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INFECTIONOUS MULTIPLE DRUG RESISTANCE
IN THE ENTEROBACTERIACEAE

ANNUAL REPORT

By

Stanley Falkow, Ph.D.

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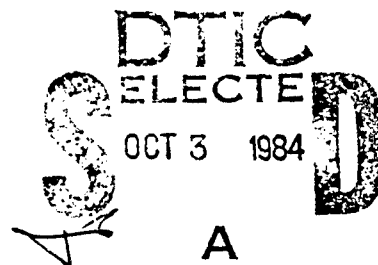
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Based on the data that there were at least two heterologous ST detectable in the infant mouse assay, we cloned and sequenced an ST gene from one of the human strains from Dacca that was not detected in our initial hybridization studies. This gene is related to the porcine ST gene but is clearly divergent possessing 30 different amino acids and 31% different base pairs.

Environmental and non-toxigenic strains of V. cholerae O-1 were examined for genes homologous to genes encoding E. coli LT. All non-toxigenic strains of V. cholerae O-1 from Louisiana, Alabama, Maryland, Guam, Brazil, Bangladesh and Great Britain showed no homology, while all toxigenic strains exhibited homology. In addition, strains of V. cholerae non O-1, "group F" Vibrios, V. vulnificus and Aeromonas hydrophila were tested and all were negative for any trace of toxin genes except for two strains of V. cholerae non O-1. The presence of plasmids did not correlate with toxigenicity. It appears therefore that these environmental isolates do not possess any genetic material encoding cholera toxin and cannot serve as a reservoir for cholera.

Studies of the molecular heterogeneity of the structural gene for V. cholerae toxin indicate that we can distinguish among different epidemic strains of classical V. cholerae and El Tor strains of V. cholerae. Such differences may lead to a molecular typing system potentially as useful epidemiologically as phage or serotyping.

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Abstract

Key words, Cholera, Enterotoxin, E. coli, Plasmid

Current methods for the detection and isolation of enterotoxigenic E. coli (ETEC) are unsuitable for assaying large numbers of isolates. An alternative method is described which is based on detection of the genes encoding the enterotoxins rather than the detection of the toxins themselves. Isolated radiolabeled fragments of DNA encoding the heat labile (LT) or heat stable (ST) toxins are used as hybridization probes for homologous DNA sequences in E. coli colonies grown and lysed in situ on nitrocellulose filters. This method was also effective for detecting the presence of ETEC in bacterial growth from directly spotted stools from patients with acute diarrhea. A study was performed in Dacca, Bangladesh in which all LT producing strains were detected by the hybridization method. Using a single ST probe (from a porcine E. coli) 12 of 17 ST producing strains were detected but only 3 of 26 ST+LT isolates were detected.

Based on the data that there were at least two heterologous ST detectable in the infant mouse assay we cloned and sequenced an ST gene from one of the human strains from Dacca that was not detected in our initial hybridization studies. This gene is related to the porcine ST gene but is clearly divergent possessing 30 different amino acids and 31% different base pairs.

Environmental and non-toxigenic strains of V. cholerae O-1 were examined for genes homologous to genes encoding E. coli LT. All non-toxigenic strains of V. cholerae O-1 from Louisiana, Alabama, Maryland, Guam, Brazil, Bangladesh and Great Britain showed no homology while all toxigenic strains exhibited homology. In addition, strains of V. cholerae non O-1, "group F" Vibrios, V. vulnificus and Aeromonas hydrophila were tested and all were negative for any trace of toxin genes except for two strains of V. cholerae non O-1. The presence of plasmids did not correlate with toxigenicity. It appears therefore that these environmental isolates do not possess any genetic material encoding cholera toxin and cannot serve as a reservoir for cholera.

Studies of the molecular heterogeneity of the structural gene for V. cholerae toxin indicate that we can distinguish among different epidemic strains of classical V. cholerae and El Tor strains of V. cholerae. Such differences may lead to a molecular typing system potentially as useful epidemiologically as phage or serotyping.

Introduction

Over the past decade there has been a growing appreciation that some extra-chromosomal elements of bacteria plasmids carry genes that directly contribute to pathogenicity^{1,2}. In some instances, the plasmid's contribution to pathogenicity may border on the accidental. For example, some plasmid-mediated proteins employed for conjugation are inserted into the bacterial envelope and bring about a relative increase in resistance to serum killing. Other plasmid-mediated determinants of pathogenicity act by providing a host bacterial cell with an alternative means of sequestering iron or, as in the case of Yersinia, may provide a host cell with a means of sensing the environment to turn on or off appropriate cellular functions. The best known examples of plasmid-mediated factors of pathogenicity are, however, the enterotoxins and colonization factors of certain E. coli serotypes.

Enterotoxigenic E. coli are important causes of diarrhea in infants, children and adults in developing countries and also traveller's to these countries. The capacity of these strains to produce enterotoxins and cause disease is largely plasmid-mediated. Plasmids called Ent³, encode for two general classes of enterotoxins, a heat stable enterotoxin (LT) which is structurally and functionally similar to cholera toxin as well as several types of heat stable enterotoxin (ST) which are non-immunogenic, small polypeptides (about 47 amino acids) which act by stimulating the production of increased levels of cyclic GMP in small bowel cells leading to fluid secretion into the bowel lumen^{4,5}.

Plasmids may carry genes for only ST, only LT or both ST and LT^{2,3,6}. While Ent plasmids are generally transmissible by cell to cell contact to recipient cells in the laboratory, naturally occurring isolates carrying Ent plasmids are usually restricted to a small handful of E. coli serotypes⁷. Thus the Ent plasmids do not appear to be widely distributed in Nature. In part this may be a misleading finding since microorganisms carrying only an Ent plasmid are often avirulent. Rather, in order for E. coli to be fully pathogenic, the presence of yet another plasmid species (or plasmid gene), the colonization or Kad plasmids, are required^{1,2,3}.

The Kad plasmids encodes for proteinacious cellular appendages which appear as bacterial pili on the cell surface. These pili adhere to certain mammalian cells, especially small bowel epithelial cells. It is the combination of both the Kad pili biosynthesis and toxin production that is necessary for enteropathogenicity in E. coli³. To be sure other (unknown) factors are involved. One cannot simply transfer an Ent plasmid and Kad plasmid to any E. coli to produce a pathogenic strain³. Nevertheless we view plasmid-mediated toxins and colonization factors as a general microbial strategy to produce disease.

Despite the global importance of enterotoxigenic E. coli as a major cause of diarrheal disease, the epidemiology of infection is not well understood. One impediment to the study of E. coli diarrhea has been the difficulty in differentiating enterotoxigenic strains from normal flora. Current methods involve the detection of enterotoxin production by biological and immunological assays. E. coli LT is detected by tissue culture^{8,9} and immunological assays¹⁰, while ST is assayed by fluid accumulation in ligated rabbit¹¹ or pig¹² intestinal loops or in infant mice¹³. All of these techniques require the preparation of cultural supernatants of individual strains or pools of strains to

be assayed for toxin production. The cost and inconvenience associated with these methods make them unsuitable for large scale epidemiological studies.

Over the past contract year we devised an alternative technique for the detection of enterotoxigenic E. coli as well as a means to examine the significance of environmental strains of Vibrio cholerae O-1. Unlike the current methods which involve the detection of enterotoxins themselves, our approach involves the detection of the genes encoding the enterotoxins. The method for E. coli was tested during a field study of diarrheal disease in Dacca, Bangladesh and has provided unequivocal data that there is a family of ST genes. Moreover, the study of V. cholerae O-1 environmental strains performed with the cooperation of the Center for Disease Control, Atlanta, Ga., throws considerable light on their potential as a reservoir for cholera in the U.S.A. and elsewhere.

Results

1. Detection of Enterotoxigenic E. coli by Colony DNA Hybridization: Report of a Field Study in Dacca.

a. Plan of the Study.

We have reported the successful characterization of LT and ST genes using recombinant DNA techniques^{14,15}. We have also noted in last years Annual Report that toxin genes in unknown strains of E. coli could be detected by using restriction endonuclease-generated fragments of DNA encoding ST and LT radiolabeled in vitro and in situ DNA-DNA hybridization¹⁶. Briefly, E. coli strains (or fecal material) are inoculated onto a nitrocellulose filter which has been overlayed onto a suitable solid growth medium. After growth of bacteria, the colonies are lysed with NaOH and the DNA is fixed on the filter. The filter was then incubated with radioactive LT or ST DNA (probe DNA) under conditions permitting DNA-DNA duplex formation with any homologous DNA sequences on the filter. The filter was then washed and exposed to X-ray film. Enterotoxigenic strains (or fecal material containing enterotoxigenic E. coli) are detected by exposure of the film over areas of the filter where DNA-DNA duplexes had formed, indicating the presence of DNA sequences homologous to the toxin gene. The precise procedure is detailed in appendix A and will be published shortly¹⁶.

Mr. Steve Moseley, a graduate student in my laboratory, traveled to the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dacca, Bangladesh during the course of this investigation.

Two groups of patients were studied. For isolation of enterotoxigenic E. coli for comparison of the colony hybridization techniques for toxin assay, patients with acute diarrhea likely to be caused by enterotoxigenic E. coli were cultured. Patients were selected as having diarrhea of probable enterotoxigenic E. coli etiology on the basis of the following criteria: 1) acute watery diarrhea with moderate dehydration, 2) absence of motile vibrios in stool as detected by dark-field microscopy, 3) absence of blood on pus cells in stool, and 4) age greater than five years (to reduce the probability of selecting patients with rotavirus diarrhea). Stools or rectal swabs from these patients were streaked on MacConkey agar. For each patient, five lactose fermenting colonies typical of E. coli were picked and examined for enterotoxin production and by colony hybridization. The "standard assays" for enterotoxin production included the Chinese Hamster ovary (CHO) cell assay for LT while ST production was detected by the infant mouse assay. These assays were performed by ICDDR,B personnel and have been in use for several years. The classification of strains as ST and/or LT was based on these assays and the results obtained by colony hybridization compared to this classification.

A second group of patients were selected for direct spotting of stool material on nitrocellulose for colony hybridization. This group consisted of 50 consecutive patients admitted to the ICDDR,B intravenous prehydration ward over a two day period. Patients are admitted to this ward on the basis of acute diarrhea with moderate dehydration. Stools or rectal swabs from each patient were directly spotted onto nitrocellulose filters overlayed on MacConkey agar (25 stools per filter). Each stool or rectal swab was also streaked onto MacConkey agar for isolation of colonies. Two isolated colonies and a pool of 5 colonies

from each culture were assayed for enterotoxin production by the standard CHO and infant mouse assay methods.

b. Detection of enterotoxigenic E. coli by colony hybridization.

Initially, a group of 42 clinical E. coli isolates (ICDDR,B) previously characterized as producing ST, LT, ST+LT, or as non-toxigenic by standard assay were examined by the colony hybridization method. Strains were inoculated on two nitrocellulose filters for separate hybridization with the LT and ST radioactive probes. For these studies the LT probe consisted of a ³²P-nick-translated 17 10,000 base pair Hind III - generated DNA fragment encoding a portion of the LT molecule. The ST probe was a ³²P-nick-translated¹⁷ 157 base pair Hinf I fragment encoding a portion of the ST molecule. Both of these toxin genes were originally isolated from Ent plasmids isolated from E. coli isolated from piglets. The data obtained by colony hybridization in comparison to standard enterotoxin assays are summarized in Table 1.

Table 1: Detection of Enterotoxigenic E. coli by Colony Hybridization.

Toxin Type*	No.	No. detected by colony hybridization	
		LT probe	ST probe
ST+LT	14	14	1
LT	4	4	0
ST	8	0	6
Nontoxigenic	16	0	0

*Toxin production characterized by tissue culture (LT) and infant mouse (ST) assays.

All 18 LT producing strains (LT-only and ST+LT) were detected by the LT probe, while the ST probe detected 6 of 8 ST strains but only 1 of 14 ST+LT strains. It is significant that none of the 16 nontoxigenic strains reacted with either probe; this illustrates the lack of DNA homologous to either probe in nontoxigenic E. coli. Moreover the data do not indicate any degree of cross-reactivity between ST and LT DNA.

c. Detection of enterotoxigenic E. coli infection in patients fecal material.

The colony hybridization method was then compared with the CHO and infant mouse assays for the ability to detect E. coli infections directly in fecal material in persons with acute diarrhea. The fecal material from 50 consecutive patients was spotted on plates and processed as described above. It is noteworthy that we required only 8 agar plates and 8 filters to process duplicate tests on all 50 patients. In contrast the standard assays used required several

hundred sucking mice and 150 tissue culture assays. The data are summarized in Table 2.

Table 2. Detection of enterotoxigenic E. coli in bacterial growth from directly inoculated stool material from patients with acute diarrhea.

Tysin Type	Standard Assay*	Colony Hybridization	
		LT Probe	ST Probe
ST+LT	12	11	2
LT	1	1	0
ST	9	0	6
Nontoxicogenic	28	0	0

* Two individual colonies and a pool of 5 colonies were picked from an isolation plate of each stool for standard assays for enterotoxigenicity. LT was detected by the CHO cell assay, and ST was assayed in infant mice.

Twenty-two of the 50 patients were found to have toxigenic E. coli infection by standard assay. Colony hybridization detected 18 patients with E. coli diarrhea. We were able to detect 11 of 12 patients infected with ST+LT strains with the LT probe as well as the one patient infected with the one E. coli elaborating only LT. Paranthetically we should note that although we failed to detect ST+LT E. coli in direct stool material from one patient, isolated colonies from the stool culture of this patient individually inoculated on nitrocellulose were detected by the LT probe. Using the ST probe we detected only 2 of 12 ST+LT infections but 6 of 9 infections by E. coli which produced only ST. Twenty-eight patients were not infected with enterotoxigenic E. coli as determined by standard assays and none of these was positive by colony hybridization.

d. Implications of the field study.

We have shown that enterotoxigenic E. coli can be detected by hybridization of radiolabeled enterotoxin genes to E. coli colonies lysed in situ on nitrocellulose filters. All LT and ST+LT strains were detected by the LT probe and 12 of 17 (71%) of ST only strains were detected by the ST probe.

In addition to the strains examined in this study, we have also examined numerous LT producing isolates from diverse geographic locations and from animal origin. In all cases, strain producing LT as determined by tissue culture assay (or by immunologic means) were also detected by colony hybridization with the LT probe, even though this particular probe DNA came from a plasmid of a porcine E. coli isolate. These data suggest that the LT toxins of E. coli are homologous as has been suggested by serological data^{18,19}. Whether one can distinguish the LT genes from animal and human strains or whether one can use internal differences in the LT gene to distinguish between particular human isolates remains to be seen.

The failure of the ST probe to detect all ST producing E. coli suggests that human isolates of E. coli can elaborate at least two distinguishable heat stable toxins detectible in the infant mouse assay. Although the number of strains we examined was relatively small, the indication is that most, but not all, ST-only E. coli isolated from human disease produce a heat stable toxin homologous to the

porcine toxin encoded by the ST DNA probe, while only a small proportion of ST+LT strains (3 of 26) produce this toxin.

Burgess et al.²⁰ and Gyles²¹ have reported two different types of ST activities produced by animal isolates of E. coli. These have been termed STa and STb by Burgess and coworkers²⁰. Only STa is detected in the infant mouse assay while STb is detected only in ligated piglet or rabbit intestinal loops. Therefore it is unlikely that the heterologous STs observed in the present study represent STa and STb since in this study only the infant mouse assay was used to detect ST production. Rather our data indicated that there appear to exist at least two heterologous STs of STa type among human E. coli isolates.

- d. Direct molecular evidence for at least two distinguishable ST genes from human E. coli.

Based on the data we obtained from the field study, Mr. Moseley selected an ST producing E. coli that failed to react with our ST probe. Plasmid DNA was isolated from this strain and a gene encoding ST was isolated by recombinant DNA methods^{14,15}. Initially the gene was located as an 800 base pair TaqI fragment DNA and we were to sequence the gene by the Maxim Gilbert method²² of nucleotide sequencing. Our data, show unquestionably that this ST is clearly distinguishable from the porcine isolate²³ used as a probe in our initial studies (see Figure 1).

Moseley also examined the DNA-DNA homology our original probe DNA under varying conditions hybridization against purified plasmid DNA from ST producing strains not detected in the field study. It is important to emphasize that the conditions we initially selected under which the colony hybridizations were carried out were stringent (see appendix A) and therefore demanded a high degree of homology for binding the probe DNA. This seemed necessary since we were unsure to what extent potentially cross-reacting DNA sequences might be found in non-toxigenic bacteria. However, if one changes the parameters of hybridization one can 'relax' the stringency required for DNA-DNA duplex formation and detect partially related sequences. We began therefore to hybridize our original probe DNA against a collection of ST-only, ST+LT, and non-toxigenic strains by varying the concentration of formamide. We were able to achieve conditions under which our probe DNA could now detect sequences in many ST strains not previously detected while still failing to react with non-toxigenic or LT-only producing E. coli. This is illustrated in Figure 2.

These data point out that there are distinct classes of STa genes but that they bear partial homology to each other. We suspect that these regions of partial homology reflect the nucleotides encoding the toxic moiety of ST. At a different level, our ability to distinguish between ST genes permits us to further explore elements of toxigenic E. coli epidemiology, for example, is one ST type more prevalent in one geographical area?; in a particular age group?; in a particular serotype etc.? We propose to explore these questions in the next contract period.

Figure 1. COMPARATIVE NUCLEOTIDE SEQUENCE OF BOVINE AND HUMAN ST GENES

Bovine ST	ATG AAA AAG CTA ATG TTG GCA ATT TTT ATT TCT GTA TTA TCT TTC CCC TCT TTT AGT CAG	5	10	15	20
	met lys lys leu met leu ala ile phe ile ser val leu ser phe pro ser phe ser gln				
Human ST	ATG AAG AAA TCA ATA TTA TTT ATT TTT TCT CTA TTG TCT TTT TCA CCT TTC CCT CAG				
	met lys lys ser ile leu phe ile phe leu ser val leu ser phe ser pro phe pro gln				
Bovine ST	TCA ACT GAA TCA CTT GCA TCT TCA AAA GAG AAA ATT ACA TTA GAG ACT AAA AAG TGT GAT	25	30	35	40
	ser thr glu ser leu asp ser ser lys glu lys ile thr leu glu thr lys lys cys asp				
Human ST	GAT GCT AAA CCA GTA GAG TCT TCA AAA GAA AAA ATC ACA CTA GAA TCA AAA AAA TGT AAC				
	asp ala lys pro val glu ser ser lys glu lys ile thr leu glu ser lys lys cys asn				
Bovine ST	GTT GTA AAA AAC AAC AGT GAA AAA AAA TCA GAA AAT ATG AAC AAC ACA TTT IAC IGC TGT	45	50	55	60
	val val lys asn asn ser glu lys lys ser glu asn met asn asn thr phe tyr cys cys				
Human ST	ATT GCA AAA AAA AGT AAT AAA AGT GGT CCT GAA AGC ATG AAT AGT AGC AAT TAC TGC TGT				
	ile ala lys lys ser asn lys ser gly pro glu ser met asn ser ser asn tyr cys cys				
Bovine ST	GAA CTT TGT TGT AAT CCT GCC TGT GCT GGA TCT TAT TAA	65			
	glu leu cys cys asn pro ala cys ala gly cys tyr stop				
Human ST	GAA TTG TGT TGT AAT CCT GCT TGT ACC GGG TGT CAT TAA				
	glu leu cys cys asn pro ala cys thr gly cys his stop				

An *E. coli* ST isolate, SLM1, from a patient in Bangladesh was selected for study. This strain failed to react with a DNA probe prepared from a bovine *E. coli* strain under stringent conditions of hybridization but showed homology under reduced stringency. Plasmid DNA was extracted from the strain and a Taq I fragment containing the structural gene for ST was cloned into the vector pBR322 as described previously^{14,15}. The cloned fragment was cleaved with the enzyme HpaII and fragments were sequenced by the method of Maxam and Gilbert²². The DNA sequence was translated into corresponding amino acids using a computer program that searched the sequence in three reading frames for both strands of DNA. An open reading frame corresponding to 216 base pairs was found and compared to the published DNA and amino acid sequences for a bovine ST described by So and McCarthy²³.

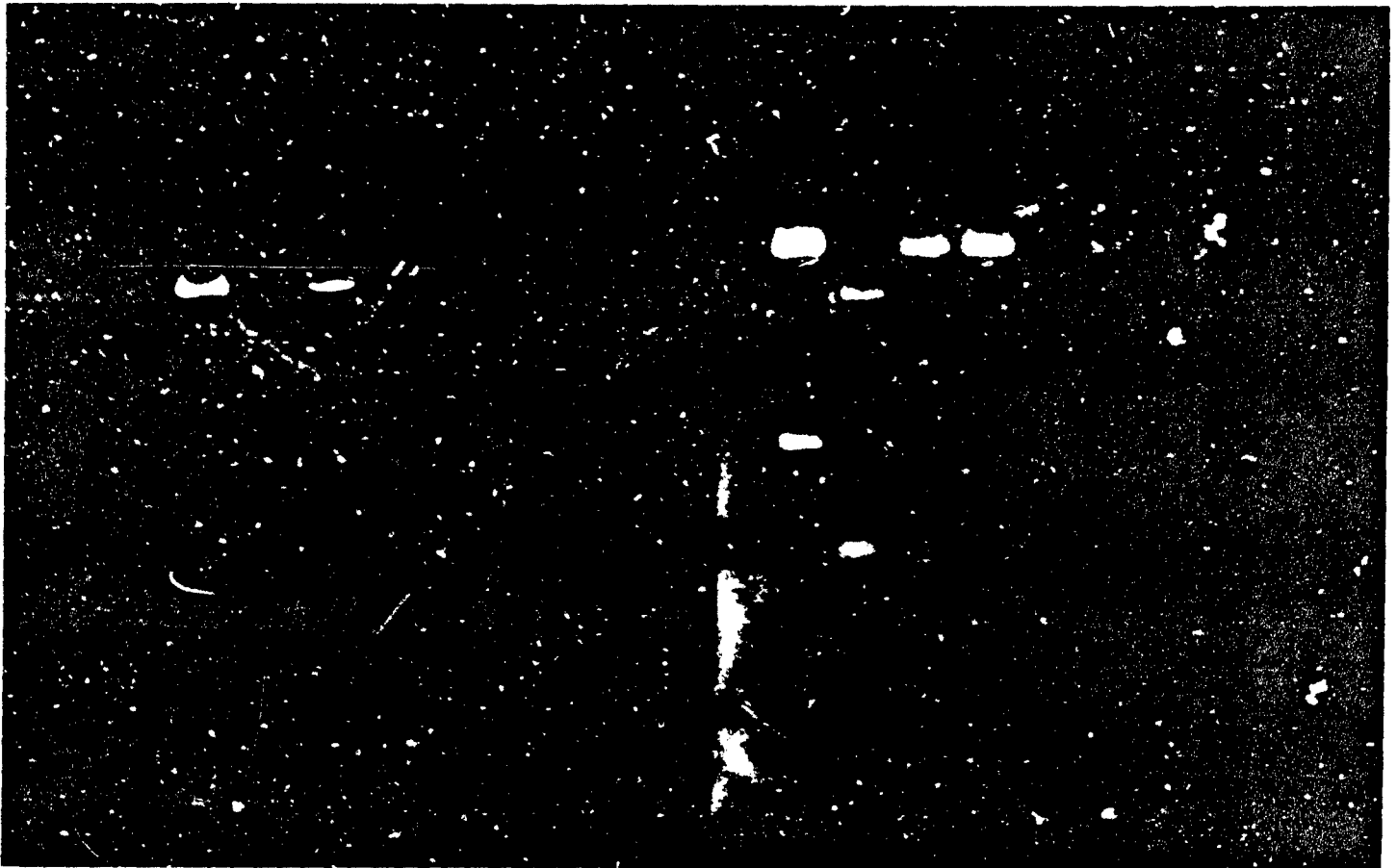


Figure 2. Detection of ST-specific sequences of DNA-DNA hybridization.

Four strains of toxigenic *E. coli* from humans which failed to be detected in Dacca with an ST DNA probe isolated from a bovine *E. coli* were hybridized against the same probe in 50% (left) and 20% (right) formamide. At 50% formamide only duplexes with about 10% mismatched sequences can be detected. In 20% formamide DNA-DNA duplexes with as much as 30% mismatching can be detected.

This figure shows that ST-specific sequences (the lower bands in all cases) can be detected only when the hybridization is performed in the 20% formamide hybridization solution.

2. Characterization of Environmental and Non-toxicogenic Strains of Vibrio cholerae.

a. Background information.

A persistent mystery in the epidemiology of cholerae is the repeated isolation of Vibrio cholerae 0-1 which is non-toxicogenic. These non-toxicogenic strains are usually isolated from environmental sources such as sewage, shellfish, and brackish water from such diverse areas as Bangladesh, Brazil, Guma, Great Britain and the United States, often in the absence of cholera in the nearby community^{24,25}. The significance of these isolates in terms of an environmental reservoir of cholera is uncertain. It has been postulated that they would be mutants of toxicogenic strains which have the potential to revert to toxicogenicity and therefore serve as a source of infection. Conversely, they could be merely natural inhabitants of aquatic environments which are aberrant not in their toxicogenicity, but rather in their serological characteristics.

An issue closely associated with non-toxicogenic strains of V. cholerae 0-1 is the toxicogenicity of strains of V. cholerae serotypes other than 0-1, the so-called "nonagglutinable" (NAG) or "noncholera vibrio" (NCV). These strains are commonly found in the aquatic environment and have been implicated in diarrheal and nondiarrheal illnesses on numerous occasions^{26,27}. Some of these strains possess a "cholerae-like toxin", but most do not²⁸. Again the same question arises about the basis of toxicogenicity or nontoxicogenicity in these strains. Are these strains mutants for toxin or are other factors such as plasmids, bacteriophage or environmental conditions responsible for toxin production or even repression of toxin production? The presence of additional cytotoxins has also confused the detection of enterotoxin in assays such as Y-1 adrenal cell system and significant strain-to-strain variation exists for optimal growth and testing conditions for toxin production for both V. cholerae 0-1 and non 0-1. All of the above factors influence determination of toxicogenicity by any assay which depends upon a phenotypic expression of toxin, antigen, or enzymic activity.

As we noted last year the nucleotide sequence of LT cistrons have been found to be very similar to that of cholera toxin²⁹. Moreover, not only can a molecular probe of LT genes detect toxicogenic E. coli but this probe can hybridize with the toxin genes of V. cholerae under appropriate conditions³⁰. Consequently over the past contract period we utilized molecular hybridization to examine the genes of V. cholerae as well as NCV and NAG strains.

b. Plan of the study.

A number of strains of V. cholerae 0-1 and non 0-1 were examined for the presence of genes homologous to E. coli LT genes. Strain numbers and sources are given in Table 3 along with the results we obtained using colony hybridization methods similar to those reported above and in appendix A. To test the sensitivity and accuracy of the colony hybridization method a set of 32 vibrio strains were received from the Center for Disease Control, Atlanta, Ga. which were coded as to identity and toxicogenicity as a blind study. In addition, a number of strains of V. vulnificus (lactose + vibrios, "group F" or "EFG" vibrios, V. parahaemolyticus and Aeromonas hydrophila were examined. Virtually all strains were examined for plasmid DNA by a method detailed in last years Annual Report.

Table 3. Summary of hybridizations of E. coli LT DNA with strains of V. cholerae

Strain Number	Source	Homology With LT
<u>V. cholerae</u> 0-1:		
3784, 3786, X725, X392, X316	EN ^a , Guam	-
1196-74, 1074-78, 2634-78, 2633-78	EN, Brazil	-
MP19812	EN, Bangladesh	-
V69	EN, Maryland	-
VL5962, VL6007, VL6085	EN, England	-
692-79, 1094-79, K5, 1528-79, 1717-79 1742-79, 1954-79, 1955-79, 1956-79, 2974-79, 2075-79	EN, Louisiana, 1979, 1980	-
1077-79	EX, Louisiana, 1979	-
1175-77	EX, Alabama	-
N-20, N-32, N-44, E7708, E8500, E7626	EN, Louisiana, 1978	+
E73 ³ , E7657, 4808	FC, Louisiana, 1978	+
569B, 395, ATCC 14035	FC, India	+
30167, 62746, 1944	FC, Bangladesh	+
<u>V. cholera</u> non 0-1		
V10, V37	EN, Maryland	-
VL3944, VL6214	EN, England	-
D1560, C6487, C7431, C6713, C9414, C4750	EX, U.S.	-
C5852, C7037, C6770	FC, U.S.	-
S-21	FC, Sudan	+
61956	FC, Bangladesh	+

^aAbbreviations: EN = environmental, EX = extraintestinal infection, FC = feces.

The structure of V. cholerae toxin genes was also studied in some detail. For colony hybridization we employ an LT probe that contains the genetic material encoding both the A and B subunits of LT toxin. We also prepared probes consisting of a 1200 base Hinc II fragment encoding only for the A toxin subunit and a 400 base pair Eco RI - Hind III fragment encoding only the B toxin subunit.

In order to examine the vibrio toxin gene in detail we employed hybridization of the probes against extracted whole vibrio chromosome DNA. One microgram amounts to the Vibrio DNA were digested with an appropriate restriction endonuclease. The cleaved DNA was electrophoresed through a 0.7% agarose gel in Tris-acetate buffer pH 8. After electrophoresis the gel was denatured in NaOH, neutralized and the DNA fragments transferred to nitrocellulose sheets according to the technique of Southern³¹. After transfer, the nitrocellulose sheet is baked at 80C, and hybridized with the appropriate radiolabeled DNA probe. Following hybridization the nitrocellulose sheet is washed, air dried and placed against Kodak X-omat R film. The film is developed in 48 hrs. Exposed areas define the DNA fragments on the nitrocellulose that are homologous to the probe (in this case the toxin genes of V. cholerae).

c. Results.

A total of 21 coded strains received from CDC were tested with an LT probe. All toxigenic strains (5 out of 21) were detected by the probe with no false positives or false negatives thus showing 100% agreement with conventional methods for detecting CT. No correlation was seen for the presence of plasmids and toxigenicity.

All strains of V. cholerae O-1 which gave a positive Y1 adrenal cell test were found to possess DNA sequences homologous to E. coli LT genes whereas all strains which were Y1 adrenal cell negative failed to give any sign of homology with LT DNA. The results are summarized in Table 3.

All environmental isolates of V. cholerae O-1 from Brazil, Bangladesh, England and Maryland were negative as were an isolate from a gall bladder. All 1978 environmental strains from Louisiana isolated in conjunction with the epidemiologic investigation there following an outbreak of cholera, were positive for genes homologous to LT. However, all 1979 and 1980 isolates from Louisiana tested to date, including strains from sewage, water, shellfish and a leg ulcer were negative. The A and B subunit probes gave identical results and no strain possessed genes for one subunit only.

Among serotypes of V. cholerae other than O-1 (the NAG or NCV vibrios), strains S-21 and 61956, stool isolates from the Sudan and Bangladesh, respectively, possessed sequences homologous to LT. All other V. cholerae non O-1 strains tested were negative. All strains of group F vibrios, V. vulnificus, V. parahaemolyticus and A. hydrophila tested were negative for sequences homologous to LT.

When DNA extracted from toxigenic strains of V. cholerae were examined by use of restriction endonucleases, certain differences became apparent (Figure 3). All strains of the El Tor biotype possessed only one Hind III restriction fragment homologous to LT whereas classical strains demonstrated at least two homologous bands greater than 5000 nucleotide pairs in size. Digestion with EcoRI

yields at least 4 large fragments in classical strains. This may indicate the presence of multiple gene copies in classical strains³⁰. An exception to the pattern was seen with strain 4808, an El Tor isolated from the 1978 outbreak in Louisiana³². This El Tor strain yielded two small fragments when cut with the enzyme Hind III, rather than the single fragment seen with the other El Tor strains. The sum of the molecular weights of the two smaller fragments of 4808 was approximately equal to that of the single large fragment of other El Tor strains. When cut with other enzymes such as EcoRI, strain 4808 yielded only one fragment as did other El Tors. Thus the Louisiana strain possessed a unique Hind III restriction site within the toxin gene. Such differences may lead to a molecular typing system potentially as useful epidemiologically as phage typing. In this latter vein it may be noted that we have shown that a single case of cholera in Texas that proceeded the Louisiana outbreak by five years is of the same molecular type as the Louisiana strains.

d. Implications of the study of environmental and NAG strains.

Non-toxicogenic strains of V. cholerae O-1 have been reported only in recent years, undoubtedly due in part to the development of toxin assays suitable for large scale testing e.g., the Y-1 and CHO cell assays and ELISA tests. Outbreaks in nonendemic areas such as Louisiana and Guam have also spurred interest in environmental sources of V. cholerae. In part because media and incubation conditions can affect toxin production, it was not clear if these strains produced no toxin at all, or if they produced toxin below detectable amounts or even if a partial or defective toxin was produced. If these strains produced absolutely no toxin, then it was uncertain whether a functional structural or regulatory gene was lacking.

The use of a molecular probe derived from structural genes encoding LT indicate that the 28 non-toxicogenic strains of V. cholerae tested in this study simply lack any trace of structural toxin genes. The absence of structural genes applies to both the A and B subunits of toxin and therefore, defects in regulatory genes or subunit assembly are not relevant.

Some of the non-toxicogenic V. cholerae O-1 strains produce substances which may react in one or more assays for CT. For example Spira and coworkers³³ found that strains from Guam and Alabama elicited fluid accumulation in rabbit ileal loops. Other strains tested by Merson et al.²⁵ have produced small amounts of permeability factor (PF). None of these satisfy a polyphasic approach, viz., PF, cell culture results, ELISA assays, neutralization by specific antisera etc., necessary to satisfy the criteria for the possession of CT genes. The converse does not hold true, however. A non-toxicogenic strain could be defective in regulatory, not structural genes and would thus give a positive result with the LT probe. Similarly, a small deletion or missense mutation in the structural genes would react with the probe since enough nucleotide sequences remain to hybridize with the probe. It is in this regard that our negative results are quite informative, since there is no possibility of the organism serving as a gene reservoir for cholerae toxin.

It is of interest that none of the strains of V. cholerae non O-1 isolate from clinical samples in the U.S. possessed demonstrable toxin genes whereas strains from the Sudan and Bangladesh were toxigenic by both the Y1 adrenal test and by DNA hybridization. Spira et al.²⁸ reported that 24% of 72 diarrheal isolates of V. cholerae non O-1 from around the world produced cholera or a

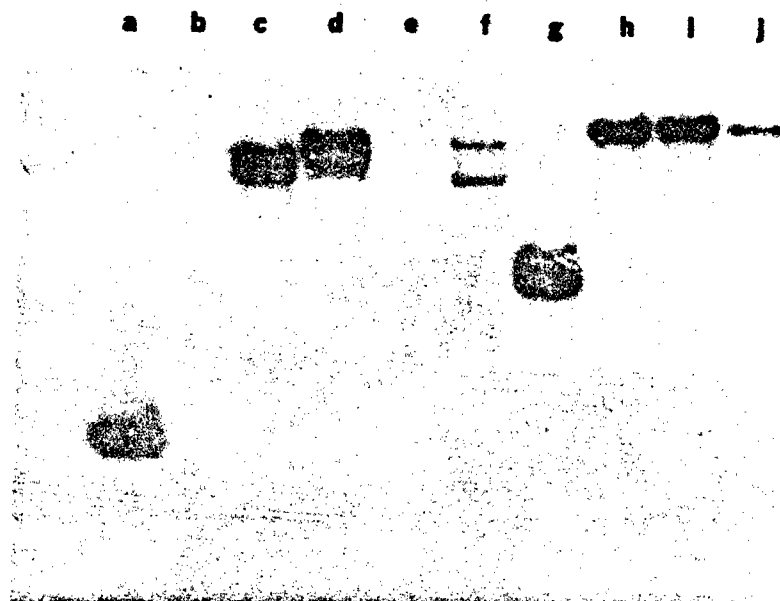


Figure 3. Molecular heterogeneity of the *V. cholerae* toxin gene.

A 780 base pair *Hind* III fragment of DNA encompassing the sequences encoding the *E. coli* bent stable A toxin subunit was hybridized with 1 μ g of *Hind* III cleaved whole chromosome DNA. The observed bands correspond exclusively to gene(s) encoding the *V. cholerae* enterotoxin.

- A = Control hybridization against *E. coli* P307 plasmid.
- B = Control hybridization against *E. coli* F⁻ strain.
- C = Classical cholera strain 569B (Inaba)
- D = Classical cholera strain ATCC 14035.
- E = Non-toxigenic *V. cholerae* O1 environmental isolate.
- F = Classical cholera strain 395 (Ogawa).
- G = El Tor strain 4804 isolated from Louisiana.
- H = El Tor strain 30167 from Bangladesh.
- I = El Tor strain 62746 from Bangladesh.
- J = El Tor strain 1944 from Bangladesh.

cholera-like toxin. These investigators also found fewer environmental strains producing CT, only 7%. The fact that 68% of the diarrheal V. cholerae non O-1 strains studied by Spira and coworkers produced enteritis without demonstrable LT or CT while 24% produced CT and only 8% were avirulent indicates that the most important virulence factor(s) for these strains remain to be elucidated. In addition, no other Vibrio species we tested possessed a CT or LT-like toxin indicating that the pathogenesis of infections caused by these species remains to be resolved.

Despite the wealth of information that exists on the pathogenesis of cholera, the reservoir of the disease is still uncertain. If humans are the sole carriers of cholerae, then the disease would be maintained through long-term carriers, which appear to be rare, or by continuous transmission involving asymptomatic cases. The other possibility is an environmental reservoir of free living V. cholerae. The possibility of an environmental reservoir was demonstrated in the 1978 Louisiana cholerae outbreak which was traced to improperly prepared shellfish³². As noted above, we find the Louisiana strain and the single Texas case of 1973 to have a unique toxin gene structure. Certainly environmental strains of V. cholerae from the environment during 1978 gave us positive evidence for this unique molecular marker, but environmental isolates from the same area after the epidemic subsided failed to show a reservoir of these strains in nature. We presume the strain has been endemic in the gulf coast of the U.S. since 1973 - but where? One must speculate that the strain, if it is in nature, must be rare indeed. Certainly the idea of an environmental reservoir is a more appealing explanation to us than the hypothesis that sporadic cases of cholera are caused by effluent discharged from aircraft³⁴. Proof of the environmental reservoir of cholerae will require rigorous monitoring. We believe that the molecular technique we employed in the present study can be of significant value in deciphering this and other epidemiological puzzles. Moreover, may not the appearance of positive environmental isolates predict the potential for epidemic?

Discussion

Over the past contract year we have successfully applied some of our basic findings on the molecular structure of E. coli enterotoxin production to practical aspects of the epidemiology of enterotoxigenic E. coli infection as well as V. cholerae infection. As we had hoped these practical studies have proved useful to better focus our interests.

One of the most interesting findings of our work is the discovery of a second subclass of human E. coli ST. Several years ago we first isolated by recombinant DNA methods the ST gene from a plasmid found in an E. coli of bovine origin¹⁴. Subsequent work by So et al.²³ showed that this ST gene was a transposable element. This important finding seemed to explain the ubiquity of the ST gene and the heterogeneity of Ent ST plasmids^{6,14,23}. Our epidemiological studies this year show a second subclass of ST found in human E. coli strains. These strains give a strongly positive infant mouse test for ST and hence do not appear to be the so-called STb toxin which reacts only in ligated intestinal loops²⁰. For convenience therefore we call the initial ST gene of bovine origin STa1, and the second subclass identified by us in the human E. coli as STa2.

Our DNA sequencing (Figure 1) suggests that the C-terminus of both STa1 and STa2 are relatively homologous. This portion of the toxin molecule is noteworthy for the number of cysteine residues it possesses. Perhaps this is related to the toxic properties and/or the host stability of the ST. However, we have also found that one of our STa2 clones is lacking the sequences encoding the last four C-terminal amino acids. Yet the toxin produced by this strain gives a strongly positive infant mouse test. One of these four terminal amino acids is a cysteine; of course there are still 5 additional cysteine residues remaining from amino acid 59 through 68. It should be interesting to determine whether the heat stability of the molecule is affected by the loss of this single cysteine residue. It will also be worthwhile to see whether a fragment of the C-terminus of ST (encompassing amino acids 53-72) cloned into a carrier peptide possesses measurable toxic properties; this experiment is currently in progress.

The other areas of homology between STa1 and STa2 include amino acids 1-20 which has the properties of a signal sequence not present in the functional toxin. Although 13 of these 20 amino acids are identical 18 of the 60 nucleotides are mismatched. These similarities in the leader sequence presumably reflect that a certain proportion of the same hydrophobic amino acids are needed to transport the toxin across the cell membrane. As noted by So and McCarthy²³ this leader sequence is similar to the bacteriophage fd minor co protein signal sequence.

In the 13 amino acids at positions 27-39, which probably comprise the beginning of the functional toxin molecule we find identical residues except for a serine in place of a threonine at position 36. In this stretch of 39 base pairs only 7 are mismatched. Thus the N terminus and C terminal amino acids of STa1 and STa2 are highly conserved; one or both in concert presumably contain the important physiological component of ST.

Despite these obvious areas of amino acid similarity we find that of the 216 base pairs in STa2 some 66 (31%) were different from STa1 and that 30 of the 72 amino acids are different. This is most striking over the stretch of DNA encod-

ing the 18 amino acids 40-57 in which 25 of the 54 base pairs are mismatched and 14 of the amino acids in STa1 and STa2 are different.

As one examines the DNA sequence of STa2 in detail it becomes clear why despite the similarities between STa1 and STa2 we failed to detect significant DNA-DNA homology under normal hybridization conditions (i.e. high stringency). The longest stretch of DNA present in both genes that does not contain at least a single mismatch is only 14 base pairs in length. Since hybridization under high stringency require several regions of at least 12 exactly matching nucleotide pairs to form a stable duplex it is not surprising we failed to detect any hybridization. Under our conditions of reduced stringency 1 mismatch in 5 base pairs could be tolerated to give stable duplex formation without loss of specificity. Both ST genes also have a high proportion of AT bases (64% for STa1 and 64% for the human ST gene) which also affects the efficiency of hybridization in that the higher the G+C content the more stable the duplex.

One final point of disparity between STa1 and STa2 should be noted. So and McCarthy²³ noted that the DNA sequences at the functions of the STa1 gene contained inverted-repeated IS1 sequences which are undoubtedly responsible for transposability of the toxin gene. The DNA sequences adjacent to STa2 do not contain IS1 DNA nor indeed do we find that the gene is bound by any significant inverted repeated DNA sequences. Hence the STa1 gene may be more prevalent in bacterial populations because it is part of a transposable element; this might also explain why the same gene is found in such a high proportion of human and animal E. coli strains.

Our finding of a 'new' ST gene prompts us to return to Bangladesh armed with new ST probes to determine whether we can detect a greater proportion of ST strains in clinical material. Moreover it would be useful to use the large scale screening potential of our method to examine patient contacts and environmental samples to better appreciate the epidemiology of ST infection. Moreover as we noted above it could be of considerable interest to determine if there is any epidemiological significance in the divergence of ST genes.

It is not our intent to continue screening strains of environmental V. cholerae. It is not that the questions we asked have been answered unequivocally. Rather the CDC and FDA have indicated that they plan to go ahead with large scale screening utilizing the methods we have developed over the past contract period. We are interested, however, in the apparent molecular diversity in cholera toxin genes. This seems worthwhile as a continuing effort, particularly if one can use the molecular heterogeneity to pinpoint particular subtypes of V. cholerae in different epidemics and from endemic areas of the world.

Above all, as a general philosophy, we should like to continue to use the clues provided by our basic molecular studies to probe practical matters. To us the application of molecular genetics to the study of microbial pathogenicity represents an exciting avenue of research.

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Appendix A.

Methods Employed for in situ Hybridization

Preparation of ^{32}P -labeled probe DNA. "Probe DNA" refers to radiolabeled, specific DNA fragments from enterotoxin genes which are used to probe for homologous DNA sequences in strains being assayed. LT probe DNA was prepared from EWD299 plasmid DNA, and consisted of a 0.5 megadalton HindIII-generated fragment encoding a portion of the LT molecule¹⁵. ST probe DNA was prepared from CLS-2 plasmid DNA consisted of a 157 base pair HinfI fragment encoding a portion of the ST molecule²³. Each DNA fragment was isolated by polyacrylamide gel electrophoresis of restricted DNA. The appropriate fragment was cut out of the gel, and the DNA removed from the polyacrylamide by electroelution. The isolated DNA fragments were phenol extracted twice, ethanol precipitated, and labeled in vitro with α - ^{32}P deoxynucleotide triphosphates (New England Nuclear, Boston, MA) by nick translation¹⁷ to a specific activity of $2.5\text{--}5 \times 10^7$ cpm/ μg DNA.

Preparation and hybridization of nitrocellulose filters. Nitrocellulose discs (9 cm, BA-85, Schleicher and Schuell, Keene, N.H.) were boiled in water for two minutes, then individually wrapped in paper and autoclaved. A single sterile filter was placed on the surface of MacConkey agar and directly inoculated with isolated colonies or spotted with stool material. After overnight incubation at 37°C , the filter (on which colonies had formed) was removed from the agar. The filter was placed (colony side up) onto a double layer of Whatman No. 3 paper saturated with 0.5 M NaOH. After ten minutes the filter was transferred to a double layer of Whatman No. 3 paper saturated with 1 M Tris, pH 7 for one minute. After two additional transfers on fresh 1 M Tris, pH 7 saturated paper for one minute each, the filter was transferred to a double layer of Whatman No. 3 paper saturated with 1 M Tris, pH 7, 1.5 M NaCl for ten minutes. The filter was then removed from the Tris-NaCl, allowed to air dry, and baked overnight at 65°C . After baking, the filter may be stored before use for at least 16 weeks. Before performing in situ hybridization with radiolabeled DNA fragments, the filter was incubated at 37°C for three hours in plastic wrap (Saran Wrap) containing a sufficient volume of the following hybridization solution to thoroughly wet the filter: 50% formamide, 5 x SSC (1 x SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 x Denhardt's solution (0.02% ficoll, MW 400,000; 0.02% polyvinylpyrrolidone, MW 360,000; 0.02% bovine serum albumin) (16). The filter was then placed in fresh hybridization solution containing approximately 1×10^5 cpm/ml probe DNA (heat denatured) and 75 $\mu\text{g}/\text{ml}$ heat denatured calf thymus DNA sheared to an average size of 2.5×10^5 daltons by sonication. Hybridization was carried out for 24 hours at 37°C . The filter was then washed in 5 x SSC, 0.1% SDS for 45 minutes at 65°C , rinsed in 2 x SSC at room temperature, and allowed to air dry. The filter was exposed to Kodak Xomat-R X-ray film with a single DuPont Cronex Lightning-Plus intensification screen (DuPont De Nemours, Wilmington, Delaware) for 24 hours at -70°C . The film was developed according to the manufacturer's instructions.

As many as ten filters were processed consecutively on the same set of Whatman No. 3 double layers. The paper was kept saturated with fresh NaOH or buffers. As many as 50 filters were preincubated and hybridized together. Care

was taken to ensure a sufficient volume of hybridization solution to thoroughly wet all filters.

For hybridization under reduced stringency the concentration of formamide was reduced to 20% or 25%. Following hybridization the filters were rinsed in 5 x SSC, 0.1% SDS at 54.4°C and then washed for 1 hour in fresh 5 x SSC, 0.1% SDS at 54.5°C. After a final rinse in 2 x SSC at room temperature, the filters were air dried and developed as described above.

Hybridization of labeled genes with the chromosome. Chromosomal DNA was extracted as previously described¹⁴. One microgram amounts of the DNA preparations were digested with restriction endonucleases for approximately 3 hours at 37°C in the buffers recommended by the manufacturers. The reactions were stopped by adding 4 µl of 0.07% bromophenol blue, 7% SDS in 20% Ficoll and samples were layered on top of 0.7% agarose gels. Electrophoresis was performed in Tris-acetate buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 8) at 40 volts for ca. 16 hours. After electrophoresis, the gels were denatured in 0.2 M NaOH, 0.6 M NaCl, pH 7.5 for 45 minutes and the DNA fragments transferred to nitrocellulose filters according to the technique of Southern³¹. After overnight transfer, the filters were baked at 80°C in vacuo and stored until hybridization was performed as described above for colony filters.

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