal isolates. Results of the screening indicated that several strains exhibited homology with both K88 and F41 probes indicating possible genetic relatedness of the fimbrial genes. Some strains were found to contain both the K99 and F41 genes upon hybridization with the respective probes. This result was significant since this was the first indication there were plasmid-encoded and chromosomally-encoded fimbrial genes present in the same isolate.

Incidence of F41 in calf and lamb diarrheal outbreaks has not been studied. As stated previously, F41 is commonly found on ETEC strains also producing K99. This makes it difficult to detect strains expressing $F41^+$ antigen exclusively. Detection of ETEC strains expressing F41 has relied upon serological identification. A more sensitive detection system has been used for $F41^+$ strains through the use of DNA probe hybridization. Although this method can be used to detect F41 DNA in an ETEC strain, it is not reliable since these probes will also hybridize with K88 genes.

987P

The 987P antigen was first discovered on neonatal porcine EPEC lacking K88 antigen (45, 75, 76). This was a previously undescribed antigenic type that caused diarrhea and was produced by the EPEC strain

987. These investigators observed that after a few passages on agar plates, these bacteria did not adhere to isolated intestinal epithelial cells or react with antiserum in vitro. Conversely, these E. coli strains adhered to porcine epithelial cells in vivo causing disease. These strains were found to produce low numbers of fimbriated cells and were introduced into hysterectomy-derived colostrum deprived piglet hosts. Following reisolation, there was an approximately 50-fold increase in 987P fimbrial production. The adhesin present on E. coli strain 987 was named 987P. Nagy et al. (76) explained that growth conditions were responsible for the differences in adhesiveness between in vitro cultures and in vivo E. coli 987 strains. When bacteria were passaged through a porcine host the 987 bacteria isolated were richly fimbriated, possibly due to available nutrients and low oxygen tension. On laboratory media, 987 bacteria tended to lose their ability to produce fimbriae, and thus lost their adhesiveness. Therefore, they concluded that fimbrial production was a trademark of virulence among E. coli 987 strains.

Purification and further characterization of 987P fimbriae were carried out by Isaacson and Richter (46). Morphologically, 987P fimbriae have a rigid structure identical to Type 1 fimbriae. Differences were found in subunit size; the molecular weight of 987P subunits was found to be 20,000 daltons and that of Type 1 was found to be approximately 18,000. The isoelectric point of 987P and Type 1 subunits are similiar at 3.7 and 3.9, respectively. Bacteria

expressing 987P failed to hemagglutinate erythrocytes from any of several animal species (45, 46) in contrast to Type 1 fimbriae (13, 14, 26). Although hemagglutination with untreated erythrocytes does not occur with 987P fimbrial antigen, a recent report by Ike et al. (41b) found erythrocytes pretreated with gluteraldehyde could be agglutinated by 987P fimbrial antigen. Erythrocytes from guinea pigs and chickens reacted strongly at the highest dilution. They postulated that the receptor for 987P fimbriae exists on the inner side of the erythrocyte membrane and glutaraldehyde treatment exposes the receptor.

Incidence of 987P among porcine diarrheal outbreaks has been addressed in the same studies as those mentioned previously concerning K88 (4, 103). Demonstration of 987P on ETEC strains found in porcine diarrhea has relied on serologic testing. Like F41, sensitive detection of 987P genes will probably be through the use of DNA probes specific for those fimbrial genes.

Genetics of 987P fimbriae

Intestinal colonization by porcine ETEC strains bearing 987P fimbriae has been investigated (31, 44, 45, 76). Manipulation of E. coli 987 suggested fimbrial genes were chromosomally-encoded since the subunit purification procedure and morphology resembled Type 1 fimbriae (65b). Fimbrial phase variation is characteristic of strains belonging to the 987 group (65a, 76). Because of this ability to "switch" from fimbriated to non-fimbriated forms, Brinton (5) suggested the genes for

987P fimbrial production were similiar to Type 1 genes. Type 1 fimbriae exhibit fimbrial variation and are common among bacteria inhabiting the mammalian intestine. The genes encoding the Type 1 subunit reside on the chromosome and Brinton postulated the genes for 987 also must reside on the chromosome. In 1986, Morrissey and Dougan (71) cloned chromosomal DNA from an E. coli strain producing 987 fimbriae into a K-12 host and were able to detect expression of 987 fimbriae on the host cell surface. Precise location of the 987P fimbrial genes has not been reported, although it is generally accepted they reside on the chromosome.

Pedersen et al. (85) compared the 987P amino acid sequence of the amino terminal end of the protein subunits of different fimbrial types found in animal and human diarrheal disease. Little homology existed between the antigenic types investigated. Comparing 987P and Type 1 amino acid sequences indicated no homology between their protein sequences. Since these sequences were unique to each fimbrial type, a synthetic oligonucleotide probe was engineered that was specific for the 987P subunit genes. De Graaf and Klaasen (18) described the nucleotide sequence of the gene encoding the 987P fimbrial subunit. Mature subunits were approximately 17,200 daltons while the subunit precursor contained a 21 or 23 amino acid signal sequence. They compared the primary structure of the 987P subunit with the amino acid sequence of other fimbrial subunits and found significant homology between 987P and Type 1A and Type 1C strains. Complete nucleotide

sequencing of a 954 base-pair DraI-ClaI fragment of the 987P subunit gene revealed a ribosome binding site and -10 and -35 promoter consensus sequences.

Organization and expression of 987P fimbrial genes was examined by De Graaf and Klaasen (17). They cloned a segment of the chromosome from an E. coli strain expressing 987P into a cosmid vector and subcloned into a plasmid vector. E. coli minicells containing the recombinant DNA expressed 5 polypeptides associated with 987P production. Location of the genes encoding for the polypeptides was determined by insertional mutagenensis, using transposon Tn5, and restriction endonuclease mapping. A 12,000 base-pair fragment was found to contain genes for proteins with molecular weights (in daltons) of; 81,000, 39,000, 28,500, 20,500, and 16,500.

De Graaf and Klaasen compared the size and function of 987P polypeptides to other fimbrial type polypeptides whose function was already known. The largest protein of 81,000 daltons probably serves to "anchor" growing fimbriae or transport fimbrial subunits to the outer surface of the cell. The function of the 39,000 dalton protein is not yet known. Tn5 insertional mutagenesis in the 28,500 dalton polypeptide gene causes degradation of the mature fimbrial subunit. Therefore, the 28,500 dalton polypeptide probably is responsible for transport of the subunit through the periplasmic space. Through the use of minicell preparations and serological screening, the 20,500 dalton protein was found to be the 987P subunit. Although a 16,500

dalton polypeptide was detected, the gene encoding for it was not located. De Graaf and Klaasen suggest this protein is either a degradation product of a polypeptide previously mentioned or is rapidly degraded when there is a deficiency of another mature polypeptide.

Use of diagnostic DNA probes

Use of DNA probes to detect genes encoding virulence traits important in human diseases has increased in recent years with the advent of improved molecular biology techniques (97). In veterinary diagnostics, progress has been slower in developing DNA probes for detecting pathogenic traits of bacteria. The idea of using DNA encoding for all or part of a virulence trait to detect the presence of those traits in a pathogenic organism is precise. This screening technique concentrates on finding nucleic acids that are always present as opposed to their gene products that are detected in biochemical assays. The first reported use of DNA as a probe for virulence factors in ETEC was by Moseley et al. (73). Radiolabeled fragments of DNA encoding heat-labile (LT) and heat-stable (ST) toxins were used to screen ETEC isolates from human and animal diarrheal outbreaks. The ST probe was sensitive enough to detect two heterologous types of ST among human isolates. These findings were confirmed by other investigators (8, 36, 52a).

Currently only probes detecting ETEC toxin genes are available for veterinary diagnostic use. There have been two recent reports on the

use of genes encoding for fimbrial subunits as DNA probes. Lanser and Anargyros (56) utilized the DNA from recombinant strains, containing the subunit genes of K88 and K99, as probes. They screened 645 E. coli isolates from a variety of animal sources. Strains were tested for K88 variants and K99 by enzyme immuno assay (EIA) testing. Comparisons between DNA hybridization and EIA, and DNA hybridization and slide agglutination were made. Results of these comparisons found agreement with the DNA hybridization, and the slide agglutination and EIA tests with the K99 adhesin. The DNA hybridization was more sensitive in detecting K88 adhesin than the other two tests. Lanser and Anargyros concluded the use of DNA probes was more sensitive than the other tests mentioned since any change in environmental factors may affect the results of these tests.

Another use of DNA probes to detect ETEC adhesins was reported by Mainil et al. (60). They used a 500 base-pair probe to detect the loss of genes encoding the 18,200 dalton K99 subunit. Neonatal pigs were inoculated with a K99-producing E. coli strain and fecal samples collected. Colonies of this strain were re-isolated from the feces. Recovered colonies were screened for presence or absence of K99 subunit genes using the K99-derived 500 base-pair DNA probe. Twenty to thrity-six percent of the isolates recovered 3 to 5 days after inoculation were variants. The variants were determined by the lack of hybridization with the 500 base-pair probe. This may have indicated a selective advantage for strains having lost the K99 genes.

Other DNA probes recognizing K88, K99, and F41 fimbrial subunit genes are used for screening diarrheal outbreaks in some laboratories. These probes have yet to become commercially available.

Escherichia coli bearing 987P fimbriae can be isolated from porcine neonatal diarrhea cases. It is possible 987P fimbriae also play a role in post-weanling diarrheal disease in swine. Death loss of piglets due to these diarrheal diseases is of economic importance to the swine industry. Therefore, it would be advantageous to have a DNA probe specific for 987P strains to study the incidence of 987P fimbriae in porcine diarrheal outbreaks.

Morrissey and Dougan (71) recently cloned the genes encoding for 987P into cosmid vector pHC79 and named the recombinant pPM200. The objectives of the research described in this thesis were to subclone 987P genes from pPM200 and develop a highly specific probe for use as a diagnostic reagent.

MATERIALS AND METHODS

Bacteria

Bacterial strains HB101 (61), JM107 (105), and DH1 (61) were used in this study as a host for plasmid transformation. Other bacterial and recombinant strains are listed in Table 1.

Media

Bacteria were routinely grown in L broth or on L agar (63) unless otherwise specified. Special media for static cultures included: Trypticase Soy Broth, Z-1 (77), and M-9 minimal media (63). Cells containing pBR325 or its derivatives were selected and maintained in the presence of 100 ug\ml ampicillin. Amplification of plasmids was by the addition of chloramphenicol 170 ug\ml to the culture medium.

Enzymes

Restriction endonucleases, T4 DNA ligase, T4 kinase, and buffer were purchased from Bethesda Research Laboratories (BRL, Bethesda, MD). DNA was digested, ligated, and kinase labeled according to manufacturers directions. Plasmid pBR325 was dephosphorylated with excess amount calf alkaline phosphatase (Boehringer Mannheim, West Germany) using the method of Maniatis et al. (61).

Table 1. Bacterial Isolates Used in Colony and Dot Blot Hybridization

Strain	Serotype	Source	Fimbrial Type
987	O9:K103:NM	Swine	987P
1413	O20:K?:NM	Swine	987P
1636	O8:K85:NM	Swine	987P
1635	O141:K?:NM	Swine	987P
1879	?	Swine	987P
1786	?	Swine	987P
1682	O-:K76:NM	Swine	987P
1722	O9:K+:NM	Swine	987P
2040	O?:K?	?	987P
1476	O?:K?	?	K88ac
2041	O157:K?	Swine	K88ac
1472	O9:K30:NM	Calf	K99
1474	O?:K?	?	K99
1471	O101:K?	Calf	K99, F41
431	O101:K30:NM	Swine	K99, F41
1751	O101:K27:NM	Swine	F41
1706	O101:K30:NM	Swine	F41
1676	O101:K30:NM	Swine	F41
H10407	O78:K80:H11	Human	CFAI
123	O43:K-:H28	Swine	?
1475[a]	?	Human	Type 1
1794	?	?	Type 1
HB101(pHC79)	-	-	Type 1
HB101(pSLM204)[b]	-	Recombinant	F41
DS912(pMK005)[c]	-	Recombinant	K88
C600(pFK99)[d]	-	Recombinant	K99

[a] *E. coli* K-12 parent strain of 1474 and 1476.
[b] Reference 72.
[c] Reference 51.
[d] Reference 20.

Plasmid and chromosomal DNA extraction

All plasmids were amplified as described above, and extracted by a modified method of Clewell and Helinski (11). Cells were harvested in a Sorvall RC-5B centrifuge using a GSA fixed angle rotor at 10,000 x g for 15 min at 4°C. Cells were resuspended in 10 ml cold 25% sucrose and 0.05 M Tris hydrochloride (pH 8.0). Lysozyme (10 mg\ml) and 2.0 ml 0.5 M EDTA (pH 8.0) was added simultaneously and the mixture kept on ice for 45 min. After lysis, 13 ml of detergent solution containing 10% Triton X-100 (Sigma Chemical Co., St. Louis, MO), 0.05 M Tris hydrochloride (pH 8.0), and 0.0625 M EDTA was added. After 45 min in an ice bath, the lysate was spun in a Sorvall centrifuge using an SS-34 rotor at 36,000 x g for 30 min at 4°C. The supernatant was recovered and plasmids purified by ethidium bromide-cesium chloride density gradient centrifugation (61). Rapid extraction of plasmid DNA was by the method of Birnboim and Doly (2b) or minipreparation method of Holmes and Quigley (38). Chromosomal DNA from cells was extracted using the method of Hull et al. (41a).

Analysis of DNA

Approximately 0.5 ug of DNA was digested with an excess amount of enzyme according to manufacturers specifications. Unless otherwise stated, electrophoresis of DNA was in 0.7% agarose (SeaKem GTG Agarose, FMC BioProducts, Rockland, ME) on a horizontal gel unit at 50 V for 6 hours in Tris-Borate buffer (0.089 M Trizma base, 0.089 M Boric acid,

0.0025 M EDTA, pH 7.5). Separation of DNA fragments was in a 5 % acrylamide gel (Bio-Rad, Richmond, CA) at a concentration of 40 : 1 acrylamide to bis-acrylamide (Bio-Rad, Richmond, CA) on a vertical gel unit. Acrylamide gels were run at 50 V for 4.5 hours.

Gels were stained 30 min with ethidium bromide 10 ug\ml in dH_2O.

Transformation procedure

Transformation, using plasmid DNA, was performed by a modification of Lederberg and Cohen (57). Cells were harvested in a Sorvall RC-5B centrifuge using an SS-34 fixed angle rotor at 6000 x g for 10 min at 4°C. Cells were resuspended to their original volume in 0.1 M $CaCl_2$ and 0.05 M $MgCl_2$ and centrifuged as described above. Cells were resuspended to half original volume in 0.1 M $CaCl_2$, incubated in an ice bath for 10 min and centrifuged as above. Cells were resuspended in 2.5 ml 0.1 M $CaCl_2$ and DNA was added. The mixture was chilled 20 min in an ice bath and heat shocked 5 min at 44°C. Approximately 3.0 ml pre-warmed LB broth was added and the transformed cells incubated in a shaker water bath at 37°C for 90 min.

Slide Agglutination

Colonies checked for the presence of fimbriae on their surface were picked with a toothpick and mixed with 50 ul Tris-EDTA buffer on a glass slide. A 1 : 100 dilution of rabbit antiserum prepared against 987P fimbriae (76) was added in equal volume and mixed thoroughly with

the bacterial cell suspension. Agglutination was read after placing the slide on a horizontal platform shaker for 5 minutes at room temperature.

Preparation of synthetic probe

Synthetic oligonucleotide probes were generated on a DNA synthesizer (Biosearch Inc., Model 8750, New Brunswick Scientific Co., San Rafael, CA) by Dr. Thomas Casey. Probes were synthesized and purified according to manufacturers specifications. Probes were radiolabeled by adding 1 ul DNA (100 ng \ ul), 1 ul 10X kinase reaction buffer (61), 1 ul [γ-^{32}P]ATP, 7 ul dH_2O, and 1 ul T4 kinase (BRL, Bethesda, MD). The mixture was incubated for 1 hour at 37°C and the reaction stopped by addition of 5 ul 0.5 M EDTA and 100 ul dH_2O. The solution was passed through a small column of G-25 Sephadex (Pharmacia, Piscataway, NJ) column to remove unincorporated label.

DNA Unblot Method

Preparation of agarose gels and reagents used in the unblot method were performed according to Meinkoth and Wahl (62). Gels were dried on a Drygel Sr. (Hoeffer Scientific Instruments, Model 1160, San Francisco, CA) at 52°C for 1.5 hours.

Preparation of DNA probes from pPM200

Plasmid pPM200 was digested with enzymes HindIII and SalI according to manufacturers specifications. Each fragment was separated by electrophoresis, stained with ethidium bromide and observed under an ultraviolet light source as described above. Each fragment was extracted from the gel by making a cut ahead of the band and inserting NA-45 anion exchange paper into the gel (Schleicher and Schuell Inc., Keene, NH). Fragments were electrophoresed into paper by applying 100 V through the gel for 10 min. Under hand-held ultraviolet light, excess paper was removed around fluorescent material. Paper was placed in 200 ul of a solution containing 1.0 M NaCl, 0.1 M EDTA and 0.02 M Tris hydrochloride (pH 8.0), and eluted by incubating 20 to 50 min at 65°C. The DNA was concentrated with one volume of n-butanol, the aqueous layer removed and the total volume increased to 500 ul with dH_2O. DNA was precipitated with two volumes 95% ethanol and resuspended in 50 ul Tris-EDTA buffer (0.01 M Tris HCl, 0.001 M EDTA, pH 7.6). The DNA was extracted once with a 50:50 mixture phenol and chloroform and once with chloroform and precipitated as before. It was resuspended to a final volume of 20 ul in TE buffer. Fragments of pPM200 were labeled using [α-^{32}P]ATP and [α-^{32}P]CTP (Pharmacia Oligolabeling Kit, Pharmacia Chemicals, Piscataway, NJ). The labeled fragments were passed through a G-50 Sephadex (Pharmacia, Piscataway, NJ) column to remove unincorporated label.

Southern blot hybridization

Southern hybridization was performed by the method of Maniatis et al. (61). Hybridization solution for DNA probes was according to Maniatis et al. (61). Hybridization solution for synthetic oligonucleotide probe contained 6X SSC, 5X Denhardt's solution, 1.0 mM EDTA (pH 8.0), and 2.0 mg calf thymus DNA (Sigma Chemical Co., St. Louis, MO). The hybridization temperature for DNA and synthetic oligonucleotide probes was 37°C. All blots were washed twice in a solution containing 0.9 M NaCl and 0.09 M sodium citrate (pH 7.0) for 1 hour each. The hybridization temperature was 65°C for DNA probes and 40°C for synthetic probes. The X-ray films were exposed to the blot at -70C for varying lengths of time depending on probe activity. The films were subsequently developed.

Colony blot hybridization

Preparation and hybridization of colony blots was performed as previously described (72), except the neutralizing buffer consisted of a solution of 1.0 M Tris hydrochloride (pH 7.0) and 1.5 M NaCl. Conditions for hybridization of DNA probes and washing were the same as for Southern hybridizations. Radiolabel was removed from blots was by washing in 0.5 M NaOH for 30 minutes and then the blots were transferred to a solution of 2 X SSC and 0.1 % SDS (w/v) for 30 minutes. Blots were air dried and stored at room temperature in sealed bags.

Dot blot hybridization

Preparation and hybridization of nitrocellulose dot blots was performed by a modification of a procedure previously described (49). Cell suspensions were inoculated in a recorded pattern onto pre-moistened filters on top of agarose plates and incubated for 4 hours at 37°C. Cells were lysed by floating filters colony side up on a solution of 0.5 M NaOH for 5 minutes. The filters were removed and placed on fresh 0.5 M NaOH for the same time. The filters were then floated colony side up on a solution of 1.0 M Trizma base (pH 8.0) for 5 minutes. This was repeated a second time with fresh buffer. Filters were neutralized by placing them colony side up onto a solution of 1.0 M NaOH and 1.5 M NaCl for 5 minutes. The filters were dried under vacuum at 65°C for 1.5 hours. Conditions for hybridization of synthetic oligonucleotide probes and washing were the same as for Southern hybridizations.

Recovery of DNA fragments from acrylamide gel

DNA digest fragments were recovered from acrylamide gels using the procedure of Smith (90).

RESULTS

Instability of pPM200

Initial attempts at restriction digestion analysis of pPM200 revealed several secondary digest fragments. These bands were initially thought to be partially digested plasmid DNA. Growth conditions were suspected as possibly contributing to instability of the plasmid. The recombinant strain containing pPM200 was inoculated into three types of media: Trypticase Soy Broth (TSB), M-9 minimal media, and Z-1 broth. Each of the above media were supplemented with Ampicillin, inoculated, and left under static conditions. Static conditions enhance 987P fimbrial production. No pellicle formation was observed at the air-broth interface. Colonies were checked for fimbrial expression by slide agglutination with rabbit 987P antiserum. No colonies were found to express fimbriae. Electron micrographs of samples taken from TSB and M-9 minimal media static broth cultures revealed little or no fimbriae on the surface of the cell.

The digestion parameters were changed to attempt to eliminate partial digestion of pPM200. These parameters included: diluting the DNA preparation, altering restriction enzyme buffers, preparing fresh enzyme buffer, extending incubation time, amplification versus no amplification in large scale plasmid preparation, and testing enzymes for reactivity. Comparison was made between plasmid preparations of the pPM200 recombinant originally received and a recent preparation.

None of these modifications eliminated the unidentifiable fragments.

Stability of pPM200 in a different host strain (E. coli DH1) was compared to the stability of pPM200 in HB101. After introduction of pPM200 into DH1, fimbrial expression was detected by agglutination with antiserum against 987P. Initial results using restriction enzyme digestions revealed DH1(pPM200) to be a stable recombinant until prepared in large scale culture. Subsequent digestion and electrophoresis of pPM200 revealed partial digest fragments again, although the bands were not the same sizes as previously observed from the recombinant prepared from HB101. A preparation of plasmid pPM200, kindly provided by Robert Schneider, was found to give a stable digestion pattern and was used throughout the remainder of the study.

Synthetic probe

Three DNA oligonucleotide sequences were synthesized based upon the published amino terminal sequence of the 987P subunit genes. These sequences were kinase-labeled with ^{32}P and used as probes to screen unblots and Southern blots. Restriction enzyme digests of 987P chromosomal DNA and pPM200 were electrophoresed and three identical unblots made. Each of the three synthetic oligonucleotide probes was used to screen the unblots. The probe that hybridized the strongest was selected for further analysis. Results were checked by repeating the digests, performing electrophoresis, and Southern blotting. The same probe hybridized the strongest. This probe was used to screen an

unblot of a gel containing pPM200 DNA digested with several different enzymes. The smallest band in the digests that hybridized with the synthetic oligonucleotide probe was a HindIII / SalI double digest of approximately 1.8 kb in size (Figure 1).

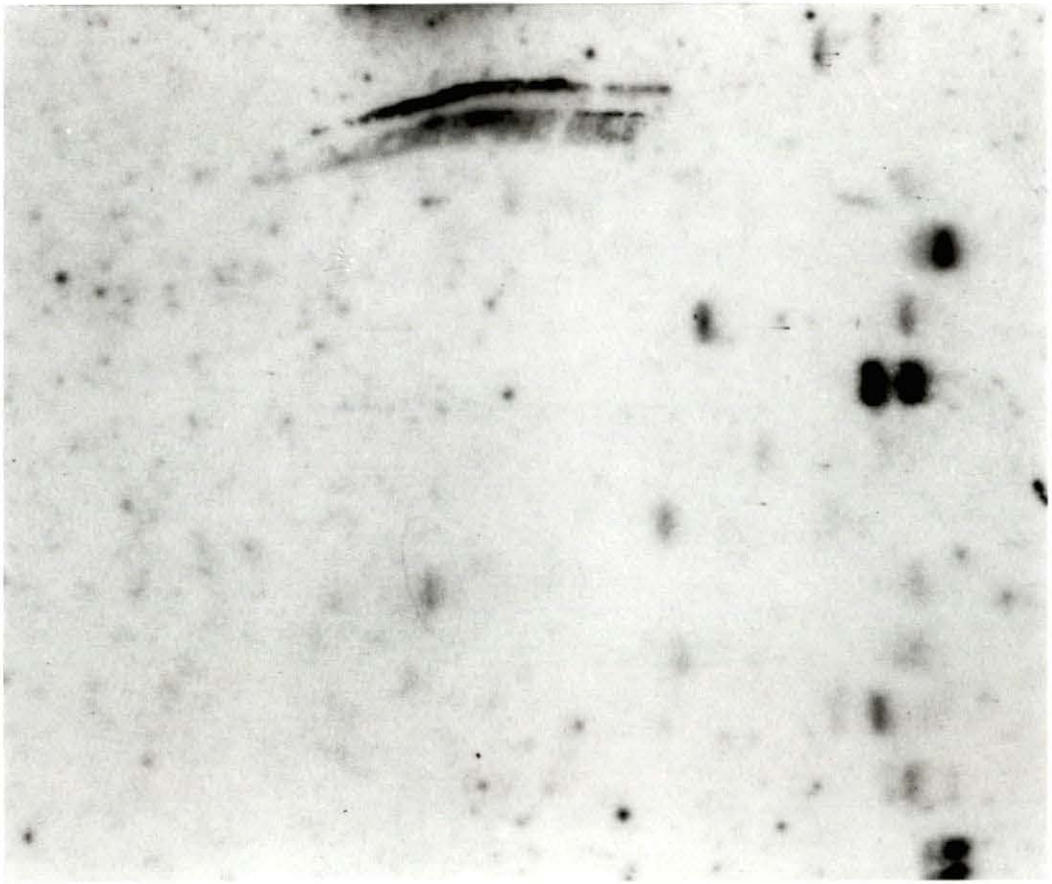
Subcloning of pPM200

Recombinant pPM200 was digested with enzymes: BamHI, HindIII, BglII, SalI, EcoRI, and PstI. The fragments were electrophoresed and an unblot was made of the gel. The unblot was probed with the synthetic oligonucleotide probe and a fragment in the HindIII / SalI digest (approximately 1.8 kb) hybridized. To isolate this fragment, pPM200 was subcloned into pBR325.

The ends of the 9 fragments in the HindIII and SalI double digest were identified as either HindIII / HindIII ends or HindIII / SalI. The vector pBR325 was digested with either HindIII or HindIII / SalI and resulting ends dephosphorylated. Insert fragments of pPM200 and vector pBR325 were ligated and transformed into either host strain HB101 or JM107 and selected on ampicillin. Colonies from the transformation were transferred to an agarose plate in a pattern grid and colony blots were made from each plate.

Each of the HindIII / SalI digest fragments was isolated and purified using anion exchange paper and radiolabeled. Each fragment was used as a probe to screen colony blots. Colonies exhibiting homology with the probes were subjected to a rapid plasmid screening

Figure 1. Digestion fragments of pPM200 hybridizing with the 20-bp synthetic oligonucleotide probe.


EcoRI / Bam HI
EcoRI/Bgl II
Sal I/Bgl II
Sal I
Bgl II
Hind III
EcoRI/Sal I
Hind III/Bgl II
Hind III/Sal I
Hind III/Bam HI
Hind III/EcoRI
EcoRI
Bam HI

and electrophoresed. Colonies with single inserts were picked and stored at -70°C. In one instance a clone with a double insert had to be selected due to lack of a single insert.

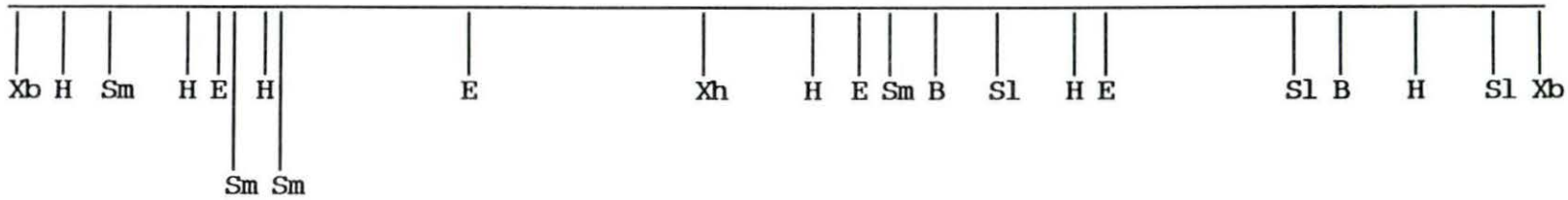
A single clone hybridizing with the 1.8 kb fragment probe was digested with HindIII / SalI and electrophoresed. A Southern blot of this gel was probed with the synthetic oligonucleotide probe and did not hybridize with the inserted fragment. Subsequently, another double digest of the clone was made and electrophoresed at a higher voltage for a longer time. Closer observation of the fragments revealed another band of similiar size, differing in approximately 100 base-pairs. To identify a clone with only the fragment of interest, colonies hybridizing with the double insert probe were screened with the synthetic probe by dot blot hybridization. Colonies hybridizing with the synthetic probe were subjected to rapid plasmid extraction and electrophoresed. Southern hybridization was performed to verify the presence of the fragment of interest. Plasmids from three colonies containing minimal number of fragments were digested, subcloned into pBR325 and transformed into JM107. Plasmids were extracted from resulting colonies, electrophoresed, and Southern blots screened with the oligonucleotide probe. Several colonies carrying only the desired 1.8 kb insert were identified.

Mapping of HindIII and SmaI sites in pPM200

Single and double digestions with BamHI, EcoRI, HindIII, SalI, SmaI, and XhoI of pPM200 were performed. Digestion of lambda DNA with enzyme HindIII was used as a standard for fragment size comparison. Determination of fragment sizes from digestions was by the method of Schaffer (89). A restriction map was derived from best-fit analysis of the fragment sizes (Figure 2).

Manipulation of the 1.8 kb fragment

The 1.8 kb fragment was radiolabeled and used as a probe to screen colony blots containing 987P-expressing strains and strains not expressing 987P. Results of this screening proved this fragment detected 987P-expressing strains as well as other strains not expressing 987P (Figure 3). This 1.8 kb fragment was then digested with several enzymes and electrophoresed. A Southern blot of the gel was made and probed with the synthetic oligonucleotide probe. Four different fragments from four different enzyme digests (AluI, HaeIII, SspI, and ThaI) of approximately 1.0 kb or less in size hybridized with the synthetic oligonucleotide probe. Three of the four digest fragments (AluI, SspI, and ThaI) were extracted from agarose gel with anion exchange paper and radiolabeled for use as probes. Colony blot hybridization using each of the three probe fragments again revealed hybridization with 987P-expressing strains and strains not expressing 987P. The HaeIII digest was electrophoresed in an acrylamide gel for



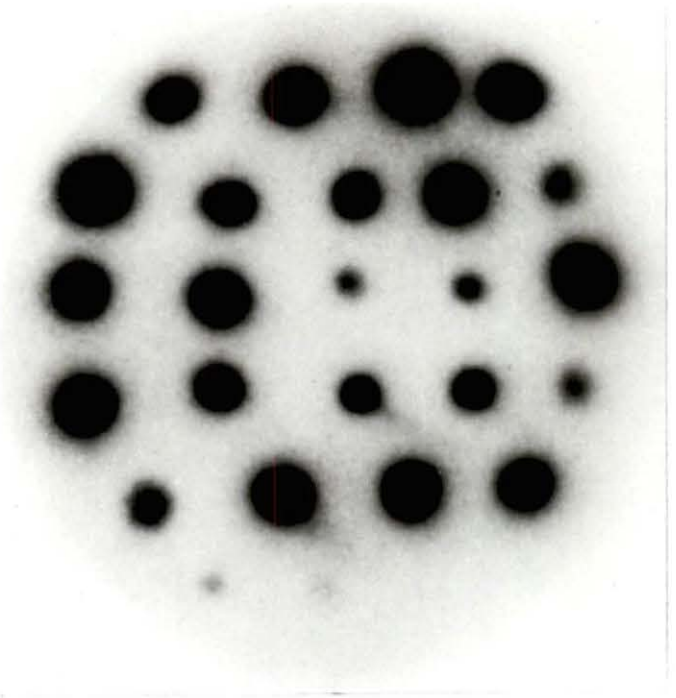
1.8 kb = 1 cm

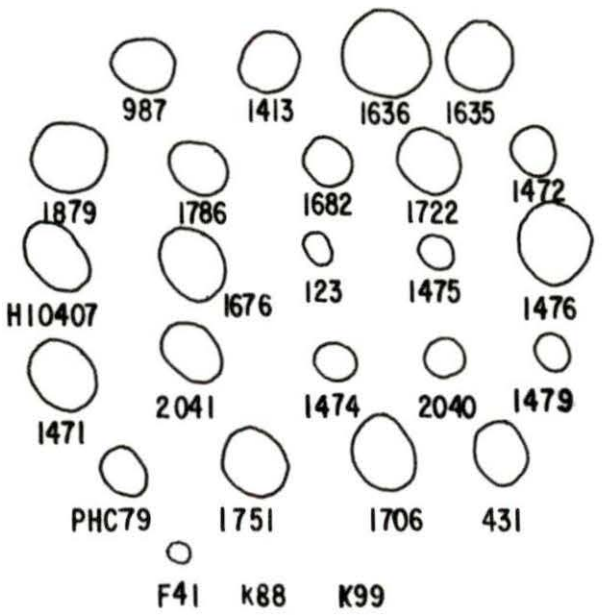
Legend

Xb = XbaI
H = HindIII
Sm = SmaI
E = EcoRI
Xh = XhoI
B = BamHI
Sl = SalI

Figure 2. Restriction mapping sites of HindIII and SmaI in pPM200

Figure 3. Colony blot filters probed with the 1.8 kb fragment. Bacterial strains listed in Table 1

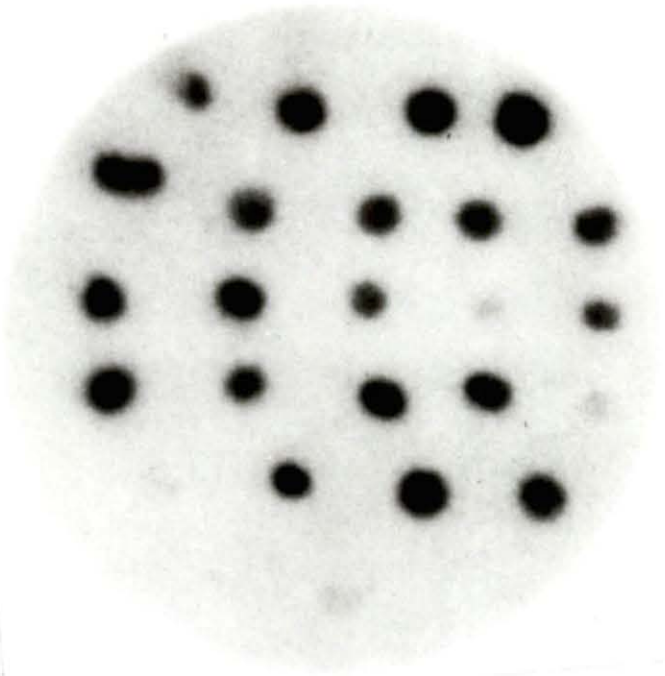


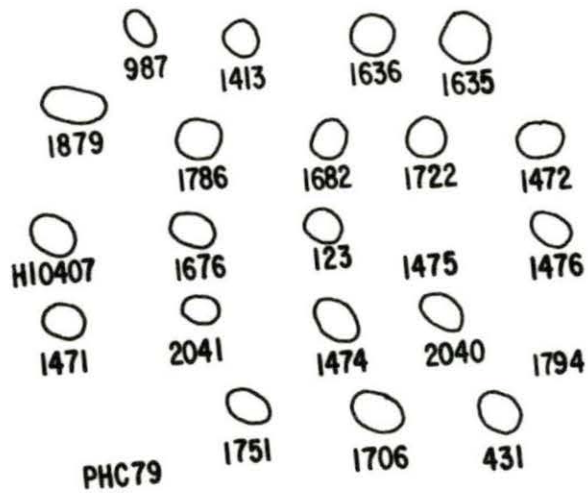

987
1413
1636
1635
1879
1786
1682
1722
1472
H10407
1676
123
1475
1476
1471
2041
1474
2040
1479
PHC79
1751
1706
431
F41
k88
K99

better separation and recovery of the 520 base-pair fragment that had been found to hybridize with the synthetic oligonucleotide probe. The fragment was recovered from the gel and radiolabeled for use as a probe. Colony blots used in the three previous fragment screenings were probed with the HaeIII digest fragment. The HaeIII fragment hybridized with the 987P strains as well as with other strains not expressing 987P on their surface (Figure 4). The HaeIII fragment proved to be more specific than the other three fragment probes (AluI, SspI, ThaI) tested. Specificity of the HaeIII digest fragment was checked using the synthetic oligonucleotide probe on the same colony blots previously used. Results of this screening demonstrated it is not possible to use this synthetic oligonucleotide probe on colony blots because it washes off the filter. Dot blot hybridization using the synthetic oligonucleotide probe was performed. Results of this screening revealed non-specific binding of the probe to 20 of the 26 strains on the dot blot. Stringency conditions were altered and the dot blot was washed twice again at 42°C. Exposed film of the dot blot revealed most of the probe was removed from all strains on the filter during the second wash.

A second synthetic probe was made from the published nucleotide sequence of the 987P fimbrial subunit reported by De Graaf and Klaasen. This probe was composed of a 21 base-pair sequence encoding for the amino terminal end of the fimbrial subunit genes. There was a 2 base-pair difference between the 20 base-pair synthetic oligonucleotide

Figure 4. Colony blot filters probed with the HaeIII fragment. Bacterial strains listed in Table 1




987
1413
1636
1635
1879
1786
1682
1722
1472
H10407
1676
123
1475
1476
1471
2041
1474
2040
1794
1751
1706
431
PHC79
F41
K88
K99

A: 20 bp synthetic probe sequence

GCT CCG GCT GAA AAC AAC AC

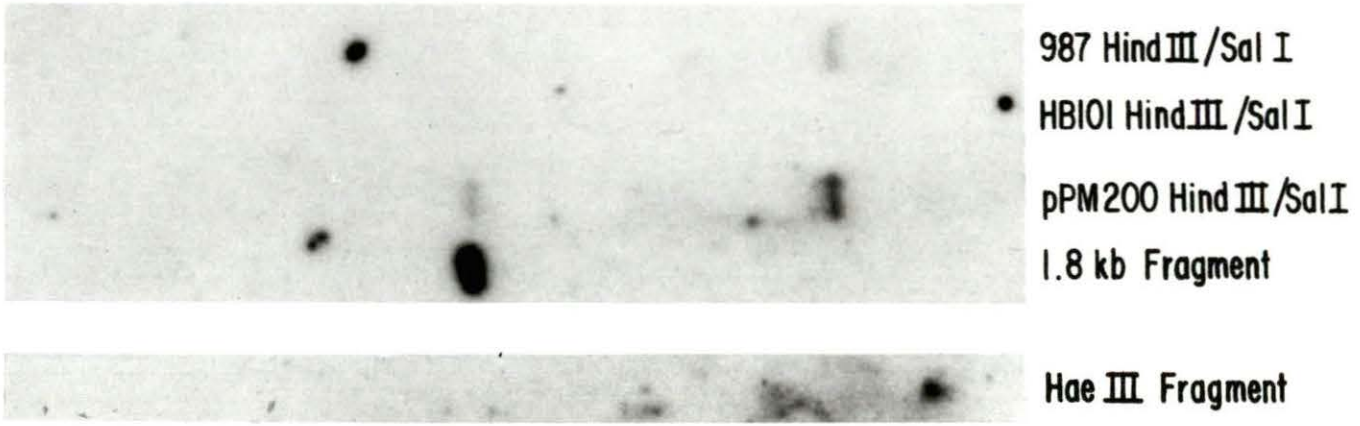
B: 21 bp synthetic probe sequence

GCG CCC GCT GAA AAC AAC AAC

Figure 5. Synthetic oligonucleotide probes. Sequence A based upon 987P amino acid sequence published by Pedersen et al., 1986. Sequence B based upon 987P nucleotide sequence published by De Graaf and Klaasen, 1987

sequence previously used and the new 21 base-pair synthetic oligonucleotide sequence (Figure 5). The probe was used to screen 2 Southern hybridization filters. One Southern hybridization filter contained DNA from 987, HB101, pPM200, the 1.8kb fragment, and strains that were K88, K99, or F41 positive. The other Southern hybridization filter contained the HaeIII fragment thought to contain the 987P fimbrial subunit genes. This synthetic probe did not hybridize to DNA present on either filter. A new 21 base-pair synthetic oligonucleotide probe containing the same sequence as the 21 base-pair probe mentioned above was prepared. This second probe was purified more carefully than the one made previously. Another Southern hybridization filter was prepared containing pPM200, HB101, the 1.8 kb fragment, HaeIII fragment, and a 987P strain. This filter was probed with the new 21 base-pair probe. The synthetic probe hybridized with strain 987, pPM200, and the 1.8 kb fragment from the HindIII / SalI digest of pPM200 (Figure 6).

Figure 6. Southern blot filter containing digests of DNA from bacterial strains. Filter probed with the 21-bp synthetic oligonucleotide probe


987 Hind III/Sal I
HB101 Hind III/Sal I
pPM200 Hind III/Sal I
1.8 kb Fragment
Hae III Fragment

DISCUSSION

Cosmids are derivatives of bacteriophage lambda that retain the cos (cohesive end sites) of lambda yet lack a large portion of non-encoding DNA. Cosmids became very popular because they offered several advantages as cloning vectors. They offer several different restriction enzyme sites for insertional inactivation as well as the ability to clone large fragments of foreign DNA for a cDNA library. Cosmid pHC79 (37) is approximately 6.4 kb with a cloning capacity of 29-46 kb and has a high replicative copy number of 50 per cell. The recombinant plasmid pPM200 was constructed by inserting DNA from E. coli strain 987 into cosmid vector pHC79 and transforming into E. coli host HB101 (71). The recombinant plasmid pPM200 contains an approximately 33 kb insert of E. coli 987 chromosomal DNA.

Initial restriction digestion and subsequent electrophoresis of the fragments of pPM200 revealed the presence of several secondary or "ghost" bands. A normal restriction digestion pattern would reveal a gradual dimunition of intensity as the molecular weight of the fragments decreases. Secondary digest fragments are usually observed as fainter bands interspersed with the darker, more prominent bands. The secondary digest fragments result from problems with the DNA, not the enzymes utilized. These undesirable bands could be explained by one of two theories: Either there was a partial digestion of pPM200 or the plasmid was unstable. Partial digestion of plasmid or low

molecular weight DNA can be accomplished experimentally by taking aliquots of the digestion mixture and inactivating the enzyme at periodic intervals (86). High molecular weight DNA (chromosomal DNA) requires extended incubation periods because the restriction sites are not readily available for enyzmatic cleavage. Since pPM200 is not high molecular weight DNA, extending the incubation period should have eliminated the undesirable secondary fragments. After increasing the incubation time to 2 hours, the secondary bands were still visible upon electrophoresis. This suggested pPM200 was unstable.

The host E. coli strain HB101 carrying pPM200 expresses 987P fimbriae on its surface. The fimbriae can be detected with electron micrographs and by agglutination with antisera specific for 987P (71). Electron micrographs and serologic testing were carried out on the stock laboratory strain of HB101 containing pPM200. It was determined that the laboratory strain of HB101(pPM200) was not expressing 987P fimbriae.

Growth conditions of E. coli 987 are an important contributing factor to the production of this fimbrial type. Isaacson et al. (45) found 987P-expressing strains form a pellicle at the broth interface when inoculated into Trypticase Soy Broth and incubated over 24 hours under static conditions. Alteration of previous growth conditions was attempted with HB101 (pPM200) to stabilize the recombinant plasmid and promote expression of 987P on the host surface. These efforts did not induce pellicle formation nor remove the secondary bands present in

restriction digest analysis.

It is possible that pPM200 became unstable sometime between the initial studies by Morrissey and Dougan and manipulations in this laboratory. A closer examination of HB101 genotype revealed it contained a mutation in the recA gene. The new host strain E. coli DH1 was chosen since it also contained a recA mutation. This DH1 recA mutation is in a different allele than the HB101 recA mutation. It was thought that by introducing pPM200 into a new host, plasmid pPM200 would stabilize and normal fimbrial expression occur. Restriction enzyme analysis of DNA from the new DH1 transformants again revealed the presence of partial digest bands. Therefore, it was possible that pHC79 could not replicate E. coli 987P fimbrial genes. Subsequent subcloning of the fimbrial genes was thought necessary.

Cosmid instability has been observed by several investigators (15, 50, 59). Cosmids may be unstable with or without insert DNA. Kaiser and Murray (50) state that some cosmids are affected by DNA insert size and this affects their ability to reproduce effectively. The larger the insert DNA size, the more unstable the recombinant clone becomes. This unstable recombinant, reproducing inefficiently, results in deletions in the cloned DNA. As stated previously, recombinant pPM200 contains a 33 kb insert which cosmid pHC79 should be capable of replicating without problem. There are possible explanations of the inability of pHC79 to replicate 987P fimbrial genes. A large insert DNA fragment would alter the cosmid from a high to low copy number

entity. The proteins necessary for replication of high copy number plasmids may be different than those required for faithful replication of low copy number plasmids. Therefore, there would be fewer plasmids per cell and these would be further reduced in number through host cell replication. Another possible explanation for cosmid instability is that the proteins responsible for replicating the cosmid vector are not recognizing certain sequences and terminating when reading a sequence of foreign DNA. This may account for the different size fragments in the digest corresponding to different lengths of 987P insert DNA.

Other investigators have found some cosmid vectors are unstable and will spontaneously rearrange 1-5% of the time (15, 59). Little (59) theorizes that cosmids may cause host cell growth to slow, generate repressor binding sites, or generate inverted duplications leading to instability of cosmid recombinants. Perhaps host strains HB101 and DH1 possess proteins that cause instability of pHC79 by not allowing faithful replication. These proteins may not be endonucleases but proteins that bind to promoter sequences or RNA polymerase binding sites. The recombinant would slowly break down over time due to its large size and fragments would result.

The presence of digestion fragments of different molecular weights suggests the possibility of two populations of plasmids. Comparing fragment sizes from the same enzyme for different plasmid preparations resulted in a variety of fragment band patterns being observed. Only a few bands were consistently observed from all

digestions. Secondary bands were rarely present in the same location from one digest to another. It is possible that the second population of plasmids consisted of deletions of insert DNA and, subsequently, the sites for endonuclease activity were altered. The deletion of DNA may have resulted in a loss of an endonuclease digestion site. This could have resulted in the production of different size fragments from the plasmid DNA.

Explanation of pPM200 instability would involve require further investigation. Two possible experiments may provide information on pPM200 instability. First, preliminary investigation into the number and size of secondary fragments present may employ the use of a densitometer. Digestions of pPM200 could be electrophoresed and photographs taken for measurement on the densitometer. Densitometer readings from negatives of the photographs would measure the intensity of the secondary bands (along with the primary bands) which gives a quantitative analysis of a particular fragment present in the digest. When several densitometer measurements were taken from similar digestions the graphed results could be superimposed for comparison. The conditions of electrophoresis and the amount of DNA present in the digest must be standardized in order for the comparisons to be accurate. This information would reveal if the postulated recombinant breakdown were always in the same region or if it were a random occurrence. Secondly, determination of sequences at the end of the undesirable secondary fragments would provide useful information

although the procedures would be very time consuming. It would be necessary to isolate these secondary fragments. Once the fragments were purified, their nucleotide sequences could be determined. This procedure would need to be repeated several times to insure that fragment sequences remained consistent and that there were no changes in the fragment length. A comparison of the sequences could explain if the breakdown in the recombinant was a random event or specific for certain sequences.

Synthetic oligonucleotide probes have proven useful in the study of a number of bacterial and viral pathogens (97). To subclone only those genes encoding for the 987P subunit, a probe specific for 987P genes was needed. Pedersen (85) published the amino acid sequence of the amino terminal end of different fimbrial subunit types. Comparison of the different fimbrial sequences revealed almost no homology of any other fimbrial type with the 987P sequence. The first seven amino acids were chosen for the oligonucleotide probe sequence. Since there is degeneracy from the protein to the nucleic acid codon, two of the amino acids had several possiblities for the last nucleotide position in the triplet. Nucleotides filling these positions were chosen on the basis of results obtained from a computer assistance program. The program PCGene (IntelliGenetics, Mountain View, CA) contains a file CDUSAGE that was employed. This program provides information on the relative amounts of the 4 nucleotides and the frequency of the types of codon at each of the three positions. This program was used to select

the sequence for the synthetic oligonucleotide probe.

The synthetic oligonucleotide probe was found to hybridize with an approximately 1.8 kb HindIII / SalI fragment from a digest of pPM200. Results of the colony blot screenings proved the 1.8 kb fragment hybridized to strains expressing 987P but also to other strains on the filter. Therefore, it was thought that this fragment was too large for use as a probe because it lacked specificity. It is possible the fragment contained part or all of the subunit genes but included other non-specific 987P genes. These non-specific genes could possibly have encoded for anchoring or periplasmic transport proteins. These would not necessarily be specific for 987P-expressing strains but merely for fimbrial production in the other strains.

Further digestion of this 1.8 kb fragment was necessary to find a smaller fragment that continued to hybridize with the synthetic oligonucleotide probe. Since the 1.8 kb fragment was smaller in size, enzymes were chosen that had an endonuclease target sequence of 4 base-pairs rather than the standard 6 or 8 base-pairs. The smaller target sequence enzymes increased the probability of sites being present and, therefore, the probability of more fragments resulting from the digestions. The 1.8 kb fragment was digested with 12 enzymes, electrophoresed and a Southern blot made. The blot was probed with the synthetic oligonucleotide probe and 4 fragments from different digests, all under 1.2 kb, were found to hybridize. Each of these fragments was purified and radiolabeled for use as probes on colony blot filters

containing 987P, K88, K99, F41, Type 1, and PAP (pyelonephritis associated pili)-expressing strains. The fragment that hybridized consistently with 987P expressing strains was from the HaeIII enzyme digest. This HaeIII fragment also hybridized with other strains not expressing 987P. Two explanations for this hybridization pattern are possible. The other strains (known to express either K88, K99, F41, or K99 and F41) may contain part of the genes necessary for 987P fimbrial production but are not able to be express fimbriae. The inability to express 987P fimbriae may result from deletions or mutations in the subunit genes. Another explanation for hybridization to these strains may be that the HaeIII fragment contains only a limited number of base-pairs encoding for the 987P fimbrial subunit. Enough base-pairs are present to hybridize with the 20 base-pair synthetic oligonucleotide probe but the remaining portion of the fragment possibly encodes for non-subunit proteins. The genes encoding for these non-subunit proteins would be necessary for fimbrial production in the non-987P-expressing strains. These genes may be hybridizing with regions found in the HaeIII fragment.

At this point, it was discovered there was a published nucleotide sequence of the 987P subunit genes (18) available. Comparison between the first 20 base-pairs of the synthetic oligonucleotide probe previously used and the newly published nucleotide sequence revealed significant differences. There was a base-pair difference at the third position, in the first and second amino acid sequences, between the

original synthetic oligonucleotide probe and the newly published sequence. This mismatch between the sequences was understandable since the original synthetic oligonucleotide probe sequence was derived from an amino acid sequence. Since there is degeneracy in the codon at the third or "wobble" position, several nucleotides could satisfy the amino acid encoding requirements. As stated previously, the base-pairs chosen in these positions were derived from the information on the frequency of codon usage by E. coli since the published nucleotide sequence was not available at the time.

The second oligonucleotide probe was a 21 base-pair fragment synthesized based on the published results of De Graaf and Klaasen (18). When this 21 base-pair synthetic probe was used to screen Southern hybridization filters, there appeared to be no hybridization. Reasons for this may include error in extraction of the synthetic probe, improper filter preparation, or a failure on the part of the DNA synthesizer to faithfully manufacture the probe. It was decided to duplicate the filters (omitting the non-987P-expressing strains) and probe them. Another 21 base-pair synthetic oligonucleotide probe was made to insure machine error was not a problem. The second 21 base-pair synthetic probe was shown to hybridize to pPM200 and the 1.8 kb fragment, but not to the HaeIII fragment.

HaeIII fragment hybridization with the original 20 base-pair synthetic oligonucleotide probe was specific for that probe but not specific for the 21 base-pair probe. Since there is a 10% mismatch

between the two probe sequences, this may be enough to prevent hybridization of the 21 base-pair synthetic probe and the HaeIII fragment. Therefore, if De Graaf and Klaasen's published sequence for the 987P subunit gene is correct then the HaeIII fragment cannot be used as a probe for detecting the 987P fimbrial subunit gene.

Although the HaeIII fragment cannot be used as a probe, the fact that the 1.8 kb fragment hybridizes with the 21 base-pair synthetic probe is useful information. This fragment may contain all of the genes for the 987P fimbrial subunit. Use of the 1.8kb fragment would preclude having to screen other subcloned fragments of pPM200. Further enzyme digestion of the 1.8 kb fragment and screening with the synthetic probe should reveal a smaller fragment that will be useful as a probe in detecting the 987P fimbrial subunit gene.

SUMMARY

Construction and cloning of a gene probe specific for the 987P subunit of enterotoxigenic Escherichia coli was attempted. Recombinant plasmid pPM200 carries the genes encoding for the 987P fimbrial subunit. The pPM200 plasmid was digested with enzymes and the fragments electrophoresed. A 20 base-pair synthetic oligonucleotide probe, derived from the amino terminal end of the amino acid sequence of the 987P subunit gene, was used to probe the digested fragments of pPM200. This probe hybridized with an approximately 1.8 kb fragment in the HindIII / SalI digest. This 1.8 kb fragment was not specific for 987P-expressing strains. Further digestion of the 1.8 kb fragment with the enzyme HaeIII revealed a fragment, approximately 520 base-pairs, that hybridized with the synthetic oligonucleotide probe. This 520 base-pair fragment hybridized with all 987P strains tested but exhibited homology with strains not bearing 987P fimbriae.

A second 21 base-pair synthetic oligonucleotide probe was made from the nucleotide sequence published by De Graaf and Klaasen (18). This probe hybridized with the 1.8 kb fragment originally cloned from the HindIII / SalI digest of pPM200, but not the 520 base-pair fragment of the subsequent HaeIII digest. Further enzymatic digestion of the 1.8 kb fragment and screening with the 21 base-pair synthetic oligonucleotide probe should identify another smaller fragment for use as a probe in detecting the 987P fimbrial subunit gene.

LITERATURE CITED

1. Awad-Malalmeh, M., H. W. Moon, P. L. Runnels, and R. A. Schneider. 1982. Pilus Production, Hemagglutination, and Adhesion by Porcine Strains of Enterotoxigenic Escherichia coli Lacking K88, K99, and 987P Antigens. Infect. Immun. 35:305-313.

2a. Bemis, D. A., H. A. Greisen, and M. J. G. Appel. 1977. Bacteriological Variation Among Bordetella bronchiseptica Isolates from Dogs and Other Species. J. Clin. Microbiol. 5:471-480.

2b. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.

3. Bovre, K, and L. O. Froholm. 1970. Correlation between the Fimbriated State and Competence of Genetic Transformation in Moraxella nonliquefaciens strains. Acta Pathol. et Microbiol. Scand., Section B 78:526-528.

4. Brinton, C. C., Jr., P. Fusco, S. Wood, H. G. Jayappa, R. A. Goodnow, and J. G. Strayer. 1983. A complete vaccine for neonatal swine colibacillosis and the prevalence of Escherichia coli pili on swine isolates. Vet. Med./Small Animal Clin. 78:962-966.

5. Brinton, C. C., Jr. 1978. The Piliation Phase Syndrome and the Uses of Purified Pili in Disease Control. pp. 34-70. In C. Miller (ed.), 13th U. S.-Japan Conference on Cholera, Atlanta, Ga., September, 1977. National Institutes of Health, Bethesda, Md.

6. Brinton, C. C., Jr. 1965. The Structure, Function, Synthesis and Genetic Control of Bacterial Pili and a Molecular Model for DNA and RNA Transport in Gram Negative Bacteria. Trans. N. Y. Acad. Sci. 27:1003-1053.

7. Brinton, C. C., Jr., A. Buzzell, and M. A. Lauffer. 1954. Electrophoresis and Phage Susceptibility Studies on a Filament-Producing Variant of the E. coli B Bacterium. Biochimica et Biophysica Acta 15:533-542.

8. Burgess, M. N., R. J. Bywater, C. M. Cowley, N. A. Mullan, and P. M. Newsome. 1978. Biological Evaluation of a Methanol-Soluble, Heat-Stable Escherichia coli Enterotoxin in Infant Mice, Pigs, Rabbits, and Calves. Infect. Immun. 21:526-531.

9. Burrows, M. R., R. Sellwood, and R. A. Gibbons. 1976. Haemagglutinating and Adhesive Properties Associated with the K99 Antigen of Bovine Strains of Escherichia coli. J. Gen. Microbiol. 96:269-275.

10. Chaconas, G., and J. H. van de Sande. 1980. Methods in 5′ - ^{32}P Labeling of RNA and DNA Restriction Fragments. Enzymology 65:75-85.

11. Clewell, D. B., and D. R. Helinski. 1970. Properties of a Supercoiled Deoxyribonucleic Acid-Protein Relaxation Complex and Strand Specificity of the Relaxation Event. Biochemistry 9:4428-4440.

12. Cohn, F. 1872. Ueber den Brunnenfaden (Crenothrix polyspora) mit Bemerkungen uber die mikroskopische Analysedes Brunnenwassers. Beitr. Biol. Pilanz. 1:108-131.

13. Collier, W. A., and M. Jacoeb. 1955. Bacterien-Haemagglutination. I. Versuche Mit Einem Haemagglutinirenden Stamm Von E. coli. Antonie Van Leeuwenhoek 21:113-123.

14. Collier, W. A., and J. C. De Miranda. 1955. Bacterien-Haemagglutination. III. Die Hemmung Der Coli-Haemagglutination Durch Mannose. Antonie Van Leeuwenhoek 21:124-132.

15. Dahl, H. H., R. A. Flavell, and F. G. Grosveld. 1981. The use of genomic libraries for the isolation and study of eukaryotic genes. Pages 49-127 in R. Williamson, ed. Genetic Engineering, Vol. 2. Academic Press, New York.

16. Darfeuille-Michaud, A., C. Forestier, B. Joly, and R. Cluzel. 1986. Identification of a Nonfimbrial Adhesive Factor of an Enterotoxigenic Escherichia coli Strain. Infect. Immun. 52:468-475.

17. De Graaf, F. K., and P. Klaasen. 1986. Organization and expression of genes involved in the biosynthesis of 987P fimbriae. Mol. Gen. Genet. 204:75-81.

18. De Graaf, F. K., and P. Klaasen. 1987. Nucleotide sequence of the gene encoding the 987P fimbrial subunit of Escherichia coli. FEMS Microbiol. Letters 42:253-258.

19. De Graaf, F. K., P. Klemm, and W. Gaastra. 1980. Purification, Characterization, and Partial Covalent Structure of Escherichia coli Adhesive Antigen K99. Infect. Immun. 33:877-883.

20. De Graaf, F. K., B. E. Krenn, and P. Klaasen. 1984. Organization and Expression of Genes Involved in the Biosynthesis of K99 Fimbriae. Infect. Immun. 43:508-514.

21. De Graaf, F. K., and I. Roorda. 1982. Production, Purification, and Characterization of the Fimbrial Adhesive Antigen F41 Isolated from Calf Enteropathogenic Escherichia coli Strain B41M. Infect. Immun. 36:751-758.

22. Duguid, J. P. 1959. Fimbriae and Adhesive Properties in Klebsiella Strains. J. Gen. Microbiol. 21:271-286.

23. Duguid, J. P., E. S. Anderson, and I. Campbell. 1966. Fimbriae and Adhesive Properties In Salmonellae. J. Pathol. Bacteriol. 92:107-138.

24. Duguid, J. P., S. Clegg, and M. I. Wilson. 1979. The Fimbrial and Non-Fimbrial Haemagglutinins of Escherichia coli. J. Med. Microbiol. 12:213-227.

25. Duguid, J. P., and R. R. Gillies. 1957. Fimbriae and Adhesive Properties in Dysentery Bacilli. J. Pathol. Bacteriol. 74:397-411.

26. Duguid, J. P., I. W. Smith, G. Dempster, and P. N. Edmunds. 1955. Non-Flagellar Filamentous Appendages ("Fimbriae") and Haemagglutinating Activity in Bacterium coli. J. Pathol. Bacteriol. 70:335-348.

27. Dykes, C. W., I. J. Halliday, M. J. Read, A. N. Hobden, and S. Harford. 1985. Nucleotide Sequences of Four Variants of the K88 Gene of Porcine Enterotoxigenic Escherichia coli. Infect. Immun. 50:279-283.

28. Evans, D. G., R. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach. 1975. Plasmid-controlled colonization factor associated with virulence in Escherichia coli enterotoxigenic for humans. Infect. Immun. 12:656-667.

29. Evans, M. G., G. L. Waxler, and J. P. Newman. 1986. Prevalence of K88, K99, and 987P pili of Escherichia coli in neonatal pigs with enteric colibacillosis. Am. J. Vet. Res. 47:2431-2434.

30. Ewing, W. H., H. W. Tatum, and B. R. Davis. 1958. Escherichia coli Serotypes Associated with Edema Disease of Swine. Cornell Vet. 48:201-206.

31. Fusco, P., A. To, S. To, and C. Brinton. 1978. The purification and characterization of four types of E. coli pili and the specificity of E. coli pili for immunity, colonization and adhesion. pp. 60-70. In C. Miller (ed.), 13th U. S.- Japan Conference on Cholera, Atlanta, Ga., September, 1977. National Institutes of Health, Bethesda, Md.

32. Gaastra, W., P. Klemm, and F. K. De Graaf. 1983. The nucleotide sequence of the K88ad protein subunit of porcine enterotoxigenic Escherichia coli. FEMS Microbiol. Lett. 18:177-183.

33. Gaastra, W., P. Klemm, J. M. Walker, and F. K. De Graaf. 1979. K88 fimbrial protein: Amino and Carboxyl Terminal sequences of intact proteins and cyanogen bromide fragments. FEMS Microbiol. Lett. 6:15-18.

34. Gaastra, W., F. R. Mooi, A. R. Stuitje, and F. K. De Graaf. 1981. The nucleotide sequence of the gene encoding the K88ab protein subunit of porcine enterotoxigenic Escherichia coli. FEMS Microbiol. Lett. 12:41-46.

35. Guinee, P. A. M., and W. H. Jansen. 1979. Behavior of Escherichia coli K Antigens K88ab, K88ac, and K88ad in Immunoelectrophoresis, Double Diffusion, and Hemagglutination. Infect. Immun. 23:700-705.

36. Gyles, C. L. 1979. Limitations of the Infant Mouse Test for Escherichia coli Heat Stable Enterotoxin. Can. J. Comp. Med. 43:371-379.

37. Hohn, B., and Collins, J. 1980. A small cosmid for efficient cloning of large DNA fragments. Gene 11:291-298.

38. Holmes, D. S., and M. Quigley. 1981. A Rapid Boiling Method for the Preparation of Bacterial Plasmids. Anal. Biochem. 114:193-197.

39. Houwink, A. L., and W. Van Iterson. 1950. Electron Microscopical Observations on Bacterial Cytology. II. A Study on Flagellation. Biochimica et Biophysica Acta 5:10-44.

40. Hu, P. C., R. M. Cole, Y. S. Huang, J. A. Graham, D. E. Gardner, and A. M. Collier. 1982. Mycoplasma pneumoniae Infection: Role ofa Surface Protein in the Attachment Organelle. Science 216:313-315.

41a. Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow. Construction and Expression of Recombinant Plasmids Encoding Type 1 or D-Mannose-Resistant Pili from a Urinary Tract Infection Escherichia coli Isolate. Infect. Immun. 33:933-938.

41b. Ike, K., M. Nakazawa, M. Tsuchimoto, S. Ide, and M. Kashiwazaki. 1987. Hemagglutination by pilus Antigen 987P of Enterotoxigenic Escherichia coli. Microbiol. Immunol. 31:1255-1258.

42. Isaacson, R. E. 1977. K99 Surface Antigen of Escherichia coli: Purification and Partial Characterization. Infect. Immun. 15:272-279.

43. Isaacson, R. E. 1978. K99 Surface Antigen of Escherichia coli: Antigenic Characterization. Infect. Immun. 22:555-559.

44. Isaacson, R. E., P. C. Fusco, C. C. Brinton, and H. W. Moon. 1978. In Vitro Adhesion of Escherichia coli to Porcine Small Intestinal Epithelial Cells: Pili as Adhesive Factors. Infect. Immun. 21:392-397.

45. Isaacson, R. E., B. Nagy, and H. W. Moon. 1977. Colonization of Porcine Small Intestine by Escherichia coli: Colonization and Adhesion Factors of Pig Enteropathogens That Lack K88. J. Infect. Dis. 135:531-539.

46. Isaacson, R. E., and P. Richter. 1981. Escherichia coli 987P Pilus: Purification and Partial Characterization. J. Bacteriol. 146:784-789.

47. Jann, D., G. Schmidt, E. Blumenstock, and K. Vosbeck. 1981. Escherichia coli Adhesion to Saccharomyces cerevisiae and Mammalian Cells: Role of Piliation and Surface Hydrophobicity. Infect. Immun. 32:484-489.

48. Jones, G. W., and J. M. Rutter. 1972. Role of the K88 antigen in the pathogenesis of neonatal diarrhea caused by Escherichia coli in piglets. Infect. Immun. 6:918-927.

49. Kafatos, F. C., C. W. Jones, and A. Efstratiadis. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot blot hybridization procedure. Nucleic Acids Res. 7:1541-1552.

50. Kaiser, K, and N. E. Murray. 1985. The use of Phage Lambda Replacement Vectors in the Construction of Representative Genomic DNA Libraries. Pages 1-47 in Glover, D., ed. DNA Cloning, Vol. 1. IRL Press, Washington, D. C.

51. Kehoe, M., R. Sellwood, P. Shipley, and G. Dougan. 1981. Genetic analysis of K88-mediated adhesion of enterotoxigenic

Escherichia coli. Nature 291:122-126.

52a. Kennedy, D. J., R. M. Greenberg, J. A. Dunn, R. Abernathy, J. S. Ryerse, and R. L. Guerrant. 1984. Effects of Escherichia coli Heat-Stable Enterotoxin ST_b on Intestine of Mice, Rats, Rabbits, and Piglets. Infect. Immun. 46:639-643.

52b. Klemm, P. 1985. Fimbrial Adhesins of Escherichia coli. Reviews of Infect. Dis. 7:321-340.

53. Klemm, P. 1981. The complete amino acid sequence of the K88 antigen, a fimbrial protein from Escherichia coli. Eur. J. Biochem. 117:617-627.

54. Koch, R. 1877. Untersuchungen uber Bacterien. VI. Verfahren zur Untersuchung, sum Conserviren und Photographien der Bacterien. Beitr. Biol. Pflanz. 2:399-434.

55. Kuramitsu, H. K. 1974. Adherence of Streptococcus mutans to dextran synthesized in the presence of extracellular dextransucrase. Infect. Immun. 9:764-765.

56. Lanser, J. A., and P. A. Anargyros. 1985. Detection of Escherichia coli Adhesins with DNA Probes. J. Clin. Microbiol. 22:425-427.

57. Lederberg, E. M., and S. N. Cohen. 1974. Transformation of Salmonella typhimurium by Plasmid Deoxyribonucleic Acid. J. Bacteriol. 119:1072-1074.

58. Levine, M. M. 1987. Escherichia coli that Cause Diarrhea: Enterotoxigenic, Enteropathogenic, Enteroinvasive,

Enterohemmorrhagic, and Enteroadherent. J. Infect. Dis. 155:377-389.

59. Little, P. F. R. 1987. Choice and use of cosmid vectors. Pages 19-42 in Glover, D., ed. DNA cloning, Vol. 3. IRL Press, Washington, D. C.

60. Mainil, J. G., P. L. Sadowski, M. Tarsio, and H. W. Moon. 1987. In Vivo Emergence of Enterotoxigenic Escherichia coli Variants Lacking Genes for K99 Fimbriae and Heat-Stable Enterotoxin. Infect. Immun. 55:3111-3116.

61. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

62. Meinkoth, J., and G. Wahl. 1984. Hybridization of Nucleic Acids Immobilized on Solid Supports. Anal. Biochem. 138:267-284.

63. Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, New York.

64. Mooi, F. R., and F. K. De Graaf. 1979. Isolation and characterization of K88 antigen. FEMS Microbiol. Lett. 5:17-20.

65a. Moon, H. W., E. M. Kohler, R. A. Schneider, and S. C. Whipp. 1980. Prevalence of Pilus Antigens, Enterotoxin Types, and Enteropathogenicity Among K88-Negative Enterotoxigenic Escherichia coli from Neonatal Pigs. Infect. Immun. 27:222-230.

65b. Morgan, R. L., R. E. Isaacson, H. W. Moon, C. C. Brinton, and C.-C. To. 1978. Immunization of Suckling Pigs Against Enterotoxigenic Escherichia coli-Induced Diarrheal Disease by Vaccinating Dams with Purified 987 or K99 Pili: Protection Correleates with Pilus Homology of Vaccine and Challenge. Infect. Immun. 22:771-777.

66. Morris, G. K., M. H. Merson, D. A. Sack, J. G. Wells, W. T. Martin, W. E. Dewitt, J. C. Feeley, R. B. Sack, and D. M. Bessudo. 1976. Laboratory investigation of diarrhea in travelers to Mexico. An evaluation of methods for detecting enterotoxigenic Escherichia coli. J. Clin. Microbiol. 3:486-495.

67. Morris, J. A., A. E. Stevens, and W. J. Sojka. 1977. Preliminary Characterization of Cell-free K99 Antigen Isolated from Escherichia coli B41. J. Gen. Microbiol. 99:353-357.

68. Morris, J. A., A. E. Stevens, and W. J. Sojka. 1978. Anionic and Cationic Components of the K99 Surface Antigen from Escherichia coli B41. J. Gen. Microbiol. 107:173-175.

69. Morris, J. A., C. Thorns, A. C. Scott, W. J. Sojka, and G. A. Wells. 1982. Adhesion In Vitro and In Vivo Associated with an Adhesive Antigen (F41) Produced by a K99 Mutant of the Reference Strain Escherichia coli B41. Infect. Immun. 36:1146-1153.

70. Morris, J. A., C. J. Thorns, and W. J. Sojka. 1980. Evidence for Two Adhesive Antigens on the K99 Reference Strain Escherichia coli B41. J. Gen. Microbiol. 118:107-113.

71. Morrissey, P. M., and G. Dougan. 1986. Expression of a cloned 987P adhesion-antigen fimbrial determinant in Escherichia coli K-12 strain HB101. Gene 43:79-84.

72. Moseley, S. L., G. Dougan, R. A. Schneider, and H. W. Moon. 1986. Cloning of Chromosomal DNA Encoding the F41 Adhesin of Enterotoxigenic Escherichia coli and Genetic Homology between Adhesins F41 and K88. J. Bacteriol. 167:799-804.

73. Moseley, S. L., I. Huq, A. R. M. A. Alim, M. So, M. Samadpour-Motalebi, and S. Falkow. 1980. Detection of Enterotoxigenic Escherichia coli by DNA Colony Hybridization. J. Infect. Dis. 142:892-898.

74. Mukasa, H., and H. D. Slade. 1973. Mechanism of adherence of Streptococcus mutans to smooth surfaces. I. Roles of insoluble dextran-levan synthetase enzymes and cell wall polysaccharide antigen in plaque formation. Infect. Immun. 8:555-562.

75. Nagy, B., H. W. Moon, and R. E. Isaacson. 1976. Colonization of Porcine Small Intestine by Escherichia coli: Ileal Colonization and Adhesion by Pig Enteropathogens That Lack K88 Antigen and by Some Acapsular Mutants. Infect. Immun. 13:1214-1220.

76. Nagy, B., H. W. Moon, and R. E. Isaacson. 1977. Colonization of Porcine Intestine by Enterotoxigenic Escherichia coli: Selection of Piliated Forms In Vivo, Adhesion of Piliated Forms to Epithelial Cells In Vitro, and Incidence of a Pilus Antigen Among Porcine Enteropathogenic E. coli. Infect. Immun. 16:344-

352.

77. Novotny, C., W. S. Knight, and C. C. Brinton, Jr. 1968. Inhibition of Bacterial Conjugation by Ribonucleic Acid and Deoxyribonucleic Acid Male-Specific Bacteriophages. J. Bacteriol. 95:314-326.

78. Orskov, I., and F. Orskov. 1966. Episome-Carried Surface Antigen K88 of Escherichia coli. I. Transmission of the Determinant of the K88 Antigen and Influence on the Transfer of Chromosomal Markers. J. Bacteriol. 91:69-75.

79. Orskov, I., F. Orskov, W. Wittig, and E. J. Sweeney. 1969. A New E. coli serotype O149:K91(B), K88ac(L):H10 Isolated from Diseased Swine. Acta Pathol. et Microbiol. Scand. 75:491-498.

80. Orskov, I., F. Orskov, W. J. Sojka, and W. Wittig. 1964. K Antigens K88ab(L) and K88ac(L) in E. coli. Acta Pathol. et Microbiol. Scand. 62:439-447.

81. Orskov, I., F. Orskov, W. J. Sojka, and J. M. Leach. 1961. Simultaneous Occurrence of E. coli B and L Antigens in Strains from Diseased Swine. Acta Pathol. et Microbiol. Scand., Section B 53:404-422.

82. Orskov, I., F. Orskov, H. W. Smith, and W. J. Sojka. 1975. The Establishment of K99, A Thermolabile, Transmissible Escherichia coli K Antigen, previously called "Kco", possessed by Calf and Lamb Enteropathogenic Strains. Acta Pathol. et Microbiol. Scand., Section B 83:31-36.

83. Page, L. A. 1962. Acetylmethylcarbinol Production and the Classification of Aeromonads Associated with Ulcerative Diseases of Ectothermic Vertebrates. J. Bacteriol. 84:772-777.

84. Parry, S. H., and P. Porter. 1978. Immunological aspects of cell membrane adhesion demonstrated by porcine enteropathogenic Escherichia coli. Immunology 34:41-49.

85. Pedersen, J. K., P. Klemm, and W. Gaastra. 1986. 987P fimbriae from porcine enterotoxigenic Escherichia coli: characterization, N-terminal amino acid sequence and immunization with purified antigen. FEMS Microbiol. Lett. 33:229-234.

86. Perbal, B. 1984. A Practical Guide to Molecular Cloning. John Wiley and Sons, New York.

87. Reichert, K. 1909. Ueber die Sichtbarmachung der Geisseln und die Giisselbewegung der Bakerien. Centralbl. F. Bakt., Abt. 1, 51:14- 94.

88. Sack, D. A., D. C. Kaminsky, R. B. Sack, I. A. Wamola, F. Orskov, I. Orskov, R. C. B. Sack, R. R. Arthur, and A. Z. Kapikian. 1977. Enterotoxigenic Escherichia coli diarrhea of travelers: A prospective study of American Peace Corps Volunteers. Johns Hopkins Med. J. 141:63-70.

89. Schaffer, H. E., and R. R. Sederoff. 1981. Improved Estimation of DNA Fragment Lengths from Agarose Gels. Anal. Biochem. 115:113- 122.

90. Smith, H. O. 1980. Recovery of DNA from Gels. Methods in Enzymol. 65:371-380.

91. Smith, H. W., and S. Halls. 1967. Observations by the Ligated Intestinal Segment and Oral Inoculation methods on Escherichia coli Infections in Pigs, Calves, Lambs, and Rabbits. J. Pathol. Bacteriol. 93:499-529.

92. Smith, H. W., and J. E. T. Jones. 1963. Observations on the Alimentary Tract and its Bacterial Flora in Healthy and Diseased Pigs. J. Pathol. Bacterol. 86:387-412.

93. Smith, H. W., and M. A. Linggood. 1971. Observations on the Pathogenic Properties of the K88, Hly and Ent Plasmids of Escherichia coli with Particular Reference to Porcine Diarrhoea. J. Med. Microbiol. 4:467-485.

94. Smith, H. W., and M. A. Linggood. 1972. Further Observations on Escherichia coli Enterotoxins with Particular Regard to those produced by Atypical Piglet Strains and by Calf and Lamb Strains: The Transmissible Nature of these Enterotoxins and of a K Antigen Possessed by Calf and Lamb Strains. J. Med. Microbiol. 5:243-250.

95. Stirm, S., F. Orskov, I. Orskov, and A. Birch-Andersen. 1967. Episome-carried Surface Antigen K88 of Escherichia coli. III. Morphology. J. Bacteriol. 93:740-748.

96. Stirm, S., F. Orskov, I. Orskov, and B. Mansa. 1967. Episome-carried Surface Antigen K88 of Escherichia coli. II. Isolation and Chemical Analysis. J. Bacteriol. 93:731-739.

97. Tenover, F. C. 1988. Diagnostic Deoxyribonucleic Acid Probes for Infectious Diseases. Clin. Microbiol. Rev. 1:82-101.

98. To, S. C. M. 1984. F41 Antigen Among Porcine Enterotoxigenic Escherichia coli Strains Lacking K88, K99, and 987P Pili. Infect. Immun. 43:549-554.

99. Tweedy, J. M., R. W. A. Park, and W. Hodgkiss 1968. Evidence for the Presence of Fimbriae (Pili) on Vibrio Species. J. Gen. Microbiol. 51:235-244.

100. Wachsmuth, I. K., S. Falkow, and R. W. Ryder. 1976. Plasmid-mediated properties of a heat-stable enterotoxin producing Escherichia coli associated with infantile diarrhea. Infect. Immun. 14:403-407.

101. Westerman, R. B., K. W. Mills, R. M. Phillips, G. W. Fortner and J. M. Greenwood. 1988. Predominance of the ac Variant in K88-Positive Escherichia coli Isolates from Swine. J. Clin. Microbiol. 26:149-150.

102. Wheeler, T. T., and W. B. Clark. 1980. Fibril-mediated adherence of Actinomyces viscosus to saliva-treated hydroxyapatite. Infect. Immun. 28:577-584.

103. Wilson, R. A., and D. H. Francis. 1986. Fimbriae and enterotoxins associated with Escherichia coli serogroups isolated from pigs with colibacillosis. Am. J. Vet. Res. 47:213-217.

104. Wistreich, G. A., and R. G. Baker. 1971. The Presence of Fimbriae (Pili) in Three Species of Neisseria. J. Gen. Microbiol. 65:167-173.

105. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.

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