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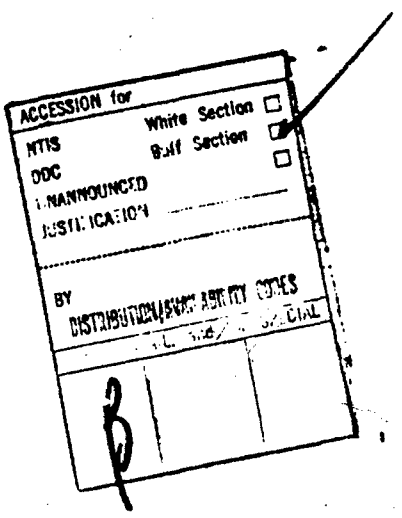
Colicin V is a protein elaborated by a variety of plasmids called collectively, ColV. It has been shown that ColV plasmids are far more likely to be found in E. coli strains isolated from extraintestinal infection than from fecal strains. We have shown that colicin V protein per se is not associated with the enhanced virulence exhibited by ColV+ bacteria. Rather, another genetic segment common to ColV plasmids is implicated as the primary virulence determinant. → over

A current serious problem in hospitals is the prevalence of enteric organisms which are resistant to the major antibiotic groups used in the treatment of gram negative infections. We have developed a rapid screening procedure that permits the identification of R plasmids within clinical isolates. This procedure together with an enzymatic "fingerprinting" method provides a rather precise tool to study the epidemiology of antibiotic resistant organisms. These methods have been useful for quickly and precisely evaluating resistance problems in the hospital setting. The findings have been of practical utility since the recognition of a single-source outbreak of nosocomial infection requires different control measures than does the appearance of unrelated multiple-resistant infection within a specific clinical setting.

We have isolated a DNA segment which can be employed as a specific probe for the E. coli LT enterotoxin gene in DNA-DNA hybridization tests. These data show that the LT gene is common to enteropathogenic E. coli strains of both human and animal origin. Of particular utility is the extension of the method to a colony hybridization technique that can be applied to direct stool isolation plates.

One of the critical steps in the pathogenesis of shigellosis is the penetration of colonic epithelial cells. Shigella flexneri strains are known to dissociate in vitro into two colonial forms, one of which, T, retains its capacity to penetrate cells while the other, O, have lost their capacity to penetrate. The T to O transition is associated with several other phenotypic changes as well. We have shown this year that the basis of the transition is the loss of two outer membrane polypeptides 100,000 and 17,000 daltons in mass, respectively.



INFECTIOUS MULTIPLE DRUG RESISTANCE IN THE ENTEROBACTERIACEAE

Annual Report

September 1978

by

Stanley Falkow, Ph. D.

Supported by

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Fort Detrick, Frederick, Maryland 21701

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Seattle, Washington 98195

Preface

In the past year we have concentrated upon the development of a simple method for the detection of E. coli heat labile enterotoxin within a single colony. Moreover, we have examined the relationship of different LT genes from various sources.

We have further found that the relationship between the ColV plasmid and enhanced bacterial virulence cannot be ascribed to colicin biosynthesis.

Our studies on the epidemiology of R plasmids has continued unabated. We have continued with our study of hospital infection and found that the plasmid complement of a particular isolate may be as useful as serotyping, phage typing or colicin typing for epidemiological purposes.

In addition, we have initiated a study to localize the molecular nature of the change associated with the mutational loss of epithelial penetration by Shigella flexneri.

As in previous years, the students in my laboratory have worked long hours to make significant contributions to this research program. I wish to particularly thank Mr. Stephen Moseley, Dr. Walter Dallas, Dr. Robert Quackenbush, Dr. Lucy S. Tompkins, Ron Gill and Dennis Schaberg for their innovative work and tireless effort.

INTRODUCTION

A plasmid is an extrachromosomal element of bacteria. Plasmids may be conveniently classified into two major types: conjugative and non-conjugative. The presence of a conjugative plasmid in a bacterial host is usually manifested by the synthesis of a specific proteinaceous cellular appendage called a sex pilus as well as other plasmid-mediated proteins which together provide a mechanism for the transmission of a plasmid from the host cell to another bacterium by direct cellular contact. In some cases, conjugative plasmids may also provide a mechanism for the transmission of host chromosomal DNA. Non-conjugative plasmids do not have the inherent ability to initiate conjugation nor do they usually encode for a sex pilus or other transfer-specific proteins. Rather, non-conjugative plasmids require that either a conjugative plasmid be also present in the same cell for their conjugative transfer (the non-conjugative plasmid is said to be mobilized) or that transmission be effected by a bacterial virus (transduction) or by direct uptake of DNA (transformation). Transduction and transformation can also act to disseminate conjugative plasmids, although the import of these two mechanisms <u>in vivo</u> remains a subject of speculation.

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 the host cell to more successfully 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, plasmid mediated resistance to antibiotics (R plasmids) have been by far the most commonly recognized and studied plasmid

determinants (1,5,6). A subject of recent great interest has also been the observation of several plasmid-mediated virulence factors in E. coli toxigenic diarrheal disease as well as E. coli invasive disease (7). In diarrheal disease the plasmids of interest are Ent plasmids associated with enterotoxin biosynthesis and Kad plasmids associated with the colonization of the small bowel by E. coli. In invasive E. coli disease, the plasmid ColV has been implicated as a significant accessory virulence factor.

In the following sections we report our progress over the past year that focuses on R plasmids in the hospital setting as well as the "molecular epidemiology" of Ent plasmids from human and animal E. coli. Moreover, we have been able to gain the first broad overview of the role of the ColV plasmid in invasive disease.

As we noted last year and explained this year we have reached a point in our work where it has become possible for us to exploit the molecular biology and molecular genetics of extrachromosomal elements to the everyday screening of clinical isolates. As we proposed last year, we have now also initiated the same kind of approach to examine the products of Shigella chromosomal genes that contribute to bacterial virulence.

RESULTS

A. The relationship between colicin V activity and virulence in Escherichia coli.

Colicin V is genetically determined by various types of plasmids (Col V factors) that occur in strains of E. coli (1,8,9). ColV plasmids vary in their molecular mass and capacity to act as conjugative sex factors (1,10). Smith and Huggins (11) observed that there was a significant association in E. coli, between the possession of a ColV plasmid and the ability to cause septicemia in livestock. Moreover, it has been further observed that E. coli strains isolated from cases of sepsis and other extraintestinal infections in Man were more likely (12% - 18%) to carry ColV than were random E. coli strains (0.5% - 1%) isolated from the stools of normal subjects.(9). Smith (9) further observed that the virulence of several laboratory strains of E. coli for laboratory animals increased when introduced and decreased when ColV plasmids were cured.

We investigated whether or not colicin V activity was the product associated with the enhancement of virulence by strains of E. coli that carry ColV plasmids. The basic approach was to compare the virulence of colicin V-producing E. coli with homogenic strains that carry ColV plasmids in which colicin V synthesis (Cva^+) had been abolished by insertional inactivation of a transposon into the Cva gene.

We have previously reviewed in some detail the use of transposons to study genetic function and to mark Ent and Kad plasmids. In essence, one exploits the fact that certain antibiotic resistance genes are carried on discrete DNA sequences that are capable of migrating between plasmids or from plasmid to chromosome independently of the normal recombinational processes of the cell (see Ref. 12 - 14). We have particularly concentrated upon transposable ampicillin resistance, TnA, which resides upon a 4.8 kilobase (about 3×10^6 daltons in mass) DNA segment flanked by inverted-repeated sequences of about 75 base pairs. TnA

may be inserted into DNA in either of two orientations and is mutagenic when inserted within a structural gene and polar (at least in one orientation) when insertion occurs within an operon (15).

In principle transposition is easy to demonstrate. One simply introduces a plasmid carrying TnA into a cell and then one monitors the transposition of TnA into other DNA segments. A variety of methods are available to determine TnA transposition within E. coli K-12 but in practice it has been difficult to exploit transposition in many E. coli strains because of the limited host range of plasmids and because of restriction-modification. During the past year we were able to isolate a temperature-sensitive derivative of the broad host range plasmid RP4 that confers resistance to ampicillin, kanamycin, and tetracycline.(16). This plasmid pMR5 (isolated in collaboration with Martin Robinson, Sandoz Forschung-institut, Vienna and Peter Bennet, University of Bristol, Bristol, England) carries TnA and has a temperature sensitive gene for the initiation of plasmid replication. When strains that carry pMR5 are grown at 42C, in the presence of carbenicillin, replication of pMR5 is inhibited thus favoring the growth of cells in which TnA has been inserted into other DNA molecules within the cell.

pMR5 was introduced by conjugation into clinical isolates of E. coli producing ColV as well as E. coli K-12 ColV strains. These strains were incubated at 42C and, subsequently, clones were screened for sensitivity to kanamycin and resistance to carbenicillin. On average 63% of clones were sensitive to kanamycin and resistant to carbenicillin, indicating the loss of pMR5 and retention of TnA. Approximately 12% of the clones presumed to carry TnA did not produce detectable levels of colicin V (cva^-). Analysis of the plasmid complement of these strains revealed that the clones had lost pMR5 but retained a ColV plasmid into which TnA had been inserted. The remaining strains were found to have lost pMR5 and retained ColV containing TnA; in rare instances, however, TnA was not inserted

into the ColV plasmid and we assume that TnA had been inserted into the bacterial chromosome. The cva$^-$ derivatives all had TnA inserted into one short segment of the ColV plasmid; the cva$^+$ ColV derivatives had TnA inserted into other regions of the ColV plasmid genome.

The virulence of cva$^-$ (colicin V negative mutants) and homogenic cva$^+$ strains was tested in Swiss-Webster adult mice following the intraperitoneal injection of about 2 x 10^7 viable cells. As shown in Table 1, colicin V activity itself is not essential for the enhancement of E. coli virulence. It is likely that the virulence enhancement trait is encoded by other ColV DNA sequences closely linked to the cva genes. This supposition is supported by our isolated of a ColV derivative which is cva$^+$ but has lost its virulence enhancement property following insertion of TnA. Over the coming year we hope to further analyze the nature of the ColV product that enhances virulence.

Table 1

Strain	Plasmid	Plasmid Properties	No. Cells Injected	Mortality Hours After Injection	
				4	18
B188	None		2 x 10^7	0/14	1/14
B188 ColV	ColV	tra$^-$, cva$^+$	2 x 10^7	2/14	13/14
B188 ColV::TnA	ColV	tra$^-$, cva$^+$, Ap^r	2 x 10^7	0/32	26/32
B188 ColV::TnA	ColV	tra$^-$, cva$^-$, Ap^r	2 x 10^7	0/40	36/40

B188 is a naturally occurring E. coli isolate which is free of plasmids. B188 ColV is a strain into which a ColV plasmid has been mobilized. B188 ColV::TnA cva$^+$ are derivatives into which TnA has been inserted; the data shown are a summary of results with four different clones. B188 ColV::TnA cva$^-$ are derivatives into which TnA has been inserted leading to a loss in colicin biosynthesis; the data shown are a summary of results with five different clones. Abbreviations are: tra$^-$, transmissibility negative; cva, colicin V biosynthesis; Ap^r, resistance to ampicillin and carbenicillin denoting presence in this case of TnA.

B. Epidemic R plasmids in Hospital Strains

R plasmid mediated resistance may become disseminated in a variety of ways (1,6). If an R plasmid gains entrance to an epidemic pathogen, Shigella dysenteriae or Salmonella typhi for example, the very communicability of the strain assures its dissemination though, of course, antibiotic selection provides selective pressure. In opportunistic microorganisms such as those encountered in a Burn Unit, in traumatic wounds or an Intensive Care Ward, the incidence of R plasmid-mediated resistance and the dissemination of the R plasmids is largely dependent upon the highly selective environment that exists because of daily antibiotic administration (a clinical necessity in most but not all cases) (17).

To be sure the majority of hospital acquired infections caused by gram negative organisms are still sensitive to most antibiotics. Yet, the emergence of nosocomial pathogens which are multiply resistant is occurring throughout the world (1,17). Moreover, organisms which were once considered to be minimally pathogenic, such as Serratia and Proteus species, have now become common cause of infection (18). Undoubtedly, this situation is relative to the increasing prevalence of hospitalized patients with impaired host defenses as well as the multiple resistance of these strains. Nor is the situation less serious in military hospitals than in civilian facilities.

A serious current problem is the prevalence of enteric organisms which are resistant to two of the most efficacious antibiotic groups used in the treatment of the gram negative infections namely, the penicillin-cephalosporins (ampicillin cephalosporin etc.) and the aminoglycosides (gentamicin, tobramycin, kanamycin and amikacin) (1,7,18). Resistance to these agents has increased in consonance with the increased in-hospital usage of these antibiotics (18). The impact of of extensive antibiotic use is also apparent in gram positive organisms. R-plasmid mediated aminoglycoside resistance in staphylococci is increasing, especially in units where these antimicrobials are employed extensively for surgical prophylaxis

or for treatment of established infection (18).

In our annual report last year we reported the direct application of the tools of the molecular biologist to the study of the epidemiology of hospital infection. Our data showed that just as there are certain strains of R-plasmid containing bacteria that cause epidemics there are certain "epidemic R-plasmids" as well. These epidemic R plasmids can be found not only in a particular epidemic strain of bacteria but also in many other enteric species causing multiply resistant nosocomial infections in the same hospital unit (19,20). We have continued our examination of nosocomial infections in different hospitals across the U.S.A. Our goal was to determine whether each local situation involved a distinct R plasmid and nosocomial strain or whether we were actually experiencing a national (or even international) plasmid epidemic. The full details of these studies will be reported next year (20). There are certain aspects of these studies that seem worthwhile reporting at this point in our investigation.

One of the most interesting aspects of our work has been the unexpected utility of agarose gel electrophoresis (AGE) as an epidemiological tool. The technique itself is straightforward (21). A colony of an organism of interest or a 3 ml overnight broth culture is lysed by a new selective method which we developed for use on clinical isolates. This lysis procedure releases plasmid DNA into supernatant fluid which is then applied to a 0.7% agarose gel. The DNA is subjected to electrophoresis for a few hours and the DNA visualized with ethidium bromide and ultraviolet light. The plasmid DNA in the preparation may then be characterized in terms of their molecular mass. In some cases the DNA may be also treated with restriction endonucleases todetermine its relationship to other plasmids in other strains. As noted last year we can usually determine the presence of an "epidemic R plasmid" in strains by the finding of a plasmid species of similar molecular mass in different isolates (18,19). In addition, we

now find the AGE plasmid profile is useful in identifying a specific epidemic microorganism (20). For example, on the basis of AGE alone we could determine that certain Serratia isolates from an nosocomial outbreak in a Seattle VA Hospital were the principal epidemic strains. Hence, the epidemic Serratia strain which were of serological type 16y and colicin type 50 by conventional epidemiological typing had identical distinctive AGE patterns. Other VA Serratia isolates, even though possessing similar antibiograms, had different AGE patterns and were subsequently found to possess different serological properties and to be of different colicin types. In six other epidemics we have examined, strains of the same species isolated concomitantly with the epidemic strain showed diverse AGE patterns from the epidemic strain (20).

These data suggest to us that AGE "fingerprinting" appears to be a rather simple, rapid and specific tool for investigating epidemics. Certainly the AGE technique does not obviate phage typing, serological typing, colicin typing or other conventional epidemiological tools. Yet, for species such as Citrobacter freundii for which no conventional typing schemes are available, the AGE method has proved to be invaluable.

The second aspect of our study that seems worthy of note is that throughout the U.S.A. and Europe most epidemic plasmids conferring significant aminoglycoside resistance in Serratia belong to a single plasmid incompatibility group, group M. The full significance of this finding remains to be determined. In any event, as we noted last year, we now have rather precise tools to study the epidemiology of antibiotic resistant microorganisms and we need no longer be limited to post facto statistics but rather can evaluate quickly and precisely current problems as well as gaining prospective indications of developing problems. Clearly this is of practical utility since the recognition of a potential outbreak of nosocomial infection requires different control measures than does the appearance of unrelated multiple-resistant infection within a specific clinical setting (18).

C. The Molecular Epidemiology of the E. coli Heat-Labile Enterotoxin Gene

Enterotoxin production by Escherichia coli isolated from humans, piglets and calves have been shown in most instances to be plasmid-mediated (7,22-24), although a recent report (25) describes phage mediated enterotoxin biosynthesis. Two broad classes of Ent plasmids have been identified (24). One plasmid class specifies the production of a heat stable enterotoxin (ST), the other class specifies the production of a heat labile (LT) enterotoxin alone or in combination with ST. The effects of both enterotoxins on the susceptible small bowel leads to the secretion of H_2O and electrolytes into the bowel lumen (24). The ST and LT toxins exert their action in different ways however. The LT toxin, like cholera toxin with which LT shares an immunological relationship, exerts its action by stimulation of adenyl cyclase activity (26). E. coli ST is now thought to exert its activity on guanyl cyclase or by some other means to lead to increased cyclic GMP and net fluid secretion. The dynamics of the response of the bowel to the E. coli enterotoxin also differs (27). The response of a sensitive small bowel to ST is characterized by an immediate accumulation of fluid in the bowel lumen but the duration of its action (unlike that observed for LT) is short lived and can be easily removed by washing the epithelial surface. The LT toxin acts for a much longer duration and, once absorbed to the epithelial surface, its effects cannot be neutralized by either washing or the use of specific antitoxin.

We have taken advantage of the techniques of recombinant DNA molecule research (molecular cloning) to focus our attention more precisely upon specific determinants of interest (28,29). The isolation of such DNA recombinant molecules involves, in one form, the ligation of an enzymatically cleaved DNA fragment to a similarly cleaved, but still functional, plasmid or phage genome in the test tube. The recombinant molecules may then be introduced into a suitable host cell by transformation where they replicate autonomously serving to 'clone' the added

DNA segments. This methodology has been successfully employed in the isolation of a variety of both prokaryotic and eukaryotic sources (29). We have employed this method to specifically isolate the ST gene from calf (30) E. coli as well as the LT gene from piglet E. coli (31).

The small plasmids carrying these cloned determinants have considerable utility. They serve to enrich for the gene of interest. Moreover, such small plasmids readily segregate into E. coli mini-cells providing an excellent model for studying the in vitro synthesis of the products of the cloned genes of interest. For example, our analysis of the products synthesized from cloned LT genes and deleted LT derivatives, have shown that the E. coli LT toxin is composed of two polypeptide subunits of 24,000 daltons and 11,500 daltons, respectively (32).

The cloned gene fragments can also be usefully employed in nucleic acid hybridization experiments to examine the distribution of the gene of interest (33). Last year we proposed to employ such genetic segments as a novel means for detecting colonies containing Ent plasmids as well as to determine the relationship between LT genes of E. coli isolated from man and livestock. Despite some initial practical problems, we have now been able to successfully perform these experiments.

Figure 1 shows the results obtained by DNA blotting techniques in which a radioactive fragment of DNA comprised solely of LT gene sequences was hybridized with endonuclease cleaved DNA from Ent plasmids of human and porcine origin. It may be seen that there is a single fragment of DNA common to all Ent plasmids which contain LT gene sequences. Though this technique is solely qualitative, it should be noted that the conditions of the hybridization dictate that the sequences show no more than 15% divergence. Hence, these studies permit us at last to state with a high degree of confidence that the LT gene of animal and human E. coli

are composed of closely related DNA sequences and probably share a common plasmid origin. Preliminary studies using filter-blot hybridization of LT DNA vs. choiera DNA suggests that these genes are related only distantly if at all. Thus, while E. coli LT and cholera toxin share immunological determinants and act by a common mode of action, they are not currently closely related at the genic level.

The detection of LT need not be solely confined to the detection of cleaved Ent plasmids. As shown in Figure 2, one may employ filter-blot hybridization to study which plasmid within a cell carries the LT specific sequence. This can be of considerable interest when studying plasmid epidemiology as will be pointed out in our next report.

Finally, we have been able to employ the LT-specific proble for colony hybridization. The method is quite straightforward really (34). One treats 90mm membrane filters by boiling with detergent and following sterilization (which causes filter shrinkage) the filter is placed on the surface of a nutrient medium. The filter surface is inoculated by spotting cells (or one may even employ a dilute suspension of feces). Following overnight incubation, colonies develop on the surface of the filter which is lifted from the surface of the nutrient medium. The colonies are then fixed, lysed in situ and the fixed DNA from the colonies hybridized with the LT-specific proble (^{32}P labelled DNA at 10^6 cpm/µg). After hybridization, the filter is treated to remove all but the hybridized radioactivity and the filter placed against a piece of x-ray film. Colonies which contain the LT gene appear as black spots where the radioactive "signal" has exposed the film. A typical example is shown in Figure 3. In this particular case, each spot tested was from cultures sent us by Dr. B. Sack of Johns Hopkins University. These strains were isolated during a survey of diarrheal disease in travellers to Morocco. The strains had been characterized by conventional ELISA methods, tissue culture tests and animal inoculation to determine if they produced ST, ST + LT or LT only. One can see from our results that the colony hybridization method permitted us to easily detect these strains which had been

previously characterized as LT^+. We should also point out that we obtained positive signals from strains producing LT only as well as ST + LT.

The colony hybridization method has considerable potential utility. We think it will be directly applicable to fecal samples to quantitate counts of toxigenic cells in the gut and to better follow the carrier state. Of course, both the filter-blot method as applied to cut and uncut DNA as well as the colony hybridization method can be applied to other genetic determinants. We hope to report our results with ST as well as Kad determinants in our next report.

D. The Identification of Specific Polypeptides Associated with the T to O Transition in S. flexneri

One of the more interesting challenges of present day infectious disease research is the elucidation of pathogenic mechanisms in terms of the molecular intereaction between the bacterial pathogen and its host. As illustrated above this can be accomplished reasonably well in the study of microbial toxins. Yet, the factors that determine the ability of a pathogen to specifically colonize a host as well as the nature of host specificity is poorly understood, even when one studies factors like the colonization antigens of toxigenic E. coli that are reasonably well delineated. Less well understood are the factors that function in an invasive disease, as exemplified by shigellosis.

It is recognized, of course, that the pathogenesis of shigellosis is dependent on the penetration of colonic epithelial cells by bacilli. It is further understood that following penetration of the epithelial barrier, there must be multiplication of bacilli within the cell (35,36). It is essential, however, to gain precise information about the mechanisms by which shigellae attach, penetrate and multiply within epithelial cells of the intestinal mucosa. Although this sequence of events for shigellae is known and despite the identification of specific genetic loci for several of these steps, the biochemical means by which the shigellae brings about these steps remains largely a matter of speculation. The difficulty

in understanding the properties of shigella related to virulence has been clearly described by Gemski and Formal.(37). They note that several bacterial attributes are likely responsible for the penetration of epithelial cells by shigella and state that such a polygenic control of invasiveness creates a geneticists nightmare, since a mutation in any one of the genes related to the property of penetration results in similar avirulent phenotype.

One way to study the factors involved in shigella penetration is the examination of spontaneous mutations which are identified as a change from a translucent (T) colonial morphology to an opaque (O) colonial morphology. Comparative studies of T and O forms of S. flexneri 2a have revealed a number of pleiotropic effects, the most dramatic of which is the loss in the ability of S. flexneri to penetrate colonic epithelial cells and cause disease. The relationship between the loss of virulence and the change in colonial morphology have not been clearly understood. Nonetheless, most of the changes associated with the T to O transition are consistent with an alteration in the cell envelope. For example, O forms show a significant increase in electronegative charge, relative increase in resistance to lysis by sodium lauryl sulfate, EDTA and lysozyme as well as loss of sensitivity to phages T1 and T4. Other more nebulous changes i.e. decrease in the capacity to oxidize TCA cycle intermediates and loss of glycerol kinase activity have also been described. Yet, there is no significant difference between virulent T and avirulent O cells in their gross lipopolysaccharide structure nor is the transition a smooth to rough mutation. Moreover, the T to O transition is not associated with the plasmid complement of Shigellae (Kopecko and Formal, personal communication).

It seemed reasonable to consider that the T to O transition reflected some change in the cell envelope that interfered with the interaction of the bacterial cell surface with the host epithelial cell. With this in mind, we undertook the study of the principal membrane proteins of S. flexneri by gel electrophoresis.

Although the membrane proteins of E. coli and Salmonella typhimurium have been studied in some detail, we were not able to find any significant publication dealing with Shigella membrane proteins. Nonetheless by employing the sodium dodecyl sulfate (SDS) polyacrylamide gel systems used to study E. coli (40-42), we have been able to readily resolve the major proteins of the cell membranes of shigella. Consequently, total membrane protein preparations of a well-defined T and O variant of S. flexneri 2a strain (kindly provided by S. B. Formal) were prepared and subjected to SDS-polyacrylamide gel electrophoresis. Figure 4 shows a gel in which the membrane proteins of the T and O form have been compared. One can readily see that there are two specific differences between the two variants. Specifically the T form possesses two polypeptides of molecular weight 17,000 daltons and about 100,000 daltons that are absent from the O form. Further studies have demonstrated that the 17,000 dalton polypeptide is a component of the inner membrane while the 100,000 dalton component is part of the outer membrane. It is not yet clear if the 17,000 dalton species is a precursor of the 100,000 dalton component or if the presence of one of these components is a necessary prerequisite for the assembly of the other in the membrane matrix. Currently, we are examining a number of independent O derivatives from a single T form to confirm that the differences that we have observed are a consistent feature of the T to O transition. In addition, we are pursuing the possibility that these same polypeptides are associated with the loss of epithelial cell penetration in O derivatives of other shigella serotypes.

Our preliminary studies also confirm the impression of Gemski and Formal (37) that penetration of epithelial cells by Shigella is likely a polygenic phenomenon. For example, we have examined S. flexneri 2a derivatives kcp^+ and kcp^-, characterized by Formal and his associates as being deficient in causing keratoconjunctivitis in the guinea pig eye. These derivatives do not show any significant

change in their membrane components relative to a fully virulent parental strain.

When we proposed last year to examine the specific molecular species associated with shigella, we noted that our studies would be simply to determine the feasibility of our experimental approach. We now believe that the approach promises to be a useful tool for the study of pathogenesis. In the coming year we hope to utilize these molecular probes in conjunction with immunological procedures and a novel approach for selecting cells from genetic crosses that possess specific antigenic determinants.

Discussion

This year we have continued with our approach to exploit the techniques of molecular biology and microbial genetics to the study of microbial pathogenesis as well as the epidemiology of infectious disease agents. Thus, we have used gene transposition to good advantage in studying the nature of the ColV factor just as last year we used transposition as a means to "mark" plasmids of medical importance. Similarly, we have exploited the agarose gel electrophoresis method to the practical study of the epidemiology of hospital infection and we have been able to utilize gene probes to study a specific determinant of microbial pathogenicity, the E. coli LT toxin. Finally, we have employed well-known methods of the membrane chemist as an approach to understand the invasive properties of shigellae.

Just as established methods of molecular biology and biochemistry can be modified to study bacterial pathogenesis so one should not overlook that suitable genetic systems for the study of the pathogenesis of E. coli, Salmonella, Shigella and V. cholerae have been available for over a decade. These genetic systems often have not been vigorously employed to study pathogenesis owing to the lack of relatively simple tests to detect the products of virulence genes among the large numbers of recombinants issuing from genetic crosses. Certainly, the use of colony hybridization as applied to the LT gene is one way that one can overcome such a problem. Yet, the fact remains that most virulence attributes of bacteria lack any of the selective properties that one wishes to employ for detection of specific classes of recombinants. This is particularly true when dealing with chromosomal determinants of virulence. It is perhaps of particular note that many of the determinants of virulence are currently characterized and identified by their immunological properties. Optimally, therefore, one should like a simple method, akin to the colony hybridization method, in which a single clone among many hundreds could be identified as harboring a specific antigenic

determinant. In this way, one could fully exploit the polypeptide differences between strains similar to those seen between the T and O forms of Shigella. Hence, in principle one should like to be able to transfer the "T-specific" polypeptides to $_{\wedge}^{T}$ and O cells and restore virulence satisfying, as it were, a molecular Koch's postulate. We believe that the means now exist to simply exploit immunological cross-reactivity so that hundreds of clones issuing from a genetic cross can be screened. In this way we hope that the progress we have made this year can be logically extended to provide us with a broader approach to the study of pathogenesis. The means by which we plan to accomplish this goal will be outlined in our proposal for the coming year.

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Figure 1. DNA-DNA blot hybridization between isolated LT gene fragment and endonuclease cleaved DNA from Ent plasmids of human and porcine origin.

Right - Photograph of DNA fragments generated following HindII digestion of Ent plasmids of porcine (P) and human (H) origin. Although the digestion patterns of these plasmids are clearly distinct it may be seen that DNA-DNA hybridization with an isolated LT gene fragment reveals a common fragment - Left hand figure.

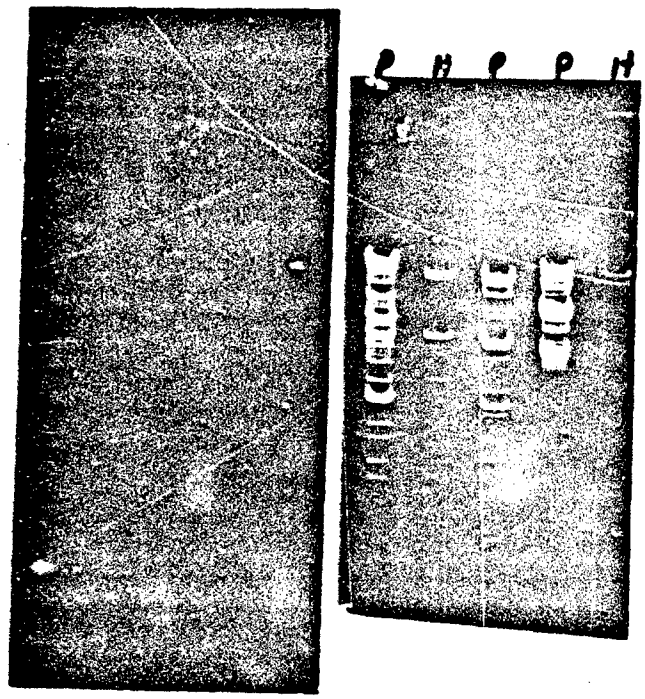

Figure 2. The identification of plasmids carrying the LT gene sequence. Plasmid DNA was extracted from 5 ml broth grown cells by a rapid lysis technique. The unpurified, crude DNA was electrophoresed through a 0.7% agarose gel. The right hand frame shows the appearance of the DNA following staining with ethidium bromide. The left hand frame shows the results obtained after transfer of the DNA from the gel to a nitrocellulose filter and hybridization against an LT^+ DNA probe. Although there is considerable background because a crude lysate was employed, it is still possible to pick up the strong LT^+ signal from the LT^+ plasmids in two of the samples. Using partially purified plasmid DNA or purified DNA from dye-bouyant density centrifugation gives more clear-cut results. This figure demonstrates results that can be obtained within 72 hr of receiving a strain for analysis.

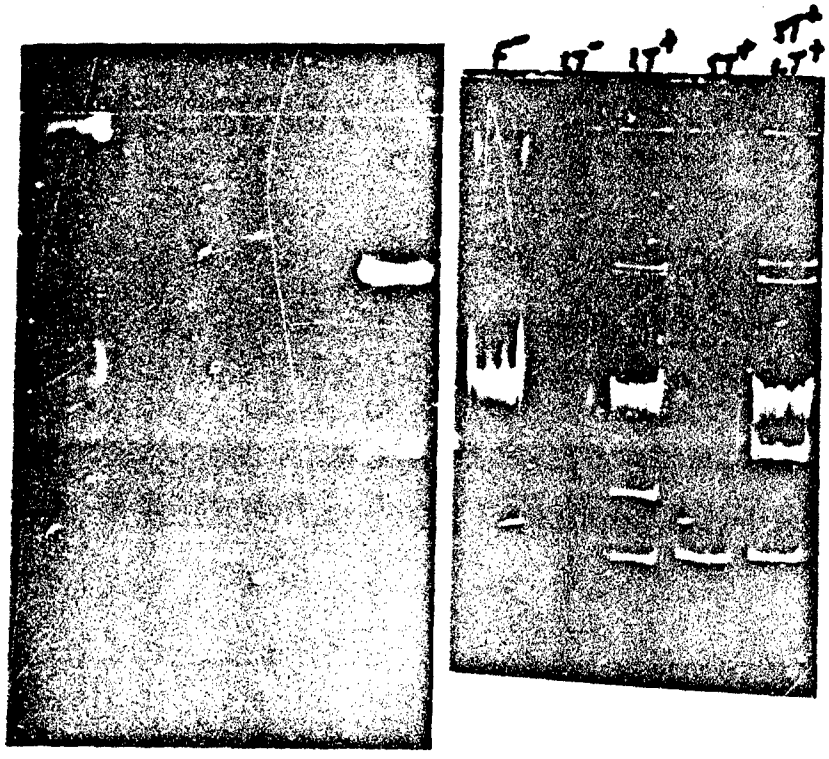

Figure 3. Colony Hybridization. Cells were spotted on the surface of a washed nitrocellulose filter placed on the surface of a nutrient agar plate. Following overnight incubation, the filter with the adherent bacterial colonies was removed from the plate. The cells were lysed *in situ*, and the DNA from the colonies hybridized to a ^{32}P-labeled DNA probe containing only LT-specific gene sequences. Following hybridization the filter was placed against x-ray film and an autoradiograph developed 48 hr later. The figure is a contact print of this autoradiograph. The colonies labeled with the M designation were all received from Dr. B. Sacks. They represented *E. coli* strains isolated from cases of Traveller's diarrhea in visitors to Morroco. All except M403 were either ST^+ LT^+ or LT^+ only. It may be seen that the colonies except for M403 give a strongly positive signal (white zone). P16 is a porcine isolate which produces only ST. The Citro and Kleb spots which are clearly negative were performed on clones of *Citrobacter freundii* and *K. pneumoniae* received from Dr. R. Guerrant and thought to be LT^+. The Y ent is from *Yersinia enterocolitica* and is clearly negative as is *E. coli* C600 F^-. Strain P307 is a porcine ST^+ LT^+ strain from which the LT^+ probe was derived. It is clearly positive.

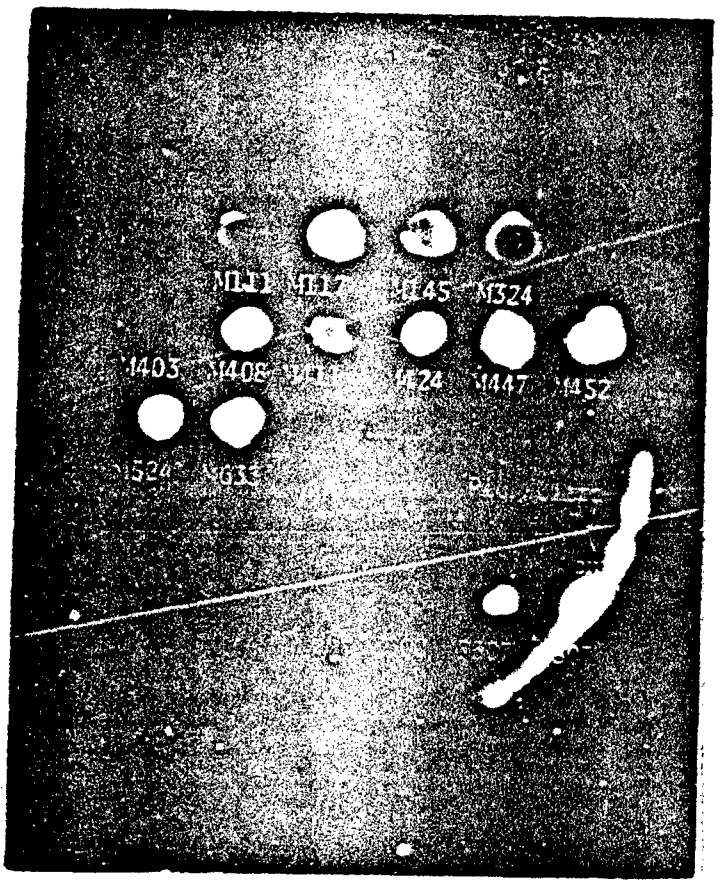
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Figure 4. Principal outer membrane proteins isolated from Shigella flexneri 2a O and T cells.

The arrows denote the polypeptide present in T cells that are absent from the O derivatives.