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Acquisition of Resistance Genes by the IncQ Plasmid R1162 Is Limited by Its High Copy Number and Lack of a Partitioning Mechanism

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R1162 is a representative member of the broad-host-range IncQ group of multicopy plasmids. Lower-copy-number derivatives of R1162 were constructed in vitro and shown to be unstable, indicating that partitioning of plasmid copies at cell division is due to random distribution and not to an active partitioning mechanism. However, the normal copy number of R1162 reduces cell fitness during growth in broth and favors the emergence of unstable, lower-copy-number variants. As a result, plasmid-borne antibiotic resistance genes active at a low copy number eventually result in plasmid loss during periods of no selection. We argue that the maintenance of R1162 in a population requires a gene that is selected only at high levels. This reduces the potential for acquiring genes from other R factors and could explain the limited variety of antibiotic resistance genes contained by naturally occurring IncQ plasmids.

R1162 is a broad-host-range IncQ plasmid (2) that is stably maintained in the *Escherichia coli* K-12 strain MV10 (7) for at least 80 generations during unselected growth in broth (Table 1). The copy number of R1162 is approximately 10 to 12 per chromosome (12), which is sufficiently high to ensure stable inheritance by random distribution during cell division (10). We constructed lower-copy-number derivatives to show that R1162 depends on its normal copy number for stability and that it does not encode a partitioning system active in *E. coli*. Our data also indicate that this dependence is likely to limit the number of different drug resistance genes carried stably by the plasmid, thus accounting for the similarity of IncQ plasmids isolated from different species (2).

We constructed pUT1485, an R1162 derivative containing both a 912-bp deletion in the gene for sulfonamide resistance and a 952-bp DNA fragment that encoded chloramphenicol resistance (4) and was cloned at the unique *Xmn*I site. The DNA fragments contained in this plasmid and in the other plasmids used in this study are shown in Fig. 1. The copy number and stability of R1162 were similar to those of pUT1485 (Fig. 2, lanes a and d, and Table 1). Within the origin of replication of these plasmids is a group of 3 1/2 20-bp direct repeats that bind the plasmid-encoded replication protein RepC (reference 6 and unpublished data). Additional copies of these direct repeats were cloned at the site of the deletion in pUT1485 (Fig. 1), in order to lower the plasmid copy number by titration of the RepC protein. The amount of plasmid DNA for pUT1396 (Fig. 2, lane e), which has two additional copies of the direct repeats, was smaller than that for pUT1485, and the amount of DNA was smaller still for pUT1415 and pUT1416, which have three and four additional direct repeats, respectively (Fig. 2, lanes f and g). These plasmids also became increasingly unstable as the number of cloned direct repeats was increased (Table 1). From densitometric analysis of the gel in Fig. 2, we estimated the plasmid copy numbers, based on a value of 6 to 8 for pSWK29 (17), to be approximately 8 to 10 for pUT1485, 5 to 7 for pUT1396, and 4 to 6 for pUT1415 and pUT1416. The corresponding level of instability of each plasmid (Table 1) was consistent with that calculated assuming a random mode of plasmid inheritance (10), and we concluded that no partitioning system is active in these plasmids.

When cells containing pUT1485 were mixed at a 1:1 ratio with plasmid-free cells and incubated with them in medium lacking antibiotics, the proportion of plasmid-containing cells decreased during serial cultivation (Table 1). Therefore, although pUT1485 is stable in MV10, cells containing this plasmid had a growth disadvantage in the absence of selection. Cells containing the lower-copy-number derivative pUT1396 were also outgrown by plasmid-free cells, but at a lower rate (Table 1). It has been demonstrated for plasmids unrelated to R1162 that the maximum growth rate attainable by *E. coli* in a variety of media decreases with increasing plasmid copy number (14). Since R1162 encodes at least 11 proteins (13), we assume that it, too, imposes a metabolic burden on the cell and that the magnitude of this burden is directly related to the copy number. Consistent with this, we found that the generation time of MV10 was 36 min in broth but was 48 min if the cells contained pUT1485. In addition, the plasmid-containing strain had a longer lag time during recovery from stationary phase after incubation overnight.

Our results suggest that if MV10(pUT1485) and MV10 (pUT1396) are serially cultured together without selection, then cells of the latter strain will predominate with time. This expectation was confirmed experimentally (Fig. 3A). The same results were obtained when the experiment was repeated with medium containing chloramphenicol (Fig. 3B). Thus, whether or not chloramphenicol was present, cells having the lower-copy-number pUT1396 overtook those containing pUT1485 in serial culture.

Taken together, these results suggest the presence of an unstable system that, if it pertained in nature, could make the plasmid vulnerable to extinction. Cells having plasmids with lower copy numbers following spontaneous mutation would come to predominate, even when cells were under selective pressure for drug resistance. During periods of no selection, these plasmids would then be lost due to the instability caused by their low copy numbers. Why, then, are IncQ plasmids, which have been isolated from a variety of species, including *E. coli*, clearly successful in nature (2)? To address this question,

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TABLE 1. Retention of plasmid DNA during serial culture of bacteria

Plasmid (relevant properties)[b]	% colonies retaining plasmid-encoded antibiotic resistance after indicated no. of generations in broth[a]			
	During unselected growth		In competition with MV10[c]	
	0	80	0	80
R1162 (Sm^r)	99.5 ± 0.5	99.5 ± 0.5	59.0 ± 16.2	12.8 ± 5.6
pUT1497 (Sm^r, 2 DR)	97.3 ± 1.4	91.8 ± 2.8	59.2 ± 7.9	34.0 ± 12.2
pUT1485 (Cm^r)	99.8 ± 0.4	98.7 ± 1.5	71.0 ± 4.2	0.0 ± 0.0
pUT1396 (Cm^r, 2 DR)	99.7 ± 0.5	94.7 ± 2.3	63.3 ± 6.1	9.2 ± 6.6
pUT1415 (Cm^r, 3 DR)	97.5 ± 2.0	84.2 ± 3.5	ND[d]	ND
pUT1416 (Cm^r, 4 DR)	91.0 ± 3.3	57.3 ± 7.1	ND	ND
pUT1429 (Cm^r Par^+, 4 DR)	99.8 ± 0.4	100.0 ± 0.0	ND	ND
pUT1517 (Sm^r, 2 DR)	99.2 ± 0.8	91.5 ± 1.0	ND	ND

[a] Averages ± standard deviations for six independent experiments; 100 to 120 colonies were tested for each time point.
[b] Sm^r and Cm^r, resistances to streptomycin and chloramphenicol, respectively; DR, additional cloned direct repeats; Par^+, contains the P1 partitioning locus (1).
[c] The initial cell ratio of the test strain to plasmid-free MV10 was approximately 1:1.
[d] ND, not done.

we inserted two direct repeats at the *Xmn*I site of R1162 (Fig. 1), a location that does not interrupt any known genes (13). The resulting plasmid, pUT1497, had a lower copy number than R1162 (Fig. 2, lanes a and b) and was slightly unstable (Table 1); both of these properties are similar to those of pUT1396. This instability, as well as that of the lower-copy-number chloramphenicol resistance derivatives, was not due to inactivation of a partitioning mechanism, cryptic at a high copy

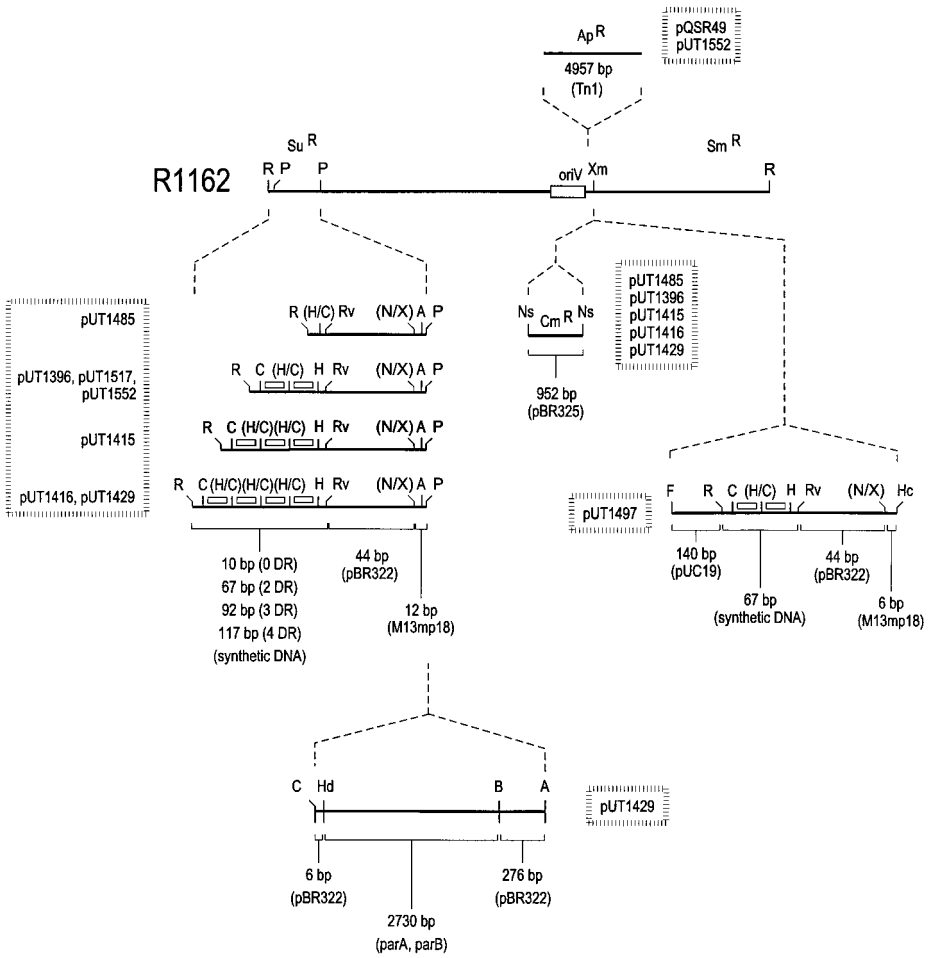

FIG. 1. Sources, sizes, and locations of DNA fragments inserted into R1162 to construct plasmids described in the text. Ap^R, Cm^R, Sm^R, and Su^R indicate regions encoding resistance to ampicillin, chloramphenicol, streptomycin, and sulfonamides, respectively, and oriV indicates the location of the origin of replication (13). Abbreviations for restriction enzyme cleavage sites: A, *Acc*I; B, *Bam*HI; C, *Cla*I; H, *Hpa*II; Hc, *Hin*cII; Hd, *Hin*dIII; N, *Nhe*I; Ns, *Nsp*V; P, *Pst*I; R, *Eco*RI; X, *Xba*I; Xm, *Xmn*I. Abbreviations separated by a slash and placed in parentheses indicate a junction involving two different cleavage sites. The synthetic DNA fragment containing a direct repeat (indicated by the open box) has been described (8); two or more copies of this DNA were used to increase the number of direct repeats in the plasmid. The R1162 derivative containing Tn*1* has also been described elsewhere as the plasmid pQSR49 (9). The P1 partitioning region (parA, parB) was from plasmid pALA801 (1). Other DNA fragments, used as linkers, were derived from pBR322 (5), pBR325 (4), pUC19 (11), and M13mp18 (11).

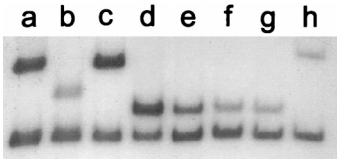

FIG. 2. Relative copy numbers of R1162 and derivatives. Plasmid DNAs were extracted (3) from mid-log-phase cells containing, in addition, pWSK29, a derivative of pSC101 with a copy number of approximately six to eight per cell (17). Cells were grown in medium with antibiotics to select for all plasmids present. The DNAs were digested with restriction endonuclease *Eco*RI, and the resulting fragments were then end labeled with [α-^{32}P]dATP and Sequenase (Amersham) and displayed by electrophoresis through a 0.8% agarose gel. The fast-migrating band in each lane is the pWSK29 DNA. *Eco*RI cleaved R1162 and the revertant plasmid in lane c once. The other plasmids were cleaved twice by this enzyme; the second, small band is not shown. The lanes contained plasmids as follows (relative copy numbers, estimated by densitometry, are in parentheses): lane a, R1162 (1.0); lane b, pUT1497 (0.6); lane c, a derivative of pUT1497 arising after serial growth of cells in medium containing streptomycin (1.3); lane d, pUT1485 (1.3); lane e, pUT1396 (0.7); lane f, pUT1415 (0.6); lane g, pUT1416 (0.6); lane h, pUT1429 (0.7).

number, by insertion at the *Xmn*I site. When the additional direct repeats were cloned at a different location, in the region normally encoding sulfonamide resistance, plasmid instability and a lower copy number were also observed (Table 1 [data for pUT1517] and data not shown).

Plasmid-free cells overtook those containing either R1162 or pUT1497 during serial culture in drug-free medium (Table 1). However, when cells of MV10(R1162) and MV10(pUT1497) were incubated together, their behavior was different from that of a mixed culture of MV10(pUT1485) and MV10(pUT1396). In drug-free medium MV10(pUT1497) predominated, but MV10(R1162) was favored when streptomycin was present (Fig. 3C and D). In addition, when MV10(pUT1485) and MV10(pUT1396) were incubated together in medium containing streptomycin instead of chloramphenicol, pUT1396 was lost from the mixed culture (data not shown).

The importance of the normal copy number of R1162 in the presence of streptomycin was demonstrated by serially propagating 10 cultures of MV10(pUT1497), inoculated from separate, isolated colonies, in both the presence and absence of this antibiotic. After 80 generations of growth, cells were collected by centrifugation and plasmid DNA was extracted from each of the cultures. There was no change in the structure of pUT1497 when cells were grown in drug-free medium, but when streptomycin was present, plasmids altered in structure were obtained from two cultures. These plasmids, one of which is shown in Fig. 2, lane c, had a higher copy number than pUT1497. Analysis of the DNA sequence revealed that this was due to a reduction in the total number of direct repeats, from 5 1/2 to 3, most likely by recombination between the cloned set and those in the origin.

Our results suggest that during growth in broth, the streptomycin resistance encoded by R1162 requires the high gene level provided by the normal copy number of the plasmid in order to be fully effective. If the copy number is lowered, then cells grow less well in the presence of the drug. As a result, selection for streptomycin resistance ensures continuation of the high-copy-number plasmid with the elimination of lower-copy-number variants. In contrast, the mechanism for chloramphenicol resistance is sufficiently active at a lower number of gene copies per cell under our growth conditions and would therefore favor cells containing plasmids with a lower copy number. In agreement with this interpretation, when cells of MV10(pUT1485) and MV10(pUT1396) were grown in mixed culture in medium containing a high concentration (400 μg/ml) of chloramphenicol, an amount insufficient to inhibit growth, cells with pUT1485 were selected (data not shown).

The preferential growth of cells containing lower-copy-number derivatives of R1162 is not unique to chloramphenicol resistance. The plasmid pQSR49 (9) is an R1162 derivative containing the transposon Tn*1*, which encodes resistance to ampicillin. We constructed a lower-copy-number derivative, pUT1552, analogous to pUT1396 in having two additional direct repeats (Fig. 1). When MV10(pQSR49) and MV10 (pUT1552) were mixed and serially cultured as before in medium containing ampicillin, cells with the lower-copy-number plasmid again came to predominant (Fig. 3F).

The potential instability of R1162 might have affected the evolution and diversification of these plasmids in nature: it is noteworthy that although IncQ plasmids have been reported for a variety of species, the majority of these appear to be

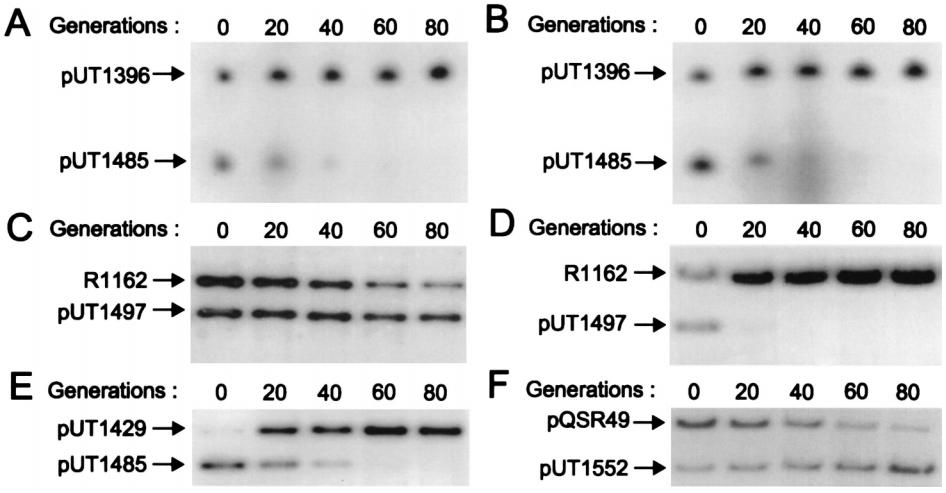

FIG. 3. Relative amounts of plasmid DNAs during serial incubation of two strains in mixed culture. The initial cell ratio was approximately 1:1. Media contained no antibiotic (A, C, and E), 25 μg of chloramphenicol per ml (B), 25 μg of streptomycin per ml (D), or 12 μg of ampicillin per ml (F). Plasmid DNAs were extracted from an equal number of total cells after 0, 20, 40, 60, and 80 generations of growth and digested with restriction enzymes *Eco*RI and *Pst*I (A and B), *Eco*RI only (C, D, and E), or *Bam*HI and *Sal*I (F). After end labeling, samples were applied to either a 5% polyacrylamide gel (A and B) or a 0.8% agarose gel (C to F).

closely related, encoding streptomycin and sulfonamide resistance and having a structure similar to that of R1162 (2). Thus, as R factors, IncQ plasmids might be a delicately balanced system in which the deleterious effects of a high copy number are counterbalanced by the selective pressure for antibiotic resistance. Under these circumstances, there would be less potential for R1162 to evolve into a diverse family of plasmids encoding different or multiple drug resistances. A few examples of naturally occurring IncQ plasmids encoding new resistances have been found by Tietze et al. (15). Interestingly, these authors showed that the new resistance genes are often different from those found in the larger (and lower-copy-number) R factors. They concluded that the pools of drug resistance genes available to IncQ plasmids and those available to the larger R factors might be different. This would be explained by our results, since resistance genes adapted to expression at low gene levels would not be stably maintained by plasmids like R1162.

One way out of the evolutionary constraint imposed by a high copy number is by the acquisition of a partitioning mechanism. When the *par* genes of the phage P1 (1) were cloned into pUT1416 (Fig. 1), the plasmid was stabilized (Table 1) without an increase in copy number (Fig. 2, lane h). In addition, in mixed cultures of MV10(pUT1429) and MV10 (pUT1485), cells with the lower-copy-number plasmid again had the growth advantage (Fig. 3E). Thus, a lower-copy-number R1162 with a partitioning mechanism would be free to acquire new drug resistance genes. One clear route to the evolution of a lower copy number, simulated by the cloning experiments reported here, would be by duplication of the direct repeats in the origin. The low-copy-number F factor has extra copies of the direct repeats found in the origin, and these are involved in copy control (16). R1162 could be a more primitive plasmid, having a similar type of origin but not yet sufficiently evolved to acquire a partitioning mechanism and a low copy number.

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