JOURNAL OF BACTERIOLOGY, Mar. 1985, p. 1042–1048
0021-9193/85/031042-07$02.00/0

Vol. 161, No. 3

Drug Resistance Gene Amplification of Plasmid NR1 Derivatives with Various Amounts of Resistance Determinant DNA

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Received 9 July 1984/Accepted 5 December 1984

Drug resistance gene amplification of derivatives of plasmid NR1 having various amounts of resistance (r) determinant DNA was examined with two types of NR1 derivatives. The first was an NR1 derivative that carried two tandem copies of the r determinant component which was isolated as an intermediate in the amplification process. The plating efficiency of host cells and restriction endonuclease analysis of the plasmid DNA indicate that plasmids with two tandem copies of the r determinant undergo spontaneous amplification to a more highly amplified state at a frequency 150-fold higher than that of wild-type NR1. The second class of derivatives consisted of plasmids in which different regions of the r determinant component had been deleted. The relationship between spontaneous amplification frequency and r determinant size was examined with these plasmids. Plating efficiency of host cells indicated that plasmids with a smaller r determinant undergo spontaneous amplification at a lower frequency than do plasmids with a larger r determinant. These results suggest that there is an ordered sequence of events in the amplification of the r determinant of NR1.

Selective gene amplification has been observed in both procaryotic (1, 3, 4, 10, 11, 15, 18, 21, 23, 25) and eucaryotic systems (for a review, see reference 19). Studies on DNA amplification in procaryotes have often involved drug resistance genes on plasmids. The amplified DNA sequences have been found to be present as tandem copies on the plasmid; autonomous copies of the amplified sequences have also been observed. Amplified plasmids may exist in cell populations that have never been exposed to antibiotics (7). This suggests that there is a subpopulation of cells containing spontaneously amplified plasmids which outgrow cells containing unamplified plasmids when the cells are cultured in medium containing high antibiotic concentrations.

The R plasmid NR1 (also called R100) is 90 kilobases (kb) and has a copy number of one to two per chromosome in enteric bacteria. NR1 carries resistance genes for chloramphenicol, fusidic acid, streptomycin, spectinomycin, sulfonamides, mercuric ions, and tetracycline. NR1 is a composite plasmid consisting of a resistance (r) determinant and a resistance transfer factor (RTF). The r determinant carries most of the resistance genes of NR1. The RTF carries genes or functions mediating plasmid replication, stable maintenance, conjugal transfer, and tetracycline resistance (12, 16, 17). A single copy of insertion element IS*1* is present at each junction between the r determinant and RTF portions of the plasmid (9). These IS*1* elements are in direct repeat orientation.

In *Escherichia coli*, NR1 is stably maintained under normal growth conditions. The culturing of host cells in medium containing a high concentration of antibiotics is selective for copy mutants of the plasmid (13). However, the culturing of *Proteus mirabilis* cells containing NR1 in medium containing high concentrations of an antibiotic to which resistance is conferred by the r determinant is selective for cells containing plasmids with multiple tandem r determinants attached to a single RTF (15, 22). Because the r determinant component of NR1 has a higher buoyant density (1.718 g/ml) than the RTF component (1.710 g/ml), there is an increase in plasmid buoyant density accompanying the increase in antibiotic resistance. This process of amplification has been referred to as a transition. If cells containing amplified plasmids are cultured in antibiotic-free medium, there is a decrease in antibiotic resistance and plasmid buoyant density, presumably because of the excision of r determinants from the poly-r-determinant R plasmids. This has been referred to as a back-transition.

Models explaining the tandem duplication of the r determinant of NR1 in *P. mirabilis* predict that two stages are involved. The first stage generates a plasmid with two tandem r determinants attached to a single RTF. Such a plasmid could be formed by three different mechanisms that all involve recombination between the IS*1* elements which flank the r determinant (Fig. 1): (i) recombination between the IS*1* elements could generate an autonomous r determinant which could then integrate into a second NR1 molecule (Fig. 1A); (ii) recombination between IS*1* elements could occur on a replicating molecule which would generate a plasmid with two tandem r determinants after the completion of replication and a second recombination to resolve the cointegrate structure (Fig. 1B); (iii) recombination between two NR1 molecules and resolution of the cointegrate by a second recombination between two IS*1* elements (Fig. 1C).

The second stage of r determinant amplification could involve recombination between IS*1* elements or any homologous regions of r determinant DNA to generate molecules carrying multiple r determinants (16, 17). Because all of the models may involve recombination between r determinant sequences after the initial IS*1*-mediated recombinational event, it would be expected that the efficiency of gene amplification of NR1 would increase in proportion to the amount of DNA available for recombination (i.e., the size of the r determinant). This two-stage hypothesis for r determinant amplification was examined in two ways. First, a clone carrying an NR1 plasmid with two tandem r determinants was isolated. This plasmid has r determinant homology available for recombination during the initial steps of gene amplification, in addition to the IS*1* element homology which

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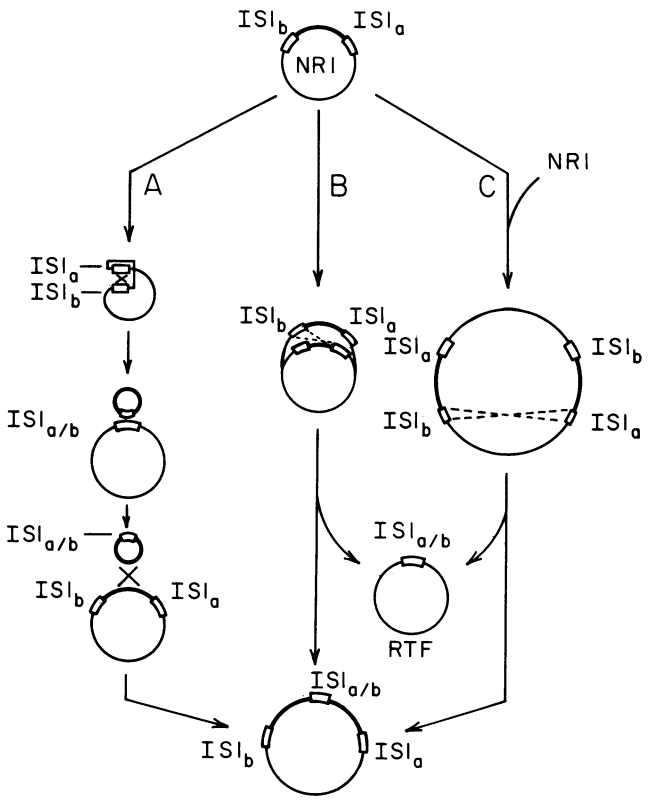

FIG. 1. Amplification models for NR1. Three possible models are shown which explain the mechanism by which tandem duplication of r determinants of NR1 could be generated. (A) Intermolecular recombination: An r determinant is dissociated by homologous recombination between IS*1* sequences. The autonomous r determinant then inserts into a second NR1 molecule. This form of amplification could also occur with dissociation of a multiple r determinant molecule from a partially amplified molecule followed by integration into a second NR1 molecule. (B) Nonreciprocal intramolecular recombination: Recombination between $IS1_a$ and $IS1_b$ on a partially replicated molecule followed by completion of replication and resolution of the cointegrate results in a molecule with two tandem r determinants on a single RTF plus an RTF alone. This process could then be repeated by using any r determinant homology for recombination. (C) Cointegrate formation: Two NR1 molecules recombine forming a cointegrate structure. An RTF is dissociated by recombination between IS*1* sequences.

is the only homology available on wild-type NR1. *P. mirabilis* containing such plasmids exhibited a much higher frequency of spontaneous gene amplification than *P. mirabilis* containing a wild-type NR1, which has a spontaneous amplification frequency of approximately 10^{-4} (7). Second, the relationship between the size of r determinant sequences available for recombination and the frequency of amplification was examined with NR1 deletion derivatives (16, 17) with different amounts of r determinant DNA. The amplification frequencies of several of these deletion mutants showed that plasmids with smaller r determinants undergo amplification less frequently. The results from these experiments may explain that which occurs during the amplification of NR1 after the initial IS*1*-mediated recombination.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. mirabilis* φS38 (*nic thy lac gal* Tet^s) (7) and NR1 have been described previously (14, 22). The r determinant deletion mutants used are shown in Fig. 2. Cells were cultured at 37°C in Penassay broth (Difco Laboratories), to which had been added 20 μg of thymine per ml. Plating efficiencies were determined by diluting stationary-phase cultures and spreading the cells onto nutrient agar or 1.5% nutrient agar (Difco) containing 20 μg of thymine per ml and various concentrations of antibiotics. Unless otherwise specified, plates consisted of 1.5% nutrient agar, which contained more NaCl (1.5% final concentration) than did the nutrient agar. Streptomycin sulfate, chloramphenicol, and tetracycline hydrochloride were purchased from Sigma Chemical Co.

Isolation, purification, and characterization of plasmid DNA. DNA was isolated from whole-cell lysates of stationary-phase *P. mirabilis* cells by a modification of the procedure of Womble et al. (24). Plasmid DNA was purified by buoyant density centrifugation in cesium chloride-ethidium bromide gradients for 48 h at 40,000 rpm in a Beckman VTi50 rotor. Alternatively, plasmid DNA was partially purified from chromosomal DNA by a sodium dodecyl sulfate-high salt precipitation procedure (6), followed by cesium chloride gradient centrifugation in Beckman VTi65 (40,000 rpm for 18 h) or VTi80 (80,000 rpm for 4 h) rotors.

Plasmid DNA was further purified for restriction endonuclease analysis by phenol extraction. An equal volume of buffer-saturated phenol was mixed with the plasmid DNA, and after phase separation, the phenol was removed. The aqueous phase was then extracted twice with chloroform-isoamyl alcohol (96:4), and the plasmid DNA was precipitated with ethanol at −20°C.

The restriction enzyme *Eco*RI was purchased from Bethesda Research Laboratories, Inc., or Worthington Diagnostic Systems, Inc. Digestion of plasmid DNA was performed in 0.1 M Tris-0.05 M NaCl-0.005 M $MgCl_2$ (pH 7.4) for 1 to 4 h at 37°C. Agarose gel electrophoresis was carried out as described previously (8). Electrophoresis was generally carried out at 10 V for 15 to 20 h or at 50 V for 4 to 5 h on vertical 0.7% agarose gels. Photographs were taken with Polaroid type 57 film or, when a negative was desired, with Polaroid type 55 film. To quantitate DNA bands in the gels, negatives were traced with a Zeineh soft laser scanning densitometer coupled to a Hewlett Packard model 3390A reporting integrator.

Assay for chloramphenicol acetyltransferase. Cells were grown to an optical density at 650 nm of 0.6, and 1 ml of the culture was harvested with an Eppendorf tabletop centrifuge. The cells were washed in cold 0.1 M Tris (pH 7.0),

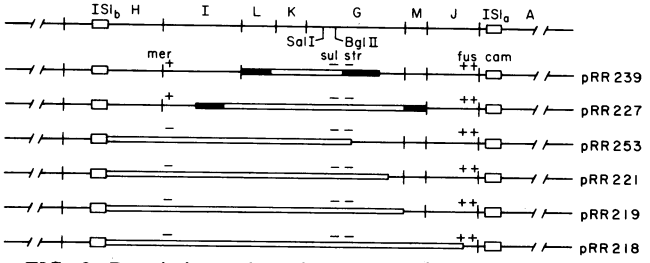

FIG. 2. Restriction endonuclease maps of r determinant deletion mutants. Open bars represent deleted regions of DNA. Solid bars are areas of uncertainty for deletion endpoints. Restriction sites for *Eco*RI are indicated (|) and letters refer to *Eco*RI fragments. Only a part of the RTF component of the plasmids is shown. Resistance genes to mercuric ions (*mer*), sulfonamide (*sul*), streptomycin and spectinomycin (*str*), fusidic acid (*fus*), and chloramphenicol (*cam*) are indicated. Resistance (+) or sensitivity (−) to each antibiotic are indicated for each plasmid. The size of the r determinant is 21 kb.

suspended 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 tabletop centrifuge for 1 min to remove cell debris. The supernatant was collected and assayed for chloramphenicol acetyltransferase as previously described (5, 20). The total protein concentration was determined by a spectrophotometric method (2).

RESULTS

Isolation of an NR1 plasmid carrying two tandem r determinants. When amplified NR1 undergoes a back-transition, there is an initial rapid decrease in both the amount and the buoyant density of the plasmid DNA to a value slightly greater than that of wild-type NR1. This rapid initial decrease is followed by a slower decrease in density to the NR1 value (7, 15). Presumably, most of the r determinants on highly amplified plasmids are dissociated rapidly because of the large amount of DNA sequence homology. The last few r determinants appear to be dissociated more slowly. This slower dissociation of the final tandem r determinants during a back-transition was exploited to isolate a clone containing a plasmid with two r determinants.

φS38(NR1) was subcultured three times in Penassay broth containing chloramphenicol (100 μg/ml) by successive 10^8-fold dilutions with growth to stationary phase. A clone was then purified by streaking the culture onto nutrient agar plates containing chloramphenicol (100 μg/ml). This clone was cultured in liquid medium containing chloramphenicol (100 μg/ml) and was shown to contain highly amplified NR1 DNA, as determined by restriction endonuclease analysis (data not shown). The culture containing amplified NR1 was subcultured four times in antibiotic-free medium by successive 10^8-fold dilutions and subsequent growth to stationary phase to allow back-transition of amplified NR1. Appropriate dilutions were spread onto antibiotic-free nutrient agar plates, and 200 colonies were tested for the presence of plasmids by replica plating onto plates containing low levels of chloramphenicol, tetracycline, and streptomycin (25, 10, and 2 μg/ml, respectively).

Ten of the drug-resistant colonies were inoculated into liquid medium containing a concentration of chloramphenicol (25 μg/ml) which was not selective for gene amplification and grown to stationary phase, and appropriate dilutions were spread onto plates containing various concentrations of streptomycin or chloramphenicol. Nonamplified φS38(NR1) (cultured in medium containing 25 μg of chloramphenicol per ml) and φS38 (containing amplified NR1 cultured in medium containing 100 μg of chloramphenicol per ml) were also plated as controls.

In the 10 cultures examined, three types of antibiotic resistance patterns were observed on plates containing the bacteriocidal antibiotic streptomycin (Fig. 3). Of the 10 cultures, 4 had plating efficiencies intermediate between that of cells containing NR1 and cells containing amplified NR1. Five of the cultures were indistinguishable from cultures containing nonamplified NR1. They had a low plating efficiency at high antibiotic concentrations. The resistance pattern of the final culture was similar to that of a culture containing amplified NR1. It had a high plating efficiency on plates containing high concentrations of the antibiotics. In these experiments, 4 to 5 μg of streptomycin per ml was judged to be the optimal concentration to distinguish between cells containing amplified plasmids from cells containing nonamplified plasmids, because it was selective for cells containing amplified NR1. At this antibiotic concentration, the clones with intermediate resistance formed colonies

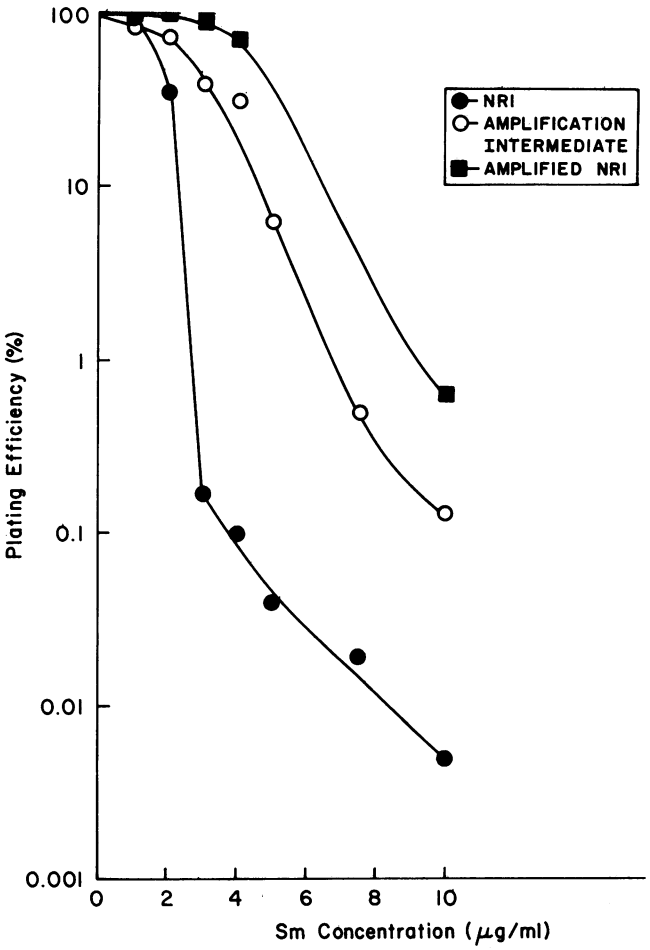

FIG. 3. Plating efficiency of NR1, amplified NR1, and an amplification intermediate on plates containing streptomycin (Sm). Cultures were grown to stationary phase, and appropriate dilutions were spread onto plates containing various concentrations of streptomycin. Colonies were counted after incubation for 18 to 20 h at 37°C.

approximately 150 times more frequently than did cells containing nonamplified NR1. Characterization of clones was also attempted on plates containing the bacteriostatic antibiotic chloramphenicol. Although the results were generally in agreement with those obtained with streptomycin, the presence of microcolonies (<1-mm diameter) on plates containing chloramphenicol (200 μg/ml) made the counting of colonies somewhat ambiguous. For this reason, plating results on chloramphenicol were not used when it was possible to use streptomycin as the selective drug.

Restriction endonuclease analysis of plasmid DNA. Plasmid DNA isolated from cells containing nonamplified NR1 or amplified NR1 or from cells with intermediate levels of antibiotic resistance were digested with *Eco*RI and analyzed on agarose gels (Fig. 4). In contrast to nonamplified NR1, plasmid DNA of amplified NR1 had r determinant fragments that fluoresced much more intensely than the RTF fragments, indicating that the r determinants had been amplified. The restriction digests of amplified NR1 also generated an amplification fusion fragment (ΔH) which was created by tandem duplication of the r determinant (22).

Plasmid DNA isolated from back-transitioned cultures with antibiotic resistance levels similar to those of cells

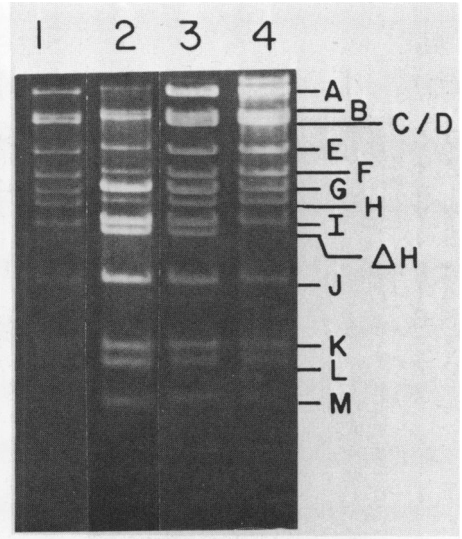

FIG. 4. Restriction endonuclease digestion and agarose gel electrophoresis of plasmid DNA. All plasmid DNA samples were digested with *Eco*RI. Lane 1, nonamplified NR1; lane 2, amplified NR1; lane 3, plasmid DNA from a culture with intermediate antibiotic resistance; lane 4, DNA from a back-transitioned clone showing levels of antibiotic resistance indistinguishable from cells containing NR1. Letters correspond to those in Fig. 2. ΔH is the novel r determinant fragment formed by recombination between IS*1*$_a$ and IS*1*$_b$ (17, 22).

containing nonamplified NR1 had no amplification fusion fragment ΔH. In contrast, plasmid DNA isolated from cultures with intermediate streptomycin resistance did have the ΔH fusion fragment. The degree of amplification was estimated by tracing the negative of the gel photograph with a scanning densitometer. Analysis of these data indicated that r determinant bands G and I from cells with intermediate resistance were present at a numerical ratio of approximately 2 to 1 as compared with the RTF bands E and F (Table 1). Fragment H and ΔH were present at one copy per plasmid, as expected for a plasmid with two r determinants.

Analysis of clones from a culture with intermediate antibiotic resistance. The plating efficiency and restriction endonuclease analysis results suggest that the colonies with intermediate antibiotic resistance consisted of cells containing a plasmid with two tandem r determinants for every RTF. The simplest way to determine the degree of amplification of the plasmids in individual cells was to examine the plating efficiency on medium containing a high antibiotic concentration. Colonies were isolated by streaking a culture with intermediate resistance onto plates containing 25 μg of chloramphenicol per ml, which is not selective for r determinant amplification (7). Whole colonies were picked off the plate and cultured in liquid medium containing 25 μg of chloramphenicol per ml, and the resistance levels were examined on plates containing a level of streptomycin (5 μg/ml) which distinguished cells containing nonamplified plasmids from cells containing amplified plasmids. Of the 40 single colonies tested, 30 had intermediate resistance levels approximately equal to that of the original population from which the clones were derived. Restriction endonuclease digests of plasmid DNA from these cells showed them to contain plasmids with r determinant DNA and the fusion fragment ΔH at a stoichiometry that indicated that there were approximately two r determinants per RTF (data not shown). Two of the clones had a plating efficiency of approximately 50% on medium containing 5 μg of streptomycin per ml, as compared with 5 to 6% for most clones showing intermediate resistance. These latter clones most likely contained plasmids with greater than two r determinants on a single RTF, as is described later for clones that grew well on medium containing 5 μg of streptomycin per ml. The remaining 8 of the 40 clones had resistance profiles indistinguishable from that of NR1. These eight colonies represented cells containing plasmids with only one r determinant, as determined by restriction endonuclease digestion (data not shown).

These data support the hypothesis that the population exhibiting intermediate resistance levels consisted primarily of cells containing plasmids with two r determinants on each RTF. The cells with plasmids carrying either multiple r determinants or a single r determinant on an RTF were not present at a frequency which could account for the plating efficiency or restriction endonuclease data. Since a single cell formed the original colony used in these experiments, the descendant cells with nonamplified NR1 or amplified NR1 must have arisen from the original cells containing plasmids with two r determinants on each RTF.

Amplification frequencies of plasmids which have had portions of the r determinant deleted. The amplification of the antibiotic resistance genes of NR1 may involve recombination between r determinant DNA sequences after the initial IS*1*-mediated recombination. If so, plasmids with less r determinant DNA would amplify less efficiently than wild-type NR1. To test this possibility, NR1 derivatives in which portions of the r determinant had been deleted were examined for the ability to undergo amplification. The structures of the plasmids used in these experiments are shown in Fig. 2. The construction of these NR1 derivatives resulted in the deletion of the streptomycin resistance gene, so chloramphenicol resistance was used. Although chloramphenicol resistance was less desirable than streptomycin resistance for the reasons discussed above, it was possible to get reproducible results by repeating the experiments three to five times.

The plating efficiencies of cells harboring various r determinant deletion mutants on plates containing 200 μg of chloramphenicol per ml are shown in Fig. 5. Cells containing

TABLE 1. Densitometer tracing from gel pictures of plasmid DNA with two tandem r determinants

*Eco*RI restriction fragment[a]	Size of fragment (kb)	Percentage of total DNA traced	Relative no. of copies of fragments[b]
E	7.6	19.8 ± 1.1	1
F	6.1	15.5 ± 0.5	1
G	5.3	26.8 ± 1.4	1.9
H	4.8	5.9 ± 1.1	0.5
I	4.1	19.9 ± 3.3	1.8
ΔH	3.6	8.1 ± 1.4	0.9

[a] Bands E through H were traced from lane 3 of Fig. 4 and the relative amounts of DNA (in percentage of the total DNA traced) in each band was determined with a densitometer and integrater. The region of the gel picture traced contains representative bands of RTF (fragments E and F) and r determinant (fragments G and I) DNA. ΔH is an amplification fusion fragment, and fragment H is shared by both the RTF and r determinant. The determination of the relative number of fragments in a band is less accurate for H and ΔH because of a low percentage of DNA in the band.

[b] The relative number of fragments in a band was calculated from the known molecular weight of fragments comprising the band and the measured intensity of the fluorescence of the band. All were normalized to RTF fragment E for each plasmid.

the plasmids with a smaller r determinant had lower plating efficiencies than cells containing NR1, which presumably reflected a lower spontaneous amplification frequency. For example, φS38(pRR218) formed colonies on plates containing chloramphenicol (200 μg/ml) 150-fold less efficiently than did φS38(NR1). The r determinant of pRR218 is one-tenth the size of the r determinant of NR1. Cells containing plasmids with r determinants which were one-third to one-half the size of the wild-type r determinant had plating efficiencies similar to cells containing NR1. Chloramphenicol acetyltransferase assays showed that the specific activities of the chloramphenicol acetyltransferase produced by the deletion mutants were indistinguishable from that of NR1. Therefore, the different plating efficiencies were not simply due to different enzyme activities.

To determine whether the colonies on plates containing chloramphenicol (200 μg/ml) contained cells with amplified plasmids, plasmid DNA was isolated from cultures inoculated with single colonies and analyzed with restriction endonucleases. Figure 6 shows representative DNA digestion patterns. Plasmid DNAs from cultures inoculated with clones from plates containing a high concentration of chloramphenicol had new amplification fusion fragments. These fragments were not present in plasmid DNA from cells grown without selection for high antibiotic resistance. The sizes of the new fragments were as expected for fusion fragments formed by tandem duplication of the r determinant portions of the plasmid DNA.

DISCUSSION

The frequencies of gene amplification of several derivatives of NR1 with altered r determinant structures were examined. The results have provided insight into the molecular mechanism of NR1 gene amplification.

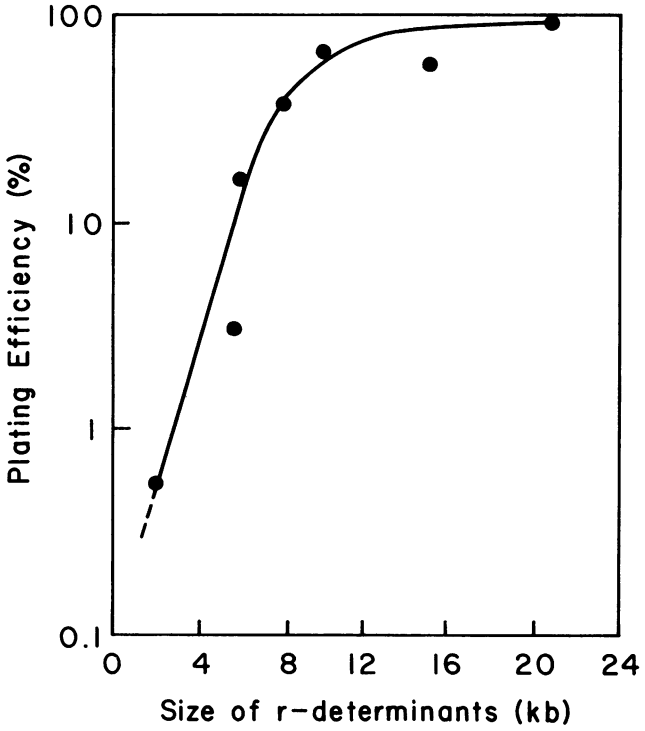

FIG. 5. Relationship between r determinant size and plating efficiency on plates containing 200 μg of chloramphenicol per ml. Strain φS38 containing various r determinant deletion mutants was cultured in liquid medium and spread onto plates containing chloramphenicol. Colonies were counted after 20 h of incubation at 37°C.

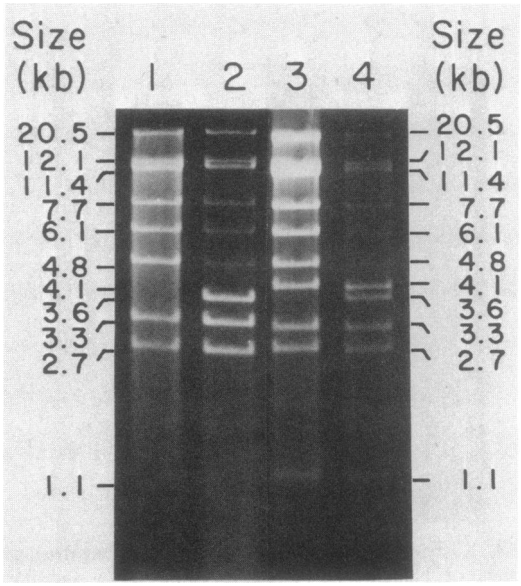

FIG. 6. Restriction endonuclease digestion and agarose gel electrophoresis of r determinant deletion plasmids. Plasmid DNAs were isolated from stationary-phase cells, purified, and digested with *Eco*RI. Cells with amplified plasmids were cultured in medium containing chloramphenicol (100 μg/ml). DNAs are pRR227 (lane 1), amplified pRR227 (lane 2), pRR239 (lane 3), and amplified pRR239 (lane 4). The six largest fragments of pRR227 and pRR239, which are not amplified, are from the RTF component (fragments A through F). The two smallest fragments of pRR227 (lane 1) and four smallest fragments of pRR239 (lane 3) correspond to nondeleted r determinant fragments. Amplified pRR227 and pRR239 contain a novel fusion fragment formed by recombination between $IS1_a$ and $IS1_b$ (17, 22). The size of the amplification fusion fragments for pRR227 and pRR239 (3.6 kb) is as expected for a tandem duplication of their r determinants, based on the expected amplified structure and the known restriction fragment sizes.

Restriction analysis of plasmid DNA isolated from cells grown on plates containing streptomycin (5 μg/ml) showed that the r determinants of these plasmids were highly amplified. The plating efficiency at this concentration of streptomycin was therefore a measure of the frequency of amplification. Clones were isolated that had plating efficiencies which were intermediate between those of cells containing NR1 or amplified NR1. Restriction endonuclease analysis of plasmid DNA isolated from the clones identified NR1 plasmids carrying two r determinants. These plasmids could amplify further to form poly-r-determinant plasmids or could delete a copy of the r determinant to form a plasmid with only one r determinant. These results suggest that a plasmid with two r determinants is an intermediate in NR1 r determinant amplification.

The ability to dissociate the extra r determinant explains the presence of nonamplified NR1 plasmids at a frequency of about 20% in colonies derived from cells harboring plasmids with two r determinants. The presence of cells in these colonies with high plating efficiencies (2 of 40 clones tested) indicates a higher frequency of spontaneous amplification than observed for NR1 itself, which is approximately 10^{-4} (7). Cells containing amplified plasmids were present, even though the cell population had not been exposed to a high concentration of antibiotic. The presence of nonamplified and amplified plasmids in these clones suggests that the

plasmids in the cells are in a dynamic state of dissociation and reassociation of the r determinants.

Because the plating efficiency of cells harboring plasmids with two r determinants on plates containing 5 μg of streptomycin per ml is only a few percent and colonies formed under these conditions contain amplified plasmids, it seems reasonable to assume that the higher plating efficiency of cells containing a plasmid with two r determinants reflects a higher spontaneous amplification frequency than previously observed for cells containing NR1. This implies that the initial IS*1*-mediated recombination may be a rate-limiting step in the amplification of plasmids with only one r determinant. Once the initial duplication has been achieved and there are two tandem copies of the r determinant, the entire r determinant is available for further recombination and amplification. The increase in amplification frequency (ca. 150-fold) was greater than the difference in size of the r determinant relative to IS*1* (27-fold). This may be due to a nonlinear relationship between the extent of sequence homology and the frequency of recombination which leads to amplification.

It is significant that clones with plating efficiencies indistinguishable from those of cells containing nonamplified NR1 were present in the back-transitioned population. Plasmid DNA from these clones had *Eco*RI restriction digestion patterns indistinguishable from those of nonamplified NR1. These results suggest that amplification of NR1 is not merely the selective outgrowth of plasmid or host mutants with enhanced amplification potential. Such mutants would be expected to have higher plating efficiencies than cells containing nonamplified NR1 in subsequent experiments.

The amplification frequencies of NR1 derivatives in which portions of the r determinant had been deleted were also examined. Plasmids with much smaller r determinants, such as pRR218, underwent amplification at a significantly lower frequency than NR1 or deletion mutants with larger r determinants. Apparently, below an r determinant size of approximately 6 to 8 kb the spontaneous amplification frequency decreases rapidly. These results suggest two alternative explanations for the lower amplification frequencies of the r determinant deletion plasmids.

First, the decrease in amplification frequency could be a result of less r determinant homology being available for the second stage of recombination proposed to be required for NR1 gene amplification. For example, a pRR218 derivative with two tandem r determinants would have 10-fold less r determinant DNA than an NR1 plasmid with two tandem r determinants.

A second reason for the lower amplification frequency could be that there is a gene or site located on the r determinant that is involved in the stimulation of gene amplification. The presence of such a gene is unlikely because, if there were a gene involved, we would expect the amplification frequencies to revert abruptly to normal once the deletion no longer inactivated the gene. We would not expect to see a gradual increase over a range of r determinant sizes as was observed.

The following model could explain these data. An initial IS*1*-mediated recombination results in a plasmid with two tandem copies of the r determinant on a single molecule. This initial recombinational event appears to be rate limiting for the overall amplification process. Once the initial r determinant tandem repeat is generated, sequence homology within any region of the r determinant can be utilized to generate additional tandem copies of the r determinant. The frequency at which further amplification occurs is proportional to the size of the r determinant DNA.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant GM30731-02 from the National Institutes of Health and by the Otho S. A. Sprague Memorial Institute. B.C.P. was a predoctoral trainee.

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