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INFECTIOUS MULTIPLE DRUG RESISTANCE IN THE ENTEROBACTERIACEAE

ANNUAL PROGRESS REPORT

by

Stanley Falkow, Ph.D.

by

U.S. Army Medical Research & Development Command, Office of The Surgeon General, Washington, D. C. 20314 in cooperation with the Commission on Enteric Infections of the Armed Forces Epidemiological Board.

Contract No. DADA17-72-C-2149
University of Washington
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Tobrymycin-resistant burn wound isolates from five different bacterial genera contained a common 68 x 10^6 dalton R plasmid that could be transmitted by conjugation to Escherichia coli K-12. It was possible by a variety of newly developed methods to detect, characterize and show molecular identity of this R plasmid in clinical isolates from patients. This study illustrates the usefulness of simple screening methods for studying the molecular epidemiology of R plasmids in the hospital setting or during an epidemic.

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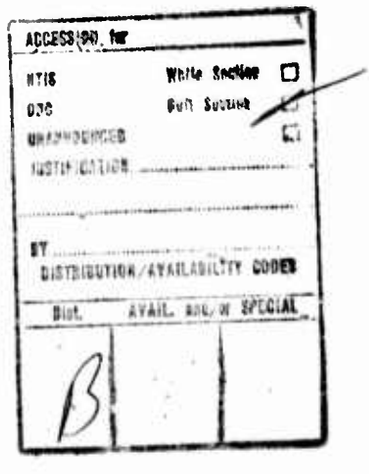
A simple screening method for identifying plasmids in a single colony has been developed during the study of a colonization plasmid, Kad (K88, Raf). The K88 adherence antigen has been found in most cases to reside on a single non-conjugative plasmid about 50 x 10^6 daltons in mass. K88 is invariably found in association with genes for raffinose fermentation (Raf). K88/Raf Kad plasmids from different sources show considerable homogenicity. The mobilization of K88/Raf Kad plasmids by conjugative plasmids is unique. Mobilization is rec^+ dependent and often involves covalent linkage with the mobilizing conjugative plasmid. In some cases, therefore, K88/Raf Kad plasmids may form cointegrates with conjugative plasmids, like enterotoxin, Ent, plasmids, and lead to new gene combinations.

Human toxigenic E. coli that have been examined in preliminary studies all harbor 1) a plasmid about 60 x 10^6 daltons in mass, encoding for heat stable enterotoxin, ST, and a colonization factor (CF^+), 2) a plasmid about 50 x 10^6 daltons in mass encoding for heat labile enterotoxin, LT, and 3) one or more other plasmids usually conjugative which are cryptic in the phenotypic sense.

PREFACE

Over the past year we have concentrated upon the initial characterization of plasmids in toxigenic Escherichia coli isolates isolated from Man. In the course of these experiments it has been necessary to devise a method in which the plasmid complement of a single colony could be examined. In addition, we have completed a study, initiated in 1976, dealing with an outbreak of tobramycin resistant microorganisms in a hospital Burn Unit.

As in previous years, the students in my laboratory have made the significant contributions to this research program. I wish to particularly thank Dr. Patricia Shipley, Mr. Walter Dallas, Dr. Gordon Dougan and Dr. Lynn Elwell for their innovative work.



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 plasmid, although the import of these two mechanisms in vivo 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 great interest recently has also been the observation of two major plasmid-mediated virulence factors in E. coli toxigenic diarrheal disease of animals (1,7). One is enterotoxin biosynthesis (Ent plasmids) and the other ability to colonize the small bowel (Kad plasmids).

In the following sections we report our progress over the past year that focuses on an R plasmid epidemic in a Burn Unit as well as the Ent and Kad plasmids of both human and animal E. coli. These studies have permitted us for the first time to gain an overview of the plasmid complement of human toxigenic E. coli.

RESULTS

A. A Common Plasmid Specifying Tobramycin Resistance Found in Enteric Bacteria Isolated From Burn Unit Patients.

Infections caused by gram-negative bacilli constitute the most frequent type of nosocomial infection, a fact that has profound implications for patients with major burns, penetrating wounds or surgical patients who of necessity require hospitalization over two weeks (8,9). A serious threat to the control of burn wound infections has been the emergence of bacteria harboring R plasmids that are selected under the pressure of intense antibiotic therapy (see 1,9). The cornerstone of therapy in cases of gram negative infection has been the use of the newer aminoglycosides, gentamicin, tobramycin and amikacin. During the course of observing the progression of microorganisms colonizing patients in a local Burn Unit, we were interested to find the simultaneous appearance of E. coli, Klebsiella pneumoniae and Enterobacter cloacae resistant to streptomycin (Sm), tetracycline (Tc), ampicillin (Ap), kanamycin (Km), gentamicin (Gm) and tobramycin (Tb). In fact, during an 11 month period, tobramycin-gentamicin

resistance emerged in five distinct genera of gram negative bacilli including Pseudomonas.

Since some R plasmids have been shown to mediate the synthesis of enzymes that modify aminoglycoside antibiotics (10), we performed genetic transfer experiments between the multiply-resistant clinical isolates and a suitably marked E. coli K-12 recipient cell line. Transfer from the clinical isolates to E. coli K-12 was found for Sm, Ap, Km, Tb and Gm.

As we have reported in earlier Progress Reports, we (11) developed an agarose gel electrophoresis method which permits us to easily examine the plasmid complement of microorganisms. As shown in Figure 1 plasmid DNA from the resistant clinical isolates and from E. coli transconjugants contained a common, 68 x 10^6 dalton, 0.50 ml fraction G + C content plasmid species which was absent from antibiotic susceptible recipients.

In order to determine whether or not these "common" plasmids (common by virtue of their similar molecular mass) were related, it was necessary to specifically label them and carry out DNA-DNA hybridization studies. As expected the common plasmids from the different species shared over 90% of their DNA sequences in common.

R plasmid mediated resistance become disseminated in several different ways (1,6). If an R plasmid gains entrance to an epidemic pathogen, Shigella flexneri or S. typhi, for example, the very communcability of the strain assures its dissemination though antibiotic selection provides selective pressure. In opportunistic microorganisms, such as those encountered in a Burn Unit or Intensive Care Ward, the incidence of R plasmid-mediated resistance and the dissemination of the R plasmids is absolutely dependent upon the highly selective environment that exists because of daily administration of antibiotics (a clinical necessity in most but not all cases). It is of paramount importance to monitor the dissemination and persistance of R plasmids in the hospital

setting. Most epidemiological studies have been largely descriptive and retrospective in nature (see 12 for discussion). More often than not R plasmids have been identified on the basis of drug resistance traits and transmissibility. The pitfall in this kind of analysis is that many antibiotic resistant genes reside upon segments of DNA (transposons) which are capable of transposing themselves from DNA molecule to DNA molecule (13). Presumably, therefore, novel R plasmid species could be generated by such a drug resistance transposon inserting itself into an indigenous, heretofore, phenotypically cryptic plasmid. In point of fact, this precise mechanism was shown (under research supported in part by this contract) to likely account for the emergence of ampicillin, tetracycline and chloramphenicol resistant H. influenzae (14).

In the present study our data probably provide evidence for the dissemination of an "epidemic" plasmid throughout a highly selective environment, presumably by in vivo transfer. The methods we have used here are the direct application of the tools of the molecular biologist to the study of the epidemiology of hospital infection or, for that matter as we have showed previously under this contract, to the study of antibiotic resistant epidemic disease. Simplification of the molecular tools (see below for example) offers a new epidemiological approach to study the antibiotic resistance problem in a manner so that it is no longer limited to post facto statistics but rather to a current or even prospective indication of developing problems. In a broader sense the study of the molecular epidemiology of R plasmids will permit the first steps to be taken to have a truly international surveillance of R plasmids and their dissemination. For example, within recent months, hospitals throughout the U.S., military, civilian, veterans's, children's have reported multi resistant Serratia isolates, (J. D. Bennett, Center for Disease Control, personal communication). In most hospitals the situation is not serious, though certainly these organisms cause considerable additional

suffering, additional costs and, unfortunately, death from sepsis. Each local situation requires individual analysis but at the national (or even international level) are we experiencing a plasmid epidemic? The dissemination of a few resistant clones? Such questions, as illustrated by our study, are now amenable to practical analysis.

B. A Single Colony Gel Electrophoresis Method

As noted in the previous section, agarose gel electrophoresis is a convenient way to analyze microorganisms for their plasmid complement (11). We use this procedure routinely to examine clinical isolates of both gram positive and gram negative species. Under ordinary circumstances we employ the growth from a 30 ml broth culture to analyze a strain for plasmid DNA. We often find it necessary, however, to obtain a rapid indication of the presence or absence of a plasmid or find that our facilities are strained because it is necessary to analyze a large number of different isolates for their plasmids. Consequently, we have developed a simple procedure that permits the identification of both large and small plasmids from a single colony from an agar plate. While the results may not have the same 'asthetic' look as our preparations using larger cell volumes and centrifugation, the results are, nonetheless, useful and suffice for our purpose over 80% of the time. For the purpose of illustration I have chosen to present the use of the single colony method to analyze a problem we encountered in the study of the Kad plasmid, K88.

As noted earlier the pathogenesis of E. coli toxigenic diarrheal disease is associated with Ent and Kad plasmids (1,7). The Kad plasmids are associated with colonization of the small bowel. In this context, colonization is synonomous with multiplication in the small bowel (for at least a limited time) and the ability of Kad^+ cells to resist the effects of perstalsis and/or villous motility. The best studied colonization factor is K88 found in porcine E. coli strains isolated from serious diarrheal disease (1,7). K88

is a large filamentous protein which contains all the common amino acids except cystein (6). K88 is manifested on the surface of the bacterial cell as a "fur of fine filaments" which can be differentiated from common pili and sex pili (1,5,6). K88 was recognized as a plasmid-mediated function in 1967. Recently, Smith and Parsell (17) reported that the K88 property was often transmitted with the ability to ferment the sugar raffinose (Raf).

Dr. Patricia Shipley of our group has recently completed the first comprehensive examination of the Kad plasmids which encode for K88 (P. Shipley, C. Gyles and S. Falkow, Infec. Immun., in press, 1978). Her data show that 1) Raf and K88 lie on a single 50 x 10^6 dalton non-conjugative plasmid, 2) Raf^+ $K88^-$ derivatives of this plasmid may be readily isolated and loss of K88 is either mutational or the result of the deletion of a homogeneous 20 x 10^6 dalton segment of DNA and 3) the K88 structural genes and Raf structural genes are distinct loci. While Kad plasmid carrying the Raf and K88 determinants are non-conjugative, they nonetheless can be mobilized by conjugative plasmids co-resident in the cell. It is in the mobilization of Kad (K88, Raf) from one cell to another that we came upon an anomaly and employed the single colony test to advantage.

When Kad (K88, Raf) was transmitted from clinical isolates to E. coli K-12 recipients we identified two classes of transconjugants. In most cases, we found a single 50 x 10^6 dalton Kad (K88, Raf) plasmid. Occasionally, however, we found a 90-120 x 10^6 dalton plasmid associated with the Kad (K88, Raf) phenotype. Moreover, these very large Kad plasmids were conjugative. The anomalous finding, however, was that none of the $K88^+$ Raf^+ clinical isolates contained a plasmid larger than 70 x 10^6 daltons. What then was the origin of 90-120 x 10^6 dalton $K88^+$ Raf^+ plasmids in E. coli K-12. We obtained some insight into this mystery when we observed that the very large $K88^+$ Raf^+ plasmids were markedly unstable in ordinary recombination proficient (rec^+)

E. coli K-12 but were more stable in recombination deficient (*rec*$^-$) *E. coli* K-12. In addition, mobilization of 50 x 10^6 dalton Kad (K88, Raf) plasmids from *rec*$^-$ *E. coli* was absent or reduced. This was a most unusual finding since plasmid mobilization has been viewed as the promotion of transfer of non-conjugative plasmid by a conjugative plasmid (1-3, 5,6). Historically, mobilization has been seen to be independent of host recombination functions (ie. *rec*$^-$) and did not involve covalent linkage between the conjugative and non-conjugative plasmid (1,5,6). Was mobilization of the Kad (K88, Raf) an exception to the rule?

A porcine clinical isolate carrying a 40 x 10^6 dalton conjugative R plasmid and a 50 x 10^6 dalton Kad (K88, Raf) plasmid was mated with an *E. coli* K-12 (rec$^-$) strain. Selection was for Raf$^+$ transconjugants. Of course, there was no way of telling which transconjugants contained the putative 90 x 10^6 dalton cointegrate plasmid without resorting to further mating procedures, in which case the cointegrate might be lost because of its instability. However, if one could but examine each transconjugant colony for its plasmid complement, it would be possible to obtain a clear view of the frequency of mobilization by cointegration.

Figure 2 shows an illustrative example of the single colony lysis technique. About one-half of a colony (no more than 48 hr old) was picked off a plate and suspended in 100 µl of Tris Borate buffer (89 mM Tris base, 87 mM boric acid containing 5 x 10^{-3}M EDTA) directly in the sample wall of an agarose gel electrophoresis apparatus (11). A sufficient quantity of 20% sodium dodecyl sulfate is added to lyse the cells (usually 20 µl) and following lysis, 2 µl of a 5 mg/ml RNAase solution is added and the entire mixture electrophoresed through a 0.7% agarose gel at 60 mA, 120V for 2 hrs. The gel was stained 15 min with ethidium bromide (0.4 µg/ml) and visualized with a long wave ultra-violet light. In Figure 2 one can see in the well marked A, a control colony

of E. coli K-12 carrying the R plasmid R1drd19 (65 x 10^6 daltons). In the wells marked B, one can see transconjugants carrying only the non-conjugative 50 x 10^6 dalton Kad (Raf K88) plasmid on the 40 x 10^6 dalton R plasmid. In the wells marked C one can see transconjugants carrying a 90 x 10^6 dalton cointegrate of the R plasmid and Kad (K88 Raf) plasmid. These latter plasmids are unstable and give rise generally (even in a rec$^-$ background) to the component plasmids. Occasionally, however, a stable plasmid cointegrate emerges. Such stable cointegrates have usually deleted part of the Kad (K88 Raf) plasmid.

These data serve to illustrate the utility of the single colony electrophoresis method. It should have many applications and in fact could be performed in the clinical laboratory to monitor, for example, R plasmids in the gonococcus (18) or the change in the plasmid complement of an epidemic strain. In the particular application chosen for presentation, the point has been to show that at least the Kad plasmids in toxigenic porcine strains have a rather unique requirement for mobilization. We are extending these observations to determine whether mobilization of a Kad plasmid with an Ent plasmid could potentially lead to the formation of single plasmid carrying both enterotoxin and specific adhesion properties. As shall be pointed out in the following section, there is reason to believe that in a number of human toxigenic isolates a Kad plasmid also carries the structural genes for E. coli heat stable enterotoxin.

C. The Plasmid Complement of Toxigenic E. coli From Man

In our research proposal submitted last year we noted tnat relatively little was known about either the Ent plasmids or Kad plasmids isolated from humans suffering from diarrheal disease. Only one Ent plasmid of human origin had been studied in any detail (19). Evidence for one putative colonization plasmid had been presented (20) but had been subject to serious question. We proposed therefore, to make a beginning effort to isolate the individual plasmid com-

ponents of human clinical isolates.

Figure 3 gives an interesting insight into our progress thus far in pursuing our proposal. We received a number of toxigenic isolates of human origin from the Center for Disease Control, from Drs. Doyle and Delores Evans as well as other diverse sources. The strains illustrated in Figure 3 represent several examples received from the Drs. Evans as well as the now classical strain, H10407. In slot A and B one can see the plasmid complement of H10407. In slot B the organism possesses the phenotype heat stable toxin positive (ST^+), heat labile positive (LT^+) and the organism possesses the ability to hemagglutinate human erythrocytes in the presence of 0.5% D-mannose (Hah) and is agglutinated in an antiserum prepared by the Evans' said to be specific for a colonization factor (CF^+). By all criteria the CF^+ and Hah^+ phenotypes are the same and treatment with CF sera block Hah. Consequently, we shall use only the CF^+ phenotype. The plasmids of H10407 in this strain are (from top to bottom) 60 x 10^6 daltons, 50 x 10^6 daltons and a small plasmid species about 4 x 10^6 daltons. In slot A one can see the plasmid complement for an H10407 derivative that is ST^- LT^+ CF^-. It may be further seen that the derivative in slot A lacks the 60 x 10^6 dolton plasmid. A similar finding was reported earlier by Evans et al. (20) to document that the 60 x 10^6 dalton plasmid was a Kad element anologous to K88 in pigs and K99 in pigs, calves and lambs (7). These data support their contention. In slot C and D one can see the plasmid complement of two derivatives of an E. coli O6 serotype isolated from a traveller to Mexico. In well D one sees the complex plasmid complement from an organism with the phenotype ST^+ LT^+ CF^+; in well C one sees the plasmid derivative from the same O6 strain that is ST^- LT^+ CF^-. One can easily observe that in this strain loss of the ST and CF phenotype is associated with the loss of a 60 x 10^6 plasmid, the same mass as that seen for H10407. Slot F and G show another example of this phenomenon with an independent O6 isolate from

another patient. The ST^+ LT^+ CF^+ derivative (well G) has a single extra 60 x 10^6 dalton plasmidthan ST^- LT^+ CF^- derivative (well G). The close similarity between the plasmid complements the two O6 strains indicate they are clonal in nature, although they were from different patients from different parts of Mexico.

Figure 4 takes this one step further. In Figure 4B one sees the plasmid complement of an O78 strain ST^+ LT^+ CF^+. Loss of ST^+ and CF^+ phenotype as before was associated with loss of the 60 x 10^6 dalton plasmid. Further loss of the LT^+ phenotype leads to the plasmid pattern seen in Figure 1A in which a 50 x 10^6 dalton plasmid has now disappeared leaving only a 55 x 10^6 dalton plasmid that is a conjugative R plasmid as well as a non-conjugative 4 x 10^6 dalton plasmid.

To be sure, the data shown in Figure 3 and 4 are but suggestive. They do tend to corroborate the view of Evans et al. (20), however, that some toxigenic E. coli of human origin possess a common Kad plasmid. In addition these data would indicate that the structural genes for ST biosynthesis reside upon this same Kad plasmid. (This result must be seen as intriguing in light of our observations on the covalent mobilization of K88). We have now marked several plasmids from the human toxigenic strains with antibiotic markers using the procedure described in our last proposal and now published (21). In addition, Mr. Steve Moseley, a graduate student, has initiated studies using isolated ST and LT gene sequences described previously (22) in our last proposal to obtain "blot" hybridization (23) visualization in agarose gels of these structural genes on their parental plasmid. The data thus far are compatible with the model that most E. coli toxigenic strains contain 1) a plasmid encoding for Kad and ST, 2) a plasmid encoding for LT only and 3) one or more conjugative plasmids, usually cryptic in the phenotypic sense. The extent to which these latter plasmids play a role in pathogenesis remains

to be seen.

The work on the toxigenic human isolates has been more difficult than we have previously experienced with animal isolates. In part, this is due to the plethora of plasmids in human isolates. Moreover, our standard method of 'marking' Ent and Kad plasmids by recombination which worked so successfully for porcine and bovine strains required modification for studies with human isolates. In part, this may reflect that porcine E. coli isolates, like their animal hosts, are more genetically homogeneous than one sees with human strains (and, of course, with humans). In any event, the analogies we learned from our study of animal Ent and Kad plasmids have been most valuable. It is clear however, from even our cursory view of human strains that the principles learned from the study of porcine and bovine plasmids will not strictly apply to the human situation.

DISCUSSION

To a great extent this has been a transition year for us. We have brought our laboratory expertise to a point where it has become possible to employ relatively difficult molecular genetic experiments in the everyday screening of clinical isolates. This year we have started to exploit these advances in our techniques. We believe that our preliminary findings with human toxigenic strains are significant and we shall propose to continue these studies for the coming contract year.

In a broader vein, we have started to take on a slightly different view towards approaches that may be taken for the study of the pathogenesis of enteric disease in general. We have had good success in focusing upon extra-chromosomal elements of bacteria which contribute to virulence. Of course, part of the reason for this success is that when dealing with plasmids it is possible to focus more precisely upon relatively few specific protein determinants

that contribute to virulence. But, of course, plasmids are but additions to the total pathogenic potential of a cell; the addition of plasmids alone do not necessarily suffice to convert a strain to form capable of causing overt disease (1,7). No, there are a variety of chromosomal genes that through their products contribute to virulence. Is it possible to apply some of the newer methods of molecular biology to identify specific host as well as plasmid products that contribute to microbial pathogenicity? We believe so, and shall outline this broader approach to the study of pathogenesis in our proposal for the coming year.

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Figure Legends

Figure 1. Agarose gel electrophoresis of ethanol precipitated DNA from cleared lysates of tobramycin resistant (Tb^r) and Tb^s strains. Procedure performed as described in Meyers et al. (11). A. *E. coli* K-12 Tb^s, B. *E. cloacae* Tb^r. C, D. *E. coli* K-12 Tb^r transconjugants from mating between *E. cloacae* and *E. coli* K-12, E. *K. pneumoniae* Tb^r, F, G. *E. coli* Tb^r transconjugants from mating between *K. pneumoniae* and *E. coli* K-12, H. Molecular weight standards.

Figure 2. Single colony agarose gel electrophoresis method for detecting plasmid DNA (see text for details).

Figure 3. Agarose gel electrophoresis of purified plasmid DNA from toxigenic *E. coli* isolates from Humans. Procedure performed as described in Meyers et al. (11). A. H10407 ST^- LT^+ CF^-, B. H10407 ST^+, LT^+ CF^+, C. *E. coli* 06 ST^- LT^+ CF^-, D. *E. coli* 06 ST^+ LT^+ CF^+, E. Molecular weight standard (from top to bottom 65 x 10^6 daltons, 40 x 10^6 daltons, 25 x 10^6 daltons, bacterial chromosome, F. *E. coli* 06 ST^- LT^+ CF^-, G. *E. coli* 06 ST^+ LT^+ CF^+. See text for further details.

Figure 4. Agarose gel electrophoresis of purified plasmid DNA from toxigenic *E. coli* isolates from Humans. Procedure performed as described by Meyers et al. (11). A. *E. coli* 078 ST^- LT^- CF^-, B. *E. coli* 078 ST^+ LT^+ CF^+. See text for further details.

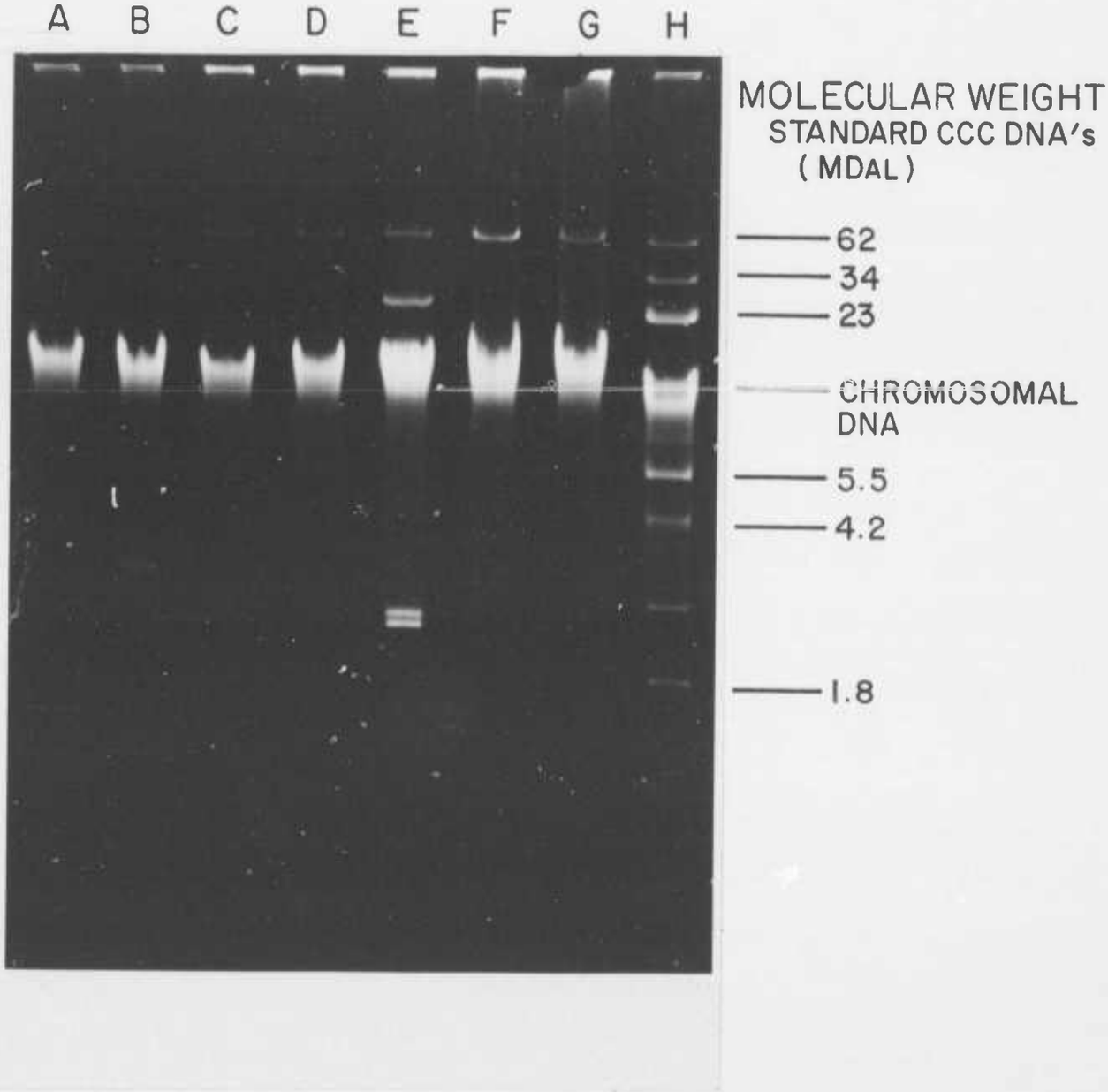

FIGURE 1
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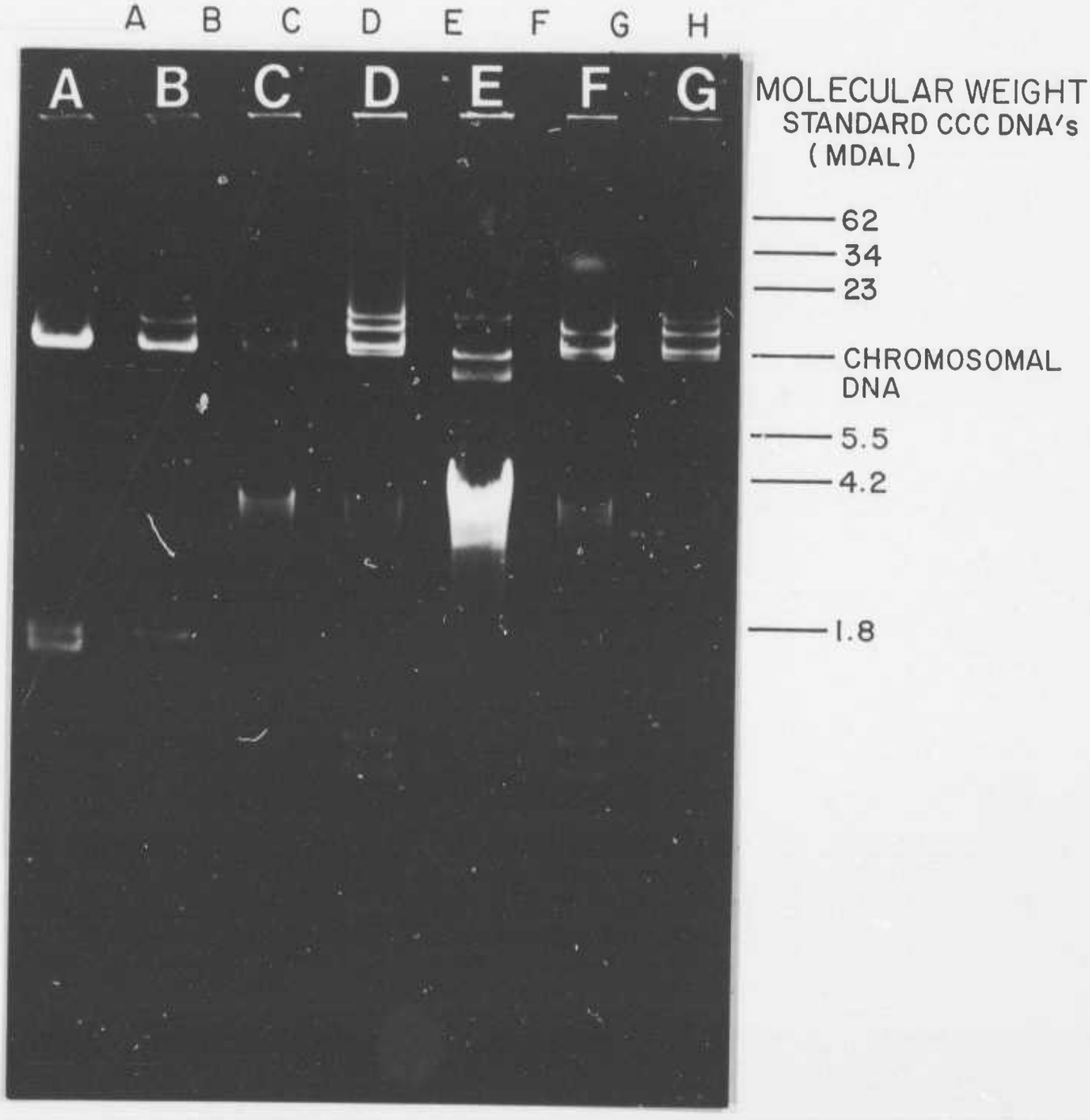
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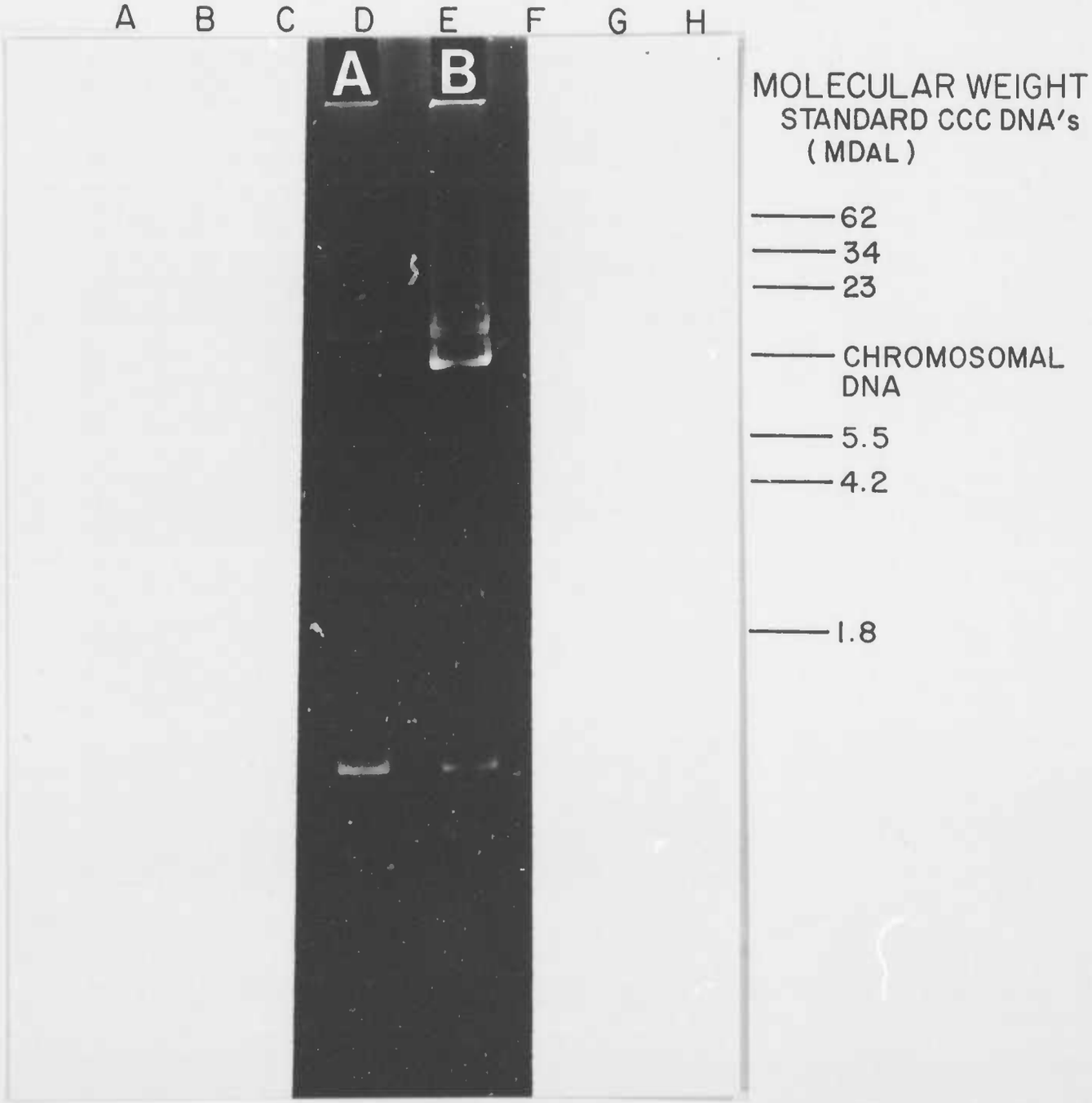
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STANDARD CCC DNA's
(MDAL)
62
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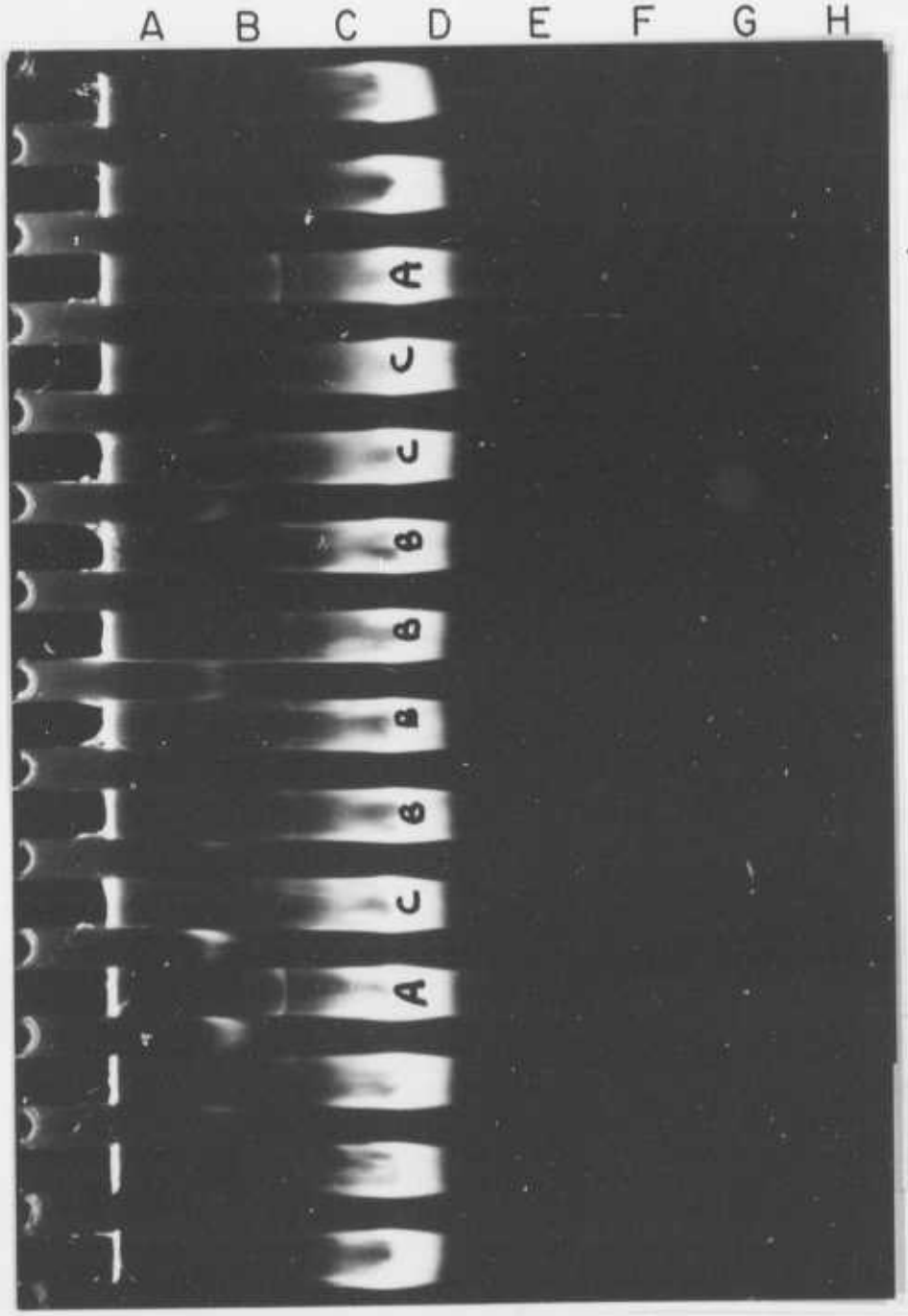
23
CHROMOSOMAL
DNA
5.5
4.2
1.8



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