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We have used the sequence-specific endonucleases Eco RI, Sma I, Bam HI, Hsu I and Hae III as identification tools in following the conjugal transfer of the well studied R plasmids Sa, R388, RP4 and R6K. Transfers were both intergenus and intragenus. Plasmid fingerprints were generated from both single and combination enzyme digests. The Sa transconjugants yielded plasmids showing consistent fingerprints for each of the respective endonucleases used while the three other R plasmid transconjugants showed both phenotypic changes as well as fingerprint changes.

Transconjugant Analysis: The Limitations On the Use of Sequence-Specific Endonucleases for Plasmid Identification

by

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TRANSCONJUGANT ANALYSIS: THE LIMITATIONS ON THE USE OF SEQUENCE-SPECIFIC ENDONUCLEASES FOR PLASMID IDENTIFICATION

Introduction

The study of R plasmid epidemiology often requires a method of deciding whether two bacterial strains harbor identical plasmids (42). A variety of biological and molecular properties have been used to characterize plasmids. Amongst these properties are the drug resistance markers carried, the size of the plasmid DNA, incompatibility (1,4,7,10,16,17,23,39,47), and DNA-DNA hybridization (19,29,46). Higher resolution techniques showing plasmid identity through heteroduplex analysis and contour length have also proven useful (14,28,40, 44,45). Investigators have also attempted to correlate R plasmid compatibility with the molecular specificities of R plasmid determined beta-lactamases (6,24,33). The limitations of some of these identification methods have been suggested by some reports concerned with normally occurring hybrids, anomalous compatibility properties and plasmid recombination (9,11,20,37). These limitations become especially significant with regard to recent reports of plasmid transposons and insertion sequences (3,5,25,27,30).

Plasmid identification has been further enhanced through the use of sequence-specific endonucleases and gel electrophoresis (13,15, 18,31,43,48). Endonucleases have also been determined to be useful in the identification of transposable DNA sequences within R plasmids (2). In this study we report our efforts to utilize the endonucleases *Eco* RI, *Sma* I, *Bam* HI, *Hsu* I and *Hae* III in transconjugant identifica-

tion of the well studied R plasmids R6K, R388, RP4 and Sa. Specifically, we address the problems of plasmid identification posed by transfer within a bacterial species, transfer between genera, transfer between genera followed by return to the "original host" species, and of transfer into environmental, laboratory and clinical strains which already contain other plasmids.

Materials and Methods

Bacterial strains and plasmids

The strains and plasmids used are described in Table 1.

Media

MacConkey agar, Simmons Citrate agar, Mueller Hinton agar and Penassay broth (Antibiotic Medium 3) were obtained from Difco Laboratories. Penassay broth was supplemented with 0.2% glucose. Penassay agar contained 1.5% Bacto agar (Difco). Glucose minimal medium was Miller's (36).

Antibiotics

Chloramphenicol (B grade), streptomycin sulfate (B grade) and nalidixic acid (B grade) were obtained from Calbiochem. Kanamycin sulfate and trimethoprim were obtained from Sigma Chemical. Sulfathiazole and tetracycline HCl were obtained from Nutritional Biochemical. Ampicillin 3 H_2O was a gift of E.R. Squibb and Sons. Chloramphenicol, streptomycin, kanamycin, nalidixic acid, tetracycline and ampicillin were used at a final concentration of 25 µg/ml. Trimethoprim was used at a final concentration of 50 µg/ml. Sulfathiazole was used at a final concentration of 200 µg/ml. Antibiotic sensitivity discs (Difco) were also used to test resistance patterns and this was evaluated on Mueller Hinton agar with discs containing the following concentrations: Tetracycline, kanamycin and chloramphenicol at 5 and 30 µg, ampicillin and streptomycin at 2 and 10 µg, and sulfathiazole at 50 and 300 µg.

Table 1

Bacterial Strains and Plasmids

Bacterial Strains	Strain Properties	Plasmid Properties	Source
E. coli C600(R6K)	lac thr leu thi	Ap^r Sm^r Inc X(27) 26×10^6 M.W.	S. Falkow
E. coli J53(R388)	pro met	Su^r Tp^r Inc W(27) 21×10^6 M.W.	S. Falkow
E. coli 711(Sa)	Nal^r lac trp pro his phe	Cm^r Km^r Sm^r Su^r(27) Inc W 23×10^6 M.W.	S. Falkow
E. coli J53(RP4)	pro met	Ap^r Km^r Te^r(5) Inc P 36×10^6 M.W.	S. Falkow
E. coli W3110N	Nal^r F^-		L. Brown
Klebsiella 190-3[a]	Nal^r Cm^r Ap^r Cb^r		R. Seidler
Salmonella enteritidis			J. Fryer
E. coli CSH 56	Nal^r F^- ara lac pro sul ($Su\ I^+$) B 1		L. Brown-CSH Collection
E. coli CSH 65	Nal^r Sm^r F^- lac leu thr B 1		L. Brown-CSH Collection

Table 1 - Continued

Bacterial Strains	Strain Properties	Plasmid Properties	Source
E. coli W3110C	F^- Cm^r Derivative of W3110N Via EMS Mutagenesis		
E. coli RY 13	r^+_{RI} m^+_{RI} Sm^r	Sm^r Su^r	L. Brown
E. coli GS[b]	Ap^r Sm^r		Good Samaritan Hospital, Corvallis, Oregon

Abbreviations

Km Kanamycin	Te Tetracycline	Cm Chloramphenicol
Nal Nalidixic acid	Sm Streptomycin	Ap Ampicillin
Cb Carbenicillin	Tp Trimethoprim	Su Sulfonamide

[a]River water isolate, 100% DNA homology with ATCC Type 3 *Klebsiella pneumoniae*

[b]Plasmid harboring, resistances not transmissible via conjugation, low Sm resistance

R plasmid transfers

Escherichia coli strains containing R plasmids were separately tested in conjugal systems to determine frequencies of transfer into E. coli W3110N, Klebsiella 190-3, Salmonella enteritidis, E. coli CSH 56, E. coli CSH 65, E. coli W3110C, E. coli RY 13 and E. coli GS. In some instances recipient strains were later used as donors in passage of the plasmid under study. E. coli and Klebsiella donor strains were first grown on MacConkey agar containing antibiotics and S. enteritidis donor strains were first grown on Simmons Citrate agar containing antibiotics to insure maintenance of all resistance markers. After overnight incubation at 37°C in Penassay broth the donors were diluted 1:40 and the recipients 1:20 into fresh broth on the day of the experiment. At a donor cell density of 2-3 X 10^8 cells/ml, the donors and recipients were mixed in a 1:1 ratio and allowed to mate overnight in a 37°C rotary incubator shaker at slow speed. Transconjugants were enumerated by plating ten fold dilutions on MacConkey agar containing nalidixic acid plus either ampicillin, streptomycin, chloramphenicol or trimethoprim (depending on the particular cross) and on MacConkey agar containing nalidixic acid to determine the total number of recipients. A total cell count was obtained by plating on MacConkey agar. Controls were included to determine the frequency of nalidixic acid resistant donor mutations and recipient mutations for the marker being initially selected for in the transfer. For R6K transfer into E. coli W3110C the counterselection agent used was chloramphenicol. Transconjugant colonies were picked to MacConkey

agar plates containing appropriate antibiotics or to Mueller Hinton agar plates containing sulfathiazole to test for all plasmid markers. The frequency of R plasmid transfer was calculated using the formula given below:

$$\text{Frequency of transfer} = \frac{\text{Number of Resistant Recipients}}{\text{Total Number of Recipients}}$$

S. enteritidus nalidixic acid resistant mutants did not grow in a dispersed manner in broth culture and exhibited rough colony morphology. Therefore enumeration of R plasmid transconjugants was performed on Simmons Citrate agar containing an appropriate antibiotic. The total number of recipients was determined using Simmons Citrate agar. Transconjugant colonies were picked to Simmons Citrate agar plates containing appropriate antibiotics or to Mueller Hinton agar containing sulfathiazole to test for all plasmid markers.

Construction of a recipient strain via EMS mutagenesis

E. coli W3110N was grown to approximately 2-3 X 10^8 cells/ml in glucose minimal medium, centrifuged, washed and resuspended in one half the original volume in minimal medium containing 0.2 M Tris, pH 7.5 with no carbon source (36). 0.03 ml EMS (ethylmethane sulfate, Eastman Kodak) was added to 2 mls of this suspension and mixed vigorously. The suspension was aerated for two hours, diluted 1:10, grown in broth overnight and 0.1 ml samples plated on Penassay agar containing chloramphenicol. Mutant stability was insured by subculture in the presence of antibiotics and the resultant strain called W3110C.

Plasmid isolation

The method of Guerry et al (21) was used with some modifications. R plamid containing strains were grown to the late logarithmic stage of growth in Penassay broth containing antibiotics. Preparation of R6K and R388 used 300 ml cultures while RP4 preparation required 600 mls and Sa 900 mls. Cells were harvested by centrifugation at 4,340 X g for 10 minutes. The pellet from a 300 ml culture was resuspended in 10 ml of 25% sucrose in 0.05 M Tris (hydroxymethyl aminomethane, Sigma) pH 8.0. To well mixed cells, 2 mls of lysozyme (5 mg/ml, Sigma egg white, in 0.25 M Tris, pH 8.0) was added and the suspension placed on ice for five minutes. The suspension was chilled an additional five minutes after receiving 4 mls of 0.25 M EDTA (ethylenediaminetetraacetate, Sigma) pH 8.0. Complete cellular lysis was achieved by the addition of 10% SDS (sodium dodecyl sulfate, Sigma) to a final concentration of 1%. Special care was taken to disperse the SDS as rapidly as possible by the use of a mechanical Vortex mixer since the solution became extremely viscous upon contact with the SDS solution. This precaution enhanced DNA yields. Sufficient 5 M NaCl was added to the mixture to a final concentration of 1 M and the lysates stored at 4°C for approximately twelve hours. The supernatant fluid (cleared lysate) was collected after centrifugation for forty-five minutes at 4°C and 27,000 X g. Prior to ultracentrifugation, the cleared lysates were assayed for the presence of plasmid DNA using gel electrophoresis (35).

Plasmid purification

Cesium chloride-ethidium bromide dye bouyant density centrifugation was used to purify the covalently closed circular plasmid DNA from the cleared lysates (41). Centrifugation was performed in a Beckman L2-65 ultracentrifuge using a SW 41 swinging bucket rotor run at 35,000 rpm at 15°C for 48 hours. The gradients were prepared by adding solid CsCl (Calbiochem Optical Quality Grade, Lot 601309) to the cleared lysate preparation in the amount of 0.87 g/ml. Sufficient ethidium bromide (Sigma) was added from a stock solution of 10 mg/ml in deionized H_2O to the mixture so that the final concentration was 50 µg/ml. The final density of the gradients was adjusted to 1.57 g/ml. After centrifugation, the presence of superhelical DNA was detected by viewing the tubes under ultraviolet illumination (8) and the lower fluorescent band collected with a 21 G needle inserted into the side of the tube. The superhelical DNA was pooled and when necessary an additional centrifugation was performed under the same conditions with the exception that a SW 50.1 rotor was used. CsCl and ethidium bromide were removed by dialysis against 1 X SSC buffer (0.15 M NaCl + 0.015 M sodium citrate) pH 7.0 for 20 hours with five one-liter changes. The purified plasmid DNA was resuspended in a buffer suitable for endonuclease digestion.

Gel electrophoresis

Specific gel concentrations, running times, miliamperes and voltages are provided in appropriate figure legends. Gels were prepared by dissolving varying percentages Wt./Vol. agarose (Sigma

Type II:Medium EEO) in a solution of E buffer composed of 0.04 M Tris, 0.001 M EDTA and 0.02 M sodium acetate, pH 7.2 (22). All gels were loaded with a 10% tracking dye mixture (2 mls E buffer, 5 mg bromo phenol blue-MCB Chemical, and 13 ml glycerol-Mallinckrodt) and run at room temperature. After steaming the agarose solution for one hour the mixture was cooled to 50°C before filling either the 30 cm X 5 mm plexiglass tubes or the slab gel assembly. Agarose was allowed to solidify at room temperature. Tube gels were stained in E buffer containing ethidium bromide at a concentration of 4 μg/ml. Vertical slab gels (Life Sciences Design and Development Facilities, U.C.L.A.) were cast with the following dimensions: 20 cm high, 22 cm wide and 4 mm thick. The slab gels contained ethidium bromide at a concentration of 1 μg/ml and were prepared by pouring the agarose between glass plates separated by silicone rubber strips (GE RTV-8111 Silicone Rubber). The E buffer used in the reservoirs contained ethidium bromide at a concentration of 4 μg/ml and was circulated through use of a peristaltic pump (Ismatec) in order to assure pH uniformity. Fluorescent DNA bands were detected in the gels by direct illumination from a long wave UV lamp (UVL 56 Blak-Ray, Ultra-Violet Products). Photography was performed with a Multipurpose Industrial MP3 Land Camera using an orange UV filter (Tiffen Photar Orange) and Type 47 film.

Endonucleases

Plasmid DNA preparations were resuspended in 0.1 M KCl, 0.01 M Tris and 0.001 M EDTA, pH 7.2 (34). Reactions were performed at 37°C

in 38 X 10 mm polypropylene vials (Eppendorf). $MgCl_2$ was added to the reactions to a final concentration of 10 mM for Eco RI, Bam HI, Hsu I and Hae III while Sma I digests contained 5 mM $MgCl_2$. Digestions with more than one enzyme were performed sequencially. Reactions were terminated via heating the mixture 10 minutes at 65°C. Generally 3 µl of Eco RI or Sma I was sufficient to cleave 1 µg of DNA in a total volume of 50 µl or less. Bam HI and Hae III reactions used 2 µl enzyme amounts and Hsu I used 1 µl. Bacteriophage λ DNA Eco RI fragments were included in the slab gels containing plasmid digests to serve as molecular weight references. Prior to loading, the λ fragments were heated at 70°C for 10 minutes in order to melt the hydrogen-bonded (cohesive) ends.

Results

Sa transfers and fingerprints

The sequence of Sa transfers is given in figure 1. The object of this series of conjugal transfers was to examine the plasmid after reisolation from a different genus as well as to analyze it following passage back to E. coli. Each recipient strain used did not contain any plasmids. Following transfer, no marker separations were observed in any of the transconjugants. However, strain E (E. coli CSH 56) showed a decrease in the level of streptomycin resistance. This plasmid was difficult to obtain from all strains and in particular strain A (E. coli 711) and strain E (E. coli CSH 56) consistently yielded a decreased amount of covalently closed circular DNA relative to the other transconjugants.

The respective fingerprints of the Sa plasmid prepared from strains A-E were consistent using Eco RI, Sma I, Bam HI and Hsu I. Eco RI produced four Sa fragments, Sma I gave one cleavage, Bam HI gave two fragments and Hsu I produced three fragments. The Hae III enzyme cleaved Sa at many sites producing numerous Sma I fragments and was therefore unsatisfactory to use with our agarose gel system. An Sma I-Bam HI digest confirmed the Sma I single site cleavage of Sa since a decrease in the size of the smaller Bam HI fragment and the appearance of a new low molecular weight fragment was observed in the combination digest. Figure 3 shows the Hsu I fragment pattern is identical for Sa prepared from strains A-E.

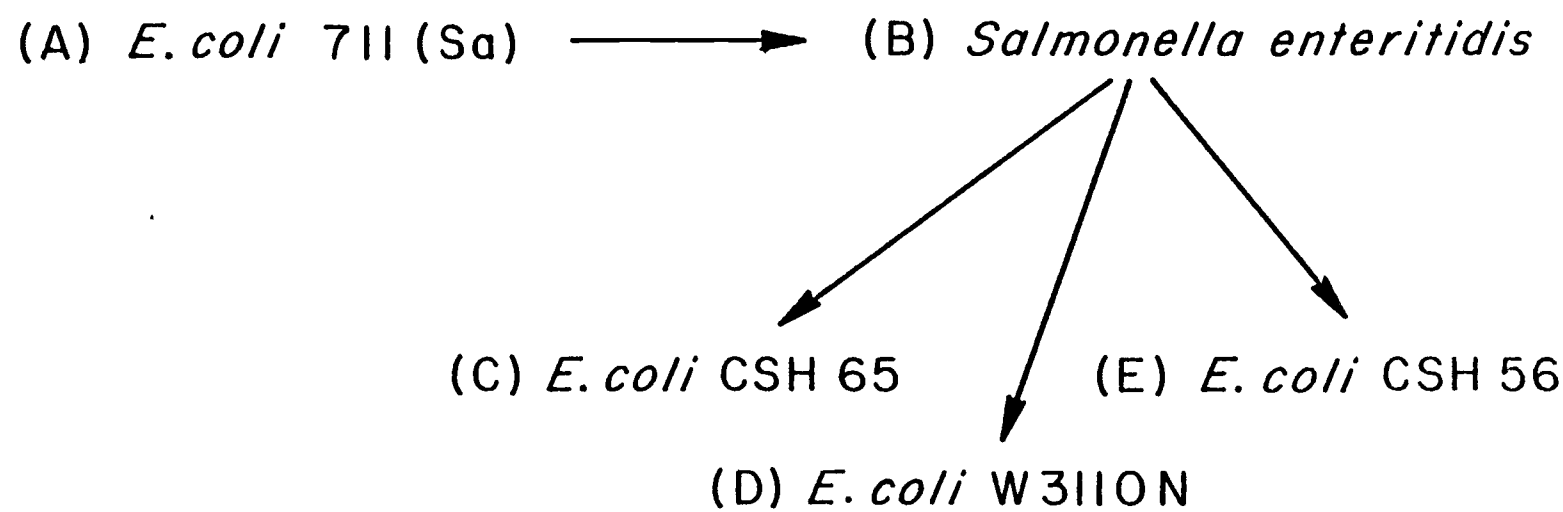


Figure 1

Sa Transfer Sequence

E. coli 711 (Sa) is designated as strain A; S. enteritidis, strain B; E. coli CSH 65, strain C; E. coli W3110N, strain D and E. coli CSH 56, strain E.

R388 transfers and fingerprints

The sequence of R388 transfers is given in figure 2. The purpose of these series of transfers was to examine the R388 plasmid after transfer into a strain containing a known restriction-modification system and to study the plasmid after reciprocal inter-genus matings. The E. coli RY 13 (R388) transconjugants were selected by the aquisition of trimethoprim resistance because the strain was already sulfonamide resistant. No marker separations were noted in any of the R388 transconjugants.

The Bam HI cleavage pattern for the R388 plasmid has previously been reported (43). This three-fragment pattern is demonstrated in figure 4 for R388 prepared from strains A-F. Digestion of R388 with Eco RI produces the fragment pattern seen in figure 5 and this pattern was identical for R388 isolated from strains A, B, C, E and F. R388 prepared from strain D (E. coli RY 13) showed a different pattern, indicating either single site or no cleavage via Eco RI. This result might be expected since E. coli RY 13 harbors the plasmid coding for the Eco RI endonuclease and modification methylase. The Hsu I enzyme produced a single cleavage in R388 isolated from strains A, B, D and E. However, both S. enteritidis R388 transconjugants, strains C and F, showed an additional four fragments not previously observed. These four bands were not present in the gels in stoichiometric amounts. R388 was uncleaved by Sma I and Hae III analysis was as unsatisfactory as with the Sa plasmid.

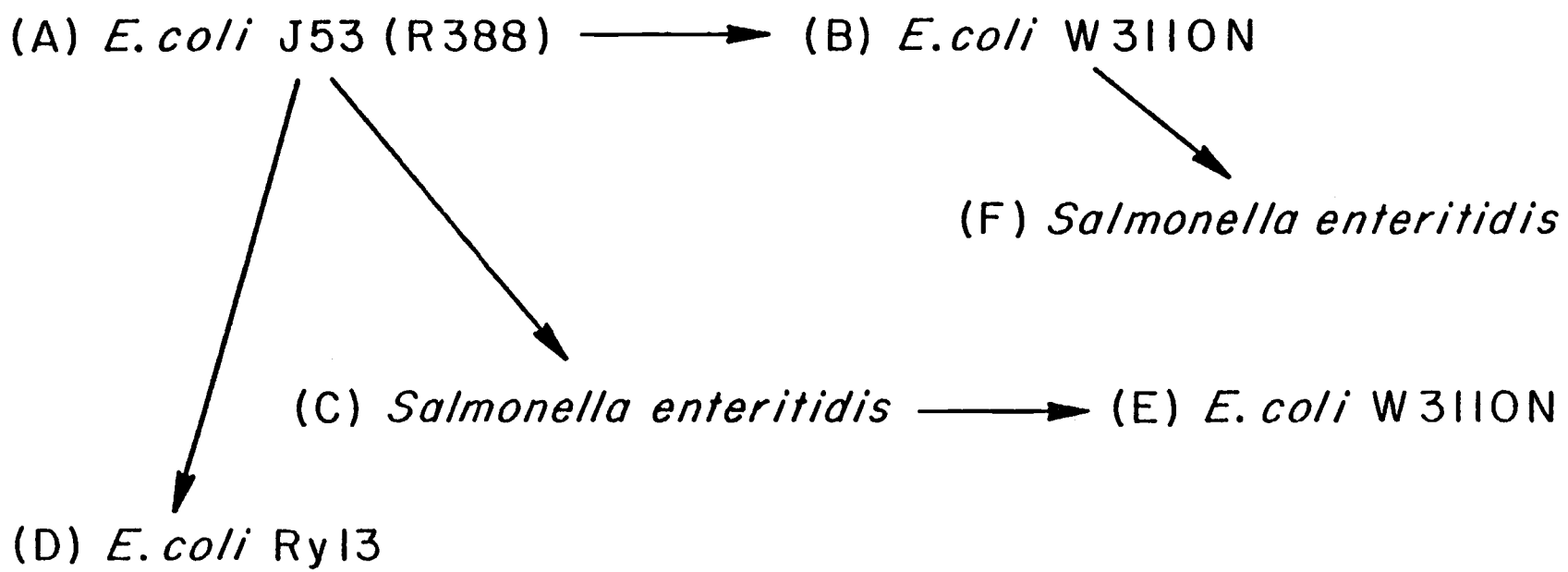


Figure 2

R388 Transfer Sequence

E. coli J53 (R388) is designated as strain A; E. coli W3110N receiving R388 from J53, strain B; S. enteritidis receiving R388 from J53, strain C; E. coli RY 13, strain D; E. coli W3110N receiving R388 from S. enteritidis, strain E and S. enteritidis receiving R388 from E. coli W3110N, strain F.

Figure 3

Sa fingerprints with endonuclease Hsu I.

This 1.2% agarose gel contains from left to right in the used wells: (1) Sa from E. coli 711-undigested, (2) Sa from E. coli 711-digested, (3) Sa from S. enteritidis-undigested, (4) Sa from S. enteritidis-digested, (5) Sa from E. coli CSH 65-undigested, (6) Sa from E. coli CSH 65-digested, (7) Sa from E. coli W3110N-undigested, (8) Sa from E. coli W3110N-digested, (9) Sa from E. coli CSH 56-undigested, (10) Sa from E. coli CSH 56-digested, (11) λ Eco RI fragments. Electrophoresis for 17.5 hours at 50 mA and 55 V.

Figure 4

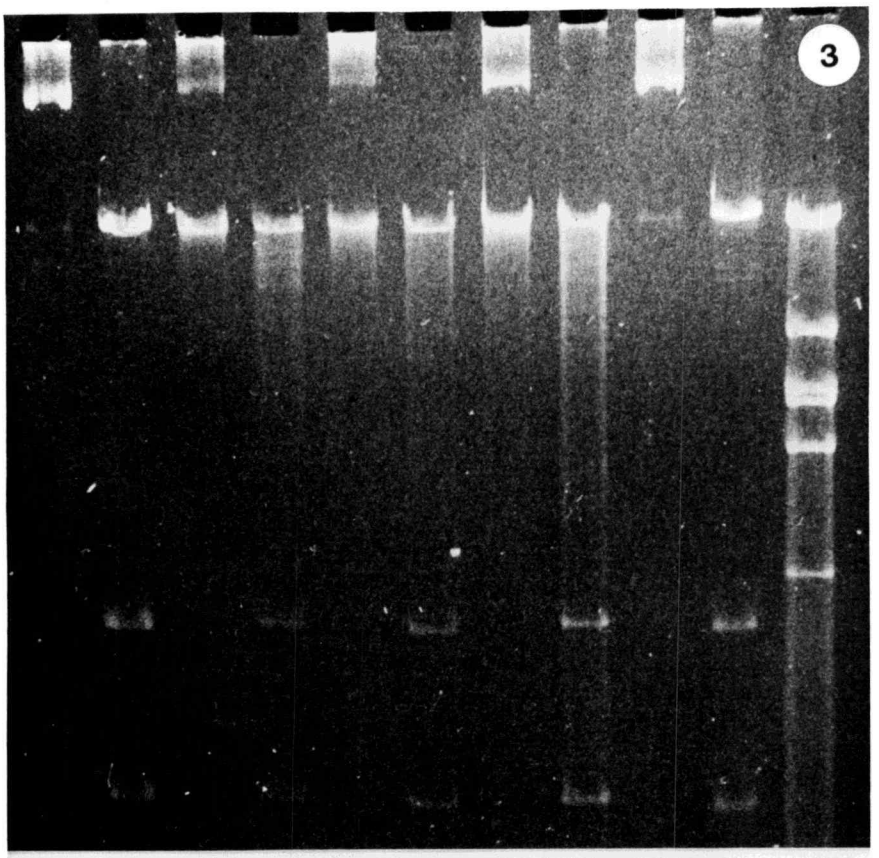
R388 fingerprints with endonuclease Bam HI.

This 1.2% agarose gel contains from left to right in the used wells: (1) R388 from E. coli J53-undigested, (2) R388 from E. coli J53-digested, (3) R388 from E. coli W3110N (strain B)-undigested, (4) R388 from E. coli W3110N (strain B)-digested, (5) R388 from S. enteritidis (strain C)-undigested, (6) R388 from S. enteritidis (strain C)-digested, (7) R388 from E. coli RY 13-undigested, (8) R388 from E. coli RY 13-digested, (9) E. coli RY 13 plasmids without R388-undigested, (10) E. coli RY 13 plasmids without R388-digested, (11) R388 from E. coli W3110N (strain E)-undigested, (12) R388 from E. coli W3110N (strain E)-digested, (13) λ Eco RI fragments, (14) R388 from S. enteritidis (strain F)-undigested, (15) R388 from S. enteritidis (strain F)-digested. Electrophoresis for 20 hours at 50 mA and 55 V.

Figure 5

R388 *Eco* RI pattern.

This 1% tube gel shows a typical three fragment pattern. Electrophoresis for 8 hours at 5 mA per tube.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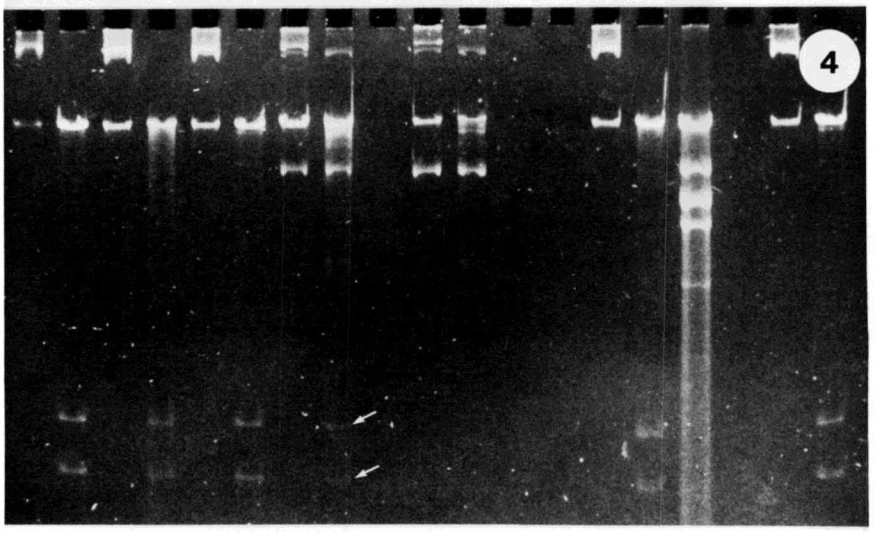
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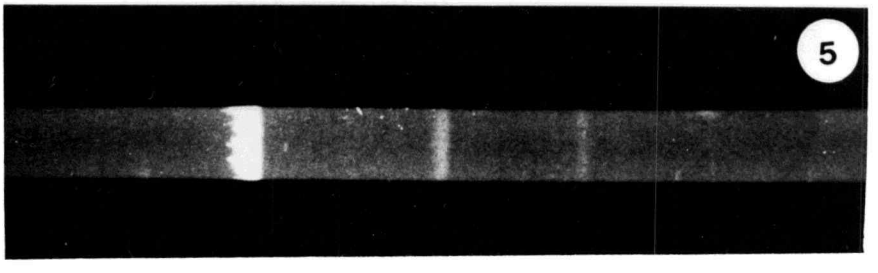

5

RP4 transfers and fingerprints

The sequence of RP4 transfers is given in figure 6. This set of transfers permitted RP4 to be fingerprinted after isolation from a clinical strain which contained other plasmids as well as to continue consideration of plasmid identification after passage through different genera. No potential marker separations were noted in any of the RP4 transconjugants although tetracycline resistance was not expressed in strain H. We confirmed the presence of RP4 in strain H by its ability to donate the plasmid to another *E. coli* strain which subsequently expressed all RP4 markers. Strain F (*E. coli* GS) was already ampicillin resistant so RP4 transfer could only be evaluated on the basis of the aquisition of tetracycline and kanamycin resistance.

Although both *S. enteritidis* RP4 transconjugants, strains D and E, could donate RP4, we were unable to isolate the plasmid from these strains and therefore they are not included in the fingerprint analysis. We considered this to be a significant result as we experienced no difficulty in isolating plasmids R388 or Sa from the same strain. We also noticed a reduced yield of RP4 plasmid DNA in strain F relative to what we obtained from other RP4 transconjugants.

The physical map of RP4 has been published (12). *Eco* RI, *Bam* HI, *Hind* III and *Hsu* I, an isoschizomer of *Hind* III (38), cleave RP4 at single sites. *Sma* I cleaves RP4 at four sites and we were able to demonstrate this pattern for the plasmid isolated from all strains. Figure 8 shows an *Eco* RI-*Hsu* I fingerprint of RP4 isolated from each strain. Two identical fragments are apparent in each case but we also

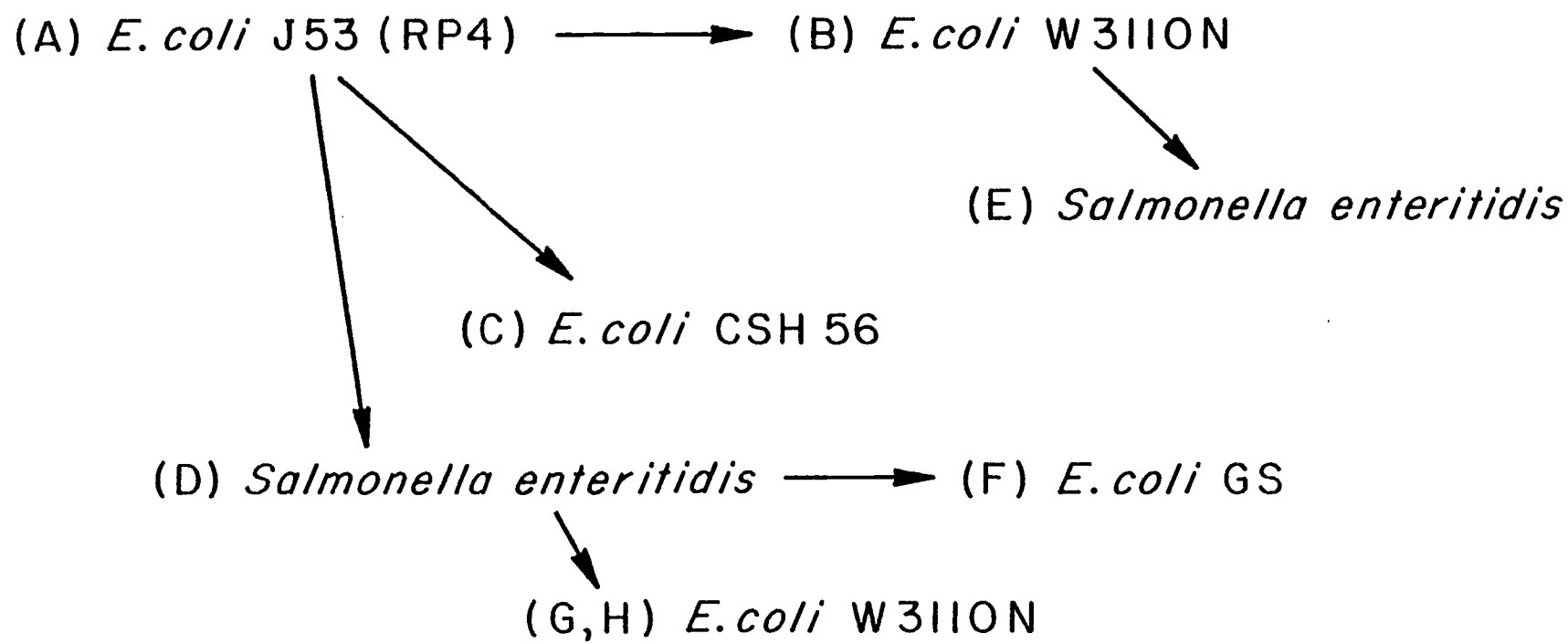


Figure 6

RP4 Transfer Sequence.

E. coli J53 (RP4) is designated as strain A; E. coli W3110N receiving RP4 from J53, strain B; E. coli CSH 56, strain C; S. enteritidis receiving RP4 from J53, strain D; S. enteritidis receiving RP4 from W3110N, strain E; E. coli GS, strain F and E. coli W3110N receiving RP4 from S. enteritidis, strain G. Strain H is an E. coli W3110N RP4 recipient from S. enteritidis which failed to express tetracycline resistance.

observed two new fragments appearing in the digest of the plasmid prepared from strain F. These two fragments are approximately 2.5 X 10^6 M.W. and less than 2.0 X 10^6 M.W. The Eco RI-Bam HI double digestion of RP4 also showed identical patterns in all strains with the smaller of the two fragments being approximately 4.5 X 10^6 M.W. Sma I-Bam HI digestion gave the expected five fragment fingerprint for RP4 isolated from each strain. The third largest RP4-Sma I fragment was cleaved in the combination digest. The production of many small fragments made Hae III digestion of this plasmid unsatisfactory to use with our agarose gel system.

R6K transfers and fingerprints

The R6K transfer sequence is given in figure 7. The object of this transfer series was to be able to prepare and fingerprint R6K from an environmental Klebsiella strain which contained other plasmids as well as to examine it after passage through E. coli strains. Strain C showed the loss of streptomycin resistance. Since strain F (Klebsiella 190-3) was already ampicillin resistant (non transmissible), R6K transfer could only be selected for on the basis of the aquisition of streptomycin resistance and this frequency was very low. Attempts to transfer either the Ap^r marker or Sm^r marker out of this transconjugant proved unsuccessful. However, cleared lysate examination of strain F did show the presence of R6K within a background of other plasmids.

As shown in figure 9, we were able to produce the two-fragment Eco RI cleavage pattern previously reported for R6K (32) with plasmids

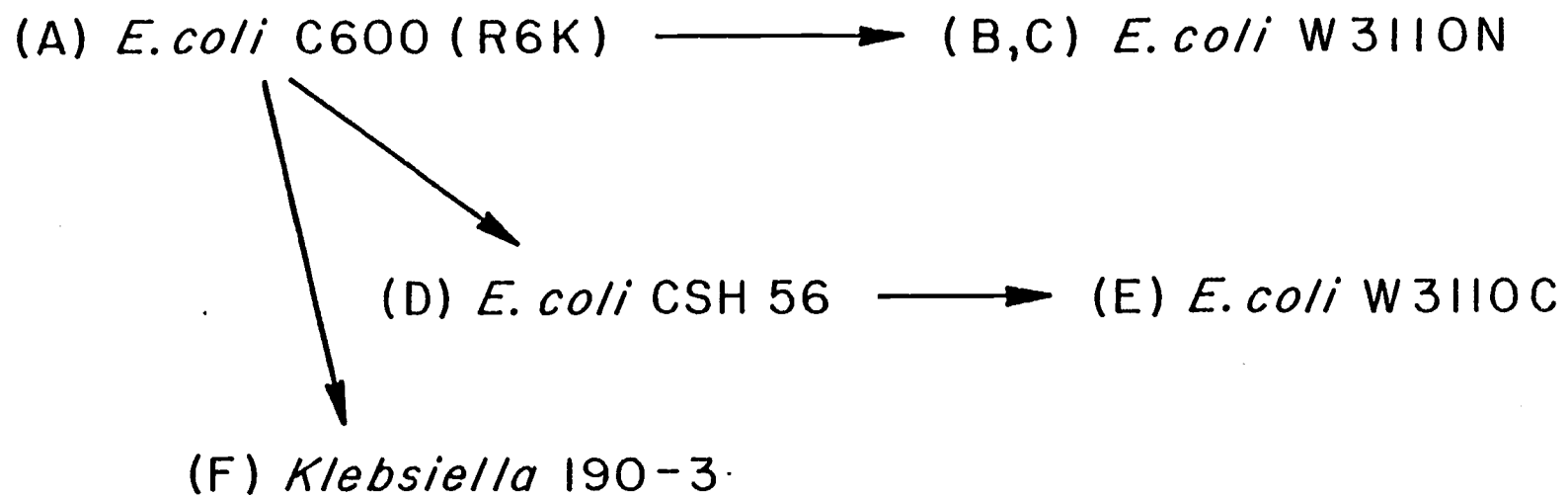


Figure 7

R6K Transfer Sequence.

E. coli C600 (R6K) is designated as strain A; E. coli W3110N, strain B; E. coli W3110N showing Sm^S, strain C; E. coli CSH 56, strain D; E. coli W3110N, strain E and Klebsiella 190-3, strain F.

prepared from strains A, B and D but plasmids from strains C and F showed only single site cleavage. Strain E gave the reported two-fragment pattern plus an additional fragment of approximately 2.8 X 10^6 M.W.

Sma I analysis indicated single site cleavage for R6K prepared from strains A, B and D, but the plasmids from strains C and F were uncleaved with Sma I. Strain E showed two Sma I R6K fragments.

The Eco RI-Sma I digests confirmed the single Eco RI site and single Sma I site patterns previously observed. The combination digests produced three fragments for R6K from strains A, B and D, while the plasmids from strains C and F showed the expected single site patterns. R6K isolated from strain E showed the three fragments observed in strains A, B and D plus an additional fourth fragment of less than 2.0 X 10^6 M.W. An expected fifth fragment from this combination digest was too small to be detected in our gel system.

Bam HI produced single site cleavage of R6K prepared from all strains except E which showed two fragments. The Eco RI-Bam HI digests confirmed the single Bam HI site patterns. R6K isolated from strains A, B and D produced three fragments from the combination digests and the plasmid isolated from strain C produced two fragments the same size as the two largest observed in the digests of the plasmids from strains A, B and D. The plasmid prepared from strain E gave three fragments identical to those just mentioned plus a fourth fragment of approximately 2.3 X 10^6 M.W. An expected fifth fragment was undetectable in our gel system due to its small size. The plasmid

isolated from strain F produced two fragments corresponding to the smaller two fragments observed in the combination digests of the plasmids isolated from strains A, B and D.

Because the Hsu I enzyme cleaved R6K at many sites, analysis of only the ten largest fragments was feasible with our gel system but the plasmids isolated from strains A, B and D had these in common. R6K prepared from strain C showed the loss of the second and third largest fragments while the plasmid from E gave the observed ten fragments plus two additional bands located between the fourth and fifth bands in the normal pattern. The plasmid from strain F produced only the first, third and tenth bands of the normal Hsu I pattern.

The Hae III enzyme was also found to cleave R6K at many sites but our comparative analysis involved the seven largest fragments and an example of a gel pattern showing these may be seen in figure 10. The plasmids from strains A, B, D and E showed these seven bands and R6K from E also produced a new eighth fragment. Strain C harbored a plasmid showing the loss of the sixth fragment and a gain in molecular weight of the first fragment. R6K isolated from strain F had only the seventh fragment in common with the patterns observed from the other strains.

Discussion

Several reports have suggested that sequence-specific endonucleases may be employed to demonstrate plasmid and transposon relatedness (2,13,15,18,48). Because the frequency of DNA transposition in nature is difficult to estimate (26), we were interested

Figure 8

RP4 fingerprints with endonucleases Eco RI-Hsu I combination digest. This 1.2% agarose slab gel contains from left to right in the used wells: (1) RP4 from E. coli J53-undigested, (2) RP4 from E. coli J53-digested, (3) RP4 from E. coli W3110N (strain B)-undigested, (4) RP4 from E. coli W3110N (strain B)-digested, (5) RP4 from E. coli CSH 56-undigested, (6) RP4 from E. coli CSH 56-digested, (7) E. coli GS plasmids without RP4-undigested, (8) E. coli GS plasmids without RP4-digested, (9) RP4 from E. coli GS-undigested, (10) RP4 from E. coli GS-digested, (11) RP4 from E. coli W3110N (strain G)-undigested, (12) RP4 from E. coli W3110N (strain G)-digested, (13) RP4 from E. coli W3110N (strain H, Te^S)-undigested, (14) RP4 from E. coli W3110N (strain H, Te^S)-digested, (15) λ Eco RI fragments. Electrophoresis for 18.5 hours at 50 mA and 55 V.

Figure 9

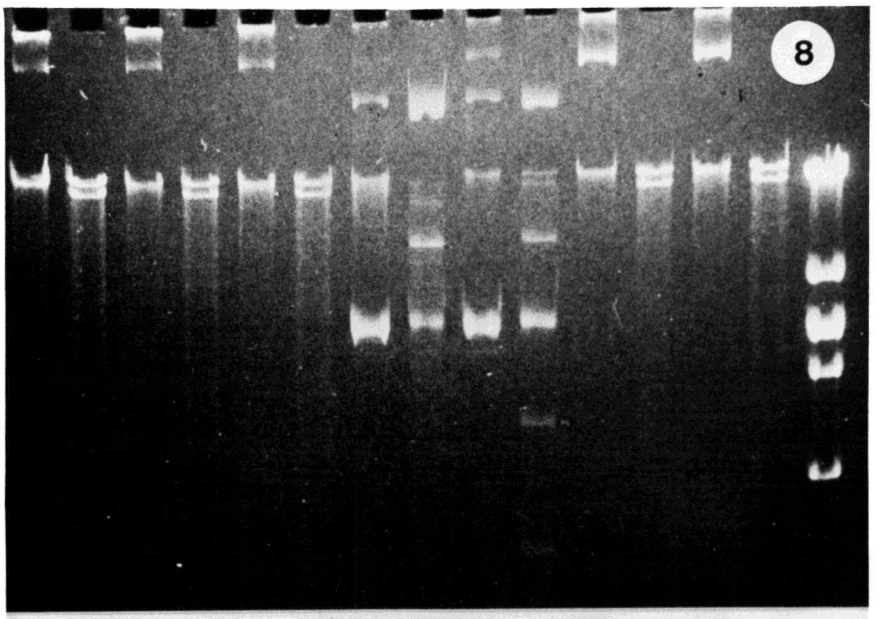
R6K fingerprints with endonuclease Eco RI.

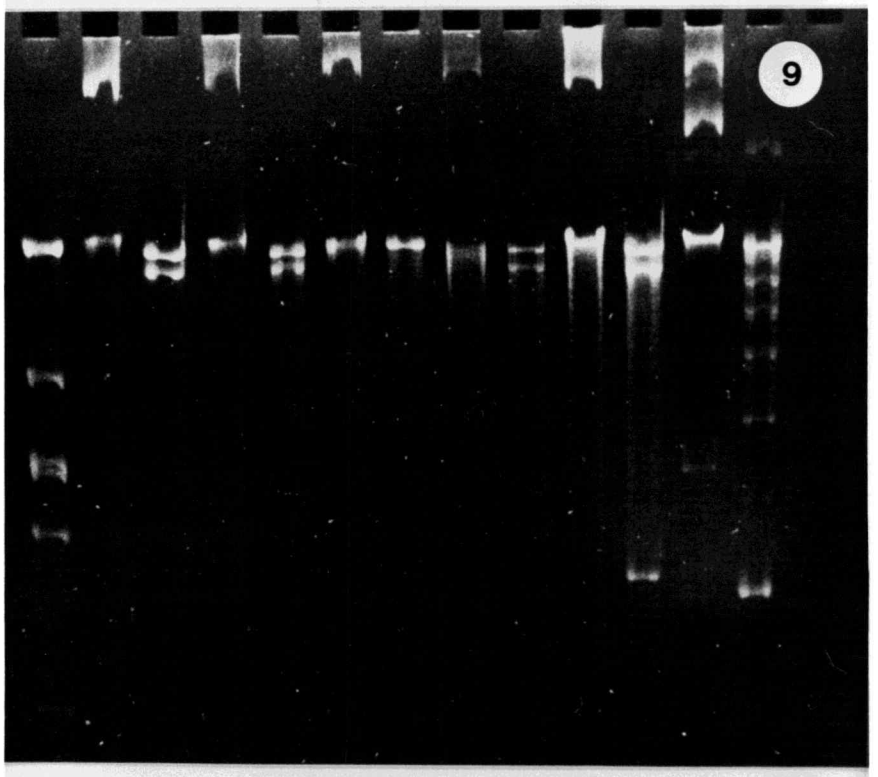
This 1.5% agarose gel contains from left to right in the used wells: (1) λ Eco RI fragments, (2) R6K from E. coli C600-undigested, (3) R6K from E. coli C600-digested, (4) R6K from E. coli W3110N (strain B)-undigested, (5) R6K from E. coli W3110N (strain B)-digested, (6) R6K from E. coli W3110N (strain C, Sm^S)-undigested, (7) R6K from E. coli W3110N (strain C, Sm^S)-digested, (8) R6K from E. coli CSH 56-undigested, (9) R6K from E. coli CSH 56-digested, (10) R6K from E. coli W3110C-undigested, (11) R6K from E. coli W3110C-digested, (12) R6K from Klebsiella 190-3-undigested, (13) R6K from Klebsiella 190-3-digested. Electrophoresis for 27 hours at 50 mA and 55 V.

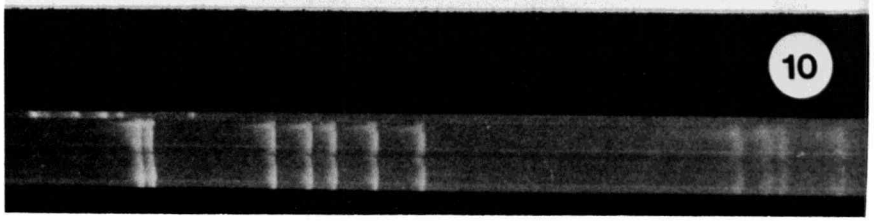
Figure 10

R6K Hae III pattern.

This 2% tube gel shows a typical pattern for the seven larger fragments used for comparative analysis. The two tubes are identical plasmid digests. Electrophoresis for 15 hours at 5 mA per tube.


8


9


10

in further evaluating the utility of endonucleases as an epidemiological tool in R plasmid identification. For this evaluation it was important to use well characterized plasmids representing different incompatibility groups, those within the same group, plasmids of limited host range, known "promiscuous" plasmids, as well as those known to contain transposons.

Plasmid identification using endonucleases was difficult following transfer into a background of other plasmids (R6K strain F, R388 strain D and RP4 strain F). The R6K fingerprints from the environmental Klebsiella strain were not identical to the R6K fingerprints produced from other transconjugants. Perhaps the dramatic differences in fingerprints reflect the limited host range of this plasmid. The very low R6K Sm^r transfer frequency into the Klebsiella strain and the inability to transfer the plasmid out suggest that R6K underwent significant change while expressing stable streptomycin resistance. Although non transmissible, the Ap^r marker must have still been present on the plasmid since there was a single Bam HI cleavage, characteristic of TnA (43), produced when R6K from the strain was digested.

The E. coli RY 13 (R388) transconjugant provided a different challenge to this identification system. In this example we placed R388 into an organism containing a known restriction-modification system. The fingerprints generated with enzymes other than Eco RI suggested that the modification system did not interfere with an identification scheme of this type.

The E. coli GS (RP4) transconjugant fingerprinting emphasized another problem in plasmid identification. In this cross we placed a stringent replicating plasmid of broad host range into a clinical isolate strain which already contained other plasmids. Possibly due to reduced replication, the yield of RP4 DNA from this strain was significantly less than that obtained from other RP4 transconjugants. This result made RP4 identification in the plasmid background difficult. Possibly the S. enteritidis strain serving as an intermediate in the passage of RP4 influenced its ability to replicate in the E. coli GS strain. Our results suggest that it may be difficult to detect and fingerprint a stringent replicating plasmid in a background of relaxed replicating plasmids.

Plasmid transfer within a species and subsequent fingerprinting demonstrated both the aquisition and loss of genetic information. R6K isolated from strain C gave patterns indicating the loss of a segment of DNA while R6K from strain E appeared to gain a segment of DNA, possibly a TnA transposon, since this strain produced a plasmid with an additional Bam HI site. The loss of genetic information for R6K from strain C is possibly correlated with its failure to express streptomycin resistance.

Plasmid transfer from a different genus intermediate back into E. coli illustrated a very positive aspect of this identification system. Sa from strain E and RP4 from strain H apparently showed their reduced level of streptomycin resistance and loss of tetracycline resistance due to a function of expression, since their fingerprints were identical with those from the plasmids isolated from the other

strains in their transfer sequences. Thus the same plasmid may yield different phenotypes in different strains but still demonstrated to be identical with this technique. R388 from strains C and F, the S. enteritidis transconjugants, both demonstrated a different and consistent Hsu I fingerprint. However, upon transfer of R388 from S. enteritidis back into E. coli the previously obtained single site cleavage pattern was obtained. Since the four additional Hsu I fragments from these two strains did not appear in stoichiometric amounts, we suggest these results to be due to a change in modification which produced a heterogeneous population of plasmid molecules.

Because of the varying sensitivities of different endonucleases used in our plasmid digests, combination digests also proved useful. This is evident from the numerous single site cleavage examples in the plasmids studied. Especially significant in this regard was R6K strain C which showed only a single Bam HI site, therefore similar to the other R6K strains except E, yet which upon more exhaustive analysis appeared to have lost a piece of DNA.

The E. coli RY 13 (R388) strain Eco RI pattern further emphasizes the requirement for multiple and combination enzyme digests as a potential fingerprint could be obscured by a given modification system. Also mentioned in this context should be enzymes producing more cleavages and thus being more sensitive. R6K from strain F, fingerprinted with Hsu I, showed more fragments common with the other R6K patterns than when fingerprinted with the other enzymes used. Therefore, to attempt to demonstrate plasmid relatedness via a single

enzyme fingerprint has the potential to produce a false positive (indicating identity) or false negative (indicating lack of identity) result.

Because we were using larger plasmids (21 X 10^6 M.W. - 36 X 10^6 M.W.) and because of the choice of endonucleases used, it was not always possible to sum the fragment molecular weights as they were out of the range of migrational linearity in our gel systems. This is a significant limitation in this type of analysis as summation of fragments proves to be a good safeguard and accounting system.

Plasmid identification using sequence-specific endonucleases has numerous limitations. The choice, combination and number of enzymes used is very crucial to avoid obtaining a false positive or false negative result. Plasmid fingerprinting in a background of other plasmids can be difficult. Identification of a plasmid prepared from a strain containing a modification system may not be possible when using a given endonuclease. Sometimes plasmid isolation may be impossible.

There are significant positive features to an identification system of this type. Plasmids showing phenotypic changes can be shown to be identical with this technique. This method can possibly detect minor changes in DNA sequences that create or destroy new recognition sequences for the respective endonucleases used and that might be undetectable by other identification methods. It is also possible that DNA transposons can be detected with this technique. Unfortu-

nately, because of the purification methodology involved, the technique cannot be quickly performed which detracts from its value as an epidemiological tool in plasmid identification.

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