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Plasmid Incompatibility and Control of Replication: Copy Mutants of the R-Factor R1 in *Escherichia coli* K-12

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Plasmid incompatibility was studied in *Escherichia coli* K-12. By double-antibiotic selection, clones were constructed that carried the two R-factors R1 and R100, both belonging to the compatibility group F_{II}. After release of the selection pressure, each of the two plasmids was lost at the same rate (8% per generation). Mutants of R-factor R1 showing an increased number of copies per chromosome (copy mutants) were tested for their incompatibility towards R-factor R100. The results indicate that plasmid incompatibility is quantitative and not just a qualitative property. All copy mutants studied affected incompatibility, and there were two classes of mutants: one increasing and one decreasing the incompatibility exerted towards the test plasmid R100. Evidence is presented that incompatibility is related to the mechanisms that control replication. The implications of such a relation on proposed models for control of replication are discussed. The data do not support the hypothesis that plasmid incompatibility is due to competition for a replicational or segregational site.

Two important properties of plasmids are their strict control of replication and their incompatibility. Plasmids are normally present in a defined number of copies per chromosome equivalent in exponentially growing populations of bacteria (5). The steady-state concentration of a plasmid is governed by the growth conditions (7, 11), the host (5, 22, 37), and the plasmid itself (7, 35). Mutations in the plasmid may increase by severalfold the steady-state concentration of the plasmid (30), suggesting that the plasmid controls its own replication. Plasmid mutants with an increased ratio of plasmid copies/chromosome equivalent have been denoted copy mutants (18, 30, 41) or round of replication mutants (27).

If two different plasmids are introduced into a cell, two situations may occur: either a stable coexistence (compatibility) or an unstable situation in which the two plasmids segregate during the subsequent cell divisions (incompatibility). Mise and Arber (26) have reported that unstable double lysogens can be formed that contain the plasmids (bacteriophages) P1CM and P1TC; these tend to segregate as single lysogens during growth. Incompatibility may even be so strong that the incoming plasmid is not replicated (9, 38). This has suggested that the plasmid incompatibility has to do with replication by competition for a replicational site or for a segregational site (17). However, since an Hfr strain cannot harbor a free F-factor, Pritchard et al. (33, 34) postulated that incompatibility is due to repressors of initiation of replication. The isolation of plasmid copy mutants offers a unique possibility of testing these aspects of incompatibility. The present paper shows that incompatibility is linked to the control of replication. A preliminary account has been published elsewhere (42).

MATERIALS AND METHODS

Bacterial strains and R-factors. The *Escherichia coli* K-12 strains D11 (*his, pro, trp, strA*) (3), EC1005 (*met, nal*) (14), and J53 (*met, pro, str*) (6) were used. The R-factors used are listed in Table 1. Plasmid R1*drd-19* is a mutant of R-factor R1 (24) derepressed with respect to conjugal transfer (25). Plasmid R1*a* is a derivative of R-factor R1 which has lost kanamycin resistance (28).

Media and growth conditions. The minimal medium was medium E of Vogel and Bonner (43), supplemented with glucose (0.2%), thiamine (1 μg/ml), and the required amino acids (25 μg of the L-epimer per ml). The complete medium was LB medium of Bertani (2), supplemented with glucose (0.2%), medium E, and thiamine (1 μg/ml). Solid medium was obtained by the addition of 1.5% agar; that derived from complete medium was called LA. The bacteria were grown at 37 C, and growth was followed by recording optical density in a Klett-Summerson colorimeter with filter no. W66.

Determination of antibiotic resistance. Antibiotic resistance was determined in single-cell tests (29). The resistance level was defined as the highest drug concentration permitting 100% cell survival.

Isolation of R-factor copy mutants. Copy mutants of R-factor R1 were isolated essentially as

described before (30) by selecting for a 2- to 10-fold-increased resistance to ampicillin. Several different mutagens were used, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, ethyl methane sulfonate, and hydroxylamine, but copy mutants were also isolated without mutagenic treatment. However, the copy mutants specifically mentioned in this paper (Table 1) were isolated after treatment of the bacteria with ethyl methane sulfonate. Copy mutants of R1 confer an increase in the number of R1 copies per chromosome equivalent. As a consequence of this increase, there is a proportional increase in the specific activities of the enzymes that metabolize ampicillin, chloramphenicol, and streptomycin and also a proportional increase in the corresponding resistances (30). The mutants isolated on ampicillin plates were tested, therefore, for increased resistance to streptomycin and chloramphenicol and for increased specific activity of β-lactamase. Mutants that showed these increases were used as donors of the R-factor to demonstrate whether the effects observed were due to chromosomal or plasmid-borne mutations. Experiments with R-factor copy mutants were always performed after transfer to a nonmutagenized host. Deoxyribonucleic acid analysis (see below) was only performed with those copy mutants that were used extensively.

Deoxyribonucleic acid analysis. The amount of plasmid deoxyribonucleic acid was determined as covalently closed circles by alkaline sucrose gradient (5 to 20%, wt/vol) centrifugation in a Beckman L2-65B ultracentrifuge with head SW40 run at 4 C for 75 min at 40,000 rpm or by ethidium bromide-cesium chloride gradient centrifugation in the same centrifuge with rotor 65 run at 20 C for at least 40 h at 40,000 rpm (14,30). The R-factor copy mutants were characterized by their copy number (Table 1), i.e., the ratio between plasmid content (plasmid deoxyribonucleic acid per chromosome deoxyribonucleic acid) in cells carrying a copy mutant R-factor and that of cells carrying the wild-type plasmid.

β-Lactamase assay. Spheroplasts were prepared from log-phase cultures, and β-lactamase activity was determined microiodometrically as described previously (20). One unit of β-lactamase hydrolyzes 1 μmol of substrate per min at 36 C.

Transfer or R-factors. The transfer of R-factors has been described before (28). To select for R-factors, the LA plates were supplemented with ampicillin (50 μg/ml) for R1*drd-19* (*amp*) and with tetracycline (25 μg/ml) for R100 (*tet*).

In transfers in which strain EC1005 was the recipient strain and strain D11 or J53 was the donor, nalidixic acid (100 μg/ml) was used for counterselection of the donor strain. Streptomycin (200 μg/ml) was used to counterselect the donor in transfers from strains EC1005 to D11.

Incompatibility test. The colony test for incompatibility was performed as follows. Clones carrying two R-factors (R1 and R100) were constructed by double-antibiotic selection on LA plates with ampicillin (50 μg/ml) and tetracycline (25 μg/ml). Ten colonies so obtained were picked, mixed, and spread on drug-free LA plates, from which 100 colonies were tested for the presence of R-factors by the replica-plating technique as described previously (4).

To determine the segregation rates of plasmids from cells carrying two incompatible R-factors, the following procedure was used. After clones containing both R-factors had been constructed (see above), colonies were picked and transferred to LB medium without any antibiotic. The cells were grown as shaken cultures, and the cell density was kept below 10^7 cells per ml by dilution into fresh, prewarmed medium. During the cultivation, samples were withdrawn and plated on solid medium. Presence of the R-factors was then tested by replica plating of at least 100 colonies.

Materials. Ampicillin was a gift form AB Astra, Södertälje, Sweden; chloramphenicol was donated by Ercopharm A/S, Vedbaeck, Denmark; streptomy-

TABLE 1. *R-factors used: their properties and origin*

R-factor	Resistance markers[a]	Copy effect[b]	β-Lactamase activity (U/mg of protein)	Reference
R1*a*	*amp, cml, str, sul*	1.0	1.0	Nordström (28)
R1*drd-19*		1.0	0.99	Meynell and Datta (25)
R1*drd-19B2*		3.5	3.7	Nordström et al. (30)
R1*drd-19B3*	*amp, cml, kan, str, sul*	2.0	2.0	Nordström et al. (30)
R1*drd-19B4*		9	8.3	This paper
R1*drd-19B42*		5	5.1	This paper
R100	*cml, str, sul, tet*	1.0	0	Egawa and Hirota (10)

[a] *amp*, Ampicillin; *cml*, chloramphenicol; *kan*, kanamycin; *str*, streptomycin; *sul*, sulfonamide; *tet*, tetracycline.

[b] Relative number of plasmid copies per genome determined as covalently closed circles by alkaline sucrose gradient centrifugation. The wild-type plasmid R1*drd-19* has been given the value of 1.0. The copy effect given is the mean value obtained in at least three different experiments. The copy effect was essentially the same when deoxyribonucleic acid was analyzed by ethidium bromide-cesium chloride gradient centrifugation.

cin was a gift from AB Kabi, Stockholm, Sweden. Tetracycline was obtained from Pfizer Inc., New York, N.Y. Lysozyme was obtained for Sigma Chemical Co., St. Louis, Mo. Ribonuclease A was purchased from the Sigma Chemical Co. Ltd., London, England. To destroy deoxyribonuclease activity, ribonuclease solutions were heated at 90 C for 10 min before use. [*methyl*-^{3}H]thymidine (5 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England. Toluene (Anala R) was obtained from Merck, Darmstadt, Germany.

RESULTS

Quantitative studies of incompatibility. The system used throughout these studies was comprised of the R-factor R1*drd-19* (wild type and copy mutants) and the test plasmid R100, both R-factors belonging to compatibility group F_{II} (16).

R-factor R100 (*tet*) was introduced into strain EC1005-R1*drd-19* (*amp*), and clones carrying both R-factors were selected on LA plates containing tetracycline and ampicillin. Colonies were picked and transferred to LB medium without antibiotics. During the subsequent cultivation, both plasmids were lost exponentially and with equal frequency: 8% per cell doubling (Fig. 1a and Table 2). Some segregation had already occurred in the colony. This is more easily observed in the results from colony tests presented in Table 3. Table 3 also shows that in a colony test the two R-factors, R1*drd-19* and R100, were lost with equal frequency. Hence, the colony test appears to offer a simple quantitative technique for studies of incompatibility. Table 3 also shows that about the same result was obtained irrespective of the direction of transfer.

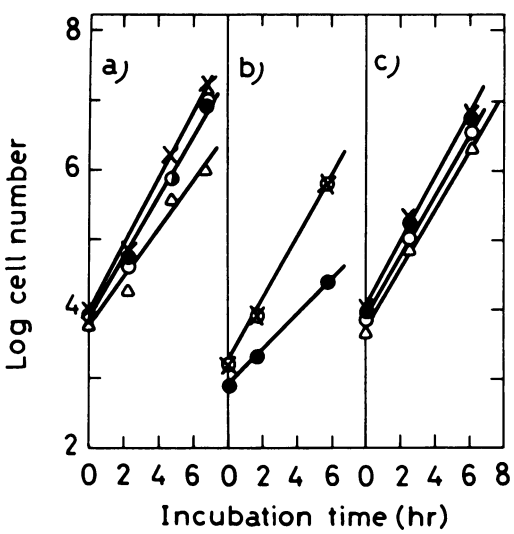

FIG. 1. *Segregation of plasmids R100 and R1 in cells carrying both R-factors. Clones of strain EC 1005 containing both R1 and R100 were selected on LA plates by double-antibiotic selection and grown in drug-free LB medium. Samples were withdrawn and tested for number of:* (×) *total viable cells,* (○) *R1 cells,* (●) *R100 cells, and* (△) *cells with both R-factors.* (*a*) *R100+R1drd-19,* (*b*) *R100+R1drd-19B2,* (*c*) *R100+R1drd-19B4.*

Incompatibility properties of copy mutants. We (30) have previously isolated copy mutants of R1*drd-19* in which the steady-state ratio of R1 copies per genome is increased two- to fourfold. The two mutants, R1*drd-19B2* (copy effect 3.5; Table 1) and R1*drd-19B3* (copy effect 2.0), were found to exert an increased incompatibility towards the test plasmid R100 (Table 2, lines 2 and 3). The increased incompatibility had two features: only R100 was lost, and the frequency of loss (26% respectively, 19% per cell doubling) was higher than that of the total loss of plasmids when the wild-type R1*drd-19* (Table 2, line 1) was used.

About 100 new copy mutants of R1*drd-19* were isolated. The plasmid was then trans-

TABLE 2. *Quantitative incompatibility test of the plasmids R1 and R100*

Incoming R-factor	Resident R-factor	Frequency of loss[a] (% per cell doubling)	
		R1	R100
R100	R1*drd-19*	8	8
R100	R1*drd-19B2*	<1	26
R100	R1*drd-19B3*	<1	19
R100	R1*drd-19B4*	5	<1
R100	R1*drd-19B42*	<1	<1

[a] Calculated from Fig. 1.

TABLE 3. *Incompatibility test of the plasmids R1 and R100: colony test*[a]

Incoming R-factor	Resident R-factor	% Colonies carrying:		
		R1 + R100	R1	R100
R100	R1*drd-19*	36	30	34
R1*drd-19*	R100	67	15	18
R100	R1*a*	35	19	46
R1*a*	R100	41	13	36
R100	R1*drd-19B2*	16	84	0
R1*drd-19B2*	R100	27	63	0
R100	R1*drd-19B4*	17	4	79
R100	R1*drd-19B42*	98	1	1
R1*drd-19B42*	R100	96	0	4

[a] Plasmid R1 and its mutants were carried by strain EC1005, and plasmid R100 was carried by strain D11.

ferred to a new host to prove that the mutation actually was located on the plasmid and also to eliminate the influence of any chromosomal mutation. In general, those R-factor mutants giving a two- to fourfold-increased ampicillin resistance were highly stable, whereas those giving a still higher resistance showed some instability; cells containing revertant R-factors were often observed in clones carrying such mutants. In some cases, R-factorless cells were more frequent in clones carrying high-copy-number R-factor mutants than in those carrying the wild-type plasmid R1*drd-19*; in a population carrying plasmid R1*drd-19*, less than 1% of the cells in a steady-state population had lost the R-factor.

The copy mutants were tested for incompatibility. About 2/3 of the copy mutants were similar to R1*drd-19B2* and *B3* in that they showed an increased incompatibility towards the test plasmid R100. The other copy mutants belong to a new class which showed a decreased incompatibility towards test plasmid R100 (Table 3, line 7). A typical example of this new class is the copy mutant R1*drd-19B4*, which was lost preferentially while R100 was retained. In liquid culture the loss of R1*drd-19B4* was 5% per cell doubling. One mutant (R1*drd-19B42*) that was essentially compatible with R100 (Table 3, line 8) was also found.

A difference in growth rate between strain EC1005 carrying R1*drd-19* and strain EC1005 carrying a copy mutant should affect the quantitative values obtained in an incompatibility test. There was a slight decrease in growth rate concomitant with increasing copy number. However, this effect was much smaller than that required to explain the values in Table 2. Furthermore, copy mutants of both classes with respect to incompatibility were found among mutants of both low and high copy number. Hence, the main effect is due to incompatibility.

However, there was a small loss of R100 from cells carrying the R-factor mutant R1*drd-19B4* (Table 2). This could be more clearly demonstrated by stressing the system. Test plasmid R100 was introduced into strain EC1005-R1*drd-19B4*. Clones carrying both R-factors were selected on LA plates containing tetracycline and different amounts of ampicillin (Fig. 2). These colonies were picked, spread on antibiotic-free LA plates, and tested for the presence of R-factors R1 and R100. The higher the selective concentration of ampicillin, the greater the fraction of cells carrying the plasmid R1 and the greater the fraction of cells lacking the test plasmid R100.

The incompatibility properties of the plasmids also affected the frequencies of colonies obtained in transfer experiments (Table 4). However, the result was essentially the same irrespective of the direction of transfer.

A complicating factor in the studies presented above is that the segregational event will be underestimated since plasmids are transferred during the experiment. However, the effect of transfer was rather small since the rate of transfer of R-factor R1*drd-19* was about 1% per generation if the recipient contained test plasmid R100 (Fig. 3). The quantitative effect of transfer between cells in a colony seems to be minute since essentially the same result was

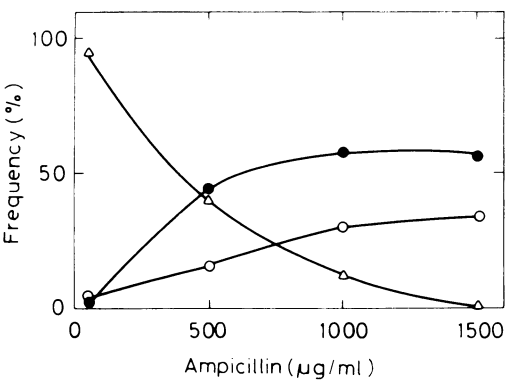

FIG. 2. *Transfer of test plasmid R100 to strain EC1005-R1drd-19B4. Selection for recipient cells carrying both R-factors was made on LA plates containing nalidixic acid, tetracycline, and different amounts of ampicillin. Symbols: △, R100 cells; ○, R1 cells; ●, cells with both R-factors.*

TABLE 4. *Conjugal transfer of R-factors*

Donor	Recipient	Frequency of recipients carrying donor plasmid (per input donor)
J53-R100	1005	7×10^{-3}
J53-R100	1005-R1*drd-19*	2×10^{-3}
J53-R100	1005-R1*drd-19B2*	4×10^{-3}
J53-R100	1005-R1*drd-19B4*	1×10^{-4}
J53-R100	1005-R1*drd-19B42*	4×10^{-4}
1005-R1*drd-19*	D11	1×10^{-1}
1005-R1*drd-19B2*	D11	9×10^{-2}
1005-R1*drd-19B4*	D11	8×10^{-5}
1005-R1*drd-19B42*	D11	2×10^{-3}
1005-R1*a*	D11-R100	4×10^{-5}
1005-R1*drd-19*	D11-R100	9×10^{-2}
1005-R*drd-19B2*	D11-R100	4×10^{-3}
1005-R1*drd-19B4*	D11-R100	$<10^{-6}$
1005-R1*drd-19B42*	D11-R100	5×10^{-5}

obtained if the derepressed plasmid R1*drd-19* was transferred to a recipient carrying R-factor R100 as if the donor carried the repressed plasmid R1*a* (Table 3).

Effect of the test plasmid R100 on the copy effect of mutant R1drd-19B4. The specific activity of the R1-mediated β-lactamase and the resistance to penicillins are proportional to the copy number (30). This is true at least up to a copy effect of 10 (Fig. 4). This fact was used to obtain the distribution of plasmids in the cells of a population (by titration). The introduction of test plasmid R100 into strain EC1005-R1*drd-19B4* not only caused a loss of the latter plasmid, but also caused a gradual reduction of the copy number in those cells that carried R1*drd-19B4* (Fig. 5). No effect on copy number was observed when test plasmid R100 was introduced into strain EC1005-R1*drd-19B2* or EC1005-R1*drd-19B42* (Fig. 6). In cells carrying only one R-factor, the wild type and copy mutants of R1*drd-19* were highly stable.

The phenotype expressed rapidly when the copy mutant R1*drd-19B2* was transferred to an R-factorless recipient. After 5 min of mating, samples were spread on LA plates containing different concentrations of ampicillin and an antibiotic to counterselect the donor. The resistance level after this short time was the same as

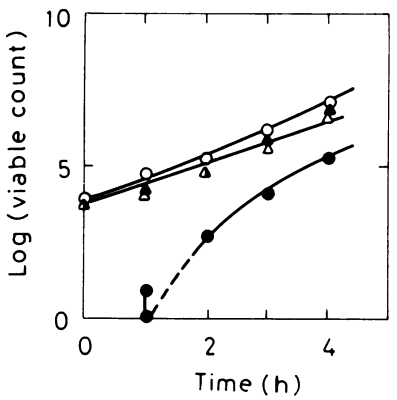

FIG. 3. *Transfer of R-factors in liquid medium. Strains J53-R100 and EC1005-R1drd-19 were pregrown in the logarithmic growth phase for at least 10 doublings. At zero time, they were diluted to 10^4 cells per ml, and equal volumes of the two cultures were mixed and incubated as shaken cultures at 37 C. Samples were taken at intervals and tested for viable count on LA plates* (○) *and on LA plates containing tetracycline (50 μg/ml;* ▲), *D-ampicillin (50 μg/ml;* △), *or tetracycline plus D-ampicillin (50 μg of each per ml;* ●).

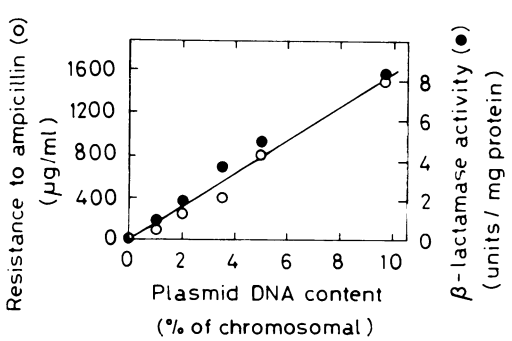

FIG. 4. *R1-mediated resistance to ampicillin and formation of β-lactamase as a function of the content of plasmid deoxyribonucleic acid (DNA) (cf. Table 1).*

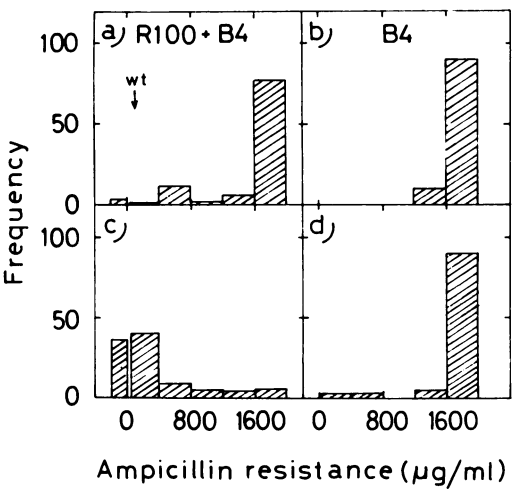

FIG. 5. *Effect of test plasmid R100 on the copy effect of copy mutant R-factor R1drd-19B4. Logarithmically growing cultures of D11-R100 and EC1005-R1drd-19B4 were mixed, and transfer was allowed to occur. After 60 min, the mating mixture was diluted into a medium that counterselected strain D11 (LB medium with 100 μg of nalidixic acid per ml). During subsequent cultivation, samples were plated on solid media, selecting for strain EC1005 carrying: R100 plus R1drd-19B4 (tet, amp), R100 (tet), or R1drd-19B4 (amp). The distribution of the R1 copy number was determined by using plates with varying concentrations of ampicillin (cf. Fig. 4), and the results are shown for zero generations (a) and 10 generations (c) after transfer was interrupted by the addition of nalidixic acid. The frequency is calculated as a percentage of tetracycline-resistant recipient cells. In the control experiment, the recipient culture (strain EC1005-R1drd-19B4), growing exponentially in LB medium, was plated on LA plates containing different concentrations of ampicillin. In this case, the result is shown at two time periods separated by ten doubling times (b and d). The single-cell resistance of strain EC1005, carrying wild-type R-factor R1drd-19, is indicated by a vertical arrow.*

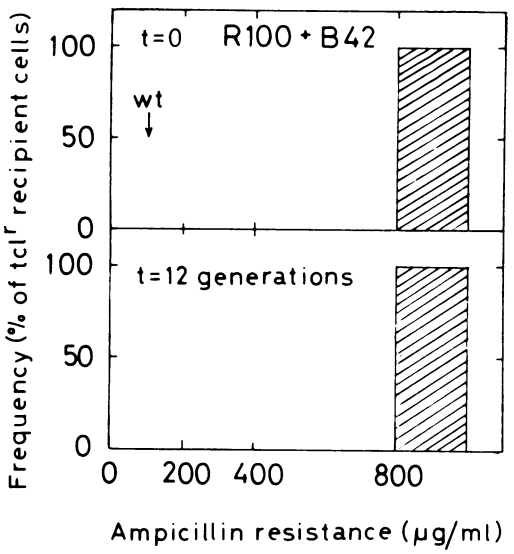

FIG. 6. *Effect of test plasmid R100 on the copy effect of the copy mutant R-factor R1drd-19B42. Experimental design was as described in the legend of Fig. 5.*

that of a clone containing R1*drd-19B2*.

DISCUSSION

All copy mutants of plasmid R1 (more than 100) studied so far in our laboratory quantitatively affect incompatibility towards the test plasmid. This indicates strongly that there is a connection between incompatibility and the mechanism that controls replication. There is no doubt that incompatibility is exerted at the level of replication (see, for example, Fig. 5c, in which the copy number of R-factor R1*drd-19B4* is shown to be reduced by the presence of the plasmid R100). Direct molecular studies of the fate of donor plasmid deoxyribonucleic acid introduced into recipient bacteria have shown that the rate of replication of the incoming plasmid is inhibited by the presence of an incompatible plasmid in the recipient (13, 19, 38).

An increased copy number causes an easily distinguished phenotype. Therefore, the obvious thing to do should be to define how many (plasmid) genes are involved in controlling the copy number and also, by complementation studies, distinguish between operator/promoter-like genes and other genes. Unfortunately, formal genetic analysis (mapping and complementation studies) of copy mutants is extremely difficult because incompatibility greatly distorts the linkage frequencies or complementation studies and plasmids are dispensable for the cell, which means that deletions in the plasmid deoxyribonucleic acid may remain undetected. Measurement of reversion frequencies is impossible since there is no means of selecting revertants as these should have a reduced antibiotic resistance compared to that of the copy mutant. However, cells carrying revertant R-factors were often found in clones carrying copy mutant R-factors. Furthermore, since all copy mutants studied affect incompatibility, we think it is fair to conclude that both effects in each mutant are due to a single mutation.

Several theories of control of replication and its correlation to incompatibility exist, but they may be grouped into two main categories: one suggesting positive (17) and the other suggesting negative control of replication (33, 34). In the positive-control model, incompatibility is explained by competition between plasmids for a replicational or segregational site (17), whereas in the negative-control model identical or cross-reacting repressors are formed by incompatible replicons (33, 34).

A theory that explains the link between incompatibility and control of replication must incorporate the following facts, as reported in this paper. (i) Two classes of copy mutants exist, one increasing and the other decreasing the incompatibility exerted towards the test plasmid. (ii) One of the classes of copy mutants is *cis* dominant. (iii) Even in a cell carrying a *cis*-dominant copy mutant, the test plasmid replicates.

The replication control models proposed in the literature postulate a number of elements: replicational and segregational sites (17, 31), activators (17), repressors (33, 34), and antirepressors (36). We will now examine how these postulated elements fit into our experimental results.

In the positive-control model proposed by Jacob et al. (17), replicon incompatibility is explained by competition for a replicational and/or segregational site. If the site were determined by the host and if its number were limited, a copy mutant should show a stronger incompatibility against a test plasmid than that exerted by the wild-type plasmid. However, the fact that copy mutations may decrease incompatibility is not explained by this model. The other possibility, namely, that plasmids code for a portion or all of their respective site, should invalidate the whole idea of competition for sites. Consequently, our results do not support the hypothesis that incompatibility is due to competition for a site.

Copy mutants may be explained by mutations in the stuctural gene for an activator of replication or in the target gene for the activa-

tor. Copy mutations in any of these two genes should lead to an increased activator concentration. Hence, irrespective of whether or not the R1 activator acts on the test plasmid R100, the existence of the two classes of copy mutants cannot be explained by the activator system.

Copy mutants may be explained by mutation in the structural gene for a repressor of replication or in the target gene for the repressor. Incompatibility should, in theory, be due to the formation of identical or cross-reacting repressors. A copy mutation in the repressor gene should cause a decreased activity of the repressor and, hence, a decreased incompatibility towards the test plasmid. Furthermore, since the repressor target remains unaffected, such a mutation should be recessive. This response was shown by plasmid mutant R1*drd-19B4*. A mutation in the repressor target should cause a copy effect if the affinity between the repressor and its target were decreased. Such a mutation should cause an increased repressor concentration and, consequently, an increased incompatibility towards the test plasmid. Furthermore, the copy effect should be essentially unaffected by the presence of the test plasmid; i.e., the mutation should be *cis* dominant (cf. copy mutant R1*drd-19B2*). Thus, the two classes of copy mutants found can be explained by the repressor theory.

In a negative-control system, the *cis*-dominant compatibility class should be the candidate for R-factors carrying operator mutations. These should then form increasing concentrations of repressor with increasing copy effect. As a matter of fact, there was a graded response in the rate of loss of test plasmid R100 as the copy effect increased in this class of mutants (Fig. 7). However, the fact that test plasmid R100 replicated at all in cells carrying copy mutant R1*drd-19B2* is puzzling, but may indicate that the control of plasmid replication is not very accurate. This is in qualitative accordance with the considerable spread in interdivision times measured in synchronous cultures (23) or by microscopy studies of individual cells in microdrops (12, 32, 39). In both cases the standard deviation was about 15 to 20% of the generation times. An inaccurate control of replication may also be compatible with the randomness in selection of R1 copies for replication reported elsewhere (15). Furthermore, it is possible that plasmids R1 and R100 form cross-reacting, rather than identical, repressors. However, unpublished experiments with R1 mutants, i.e., an isogenic system, gave essentially the same quantitative results as the heterogenic system with R100 as the test plasmid.

The plasmid R1 is a combined replicon consisting of two parts, the resistance replicon and the transfer factor. In *Proteus mirabilis*, R1 and similar plasmids are mainly present as smaller replicons that replicate autonomously (5). Copy mutations may affect either of these replicons, since mutant R1*drd-19B2* appears to be mutated in the resistance part (30), and Morris et al. (27) have isolated a copy mutant in which the mutation is in the transfer factor. This result is compatible with the Pritchard theory (33, 34). In a combined replicon, replication is governed by the replicon that has the smallest critical mass of initiation of replication. Still, both participating replicons exert their respective incompatibility by the action of their repressors.

An appropriate control of the postulate that incompatibility and control of replication are closely related should be to isolate compatible mutants and test their copy number properties. Unfortunately, the rather slow segregation of plasmids R1 and R100 makes direct selection of compatible mutants rather difficult. Maas and co-workers (8, 21) and Willetts (44) have described mutations and deletions in the F region of an Hfr which lead to decreased incompatibility towards a free F-factor. Unfortunately, it does not seem to be possible to determine the effect of these genetic changes on control of replication. Wyman and Novick (45) have recently isolated an incompatible negative mutant of a plasmid in *Staphylococcus aureus*; this plasmid mutant could not replicate as an autonomous plasmid but was integrated into

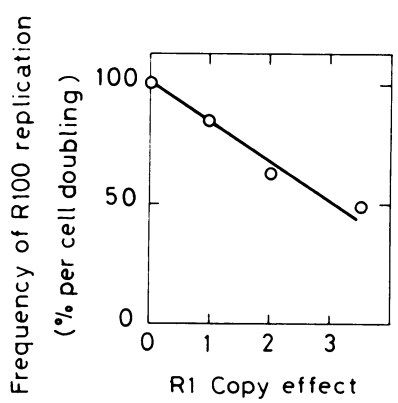

FIG. 7. *Frequency of replication of test plasmid R100 in cells carrying the wild type or copy mutants of R-factor R1drd-19. The frequency of R100 replication was estimated from the relative increase in cells carrying R100 during one doubling in cell number (see Table 2). The copy effect of R1 was taken from the data of Table 1.*

the bacterial chromosome at the nonpermissive temperature.

In most cases, R-factor copy mutants were conjugally transferred less efficiently than the corresponding wild type (Table 4). At present, it is difficult to conclude whether this is due to the copy mutation or to some other factor. Therefore, it is premature to try to include this finding in a theory for the control of plasmid replication.

The preferential loss of copy mutant R1*drd-19B4* from cells also carrying test plasmid R100 is not easily explained since the former outnumbers the latter by a factor of 10. It should be stressed that the majority of those R-factor copy mutants that showed a decreased incompatibility towards the test plasmid behaved similarly to R1*drd-19B4*. This may imply that incompatibility and control of replication are due to several factors. This is also evident from the report by Macrina et al. (22) that a chromosomal mutation may affect the copy number of the relaxed plasmid R6K.

It may also be appropriate to mention the studies of Berg (1) on replication of the λ*dv* plasmid. This plasmid contains only a small fraction of the λ genome and is present in about 20 copies per cell. Its replication is under negative control by the product of the λ gene *cro*. The *cro* gene product controls its own formation in a way similar to that in the autorepressor theory proposed by Sompayrac and Maaløe (40). The model described by Berg (1) also has basic features in common with the Pritchard (33, 34) model, in that cyclic variations in the concentration of a negative-control element governs replication.

At present it is not possible to propose a detailed model of how replication of plasmids is controlled and how incompatibility is exerted. We regard copy mutants of plasmids as useful tools in studies of control of replication and of incompatibility. We also want to stress that incompatibility is a quantitative, and not just a qualitative, property of plasmids. Since our discovery of them (30), copy mutants have been observed in plasmids with either so-called stringent (27, 30) or so-called relaxed (18) control of replication. Studies of copy mutants may reveal how many different genes are involved in control of replication and incompatibility and may demonstrate whether the corresponding gene products have positive- or negative-control functions.

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