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, Sall, BglII, HindIII, MstI, 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 carbencillin; 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 (Δtrp met his hsdR gyrA rpoB) (nomenclature is according to Bachmann [11]). Insertion mutagenesis with Tn5 and Tn10 was performed with the bacteriophages λ421 (b221 Oam29 Pam80 rex::Tn5 c1857) and λ440 (b221 Oam29 c1857 c111::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 μg/ml; chloramphenicol, 20 μg/ml; gentamicin, 15 μg/ml; kanamycin, 30 μg/ml; nalidixic acid, 15 μg/ml; rifampin, 35 μg/ml; tetracycline, 20 μg/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* Corresponding author.
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 HindIII fragments. Sizes of larger pBWH1 fragments were determined against a standard of bacteriophage λ HindIII and SalI fragments, using a 0.4% agarose gel. Fragment sizes of variants of pBWH1 were determined against a standard of pBWH1 EcoRI 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, Md.) 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 PstI or PvuII 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 EcoRI fragments denoted as bands A through O in order of decreasing size (Fig. 1). These EcoRI 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 EcoRI fragments and fragments generated by several restriction enzymes for which pBWH1 has only a few cleavage sites: BamHI, SalI, BglII, HindIII, MspI, and XhoI (Fig. 1). The genetic map included the EcoRI fragments encoding the individual antibiotic resistances, the tra (transfer) region, and the origin-of-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 1. Natural isolates of pBWH1

| Isolate | Bacterial species | Year of isolation |
|---------|------------------|-------------------|
| Cm Su Gm Ap Tra+ | Klebsiella pneumoniae | 1975 |
| pBWH1 | Enterobacter cloacae | 1981 |
| 1856C | Enterobacter cloacae | 1980 |
| 1251 | Enterobacter cloacae | 1980 |
| 42927 | Enterobacter cloacae | 1981 |
| 9725 | Klebsiella pneumoniae | 1983 |
| 1370 | Enterobacter cloacae | 1980 |
| 5580 | Enterobacter cloacae | 1980 |
| 2150 | Enterobacter cloacae | 1981 |
| 1035 | Enterobacter cloacae | 1981 |
| 5095 | Enterobacter cloacae | 1981 |
| 7207 | Enterobacter cloacae | 1980 |
| 1251 | Escherichia coli | 1980 |
| 1027 | Klebsiella pneumoniae | 1976 |
| 1010 | Klebsiella pneumoniae | 1976 |
| 1050 | Enterobacter aerogenes | 1976 |
| 1080 | Klebsiella pneumoniae | 1977 |
| 1090 | Morganella morganii | 1977 |
| 1175 | Klebsiella pneumoniae | 1980 |
| 1176 | Enterobacter aerogenes | 1980 |
| 1177 | Serratia marcescens | 1980 |
| 1178 | Serratia marcescens | 1980 |
| 24259 | Enterobacter aerogenes | 1980 |
| 3351 | Enterobacter cloacae | 1980 |
| 5095 | Klebsiella pneumoniae | 1981 |
| 1190 | Klebsiella pneumoniae | 1978 |
| 5360 | Klebsiella pneumoniae | 1980 |
| 1074 | Serratia marcescens | 1976 |
| Cm Su Gm Tra+ | Klebsiella pneumoniae | 1979 |
| 1180 | Serratia marcescens | 1981 |
| 10434 | Klebsiella pneumoniae | 1976 |
| 1055 | Morganella morganii | 1979 |
| 1382 | Serratia marcescens | 1983 |
| 1377 | Klebsiella pneumoniae | 1983 |
| Su Gm Ap Tra+ | Morganella morganii | 1979 |
| 1179 | Klebsiella oxytoca | 1980 |
| 1157 | Enterobacter cloacae | 1981 |
| 5551 | Enterobacter cloacae | 1982 |
| 3366 | Enterobacter cloacae | 1980 |
| Cm Su Gm Ap Tc Tra+ | Enterobacter cloacae | 1980 |
| 1159 | Klebsiella oxytoca | 1980 |
| 1160 | Citrobacter freundii | 1976 |
| 1092 | Citrobacter freundii | 1976 |
| 1033 | Klebsiella pneumoniae | 1976 |
| Cm Su Gm Ap Tra+ | Serratia marcescens | 1981 |
| 10311 | Enterobacter cloacae | 1981 |
| 12475 | Enterobacter cloacae | 1981 |
| 14561 | Enterobacter cloacae | 1981 |
| 14933 | Enterobacter cloacae | 1981 |
| 4989 | Enterobacter cloacae | 1981 |
| 17033 | Enterobacter cloacae | 1981 |
| Cm Su Gm Tra+ | Enterobacter cloacae | 1981 |
| 14558 | Enterobacter cloacae | 1981 |
| 14781 | Enterobacter cloacae | 1981 |
| 10025 | Enterobacter cloacae | 1981 |
| 8185 | Enterobacter cloacae | 1981 |
| 6471 | Enterobacter cloacae | 1981 |
| 14933 | Enterobacter cloacae | 1981 |

* 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.

### TABLE 2. Plasmids used in this study

| Plasmid | Phenotype* | Reference or source |
|---------|------------|---------------------|
| pMB9    | Tc         | 17                  |
| pBWH1   | Gm Su Ap Cm 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 |

* Phenotype nomenclature is that of Novick et al. (15).
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respectively:
a
a
DNA, transforming with digesting pBWH1.

2.16,

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 BamHI and BgIII, 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 Legereski 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 Sall or Xhol sites within each Tn5 insertion provided the starting point for Bal 31 degradation. In addition, digestion from the Xhol site in the E fragment of pJH20 was used.

Positions of cleavage sites for other enzymes 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+, Cm+, Tc, Tra+, and Ap+ 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. pJH3, 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.
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 and five of the six Ap 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 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 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 Cm 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
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 stably maintained in this environment. Further studies of a large number of pBWH1 variants should enable us to carry out an analysis of the evolution of this plasmid.

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