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Homologous Sequences Other Than Insertion Elements Can Serve as Recombination Sites in Plasmid Drug Resistance Gene Amplification

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A plasmid (pRR983) was constructed which has a gene coding for neomycin and kanamycin resistance flanked by direct repeats of regions of homology which contain no known insertion sequences. pRR983 does not have any homologous IS1 sequences. Growth of *Proteus mirabilis* harboring pRR983 in medium containing high concentrations of neomycin resulted in cells which were highly resistant to both neomycin and kanamycin. Plasmid DNA was analyzed by using restriction endonucleases. In most cases the neomycin resistance gene had been tandemly duplicated by using the homologous DNA sequences flanking the resistance gene as recombination sites. This is analogous to tandem duplication of drug resistance genes on NR1 using the two direct repeats of IS1 as recombination sites. The amplified plasmid DNA returned to its original structure by the deletion of amplified neomycin resistance determinants when the host cells were cultured without selection for high resistance to neomycin.

When bacterial cells which harbor certain antibiotic resistance plasmids are cultured in media containing high concentrations of the antibiotic to which resistance is conferred, there is often an increase in the copy number of the resistance genes. This increase may be a result of an increase in the copy number of the plasmid or of a selective amplification of only the specific regions carrying drug resistance genes. This selective amplification usually results in tandem duplication of the specific gene or genes on the plasmid. Selective amplification of genes carried by bacterial plasmids and some phages has been observed in several genera, including *Escherichia coli* (5, 23, 25, 45), *Proteus mirabilis* (35, 37), and *Streptococcus faecalis* (6, 47, 48).

Gene amplification has also been observed on bacterial chromosomes (2, 8); in recent years it has been observed in many eucaryotic systems after selection for high drug resistance (1, 24, 30) and also as a naturally occurring event in cellular development (40; reviewed in 41, 49). Gene amplification also seems to be associated with the presence in eucaryotic cells of homogeneously staining regions (30) and double minute chromosomes (17). Gene amplification has been specifically associated with leukemia cells (10), and homogeneously staining regions and double minute chromosomes have been observed in many tumor cell lines (12).

NR1 is a 90-kilobase (kb) bacterial plasmid of the FII incompatibility group which codes for resistance to chloramphenicol, fusidic acid, streptomycin, spectinomycin, sulfonamides, tetracycline, and mercuric ions (29). It has a copy number of 1 to 2 per chromosome in enteric bacteria. The resistance genes other than that for tetracycline are grouped together on a segment of the plasmid DNA called the resistance determinant, or r-determinant. The r-determinant is bounded on each end by a single copy of the insertion element IS1 which is in direct repeat orientation (16). In *P. mirabilis* all resistance genes of NR1 except that for tetracycline resistance are amplified when the cells are cultured in medium containing high concentrations of appropriate antibiotics (35, 37, 42). The insertion elements are the recombination sites which mediate the tandem duplication of the r-determinant genes (36, 42), which has been termed a transition (35).

Several models have been proposed for the molecular mechanism of this type of gene amplification (7, 36). Those for NR1 require a recombination between the IS1 elements as one of the initial events. Recombination between the insertion elements also mediates the deletion of the r-determinant which occurs in *Salmonella typhimurium* (36, 44).

Since insertion elements and transposons possess a number of remarkable properties, we have examined whether IS1 has an essential role in the amplification of the r-determinant of a derivative of NR1. The conclusions would pre-

sumably apply to NR1 as well. In this study, we have demonstrated amplification of an antibiotic resistance gene bounded by regions of homology which contain no known insertion sequences. This suggests that the IS1 elements are not absolutely necessary for the transition. They may simply serve as regions of homology for the initial recombinational events during a transition.

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MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Cells were usually cultured at 37°C in Penassay broth (Difco Laboratories) supplemented with thymine (20 μg/ml). Sodium ampicillin, neomycin sulfate, kanamycin sulfate, and chloramphenicol were purchased from Sigma Chemical Co. In constructing most of the plasmids used in this study, the stability (*stb*) locus of NR1 (34) was deleted, resulting in plasmids which were not stably maintained in the cell. Therefore, growth was usually carried out in the presence of a low concentration of one of the antibiotics to select for cells carrying the plasmid. Cells were plated onto nutrient agar (Difco) containing the various antibiotics.

Ligation and transformation. Transformation was performed as described by Lederberg and Cohen (19). Ligations were done as previously described (26).

Isolation and characterization of plasmid DNA. Plasmid DNA was isolated and purified from stationary-phase *E. coli* cells by a Triton X-100 cleared lysate procedure (18) or by the small-lysate procedure of Birnboim and Doly (3). DNA was isolated from whole-cell lysates of stationary-phase *P. mirabilis* cells by a modification of the procedure of Womble et al. (46). Plasmid DNA was separated by cesium chloride-ethidium bromide buoyant density centrifugation in a Beckman VTi50 rotor for 24 to 48 h at 40,000 rpm. Plasmid DNA was further purified by phenol extraction before restriction analysis.

Restriction endonucleases *Eco*RI, *Sal*I, *Pst*I, *Sst*I, *Bam*HI, and *Hin*dIII were purchased from Bethesda Research Laboratories, Inc. Digestion of plasmid DNA was performed in 0.1 M Tris–0.05 M NaCl–0.005 M $MgCl_2$ (pH 7.4) at 37°C for 1 to 4 h.

Agarose gel electrophoresis was carried out by the method of Helling et al. (15). Agarose gels (0.7 or 1%) were electrophoresed at 10 V for 15 to 20 h or at 70 V for 2 to 4 h. DNA was visualized by staining the gels with ethidium bromide (1 μg/ml) and illuminating with a short-wave UV transilluminator. Pictures were taken with Polaroid type 57 film or, when a negative was desired, with Polaroid type 55 film. To quantitate the amount of DNA in each band, negatives were traced with a Zeineh soft laser scanning densitometer coupled to a Hewlett-Packard model 3390A reporting integrator.

Assay for CAT. Cells were grown to an optical density at 650 nm of 0.6, and 1 ml of the culture was harvested by using an Eppendorf table-top centrifuge. The cells were washed in cold 0.1 M Tris (pH 7.0), resuspended in 1 ml of the same buffer, lysed by sonication for 1 min with a Fisher Model 300 sonic dismembrator, and then centrifuged in an Eppendorf table-top centrifuge for 1 min to remove cell debris. The supernatant was collected and assayed for chloramphenicol acetyltransferase (CAT) as described by Foster and Shaw (11) and Shaw and Brodsky (39). A culture of NR1-containing cells (copy number defined to be 1 per chromosome) was used as a standard. Protein concentration was determined by the method of Bradford (4).

RESULTS

Plasmid construction. Plasmid construction was carried out with *E. coli* KP245 as a host. The R plasmid pRR933 consists of the 1.1- and 1.6-kb *Pst*I fragments comprising the minimal replicator of NR1 (27) plus the 2.1-kb chloramphenicol resistance *Pst*I fragment of an NR1 deletion mutant (pRR218 [36]) which has lost all but a small part of its r-determinant. The chloramphenicol resistance *Pst*I fragment is bounded by nonhomologous partial IS1 sequences since *Pst*I, which has a site in IS1, was used in the cloning. Tn*3* was transposed into the 1.6-kb fragment of this plasmid to form pRR728. The *Eco*RI fragment of pML31 conferring neomycin and kanamycin resistance (the *neo/kan* fragment) was then inserted into pRR728 to form pRR983. pRR983 was constructed by A. Easton in this laboratory. The *neo/kan* fragment was initially derived from the R plasmid R6-5, which is closely related to NR1 (38). In addition to the *neo/kan* gene, this *Eco*RI fragment contains the part of the sequence for the *cam* gene present on *Eco*RI fragment J of NR1 plus other fragment J sequences. Therefore, insertion of the *neo/kan* fragment of pML31 into the *Eco*RI site of pRR728 with selection for chloramphenicol resistance, ampicillin resistance, and neomycin resistance selects for a reconstructed *cam* gene carried on the pRR728 replicator. In pRR983 there is 841 base pairs of directly repeated DNA sequence homology derived from *cam* DNA and part of fragment J flanking the *neo/kan* gene (Fig. 1). There is a complete *cam* gene on one side of the *neo/kan* gene and a partial *cam* gene, which is designated Δ*cam*, on the other.

We attempted to construct a plasmid which would contain the *neo/kan* gene inserted into pRR728 in the opposite orientation. This would result in a plasmid conferring resistance to ampicillin, neomycin, and kanamycin but not to chloramphenicol. Digestion of pRR728 and pML31 with *Eco*RI, followed by ligation and transformation of KP245, yielded one chloramphenicol-sensitive colony among the approximately 100 ampicillin- and neomycin-resistant colonies screened. The rest were also resistant to chloramphenicol. This plasmid (pRR312) was

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Marker[a]	Genotype or description	Reference or source
E. coli KP245		*met his trp thy lac gal*	26
P. mirabilis φS38		*nic thy lac gal* Tc^s	13
pRR933	Cm^r		27
pRR728	Ap^r, Cm^r		A. Easton, this laboratory
pML31	Kan^r, Neo^r		20
pRR983	Ap^r, Cm^r, Kan^r, Neo^r		A. Easton
pRR310	Ap^r, Cm^r, Kan^r, Neo^r	copy mutant of pRR983	This paper
pRR312	Ap^r, Kan^r, Neo^r		This paper

[a] Resistance markers: Ap, ampicillin; Cm, chloramphenicol; Neo, neomycin; Kan, kanamycin; Tc, tetracycline. Neomycin and kanamycin resistance are coded for by the same enzyme.

analyzed by gel electrophoresis, and it was found that the pML31 replicator was also inserted into the plasmid between the *cam* promotor and the *neo/kan* gene (data not shown). From this we conclude that it is not possible to insert the *neo/kan* fragment in this orientation immediately next to the *cam* promotor and be able to get replication of a plasmid conferring neomycin resistance. This may be due to interference of the transcription of the *neo/kan* gene by transcription from the strong *cam* gene promotor. Similar phenomena have been observed previously in this laboratory (R. Wu and J. Kasner, unpublished results) and in the bacteriophage lambda system (43).

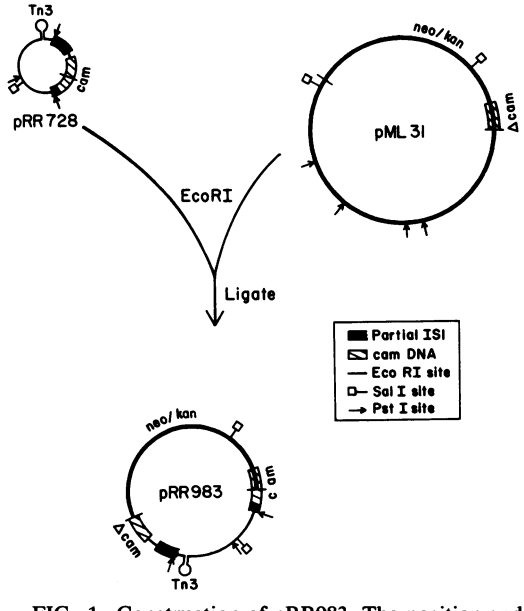

FIG. 1. Construction of pRR983. The position and orientation of Tn*3* in pRR728 were determined by digestion of pRR728 with *Pst*I, *Sst*I, and *Bam*HI and digestion of pRR983 with *Sal*I. The restriction sites of Tn*3*, not shown in this figure, were determined from its sequence (14). Tn*3* is not drawn to scale. The restriction sites of pML31 have been previously described (9, 20). There are 661 bases of *cam* DNA plus 180 base pairs of NR1 fragment J homology flanking the *neo/kan* gene. The size of the *cam* gene was determined from the sequence of Tn*9* (22). Δ*cam* is the part of the *cam* DNA downstream from the *Eco*RI site.

Amplification of the *neo/kan* resistance gene of pRR983 in *P. mirabilis*: genetic characterization. pRR983 was transformed into the *P. mirabilis* host φS38. φS38(pRR983) was cultured in Penassay broth (Difco) containing 20 μg of thymine and 20 μg of ampicillin per ml (to select for cells carrying plasmid DNA). Stationary-phase cells were spread onto nutrient agar (Difco) plates containing thymine (20 μg/ml) and various concentrations of chloramphenicol, ampicillin, neomycin, or kanamycin to determine the level of resistance to the various antibiotics (data not shown). The plating efficiencies were less than 100%, even on low concentrations of antibiotics, presumably due to the presence of some R^- cells in the population which arise due to the plasmids' lack of the *stb* locus of NR1, which confers plasmid stability (34). This was reflected in the average copy numbers, which were less than 1 per chromosome in both *E. coli* and *P. mirabilis*, as determined using CAT assays. To confirm this, colonies were picked from a drug-free plate to one containing a low concentration of ampicillin (20 μg/ml). Only 53% were found to be resistant to the antibiotic, which agrees with the CAT copy number determination, which indicated an average copy number of 0.6 per chromosome.

For colony formation on a plate containing a high antibiotic concentration, the level of resistance of the cells must be increased through a copy number mutation, a promotor mutation, a mutation in the host cell chromosome, or by gene amplification. Since the cells were relatively more sensitive to neomycin than they were to kanamycin, 200 μg of neomycin per ml was chosen to attempt to select for cells with increased levels of antibiotic resistance. Strain φS38(pRR983) had a plating efficiency of about 10^{-5} on 200 μg of neomycin per ml. φS38(pRR983) was repeatedly subcultured in

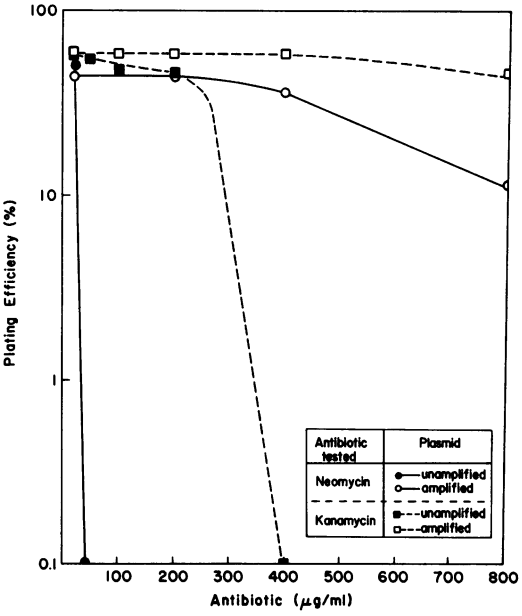

FIG. 2. Plating efficiency on neomycin and kanamycin of *P. mirabilis* containing unamplified and amplified pRR983. Cells were grown into stationary phase, and appropriate dilutions were spread onto plates. Cells containing amplified pRR983 were picked off plates containing 200 μg of neomycin per ml and grown in liquid medium containing 200 μg of neomycin per ml.

media containing 200 μg of neomycin per ml, diluted 10^6- to 10^8-fold in each subculture, and then spread onto plates containing 200 μg of neomycin per ml. Alternately, φS38(pRR983) was simply cultured in medium containing 20 μg of ampicillin per ml and spread directly onto plates containing 200 μg of neomycin per ml. The first method presumably selects for high antibiotic resistance through the outgrowth of cells which are more highly resistant, possibly through spontaneous amplification of antibiotic resistance genes. The second method presumably selects cells which have spontaneously increased their resistance without prior exposure to a high concentration of the antibiotic.

Ten single colonies were picked, cultured in medium containing 200 μg of neomycin per ml, and again spread onto plates containing various concentrations of chloramphenicol, ampicillin, neomycin, or kanamycin (Fig. 2). The level of resistance to ampicillin and chloramphenicol remained unchanged in all but one instance. This indicated that, in most cases, the expression of the *neo/kan* resistance gene had been selectively increased. These data rule out copy mutations (where we would expect all the antibiotic resistances to increase). In one case there was an increase in all the antibiotic resistances (data not shown).

Plasmid DNA isolated from highly resistant cells. The highly resistant cells from all 10 isolates were cultured in media containing 200 μg of neomycin per ml, and plasmid DNA was isolated, purified, and digested with appropriate restriction enzymes. A gel showing the restriction patterns of a representative DNA as compared to pRR983 isolated from a culture grown in a low antibiotic concentration is shown in Fig. 3A. In each digest of DNA from highly resistant cells there was a new fragment which was not present in φS38(pRR983). Tandem duplication produces a new junction at the site at which recombination has occurred between the direct repeats which is not present on the unamplified plasmid. The novel fragment in the *Sal*I and the *Hin*dIII digests are both 6.8 kb in size and that in the *Sal*I-*Hin*dIII double digest is 5.2 kb in size. These are the expected results from a plasmid with tandemly repeated *neo/kan* sequences (Fig. 3B). Of the 10 isolates, 9 had this restriction pattern. The plasmid DNA from the strain showing an increase in all the antibiotic resistances did not have any novel restriction fragments, and the bands present had relative intensities as expected for their sizes. This plasmid, pRR310, is presumably a copy number mutant of pRR983 (see below).

The intensities of the *Sal*I novel fragment bands with respect to bands representing unamplified DNA fragments in the same gel lane differed in the separate isolates of highly resistant cultures (data not shown). Negatives from these gel pictures were traced, and the relative amount of DNA in each band was determined. The estimated extent of amplification varied from a 2-fold to a 15-fold increase in the number of *neo/kan* determinants. Most were around two- to threefold.

These restriction data proved that there were multiple copies of the *neo/kan* determinant present in the cell but did not prove that the plasmid DNA consisted of tandem repeats of the *neo/kan* determinant, as presented in Fig. 3B. The previous data are also consistent with the situation in which pRR983 is present in its original form along with multiple copies of monomeric circular *neo/kan* determinants. This possibility was tested by digesting the plasmid DNAs with the restriction endonuclease *Pst*I (Fig. 4). This enzyme does not have a restriction site within the amplified region, so multiple copies of a circular monomer could be distinguishable from tandemly repeated *neo/kan* determinants. The bands corresponding to the *neo/kan* fragments migrated more slowly in the plasmid DNA isolated from highly resistant cells, indicating that the *neo/kan* determinants existed as tandem repeats.

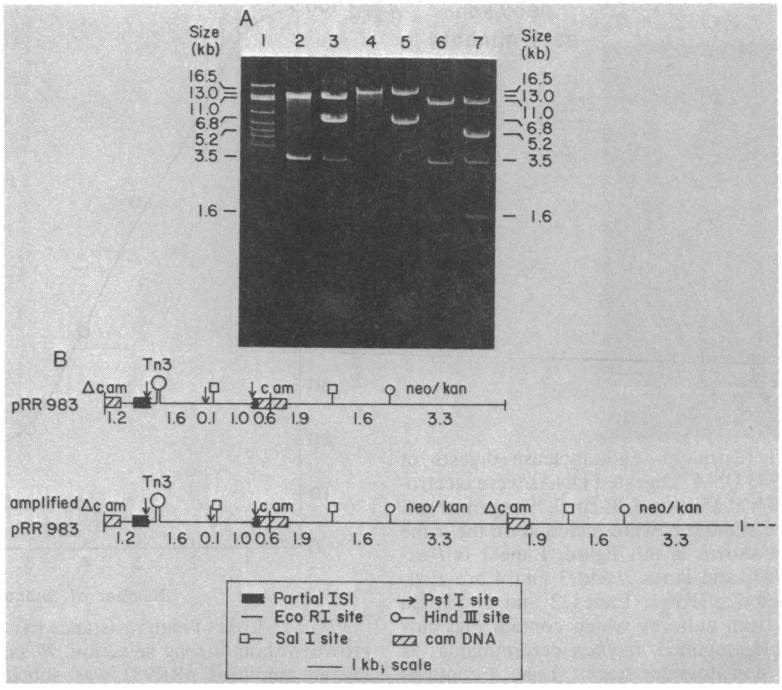

FIG. 3. Restriction endonuclease digest and map of unamplified and amplified pRR983. (A) *Eco*RI-digested NR1 DNA size standards (lane 1). pRR983 was digested with *Sal*I (lane 2), *Hin*dIII (lane 4), and *Sal*I plus *Hin*dIII (lane 6). Amplified pRR983 was digested with *Sal*I (lane 3), *Hin*dIII (lane 5), and *Sal*I plus *Hin*dIII (lane 7). (B) Restriction map of unamplified and amplified pRR983. The amplified pRR983 is shown with only two *neo/kan* determinants. Tn*3* is not drawn to scale, nor are its restriction sites shown. Numbers refer to the distance between the restriction sites in kb (not including Tn*3*, which is 4,957 base pairs in size [14]).

There was also a minor fraction of fragments corresponding to lower degrees of amplification in the cells containing highly amplified plasmids. This indicated that the DNA in a single lane represented a heterogeneous population of cells which differ in their degree of amplification, although most were of the more highly amplified type. These estimates agree with the degree of amplification *Sal*I digests as described previously.

Wild-type NR1 will not undergo drug resistance gene amplification in *E. coli*. When *E. coli* cells carrying NR1 are cultured in medium containing high concentrations of chloramphenicol or streptomycin, copy mutants are selected (28). There are mutants of NR1 which have deleted part of the r-determinant which do amplify in *E. coli* (36). Therefore, we attempted to amplify pRR983 in *E. coli* KP245. KP245(pRR983) had a plating efficiency on different levels of neomycin identical to that of ϕS38(pRR983) (data not shown). Amplification was attempted in the same ways as was for ϕS38(pRR983). DNA isolated from cells which grew on plates containing 200 μg of neomycin per ml had restriction patterns identical to that of pRR983 isolated from cells cultured in medium containing low levels of antibiotic (data not shown). Overnight cultures grown from freezer stocks of KP245(pRR983) grew very poorly in media containing 100 μg of neomycin per ml and not at all in 200 μg of neomycin per ml. ϕS38(pRR983) grew very well at either concentration.

Instability of the amplified state of pRR983. The *Pst*I-digested DNA isolated from cells grown in a high concentration of neomycin were heterogeneous in their degrees of amplification, as indicated by the multiple *neo/kan* determinant bands in DNA from a single clone (Fig. 4). This suggested that plasmid DNA in the population and in single cells was in a dynamic state in which the number of *neo/kan* determinants on a single plasmid can fluctuate. Since this seemed similar to the "back transition" of NR1 (13, 35), the analogous experiment was done for pRR983. A strain which carried a plasmid with about 15 copies of the *neo/kan* determinants (as determined by tracing negatives of a gel of *Sal*I-digested DNA) was cultured in medium containing a low concentration of ampicillin to maintain the unstable (*stb*$^-$) plasmid replicator. Each subculture was diluted 10^6-fold into fresh medium and plated onto nutrient agar containing a low concentration of ampicillin. As controls, the

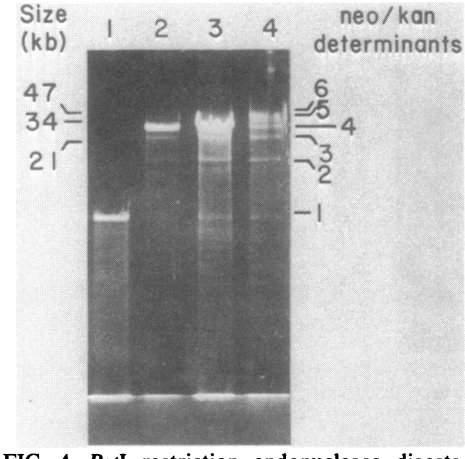

FIG. 4. *Pst*I restriction endonuclease digests of amplified pRR983 DNA. Digested DNAs were electrophoresed for 20 h at 15 V on a 20-cm 0.7% agarose gel. Lanes with size standards were included on the same gel but are not shown in this figure. Lane 1 is *Pst*I-digested pRR983, and lanes 2 and 3 and 4 are *Pst*I-digested amplified pRR983. Lanes 2 and 3 contain plasmid DNA from cultures which contain plasmids that had twofold-amplified *neo/kan* determinants, as determined by densitometer traces. Lane 4 contains plasmid DNA from a culture which contained a plasmid that was amplified fivefold, as determined by densitometer traces. The smaller fragments of the plasmid have electrophoresed off this gel. Numbers on the right indicate the location of *neo/kan* determinants of various degrees of amplification.

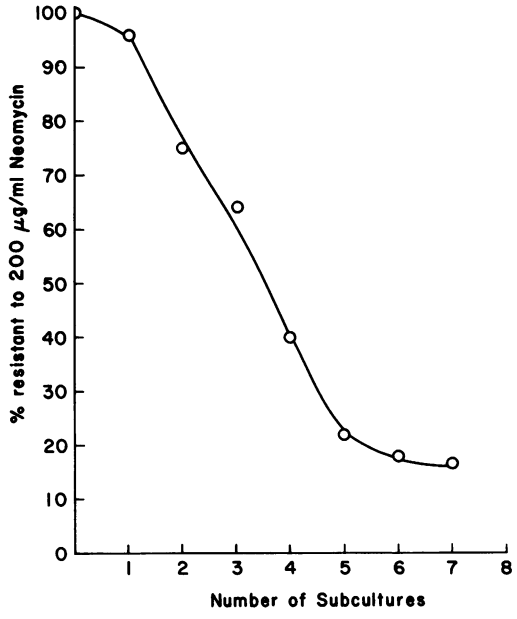

FIG. 5. Loss of high resistance to neomycin in cells grown without strong selection. *P. mirabilis* containing an amplified pRR983 was subcultured (10^6-fold dilutions) in medium containing a low concentration of ampicillin (20 μg/ml) to maintain the plasmid. Stationary-phase cells from each subculture were spread onto ampicillin-containing plates, and colonies were picked onto plates containing various concentrations of neomycin to determine the resistance level of the cells. Loss of resistance to high concentrations of neomycin corresponds to loss of *neo/kan* determinants (analogous to the back transition of NR1). The percentage indicated refers to the percentage of cells containing a plasmid which contain an amplified plasmid.

strain was also grown in 200 μg of neomycin per ml, and unamplified φS38(pRR983) was grown in media containing 20 μg of ampicillin. One hundred colonies were picked from the ampicillin-containing plates onto plates containing either 20, 100, or 200 μg of neomycin per ml to determine the level of neomycin resistance. Colonies containing unamplified pRR983 grew well at neomycin concentrations of 20 μg/ml, poorly on 100 μg/ml, and not at all on 200 μg/ml. Cells containing fully amplified pRR983 grew well on all the antibiotic concentrations (Fig. 5). These results indicate the percentage of the plasmid-containing cells which contain amplified plasmids, as defined by the screening used. The plating efficiency on a low concentration of ampicillin per milliliter was always about 65%, which presumably reflects the instability of the plasmid.

DNA from cultures which, after the nonselective growth described above, exhibited neomycin resistance similar to that of unamplified pRR983 was analyzed by restriction digests. DNA from these cultures had restriction patterns identical to that of unamplified pRR983. The decrease in antibiotic resistance was therefore a result of loss of *neo/kan* determinants.

Analysis of copy mutant pRR310. The copy number of plasmid pRR310 relative to NR1 was determined by using CAT assays. In different experiments the copy number in plasmid-containing cells varied. The copy number was 2.5 to 5.5 per chromosome when φS38(pRR310) was grown in media containing 200 μg of neomycin per ml, but only 1 to 3 per chromosome when grown in a low concentration of chloramphenicol (25 μg/ml) or ampicillin (20 μg/ml). When grown in the high antibiotic concentration, 100% of the cells contained plasmids, but only 82% of the cells grown at the lower antibiotic concentrations contained plasmids. This was taken into account in determining the copy numbers of the plasmids. Cells grown in 800 and 1200 μg of neomycin per ml had plasmid copy numbers of 4 to 6 and 5 to 9 per chromosome, respectively.

DISCUSSION

The amplification of antibiotic resistance genes on the r-determinant of the R plasmid NR1 in *P. mirabilis* involves recombination between

the IS1 sequences flanking the r-determinant. The insertion elements may play one or more roles in the phenomenon. First, they may provide sequences of homology for recombination in the initial stages of the transition. Second, they may serve as specific sequences that are acted on by other agents produced by the cell or by the plasmid which promote recombination, the end result being the tandem duplication of the r-determinant. This type of specificity of an agent for an insertion element is thought to occur in the transposition of Tn*5* (reviewed in reference 33). There is circumstantial evidence for the existence of host and/or plasmid factors in the transition of NR1. Wild-type NR1 can amplify in *P. mirabilis* but not in *E. coli*, whereas specific deletion mutants of NR1 do amplify in *E. coli* (36). Also, there are the so-called "stable mutants" of NR1 which do not undergo spontaneous amplification as frequently in *P. mirabilis* or lose r-determinants as frequently in *S. typhimurium* as wild-type NR1. Third, IS1 may itself produce an agent which is necessary for the tandem duplication to occur. IS1 is known to have sequences which could be RNA polymerase-binding sites (32) and is thought to have two protein products (21, 31). The plasmid should carry the only IS1 elements in the cell since the *P. mirabilis* chromosome does not carry any IS1 elements (J. Miller, unpublished results).

In this study we have shown that sequences other than IS1 can serve as recombination sites in the amplification of DNA on an NR1 replicator in *P. mirabilis*. The plasmid pRR983 is a derivative of NR1, which has the *neo/kan* gene of R6-5 flanked by 841 base pairs of homology derived from the structural gene for CAT and from *Eco*RI fragment J of NR1, which is unlikely to be an insertion-like element. These segments of homology were able to serve as recombination sites in the tandem duplication of DNA between them. pRR983 does have all the bases in the IS1 sequence, but the *Pst*I digest used to construct the plasmid split the IS1 sequence into two parts. Therefore, this study does not entirely rule out the possibility of an agent being produced by IS1 which could be involved in the amplification process. This seems unlikely since the DNA coding for the polycistronic mRNA thought to code for the two protein products of IS1 (21) is cleaved by *Pst*I at a site within the first gene in the message. Also, if such an agent is produced, it must allow sites other than IS1 to be used as recombination sites. Since *P. mirabilis* has no IS1 elements on its chromosome, the plasmid was the only source of any putative gene product.

The tandem duplication of the *neo/kan* gene of pRR983 is analogous to the tandem duplication of the r-determinant of NR1 in other ways. First, the *neo/kan* gene of pRR983 will not amplify in *E. coli*. Second, amplification is reversible, with nonamplified plasmids predominating in a population of cells previously containing amplified plasmids which had been grown without selection for high resistance to neomycin.

The plating efficiencies of cells containing pRR983 on various drug concentrations is also dependent on factors other than the amplification of specific genes. Since pRR983 was not stably maintained in the cell, some cells in the population do not contain a plasmid. This is the reason for plating efficiencies on low antibiotic concentrations of less than 100% for cells containing pRR983. The copy mutant of pRR983 that was isolated, pRR310, was present in a larger percentage of the cells than was pRR983, presumably due to its higher copy number. The measured plasmid copy number for a population of cells containing pRR310 was dependent on the conditions under which the culture was grown. Growth in medium containing higher antibiotic concentrations yielded higher average copy numbers and a higher percentage of cells which contained the plasmid. This is probably a result, in part, of the instability of the plasmid. The actual copy number in plasmid-containing cells also appeared to vary with growth conditions for reasons which are not understood. The plating efficiency of cells containing φS38(pRR310) which were grown in 200 μg of neomycin per ml was less on a high concentration of neomycin than that of a strain containing an amplified plasmid. The reason for this may be that, whereas a culture of φS38(pRR310) grown in high concentrations of neomycin had an average plasmid copy number greater than 1, individual cells in that population may have had fewer plasmids. Only cells containing many plasmids would form colonies on high levels of neomycin.

The study of gene amplification will probably be intensified in years to come, expecially in eucaryotic cells, and the possible relationship between gene amplification and oncogenic transformation and development will be determined. Elucidation of the mechanism by which gene amplification occurs is therefore very pertinent to our understanding of these phenomena.

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