

Genetic and Physical Characterization of IncM Plasmid pBWH1 and Its Variance among Natural Isolates

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We present a genetic and physical characterization of the IncM plasmid pBWH1. A physical map was constructed for the enzymes *EcoRI*, *BamHI*, *SalI*, *BglII*, *HindIII*, *MstII*, and *XhoI*. A series of deletions and a series of subclones of pBWH1 were constructed and used to determine the locations on this map of the transfer region; the replication region; and the genes determining resistance to beta-lactams, chloramphenicol, the sulfonamides, and gentamicin. We compared 51 different isolates, including isolates which had lost individual antibiotic resistances or the transfer phenotype, and showed that variations occurred in all regions of the plasmid genome. Frequently, correlations could be made between phenotypic variation and variation of the *EcoRI* fragments which contained the gene determining that phenotype.

The plasmid pBWH1 is a 92-kilobase IncM plasmid that has a broad host range for gram-negative members of the family *Enterobacteriaceae* and carries four antibiotic resistance genes. These genes encode resistance to the aminoglycosides gentamicin, tobramycin, and kanamycin; to ampicillin and carbenicillin; to chloramphenicol; and to the sulfonamides (16). We have been monitoring the evolution of this plasmid and its derivatives at one location. Over a 10-year period, we obtained over 2,000 different isolates of strains carrying this plasmid as we followed the epidemic spread of pBWH1 at the Brigham and Women's Hospital in Boston.

We examined phenotypic changes and restriction fragment variations. Analysis of these variations in pBWH1 may extend an important technique in the field of molecular evolution. Restriction fragment variation of, for example, metazoan mitochondria is currently being used to ascertain phylogenetic relationships (7, 13). The results of these experiments can be compared with the relationships inferred from comparative morphology and the fossil record, but molecular data are available only for the present. The molecular evolution of bacterial plasmids is much faster, with significant changes appearing over relatively brief periods. Thus it is possible to monitor changing populations of plasmids through real time, as well as to extensively sample a population at any given time.

pBWH1 has been found in strains of six different genera of the family *Enterobacteriaceae*. Screening of many of these isolates has shown that the full phenotype of pBWH1, as well as a standard pattern of *EcoRI* restriction enzyme fragments, has persisted from 1976 to the present. A number of isolates, however, have shown significant phenotypic variations, losing resistance to an antibiotic or the ability to transfer the plasmid. There have also been many variations in the pattern of *EcoRI* fragments. We refer to these isolates as the pBWH1 series of plasmids. In this report we present a genetic and restriction enzyme map of pBWH1 and discuss variations in natural isolates in relationship to this map. We

show that the variations of *EcoRI* restriction enzyme sites indicate that variations occur throughout the plasmid molecule and are not centered on one or two sites, which is in contrast to the variations in some other well-characterized plasmids (14, 18, 21). This situation makes pBWH1 an ideal plasmid for the study of molecular evolution.

MATERIALS AND METHODS

Bacterial and bacteriophage strains and plasmids. Clinical isolates of strains carrying the plasmid pBWH1 are listed in Table 1. In conjugation experiments, pBWH1 and its derivatives were transferred into the *Escherichia coli* K-12 strain SY663 ($\Delta trp met his hsdR gyrA rpoB$) (nomenclature is according to Bachmann [1]). Insertion mutagenesis with Tn5 and Tn10 was performed with the bacteriophages $\lambda 421$ (*b221 Oam29 Pam80 rex::Tn5 cI857*) and $\lambda 440$ (*b221 Oam29 cI857 cIII::Tn10*), respectively, using procedures described previously (5). Plasmids used in this study are listed in Table 2.

Growth media. LB medium was used for liquid cultures, and yeast-tryptone plates were used for solid medium (12). Antibiotics were added as needed at the following concentrations: carbenicillin, 200 $\mu\text{g/ml}$; chloramphenicol, 20 $\mu\text{g/ml}$; gentamicin, 15 $\mu\text{g/ml}$; kanamycin, 30 $\mu\text{g/ml}$; nalidixic acid, 15 $\mu\text{g/ml}$; rifampin, 35 $\mu\text{g/ml}$; tetracycline, 20 $\mu\text{g/ml}$. In sulfadiazine resistance experiments minimal medium plates made with M9 salts (12) containing the required amino acids, 0.2% glucose, and 200 μg of sulfadiazine per ml were used.

Plasmid DNA isolation. Plasmid DNA was isolated from 5 ml of late exponential phase cultures by the alkaline sodium dodecyl sulfate lysis method described by Birnboim and Doly (2). Larger quantities were prepared by proportionally scaling up the same procedure. DNA for *Bal* 31 degradation experiments was prepared from 2-liter cultures by the same procedure and then purified by centrifugation in a CsCl density gradient containing ethidium bromide.

Enzyme digestions. Restriction enzymes, T4 DNA ligase, and nuclease *Bal* 31 were obtained from New England Biolabs (Beverly, Mass.).

Restriction enzyme digestions and ligation reactions were

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TABLE 1. Natural isolates of pBWH1

Isolate	Bacterial species	Year of isolation
Cm Su Gm Ap Tra ⁺		
pBWH1	<i>Klebsiella pneumoniae</i>	1975
1856 ^a	<i>Enterobacter cloacae</i>	1981
1251	<i>Enterobacter cloacae</i>	1980
4292 ^a	<i>Enterobacter cloacae</i>	1981
9725	<i>Enterobacter cloacae</i>	1981
1370	<i>Klebsiella pneumoniae</i>	1983
5580	<i>Enterobacter cloacae</i>	1980
5095	<i>Enterobacter cloacae</i>	1981
7207	<i>Enterobacter cloacae</i>	1981
2150	<i>Escherichia coli</i>	1980
1035	<i>Serratia marcescens</i>	1976
1027	<i>Klebsiella pneumoniae</i>	1976
1010	<i>Klebsiella pneumoniae</i>	1976
1050	<i>Enterobacter aerogenes</i>	1976
1080	<i>Klebsiella pneumoniae</i>	1977
1090	<i>Morganella morgani</i>	1977
1175	<i>Klebsiella pneumoniae</i>	1980
1176	<i>Enterobacter aerogenes</i>	1980
1177	<i>Serratia marcescens</i>	1980
1178	<i>Serratia marcescens</i>	1980
24259	<i>Enterobacter aerogenes</i>	1980
3351	<i>Enterobacter cloacae</i>	1980
5095	<i>Klebsiella pneumoniae</i>	1981
1190	<i>Klebsiella pneumoniae</i>	1978
5360	<i>Klebsiella pneumoniae</i>	1980
1074	<i>Serratia marcescens</i>	1976
Cm Su Gm Tra ⁺		
1180	<i>Klebsiella pneumoniae</i>	1979
10434	<i>Serratia marcescens</i>	1981
1055	<i>Klebsiella pneumoniae</i>	1976
1382	<i>Serratia marcescens</i>	1983
1377	<i>Klebsiella pneumoniae</i>	1983
Su Gm Ap Tra ⁺		
1179	<i>Morganella morgani</i>	1979
1157	<i>Klebsiella oxytoca</i>	1980
5551	<i>Enterobacter cloacae</i>	1981
3366	<i>Enterobacter cloacae</i>	1982
Cm Su Gm Ap Tc Tra ⁺		
1159	<i>Enterobacter cloacae</i>	1980
1160	<i>Klebsiella oxytoca</i>	1980
1092	<i>Citrobacter freundii</i>	1976
1033	<i>Klebsiella pneumoniae</i>	1976
Cm Su Gm Ap Tra ⁻		
10311	<i>Serratia marcescens</i>	1981
12475	<i>Enterobacter cloacae</i>	1981
14561	<i>Enterobacter cloacae</i>	1981
14933	<i>Enterobacter cloacae</i>	1981
4989	<i>Enterobacter cloacae</i>	1981
17033	<i>Enterobacter cloacae</i>	1981
Cm Su Gm Tra ⁻		
14558	<i>Enterobacter cloacae</i>	1981
14781	<i>Enterobacter cloacae</i>	1981
10025	<i>Enterobacter cloacae</i>	1981
8185	<i>Enterobacter cloacae</i>	1981
6471	<i>Enterobacter cloacae</i>	1981
14933	<i>Enterobacter cloacae</i>	1981

^a For these isolates and for Tra⁻ isolates, plasmid DNA was prepared directly from the original isolates. For all other isolates and for Tra⁺ isolates, plasmid DNA was prepared from SY663 transconjugants.

performed according to the specifications of the manufacturer. *Bal* 31 digestions were performed at 30°C for various periods of time by the method of Legerski et al. (8).

The sizes of pBWH1 fragments of 8 kilobases or less were determined by analytical electrophoresis through a 0.7% agarose gel alongside a standard of bacteriophage λ *Hind*III fragments. Sizes of larger pBWH1 fragments were determined against a standard of bacteriophage λ *Hind*III and *Sal*I fragments, using a 0.4% agarose gel. Fragment sizes of variants of pBWH1 were determined against a standard of pBWH1 *Eco*RI fragments.

Analysis of pBWH1 variants. Isolates containing the plasmid pBWH1 were initially located by routine antibiotic susceptibility testing, using commercially prepared disks (BBL Microbiology Systems, Cockeysville, Mo.) and 150-mm-diameter Mueller-Hinton plates (GIBCO Diagnostics, Madison, Wis.). Zone diameters in millimeters were recorded, filed on a computer, and analyzed as described previously (16). pBWH1 was identified in these isolates by its characteristic antibiotype (16). Thirty-six random isolates that exhibited this antibiotype were shown by plasmid isolation and restriction enzyme analysis to contain pBWH1 (9). For a number of isolates, the relationships between the plasmids were corroborated by examining the patterns of restriction enzyme fragments produced after *Pst*I or *Pvu*II digestion.

RESULTS

Mapping of pBWH1. We used the plasmid of an early isolate with the complete phenotype Gm Su Ap Cm Tra⁺ (Tables 1 and 2) as the standard pBWH1 for developing physical and genetic maps. It had 15 *Eco*RI fragments denoted as bands A through O in order of decreasing size (Fig. 1). These *Eco*RI fragments were a total of 91.8 kilobases, which is in agreement with the previously published size of the entire plasmid (16).

We determined the order of the *Eco*RI fragments and fragments generated by several restriction enzymes for which pBWH1 has only a few cleavage sites: *Bam*HI, *Sal*I, *Bgl*II, *Hind*III, *Mst*II, and *Xho*I (Fig. 1). The genetic map included the *Eco*RI fragments encoding the individual antibiotic resistances, the *tra* (transfer) region, and the origin-replication region (Fig. 2). The techniques used to develop simultaneously the physical and genetic maps included construction of a series of plasmid deletions, cloning of restriction enzyme fragments, insertion mutagenesis with Tn5 and Tn10, *Bal* 31 degradation, and digestion with multiple restriction enzymes.

Several of the restriction enzymes for which there are only

TABLE 2. Plasmids used in this study

Plasmid	Phenotype ^a	Reference or source
pMB9	Tc	17
pBWH1	Gm Su Cm Ap Tra ⁺	16
pJH3	Cm Tra ⁻	Deletion derivative of pBWH1
pJH5	Cm Tc Tra ⁻	Subclone of pBWH1 in pMB9
pJH6	Cm Tc Tra ⁻	Subclone of pBWH1 in pMB9
pJH9	Cm Tc Tra ⁻	Subclone of pBWH1 in pMB9
pJH13	Ap Tra ⁺	Deletion derivative of pBWH1
pJH14	Ap Cm Tra ⁺	Deletion derivative of pBWH1
pJH15	Ap Cm Tra ⁺	Deletion derivative of pBWH1
pJH17	Gm Su Tc Tra ⁻	Subclone of pBWH1 in pMB9
pJH20	Gm Su Cm Tra ⁺	Deletion derivative of pBWH1

^a Phenotype nomenclature is that of Novick et al. (15).

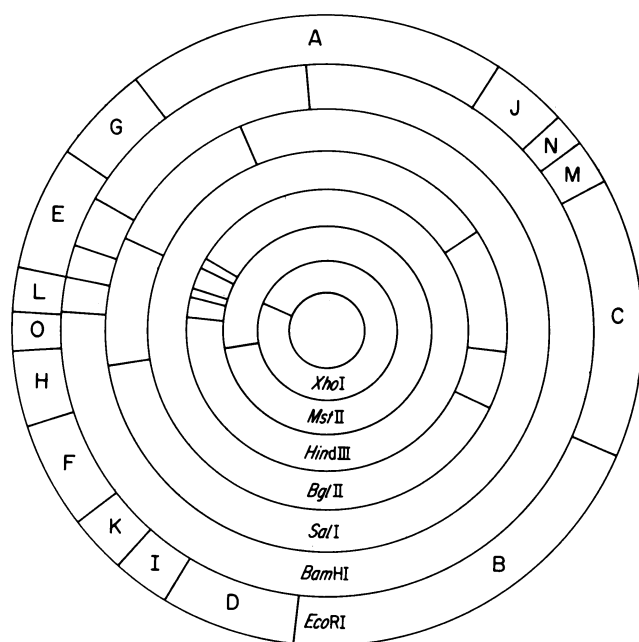


FIG. 1. Restriction enzyme map of pBWH1. The *EcoRI* fragments are designated A through O in order of decreasing size, respectively: 18.7, 17, 12.5, 8.6, 5.4, 5.1, 4.5, 4.4, 3.6, 3.3, 2.5, 1.8, 1.6, 1.3, and 1.2 kilobases.

a few cleavage sites in pBWH1 were used to construct a series of deletion plasmids (Fig. 2). This was done by digesting DNA separately with each of these enzymes, or with a combination of *Bam*HI and *Bgl*II, ligating the cleaved DNA, transforming *E. coli* SY203, selecting and purifying colonies resistant to individual antibiotics, and screening for clones that were missing at least one of the four pBWH1 resistance phenotypes. This experiment generated the deletion plasmids pJH3, pJH13, pJH14, pJH15, and pJH20 (Fig. 2 and Table 2). In addition, we prepared larger deletions of pBWH1 by cloning fragments of the plasmid into the tetracycline resistance vector pMB9 (16) to obtain pJH5, pJH6,

pJH9, and pJH17 (Fig. 2 and Table 2). These plasmids determined the order of many of the *EcoRI* fragments, leaving only the relative order of fragments within each of three sets (E-L, C-J-M-N, and D-I-K) undetermined. Because each plasmid carried only an incomplete set of antibiotic resistance genes and some were transfer deficient (*Tra*⁻), these subclones also revealed the locations of the antibiotic resistance genes and an approximation of the transfer and replication regions (Fig. 2). We positioned the replication incompatibility region in the *EcoRI* fragment B, next to the *tra* region, because a comparison of the set of pJH plasmids (Fig. 2) showed that this was the only region found in all plasmids with pBWH1 replication genes and in none of the plasmids with pMB9 replication genes.

Additional information was obtained by *Tn5* (Km) and *Tn10* (Tc) transposon mutagenesis of the deletion plasmids pJH13 and pJH20. Results of these insertions indicate that the ends of the *tra* region lie in *EcoRI* digest fragments A and B (Fig. 2).

Ambiguities in the order of some *EcoRI* digest fragments were resolved by the technique of Legerski et al. (8) by using progressive degradation by the exonuclease *Bal* 31, starting at unique restriction enzyme cleavage sites. For the plasmids pJH5-D::Tn5, pJH5-K::Tn5, pJH5-F::Tn5, pJH13-A::Tn10, M::Tn5 and pJH13-C::Tn10 J::Tn5, cleavage at the *Sal*I or *Xho*I sites within each *Tn5* insertion provided the starting point for *Bal* 31 degradation. In addition, digestion from the *Xho*I site in the E fragment of pJH20 was used.

Positions of cleavage sites for other digestions shown in Fig. 1 were determined by double digestions of the pJH deletion plasmids with *EcoRI* and each of these enzymes, as well as by double digestions with pairs of the enzymes.

Variations among derivatives of pBWH1. We examined the *EcoRI* fragment patterns of pBWH1 series plasmids from 51 separate isolates (Fig. 3). These included 26 isolates with the standard phenotype (Cm Su Gm Ap Tra⁺). The remaining isolates are grouped in five phenotypic classes: Ap^s, Cm^s, Tc, Tra⁻, and Ap^s Tra⁻. These 51 isolates do not represent a random collection from the pBWH1 series, as we specifically examined isolates with phenotypic variations. In Fig. 4A through F is summarized the restriction enzyme fragment

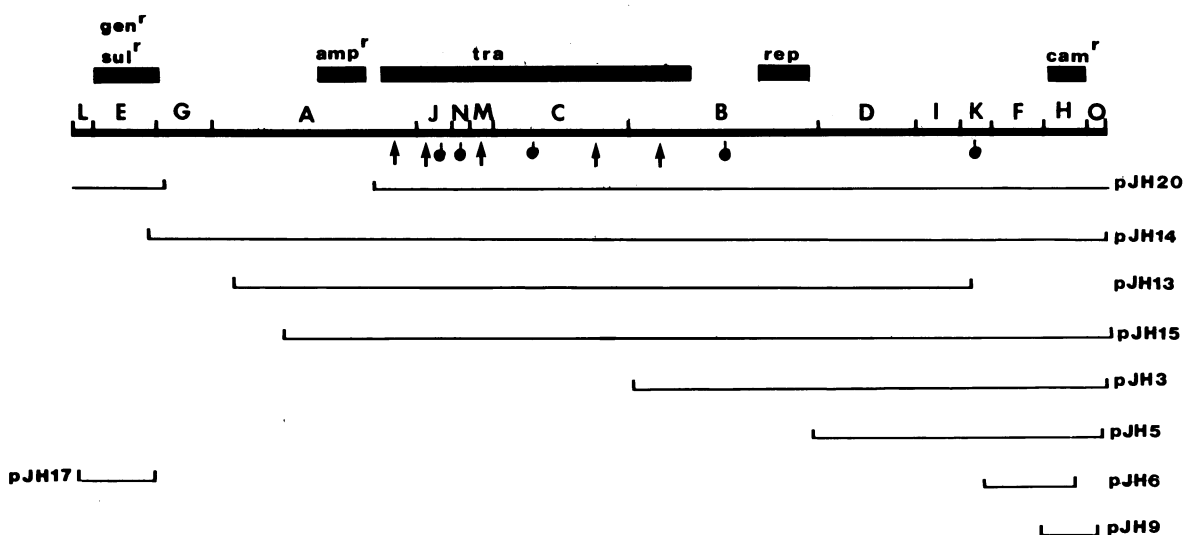


FIG. 2. Genetic map, deletion derivatives, and subclones of pBWH1 in relation to the *EcoRI* restriction enzyme fragment map. The genetic map is represented by the heavy bars above the *EcoRI* map. The lines below the *EcoRI* map show the pBWH1 DNA remaining in each pJH plasmid. pJH5, pJH6, pJH9, and pJH17 are subclones in pMB9. Phenotypes of the pJH plasmids are given in Table 2. *Tn10* inserts are denoted by arrows; *Tn5* inserts are denoted by closed circles.

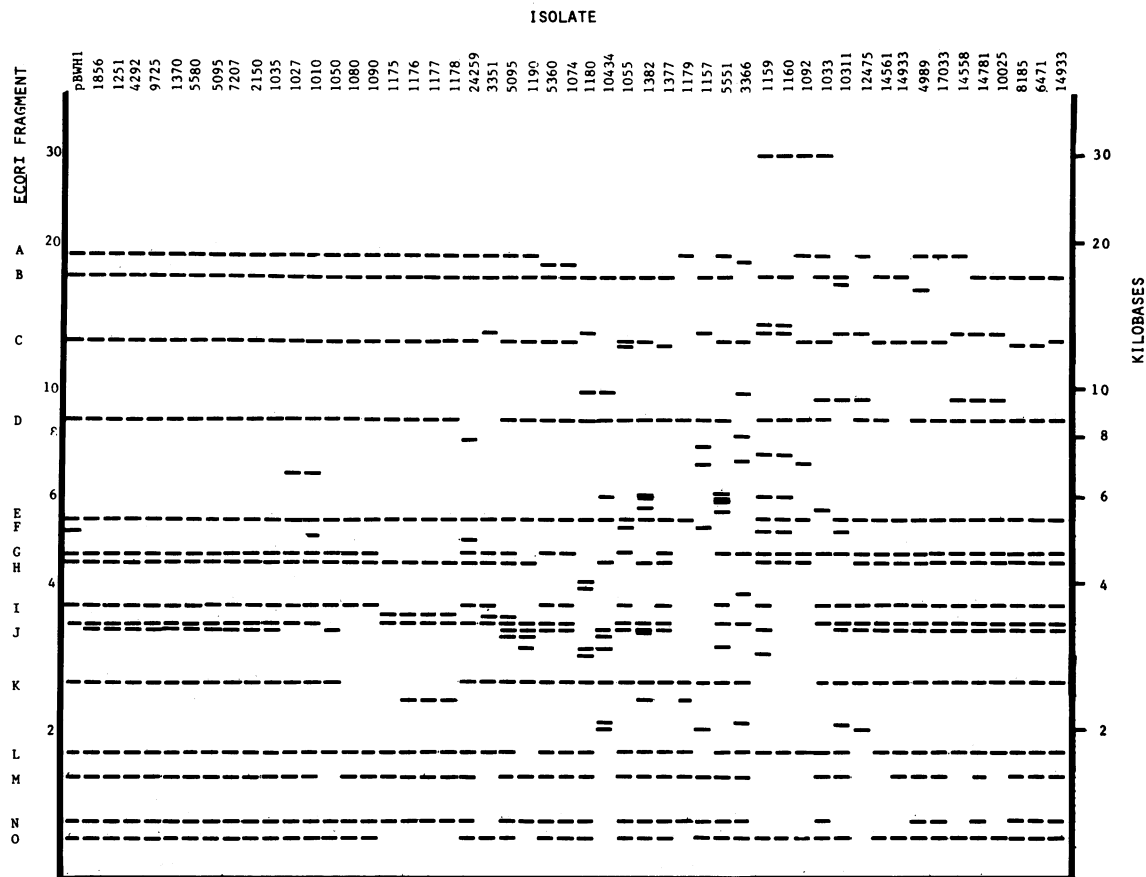


FIG. 3. DNA fragments produced on agarose gel electrophoresis of *EcoRI* digests of pBWH1 from 51 clinical isolates. The phenotype, bacterial species, and year of each isolate are given in Table 1.

data shown in Fig. 3, showing the variation of pBWH1 for each phenotype.

The 26 derivatives which retained the complete phenotype did not show a great deal of variation (Fig. 4A). Those with altered phenotypes showed a much greater degree of variation in their *EcoRI* restriction enzyme fragments, with there being some degree of clustering for each phenotype (Fig. 4B through F). In some cases the variations showed precise correlation with the phenotypic variation. For example, all five Ap^s and five of the six Ap^s Tra^- isolates showed a radical change in band A, which is consistent with the mapping data that place the TEM beta-lactamase gene in band A. All four Cm^s isolates were characterized by a change in, or loss of, band H, the site of the *cam* gene. The six Tra^- isolates with the standard antibiotic resistance phenotype showed a concentration of changes within the *tra* region, as did the Ap^s Tra^- isolates. In other ways, however, the *EcoRI* fragment variations did not exhibit a straightforward relationship with the phenotypic state. For example, most of the isolates that are Ap^s Cm^s or Tc while still Tra^+ showed extensive changes in the *EcoRI* fragments of the *tra* region. This region spans more than 20 kilobases, however, so there is ample opportunity for DNA sequence changes at *EcoRI* sites or even major deletions and insertions within *EcoRI* fragments without the loss of the Tra^+ phenotype.

DISCUSSION

The map of pBWH1 shows that the replication and origin genes of this plasmid are confined to a small region adjacent

to a single transfer region, while the antibiotic resistance genes lie in a separate segment of the plasmid genome. This structure is similar to that of F-like plasmids and contrasts with the more dispersed genetic organization of P-group plasmids (19, 20). In addition, pBWH1 has only a few recognition sites for several restriction enzymes. There is a concentration of recognition sites for several restriction enzymes in the antibiotic resistance gene region, especially in the genes for sulfonamide resistance and the 2''3-aminoglycoside nucleotidyl transferase (aminoglycoside resistance). In contrast, there are few or no cleavage sites for several restriction enzymes in the transfer and replication regions. It has been suggested that this distribution of sites reflects the evolution of broad-host-range plasmids (10, 11, 19).

The analysis of 51 variants of pBWH1 can be summarized by four points. (i) In the absence of any detectable phenotypic change, restriction fragment variations are frequently encountered; a majority of 26 isolates show at least one change from the prototype pBWH1 without the loss of any of the tested phenotypes. (ii) These changes do not cluster at any particular site, but rather, they seem uniformly distributed across the map (Fig. 4A). (iii) Those variants with altered phenotypes show a much higher degree of fragment variation (Fig. 4B through F). (iv) The variants displaying altered phenotypes usually show a clustering of restriction fragment changes.

Our results show that the pBWH1 variants analyzed in this study experienced chromosomal rearrangements such as

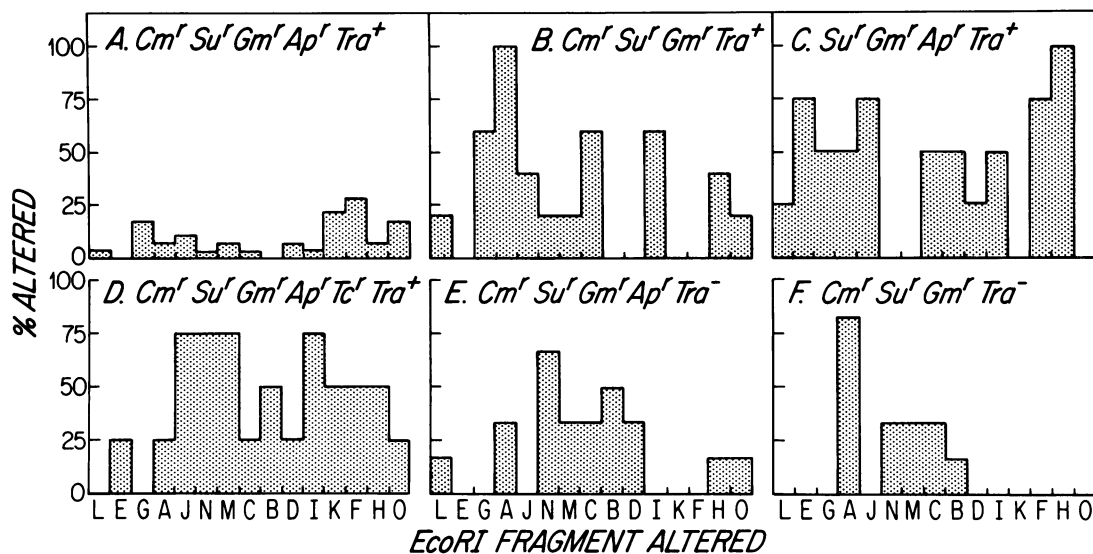


FIG. 4. Variability of *Eco*RI restriction enzyme fragments in six phenotypic classes of pBWH1 isolates. Bar heights indicate the percentage of isolates which have lost or altered a given *Eco*RI fragment. Fragments are in order but not to scale.

deletions, insertions, or inversions rather than base substitutions, since we did not find any two variants with a difference in restriction enzyme fragment pattern but no change in the plasmid size.

These properties of pBWH1 are in contrast with those of the IncW- and IncP-group plasmids. In these cases variations among a small number of isolates were analyzed by heteroduplex analysis and were found to be limited to discrete parts of the plasmid genome and often were associated with single deletions or insertions of antibiotic resistance transposons (4, 22). Results of several other analyses of plasmid variation have shown similar limited changes in plasmid structure centered on one or two insertion elements (14, 18, 21).

These previous examples fit into a picture of limited macroevolution: insertions, deletions, and, possibly, rearrangements, mediated by transposons and insertion sequence elements (3, 6, 21). Often the genetic changes directly relate to a phenotypic change, especially the gain of one (or more) additional resistance gene(s) by a transposition event. In the case of pBWH1, however, the map locations of the changes are so numerous that they cannot be explained by the presence of insertion elements at one or two sites on the plasmid genome. Finally, while many variations occurred, there persisted a single plasmid that we believe represents the ancestral plasmid for the entire pBWH1 series. It is possible that this archetypal plasmid encodes additional genes that maximize its viability in a hospital environment and that many of the variant plasmids are less stably maintained in this environment. Further studies or a large number of pBWH1 variants should enable us to carry out an analysis of the evolution of this plasmid.

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