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INFECTIOUS MULTIPLE DRUG RESISTANCE
IN THE ENTEROBACTERIACEAE

ANNUAL REPORT

By

Stanley Falkow, Ph.D.

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antibiotic resistance genes and transferred to laboratory strains of E. coli K-12. The plasmids possess a molecular mass of 92×10^6 daltons and were members of the F incompatibility complex. The preliminary molecular and genetic data suggest that the structural genes encoding a Vir-specific antigen/toxin may reside upon a transposable element.

Specific DNA sequences encoding for either E. coli heat stable toxin (ST) or heat labile toxin have been isolated. The entire DNA sequence of the cistron encoding the B subunit of LT has been determined. The translated sequence shows 80% similarity to the known amino acid sequences of the B unit of cholera toxin (CT). On the other hand, on the basis of DNA-DNA hybridization studies, LT and CT have diverged so that they show roughly 18% base sequence mismatching

Isotopically-labelled LT specific DNA has been used as a way to probe the relatedness between Ent plasmids of both human and animal E. coli. The data strongly suggest that the LT gene of animal Ent plasmids show a high degree of molecular homogeneity while the LT gene of Ent plasmids of human origin are divergent.

The LT-specific probes have also been applied to study the epidemiology of toxigenic E. coli diarrhea in the field. Moreover, our methodology has been now refined to a point where it is possible to quickly screen strains and pinpoint precise plasmid species encoding for enterotoxins or colonization antigens.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised, 1978).

Preface

In the past year we have completed and published several aspects of our research supported under this contract (see appended list of publications). The completed work has encompassed our studies of R plasmid epidemiology in a hospital setting as well as our studies on the transposition of antibiotic resistance. This is not to say that no further work is required but only that the work is completed in so far as we view the goal of this contract.

In our last research proposal we presented a detailed research plan to examine the chromosomal determinants of virulence in Shigella flexneri 2a and S. dysenteriae using a general method to detect specific antigenic determinants by in situ colony antigen-antibody reactions. Subsequent to submitting this proposal we were advised that several of our planned experiments in this sphere would, in essence, duplicate efforts currently under way at the Walter Reed Army Institute of Research. Rather than initiate redundant studies we concentrated this year upon our ongoing work concerning the molecular epidemiology of Vir, Ent and Kad plasmids. I am happy to say we made excellent progress on this problem during the past year due in part to some improvements in our methodology.

As in previous years, the graduate students and postdoctoral fellows in my laboratory have worked long hours to make a significant contribution to this research program. I wish to particularly thank Mr. Stephen Moseley, Mr. Daniel Portnoy, Mr. Tom Swanson, Dr. Lucy S. Tompkins and Dr. James Kaper for their efforts. Dr. Jose Lopez-Alvarez from Carlton Gyles' laboratory in Guelph was also a welcome colleague and our joint results are part of this report.

Abstract

Key Words: Plasmid, Enterotoxin, Colonization Factor, Enteric Bacteria

A new method for the detection of plasmid DNA in clinical isolates has been developed. The method requires only small culture volumes and can detect plasmid genomes ranging in mass from 1×10^6 to 300×10^6 daltons in mass. Moreover, the plasmid DNA is isolated in sufficient amount and purity so that multiple examinations of the molecular characteristics of the plasmid DNA is possible.

Plasmids, Vir, common to E. coli strains associated with sepsis have been characterized. The plasmids from wild-type E. coli were "tagged" with antibiotic resistance genes and transferred to laboratory strains of E. coli K-12. The plasmids possess a molecular mass of 92×10^6 daltons and were members of the F incompatibility complex. The preliminary molecular and genetic data suggest that the structural genes encoding a Vir-specific antigen/toxin may reside upon a transposable element.

Specific DNA sequences encoding for either E. coli heat stable toxin (ST) or heat labile toxin have been isolated. The entire DNA sequence of the cistron encoding the B subunit of LT has been determined. The translated sequence shows 80% similarity to the known amino acid sequences of the B unit of cholera toxin (CT). On the other hand on the basis of DNA-DNA hybridization studies, LT and CT have diverged so that they show roughly 18% base sequence mismatching.

Isotopically-labelled LT specific DNA has been used as a way to probe the relatedness between Ent plasmids of both human and animal E. coli. The data strongly suggest that LT gene of animal Ent plasmids show a high degree

of molecular homogeneity while the LT gene of Ent plasmids of human origin are divergent.

The LT-specific probes have also been applied to study the epidemiology of toxigenic E. coli diarrhea in the field. Moreover, our methodology has been now refined to a point where it is possible to quickly screen strains and pinpoint precise plasmid species encoding for enterotoxins or colonization antigens.

INTRODUCTION

A plasmid is an extrachromosomal element of bacteria. Plasmids may be conveniently classified into two major types: conjugative and non-conjugative. Conjugative plasmids endow their bacterial hosts with the capacity to act as genetic donors so that the plasmid (and occasionally other host genetic material) can be transmitted to another bacterium by direct cellular contact.

Non-conjugative plasmids do not have the inherent ability to initiate conjugation. Rather non-conjugative plasmids require that either a conjugative plasmid be also present in the same cell to "mobilize" the non-conjugative plasmid or that transmission be expected by a bacterial virus (transduction) or by direct uptake of DNA (transformation). Transduction and transformation can also act to disseminate conjugative plasmids.

Plasmids represent a reasonably stable but dispensable autonomously replicating gene pool of bacteria. Bacterial plasmids carry a wide variety of determinants which may permit their bacterial hosts to better survive in an adverse environment or permit host cell to better compete with other microorganisms of the same or different species. The general genetic and molecular properties of plasmids as well as the ecology and clinical significance of the plasmids of both gram negative and gram positive species have been the subject of several books (1-3) and reviews (4-6). Plasmids may certainly be considered to be part of the normal genetic complement of virtually all bacterial genera. From the standpoint of medicine, however, plasmids that mediate resistance to antibiotics (R plasmids) have been by far the most commonly recognized and studied plasmid determinants (1,2,5,6). A subject of great interest over the past decade has also been the observation of several plasmid-mediated virulence factors in E. coli isolated from diarrheal disease (7) as well as invasive disease (7). In diarrheal disease the plasmids of interest are Ent plasmids associated with enterotoxin

synthesis and Kad plasmids associated with the colonization of the small bowel by certain E. coli. In invasive disease the plasmids most current interest are Vir and ColV.

In the following sections we report our progress over the past year. We have found a new method for screening strains for plasmid DNA. Building upon last years results with ColV we now report the first molecular and genetic characterization of Vir plasmids. Finally, we report our ongoing work on the molecular epidemiology of Ent plasmids of human E. coli. In addition, we report the successful cloning of the E. coli K-99 gene.

RESULTS

1. An improved method for the screening of bacterial isolates for plasmid DNA.

Several years ago we (8) reported that plasmid DNA in gram negative microorganisms could be demonstrated in the agarose gel electrophoresis of extracts prepared from detergent-lysed bacterial cells. The method gained wide acceptance even though a) it was necessary to employ at least 50 ml of culture b) plasmids greater than 90×10^6 daltons were not reliably demonstrated and c) the method required several working days to compute the analysis of about 12 cultures. Subsequently, several (9,10) alternative procedures were reported that appear to work well with various bacterial species and permit the detection of bacterial plasmids of high molecular mass, although these methods required relatively large culture volumes and required several working days. Techniques designed to detect the plasmid DNA in a single colony have been popular and work reasonably well, although the best of the methods (11) results in a great deal of contaminating chromosomal DNA.

Optimally one would wish to have a method which required a small volume of culture, could detect plasmid DNA over a broad range of molecular mass, and was sufficiently free of contaminating chromosomal DNA so that one could perform restriction-endonuclease analysis. Daniel Portnoy who is a graduate student in our group, was frustrated in his attempts to detect high molecular mass plasmid DNA in clinical isolates. Consequently, he invested several months developing an improved method for screening plasmid DNA. His efforts were rewarded and listed below is the protocol that he developed for screening plasmid DNA from a 2 ml broth culture. The procedure requires less than 1 working day and the plasmid DNA is indeed of sufficient purity so that one can perform restriction endonuclease analysis. Furthermore, although only

a small volume of culture is used one obtains surprisingly large amounts of the plasmid DNA-- generally enough for at least three determinations. As illustrated in Figure 1, it is possible to detect plasmids ranging over a wide range of molecular mass. In practice, we have successfully used the method to detect plasmid DNA ranging from $>300 \times 10^6$ daltons to $<2 \times 10^6$ daltons.

We have distributed this procedure to a number of laboratories and are preparing a manuscript for publication. Several laboratories have confirmed our impression that the method works well (S. B. Formai, personal communication; R. P. Silver, personal communication).

The Portnoy Procedure for Screening Plasmids

1. Two ml of a suitable growth medium is inoculated and grown to the mid-logarithmic phase of growth. (In most cases stationary phase cells can be employed as well).
2. The cells are harvested by centrifugation in a table-top centrifuge and the cell pellet washed with 2 ml of TE (TE = .05 M Tris, .01 M EDTA, pH 8). The cells are dispersed, recentrifuged and the cell pellet resuspended in 40 μ l of TE.
3. A lysis buffer is prepared from a 4% solution of sodium dodecyl sulfate in TE. This solution is brought to a pH of 12.4 with base. It is essential that one employ a high pH tris electrode to prepare this solution. Add 0.8 ml of the lysis buffer to a 1.5 ml conical centrifuge tube (Eppendorf) and with a pasteur pipette transfer the 40 μ l of cells to this tube. The cell suspension should be mixed well in the lysis buffer. The suspension is incubated at 37C for 20 min.
4. The suspension is neutralized by the addition of 30 μ l of 2.0 M Tris, pH 7.0. The tube is inverted gently until a change in viscosity is noted.

5. Chromosomal DNA is salted out by the addition of .16 ml of 1 M NaCl. The solution should be mixed well to insure uniform dispersion of the NaCl in the viscous solution.

6. Place the solution on ice for 1 hr.

7. Centrifuge in a table top centrifuge for 5 min and decant the supernatant into another 1.5 ml centrifuge tube. Add 0.55 ml of isopropanol (at -20C) to the supernatant. Place on ice for 30 min.

8. Centrifuge the solution for 30 min; decant the supernatant and invert the tube on a paper towel (or place in a vacuum dessicator for a few minutes).

9. Resuspend the dry precipitate in 30 μ m of TE. At this point a 10 μ l may be analyzed by agarose gel electrophoresis as described by us previously (8).

10. If one wishes to perform restriction endonuclease analysis, it is often useful to resuspend the precipitate in 100 μ l of 10 mM Tris, pH 8.0 followed by 100 μ l of Tris equilibrated phenol and 100 μ l of chloroform. The tube is inverted to insure complete mixing. This solution is then centrifuged for 30 sec and the upper phase is removed with a micropipette. The plasmid DNA in this solution is then treated as described above for steps 7-9.

The procedure is rapid and gives uniformly excellent results with every bacterial species (including gram positive species) that we have examined. It is quite easy in practice for one individual to analyze 25-50 strains in six hours. Because of its utility, I felt it worthwhile to adequately document the procedure in this report.

2. Genetic and molecular characteristics of Vir plasmids of Bovine

Sypticemic Escherichia coli

On the basis of genetic evidence, two "virulence" plasmids were detected

by Smith and his colleagues (12,13) in invasive E. coli of animal and human origin. One of the two, the ColV plasmid, has been shown to endow bacteria with increased virulence by virtue of their greater ability to resist normal host defence mechanisms. The other virulence plasmid discovered by Smith in septicemic E. coli strains is the Vir plasmid. The Vir phenotype is characterized by the synthesis of toxin and a surface antigen.

Last year we described some of our studies with the ColV plasmid. At that time we noted that one of the difficulties in studying virulence plasmids is that there is usually no suitable selection method for detection of their transfer. As we noted in last years Annual Report, several methods are now available to "tag" plasmids with antibiotic resistance markers to facilitate subsequent studies. We have applied this methodology to study and characterize several Vir plasmids from wild type E. coli. We have been able to isolate two Vir plasmids in E. coli K-12 and determine their molecular and genetic properties as well as determine the relationship among different Vir plasmids. This work was performed in collaboration with Jose Lopez-Alvarez and Carlton Gyles, University of Guelph, Ontario, Canada. Dr. Alvarez performed most of these studies during a three month visit to Seattle and later used these data to satisfy, in part, his requirements for the Ph.D. degree. The work is summarized since it will appear shortly in the Journal of Bacteriology.

All of the strains used in this work were isolated from bovines suffering from sepsis. One strain, JL21 (isolated in Guelph) was untypeable while two of the strains JL10 (Guelph) and B177 (from H. Williams Smith in England) were 078:K80H⁻. Vir was detected by use of a specific antiserum prepared as described by Smith (12).

By mating strains JL10, JL21 and B177 with E. coli KJL711, transfer of the Vir antigen was observed only from JL21 (2/34 recipient.). Those K12 transconjugants receiving Vir antigen contained a plasmid species called

pJL1. Subsequently we could document the mobilization of a Vir plasmid from B177 using the F plasmid, F-Tc: The Vir plasmid from strain B177 was termed pJL2. To simplify further genetic characterization of these Vir plasmids, they were 'tagged' with the transposon Tn5 (conferring kanamycin resistance) using a method previously reported by this laboratory (14).

These tagged plasmids could then be used directly in mating experiments. The pJL1::Tn5 plasmid transferred at very high frequency, 9% in a 60 min mating. The pJL2::Tn5 plasmids were in fact found to be conjugative though their transfer frequency was estimated to range from 6×10^{-6} to 1.3×10^{-2} in 24 hr matings.

Both pJL1 and pJL2 were found to belong to the F incompatibility plasmid complex, specifically the FIV incompatibility group. In this respect the Vir plasmids are analogous to the ColV, Ent (LT) and K88 plasmids also known to contribute to E. coli virulence (see below).

By use of agarose gel electrophoresis, we found that both pJL1 and pJL2 possessed a molecular mass of 92×10^6 daltons. This is considerably larger than most Ent, ColV and K88 plasmids that have been reported. Despite their similarity in molecular mass, by both restriction endonuclease analysis and DNA-DNA hybridization, pJL1 and pJL2 were different. Indeed, pJL1 shared only 58% of its polynucleotide sequences in common with pJL2. Parenthetically, we should note that no plasmid DNA could be detected in the wild-type strain JL21 and that a rare transconjugant from matings using E. coli K-12 (pJL1) or pJL2) while showing the Vir phenotype were devoid of plasmid DNA. These observations suggest that the Vir gene(s) can occupy a chromosomal location and raises the possibility that the Vir genes may be carried on a transposon, like the E. coli ST enterotoxin genes. It may be of further parenthetical interest to note that pJL1 DNA is also related to ColV plasmid DNA. Both Vir and ColV apparently belong to the same incompatibility group. This probably explains the observation that in studies

involving literally hundreds of ColV⁺ septicemic E. coli none were found to be Vir⁺ and that no Vir⁺ isolates have been found to be ColV⁺ (12,13, 15). Recombinants between the two could be expected to arise in nature.

Now that we have been able to isolate single plasmid species carrying the Vir genes we may hope to be able to better characterize the Vir gene products. Moreover, we already have isolated several plasmid derivatives which are phenotypically Vir⁻ because we presume Tn5 has inserted into an essential Vir function. In any event, the continued characterization of these plasmids should be a useful contribution to our understanding of how plasmid-mediated products contribute to bacterial virulence. We should also hope that the availability of specific Vir DNA sequences would let us search for genetic analogs in a wide range of enteric species.

3. The molecular epidemiology of E. coli Ent and Kad plasmids.

Enterotoxigenic E. coli have been shown to make two distinct enterotoxins, ST and LT (16,17). These toxins are discernable by several characteristics including biological activity, immunogenicity and relative heat stability (17). Burgess et al. (18) have reported that there are two distinct ST's that can be distinguished by biological assays. STa elicits fluid accumulation in sucking mice while STb is only active in weaned piglets and rabbit ligated loops. Alderete et al. (19) have reported STa to be a low molecular weight protein (about 5100 daltons). LT is a larger protein which is comprised of two major protein subunits of 11,500 and 25,000 daltons (20). LT shares several characteristics with the classical toxin of V. cholerae. Both toxins share ADP-ribosylating activity and they both stimulate adenyl cyclase in eukaryotic cells (21). LT and cholera toxin also show partial immunological cross-reactivity and have similar membrane receptors (22,23).

Smith et al. (24) first used the term Ent to describe enterotoxin-encoding plasmids. Ent plasmids can encode LT only, ST only, and both LT

and ST. Work from our laboratory showed that LT + ST plasmids seemed to constitute a homogeneous group of extrachromosomal elements that shared a similar mole fraction guanine + cytosine (0.49-0.50), molecular mass (about 60×10^6 daltons) and DNA sequence homology (25). On the other hand ST plasmids were found to constitute a disparate group of plasmids. Following the first successful isolation of the ST gene in our laboratory (26), So et al. (27) reported that the ST genetic determinant was part of a transposon that was flanked by inverted and repeated IS1 elements. Presumably the transposability of ST is responsible for its dissemination among disparate plasmid species. It is important to note, however, that the ST gene isolated by us is a typical example of the *Sta* class.

In an extension of our initial work on Ent, work supported under this contract permitted us to isolate and characterize the LT determinant from a plasmid of porcine origin. A rather comprehensive report of the cistrons encoding this *E. coli* LT was reported by us during the past year (28). Over the past few months we have particularly emphasized the refinement of our methods for the development of ST and LT-specific DNA probes as well as launching a detailed examination of the sequence homology among LT genes of different origins and the relationship between LT genes and the genetic material of *V. cholerae* and other pathogenic *Vibrios*.

a. The development of specific LT and ST probes.

One of the major developments in our work has been the complete sequencing of the cistron encoding the B subunit of LT. M. So (personal communication) has also completed the sequencing of the *STa* gene. The availability of these DNA sequences now permits us to select at will DNA fragments encompassing specific parts of a gene or only sequences totally internal to a gene of interest. For example, for *STa* we now employ a 156 base pair *Hind*III DNA fragment that encompasses the Carboxy-terminal end of the *STa* gene.

In the case of the LT gene, translation of the DNA sequence indicates that the B subunit is comprised of 124 amino acids (372 base pairs). Twenty-one amino acids comprise a "leader" sequence that we presume is involved in the transition of the B subunit from the cytoplasm to the periplasmic space. The remaining 103 amino acids show a remarkable degree of identity with the known amino acid sequence of the cholera B subunit. Eighty-one of the amino acids are identical, although one cannot be certain, of course, that the DNA sequence has not shown some degree of base mismatching. The remaining amino acids are different, although in all but one case, we can explain the shift on the basis of a change in a single nucleotide pair. Fig. 2 shows the sequence of the first 13 amino acids of the functional B polypeptide of E. coli in relation to that reported for V. cholerae.

The complete sequence will be published over the coming year. Of additional interest is the observation that the overall guanine + cytosine content of the E. coli LT gene (and STa gene) is low-- about 40% overall which is significantly different than the overall composition of E. coli Ent plasmids (0.49 overall) and the V. cholerae chromosome (0.47 overall). At any rate, it may be seen that the availability of these sequences greatly simplify our molecular analysis of the relatedness between the E. coli LT and ST genes and other genomes of interest.

b. Homology shown by isolated LT gene fragments.

In last year's Annual Report, we showed our initial attempts to perform colony blot (30,31) hybridization. Fig. 3 illustrates one recent experiment in which 43 colonies representing individual isolates from travellers to Africa (obtained from R.B. Sack) were spotted on a filter paper disk overlaid on the surface of a MacConkey agar plate. Following overnight incubation, the paper (on which the colonies developed) was lifted from the surface and hybridized (31) with a ³²p-labeled-LT specific DNA fragment. This procedure

takes less than 24 hours. After drying, the filter paper is placed against a piece of x-ray film. After 24 hrs exposure time, the film is developed and colonies containing LT genes appear as exposed spots on the film. (In the figure, a positive print is shown so that the LT⁺ colonies appear as white spots). In this particular instance, each colony giving a positive "signal" was characterized as LT⁺ by standard immunological or tissue culture assay. An ST colony blot is also illustrated (Fig. 3A).

The utility of this test was shown this past summer when an Iranian student, home for a holiday, collected strains from cases of diarrhea in rural areas. He prepared the filters in Iran and carried the dried filters back to Seattle in October. Filter blot hybridization permitted us to estimate that some 10% of the diarrheal disease among 86 patients he examined were caused by LT⁺ E. coli. Among some 30 odd control patients, two showed carriage of LT⁺ E. coli. Interestingly enough, both of these patients were epidemiologically linked to one of the diarrheal cases. As noted in our research proposal submitted last year, we will be initiating an extensive field study at the Dacca Cholera Laboratory using both LT⁺ and ST⁺ DNA probes applied to direct fecal cultures. Mr. S. Moseley will leave for Dacca at the end of January 1980, and he will study, in collaboration with Dr. W.B. Greenough and his associates, hospitalized patients, patient contacts and environmental samples. The advantage of this approach is that we can effectively screen virtually every colony on a plate for the presence of toxigenic E. coli. This should be of particular interest when examining the stools of convalescent patients, patient contacts and environmental samples in which the numbers of toxigenic strains may be low.

In another vein, in order to more precisely determine the degree of homology among LT genes, we chose four Ent plasmids to study in detail. Two of the plasmids were isolated from E. coli pathogenic for humans and the other

two were isolated from porcine strains. One of the porcine Ent plasmids was P307, the plasmid from which the hybridization probe was isolated. The other porcine plasmid was pCG86 (32) which has been of interest because it was the first naturally occurring Ent plasmid isolated which carried antibiotic resistance determinants. The Ent plasmids from human sources were from H10407, the 'archetypical' human Ent plasmid, and a plasmid CI from an outbreak on a cruise ship (received from K. Wachsmuth, C.D.C.).

Each purified plasmid DNA was cleaved with a variant of restriction endonucleases. Initially, we employed a HindIII probe (comprised solely of sequences internal to the LT gene) and hybridized this probe to HindIII cleaved plasmids by the method of Southern (31). Only one fragment in each of the four samples hybridized to the probe. This result indicated that most, if not all, of the HindIII DNA portion of the LT DNA was conserved among the Ent plasmids. We performed the same type of analysis using different restriction enzymes. In this way, we studied the internal structure of the common HindIII fragment. For example, the HindIII probe was found to contain a single SmaI site. Therefore, cleavage of P307 with SmaI (followed by blotting and hybridization) resulted in the appearance of two DNA bands that hybridized to the probe (Fig. 4). Using enzymes that cleaved within the hybridization probe, we could determine if these sites were conserved in all the Ent plasmids. Similarly, by using enzymes that did not cleave within the hybridization probe, we could determine if new restriction enzyme sites were present in the LT DNA region of the Ent plasmids (Fig. 4). From this analysis, we determined that the human Ent plasmids apparently demonstrated some degree of structural divergence when compared to each other and to the hybridization probe. One human Ent plasmid was found not to have an SmaI site within the common HindIII fragment and the other human Ent plasmid did not

have an EcoRI site within the common HindIII fragment.

All in all, it appears from these preliminary results that porcine Ent plasmids are quite homogenous, whereas the human Ent plasmids comprise a more heterogenous group. It should be noted that the similarity or divergence of the sequences adjacent to the LT DNA region is indicated by the sizes of the DNA fragments that hybridized to the probe. A comparison of the sizes of the hybridized fragments of P307 and pCG86 indicated that the DNA sequences adjacent to the LT DNA regions in each of these plasmids are the same (Fig 4). In contrast, the human Ent plasmids differed markedly from each other and from the porcine Ent plasmids with respect to the sequences adjacent to the LT DNA region. We plan to continue and extend these studies during the coming contract year.

c. Locating Ent genes on plasmids.

As noted above, we can easily detect the presence of Ent genes by colony hybridization. By the same token, we can precisely locate the LT gene in isolated cleaved plasmid DNA. In many instances, it would be useful to determine which precise plasmid in a strain carried LT (or ST). By using the screening procedure for plasmid DNA documented in Part 1 of this report, we can easily demonstrate the total plasmid complement within a given isolate. Many strains (most toxigenic strains), however, contain a plethora of plasmids. We wished to be able to develop a method to permit us to specifically identify which plasmid in a multi-plasmid strain carried the gene of interest. Moreover, one is often interested whether LT and ST are carried on the same plasmid. Finally, it would be of interest to determine which plasmid within a strain encoded for a colonization antigen and whether the same plasmid encoded for a toxin gene as well.

We attempted last year to develop a method which would permit us to

detect an uncleaved plasmid in situ by blotting with a radioactive probe. The result at that time was poor. The major technical difficulty was that uncleaved plasmid DNA in the covalently-closed form cannot be denatured effectively and, hence, is a poor candidate for hybridization. The solution was a relatively straightforward matter; one can cause controlled breakage of DNA embedded in the matrix of an agarose gel by exposure to irradiation or by exposure to a mineral acid. Figure 5 shows an example in which uncleaved extracts of strains lysed in the procedure described in Part 1 of this report was appropriately treated, blotted and hybridized with LT specific DNA. One can see a quite clear-cut band in each sample corresponding in each case to the plasmid encoding for LT. One interesting sidelight of this demonstration is the heterogeneity in molecular mass of the 7 plasmids carrying LT even though all of the strains under study were from travellers to a single geographical area in Africa. Once again there is implication of more diversity in Ent plasmids than we might have suspected from our studies of animal E. coli.

d. DNA homology between E. coli LT and V. cholerae

As noted earlier in this report, the amino acid similarity between the LT-B gene of E. coli and the B unit of V. cholerae would lead us to suspect that there should be significant DNA homology between the two. Of course, one broader question was whether or not the toxin gene of V. cholerae was chromosomal (as reported for the strain 599B; 33) or was possibly plasmid mediated. Dr. J. Kaper has in fact screened over 50 Vibrio strains; (V. cholerae and other "toxigenic" Vibrio) for their plasmids. We find no evidence whatsoever that toxin biosynthesis (or any other virulence determinant is plasmid-mediated in V. cholerae or other Vibrio species. Parenthetically, we did find several interesting R plasmid species in one

cluster of strains from Indonesia.

Mr. Moseley and Dr. Kaper have also used the LT⁺-probe to determine the actual degree of DNA homology with the DNA of V. cholerae. Hybridization of the LT probe with V. cholerae DNA does give positive results, although the degree of divergence is considerable (about 18% mismatching). It is perhaps of some interest that thus far we have found positive signs of hybridization between the LT probe and both classical cholera and El Tor vibrio strains. We intend to continue this work over the next contract period.

e. The Kad plasmids and their determinants

We had successfully isolated the K88 genetic determinant about eighteen months ago (34). Since the beginning of the current contract period, we have also been successful in isolating the analogous determined K99 from toxigenic E. coli of calves. In collaboration with Dr. P. Shipley, University of Virginia, Richmond, the gene for the human cFAI colonization factor (17) has been isolated as part of the 2×10^6 dalton fragment inserted into the cloning vehicle pBR322. At this time, we have just started our detailed molecular characterization of these plasmid determinants. It is our hope that we can use the experience we have gained analyzing the E. coli LT gene to study the molecular epidemiology of the Kad plasmids. This remarkable group of genetic elements with their capacity to encode specific organelles of attachment to specific host tissue are clearly worthy of considerable emphasis over the coming contract year.

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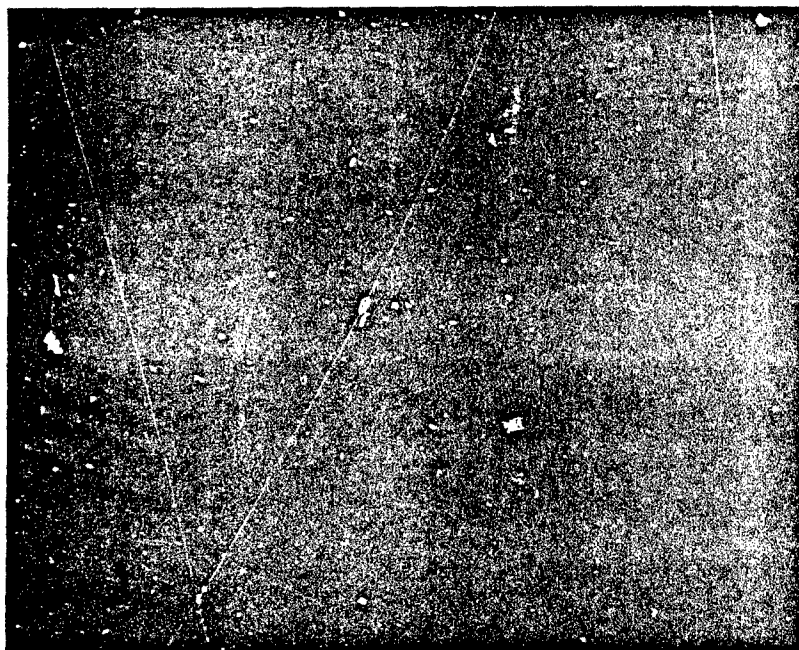
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Addendum

Papers published or accepted for publication during the current year sponsored all or in part by contract monies.

1. Dallas, W. S., Gill, D. M., and Falkow, S. 1979. Cistrons encoding Escherichia coli heat-labile Toxin. J. Bacteriol. 139:850-858.
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Legend to Figure 1. Isolation of plasmid DNA ranging over a wide range of molecular mass using a small volume of culture. A 2 ml culture of an organism (in the cases illustrated *E. coli*) was lysed and analyzed by the method described in the text. The molecular mass of the plasmids are (from left to right) 280×10^6 , 144×10^6 , 112×10^6 , 94×10^6 and 60×10^6 daltons respectively. In all but one case there is virtually no chromosomal contamination.

Figure 2

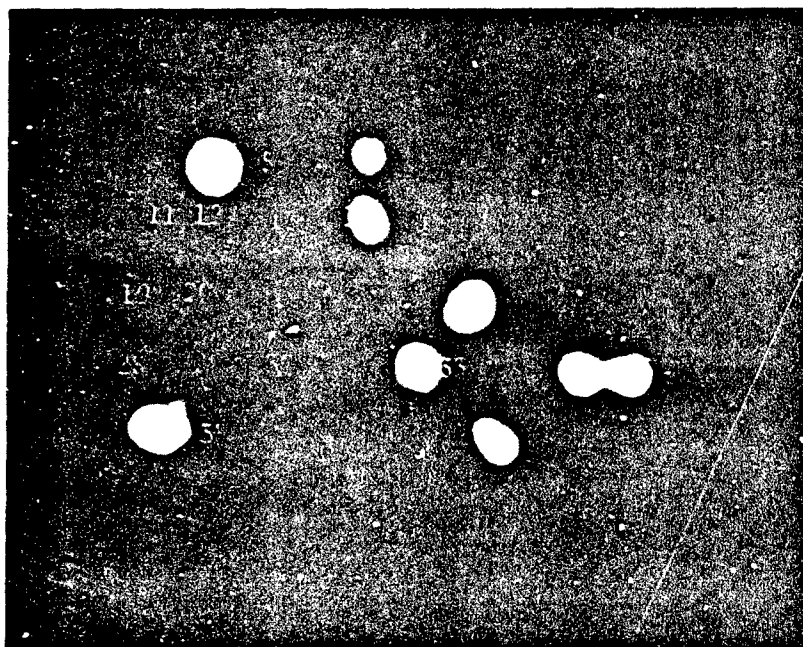
Comparison of 1st 13 Amino Acids of the
E. coli LT B Subunit Relative to the Primary Structure
of the Cholera Toxin B Chain

Sequence of LT B Gene	1	2	3	4	5	6	7	8	9	10	11	12	13
	GCT	CCC	GAG	ACT	ATT	ACA	GAA	CTA	TGT	TCG	GAA	TAT	CGA
Translated Sequence	ALA	PRO	GLN	THR	ILE	THR	GLU	LEU	CYS	SER	GLU	TYR	ARG
Reported Amino [*] Acid Sequence	<u>THR</u> **	PRO	GLN	<u>ASN</u>	ILE	THR	<u>ASP</u>	LEU	CYS	<u>ALA</u>	GLU	TYR	<u>HIS</u>

* -As reported by Kuroky, et al. (29)

** -Underlined sequences may differ by only a single nucleotide pair.

Figure 3.



Legend to Figure 3. Colony blot hybridization of strains isolated from travellers to Morocco for LT gene. Each of 43 strains isolated by Dr.

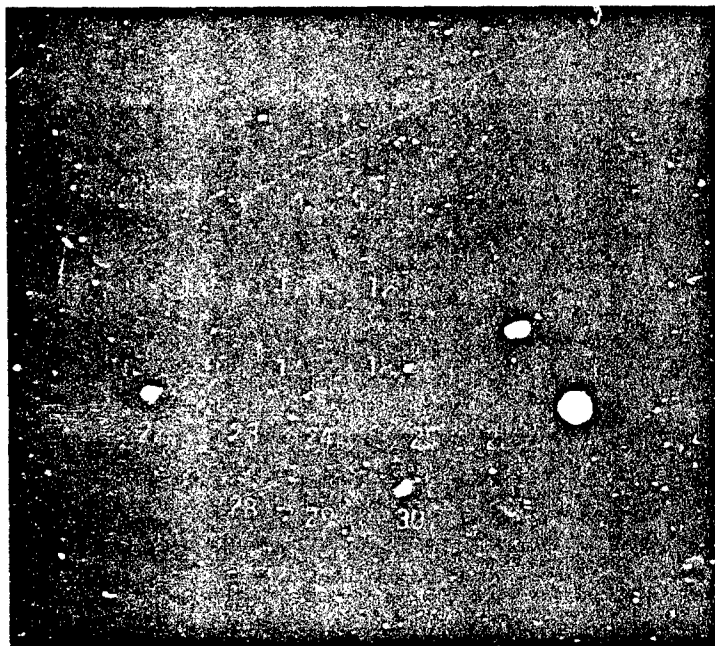
B. Sack was spotted on a filter disk and placed on the surface of an agar plate. After overnight incubation the filter was removed, the colonies lysed in-situ with base and hybridized with a LT-specific ^{32}P -probe (20×10^6 cPM/ μg .). The filter was then placed against x-ray film and placed in a freezer overnight. The film was developed and a positive print prepared. Hence each "white spot" represents the presence of LT^+ genes in the isolate.

The numbers in each case correspond to colonies on the plate. The numbers refer respectively to colonies which were characterized by tissue culture or animal experiments as:

Non-toxicogenic		ST^+		LT^+ or ST^+/LT^+	
Colony No.	Strain	Colony No.	Strain	Colony No.	Strain
2	M111C1	1	M109C2	3	M111C5 ST/LT
5	M117C5	13	M321C6E	4	M117C1 LT
6	M145C1	17	M326C3	7	M145C2 ST/LT
8	M243C1	21	M406C1	14	M324C3 LT
9	M306C1	23	M407C4	24	M408C1 ST/LT
10	M308C1	25	M409C1	32	M424C1 ST/LT
11	M309C1	26	M411C1	35	M447C4 ST/LT
15	M324C5	28	M415C1	36	M452C1 ST/LT
16	M326C1	30	M421C1	37	M633C1 ST/LT
18	No Growth	33	M443		
19	M403C1	39	M526C6B		
22	M407C1	41	M626C4		
27	M411C1				
29	M415C5				
31	M421C5				
38	M521C6A				
40	M626C1				
43	M633C3				

It may be noted that only colonies possessing the LT gene give a positive "signal". Colonies possessing both ST and LT or those harboring on LT are positive. No cross-reaction between ST and LT is noted.

Figure 3A.

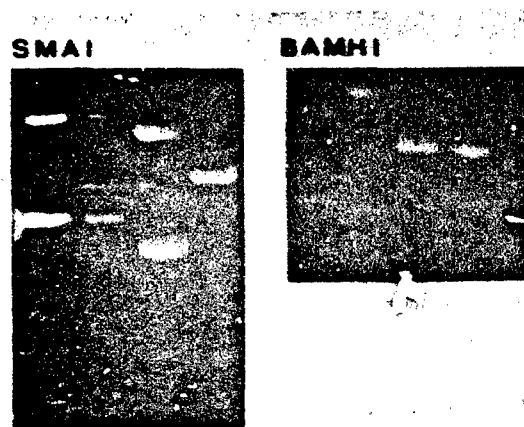


Legend to Figure 3A. Colony blot hybridization of strains isolated from travellers to Morocco for STa Gene. Each of 31 strains isolated by Dr. B. Sack was spotted on a filter disk and placed on the surface of an agar plate. After overnight incubation the filter was removed, the colonies lysed in situ with base and hybridized with an ST-specific ^{32}P -probe (30×10^6 cPM/ μg). The filter was then placed against x-ray film and placed in a freezer overnight. The film was developed and a positive print prepared. Hence each "white spot" represents the presence of STa genes in the isolate.

The numbers in each case correspond to colonies on the plate. The numbers refer respectively to colonies which were characterized by animal or tissue culture experiments as:

Non-toxicogenic		ST		LT ⁺ or ST ⁺ /LT ⁺	
Colony No.	Strain	Colony No.	Strain	Colony No.	Strain
1	M111C1	12	M321C6E	2	M116C5 ST/LT
4	M117C5	16	M326C3	3	M117C1 LT
5	M145C1	20	M406	6	M145C2 ST/LT
7	M243C1	22	M407C4	13	M324C3 LT
8	M306	24	M409C1	19	M403C3 LT
9	M308	27	M415C1	23	M408C1 ST/LT
10	M309	30	M443C1	25	M411C1 ST/LT
11	M311C6A	33	<u>E. coli</u> K-12	31	H10407 ST/LT
14	M324C5		ST ⁺		
15	M326C1				
17	M333				
18	M403C1				
21	M407C1				
26	MM411C4				
28	M415C5				
29	M421C5				
32	<u>E. coli</u> K-12 F ⁻				

It may be noted that, with one exception, strains which were ST⁺ only gave a positive signal. Moreover, not all ST⁺ strains were positive by the method. No ST⁺/LT⁺ strain from Morocco gave a positive reaction although the 'classical' H10407 ST⁺/LT⁺ strain gives a positive reaction.

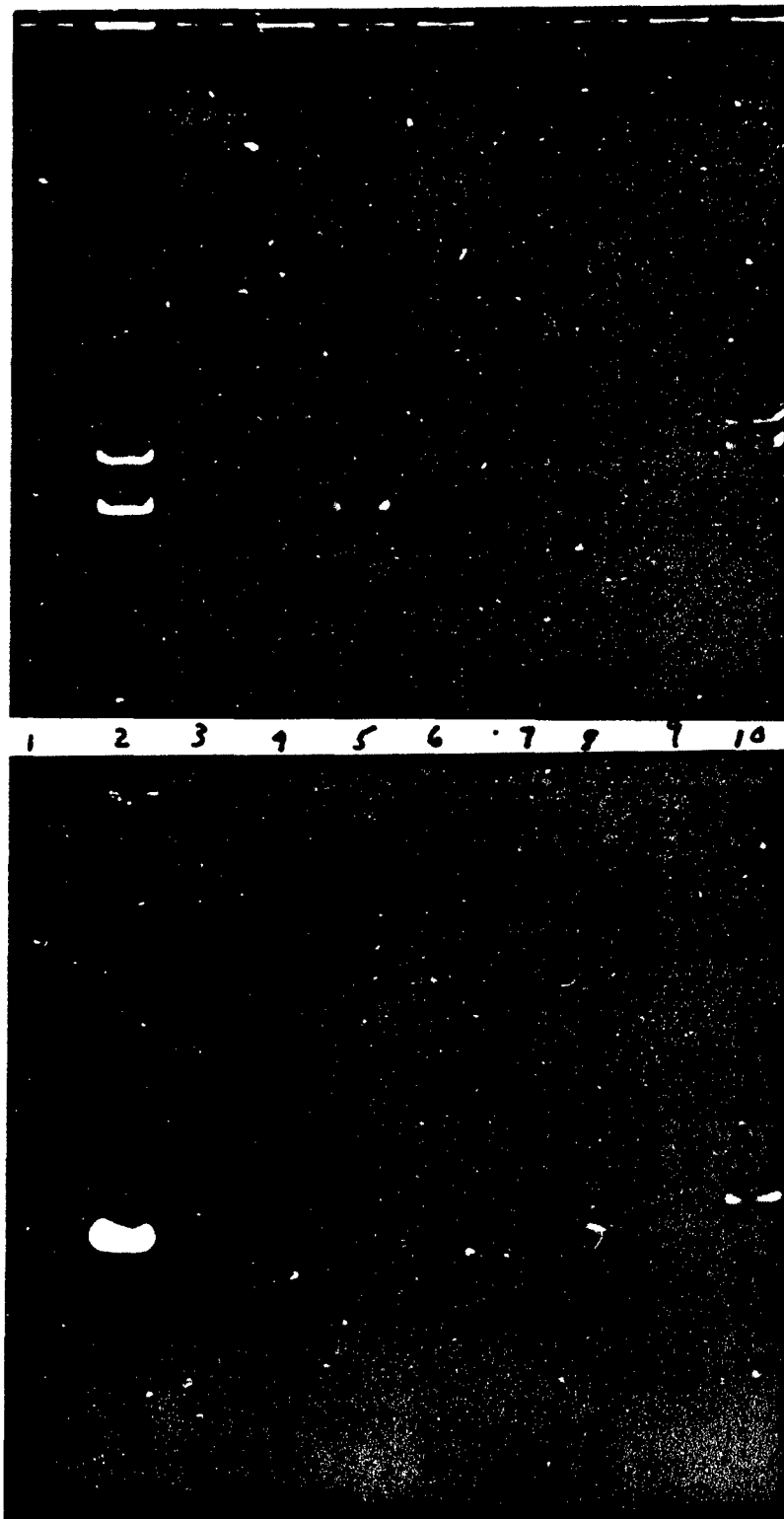


Legend to Figure 4. Autoradiographs of "Southern blots" of restricted Ent plasmids. The DNA in the first autoradiograph was cleaved with SmaI. The order of the samples is (from left to right) P307 (porcine Ent plasmid), pCG86 (porcine Ent plasmid), H10407 (human Ent plasmid), CI (human Ent plasmids).

The DNA in the second autoradiograph was cut with BamHI. The order of the samples is (from left to right) H10407, CI, pCG86 and P307.

These data are from Dallas, W. S., Moseley, S. and Falkow, S. The characterization of an *E. coli* plasmid determinant that encodes for the production of a heat-labile enterotoxin in Plasmids of Medical, Environmental and Commerical Importance (K. N. Timmis and A. Pukler, ed) Elsevier/North Holland Biomedical Press, 1979 pp 113-122.

Figure 5.



Legend to Figure 5. In-situ blotting of uncleaved plasmid DNA for the presence of the LT gene. In the upper sequence the migration in a 0.7% agarose gel of a DNA extract from a clinical isolate is shown. Each of the bands corresponds to a different plasmid in the strain. The bottom panel illustrates the results obtained when the agarose gel of the upper panel was blotted with a ^{32}P -LT-specific probe. In this panel if a band is present it represents the plasmid carrying the LT gene. Each panel corresponds to:

1. Postive control
2. H10407 ST⁺/LT⁺
3. D109 (human LT⁺ isolate received from S. Donta)
4. M145C2 ST⁺/LT⁺
5. M403C3 ST⁺
6. M111C5 ST⁺/LT⁺
7. CB64B7 Nontoxigenic E. coli
8. CB7452 Human LT⁺ strain from S. Donta
9. M524C1 ST⁺/LT⁺
10. M633C1 ST⁺/LT⁺

Note that strains 4, 6, 9, and 10 were all isolated from travellers to one geographic area.

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