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Runaway Replication of Plasmid R1 Is Not Caused by Loss of Replication Inhibitor Activity of Gene *cop*

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The replication control functions of a mutant of plasmid R1 that replicates without control at temperatures above 35°C have been analyzed. Although the mutations have not been mapped precisely, the data indicate that the gene (*cop*) previously identified on the wild-type plasmid (S. Molin and K. Nordström, J. Bacteriol. 141:111-120, 1980) as being responsible for expressing a *trans*-acting replication inhibitor, as well as for incompatibility of plasmid R1, is not affected in this mutant. Thus, the conditional lack of replication control observed in this plasmid mutant presumably is not caused by the loss of inhibitor activity of the *cop* gene.

Plasmid R1 may be mutated to elevated copy number, and conditional mutants have been isolated which show wild-type phenotype at one condition (low temperature or in suppressor strains) and copy mutant phenotype at another condition (increased temperature or lack of suppression (6).

From a temperature-dependent copy mutant, pKN301, a second mutational event resulted in the so-called runaway-replication mutant pKN400. This mutant has slightly increased copy number at 30°C and replicates without control at temperatures above 35°C. This mutant (18) and various miniplasmid derivatives (16) were described recently.

The mutations in the runaway plasmids have not been mapped precisely, but the fact that small (5×10^6 daltons) derivatives of the runaway-replication mutants include the replication region of R1 and retain the runaway-replication phenotype shows that the mutations are located near the origin of vegetative replication (11). A map of one such miniplasmid, pKN402, is shown in Fig. 1.

We have recently shown that a gene (*cop*) expressing an inhibitory activity on plasmid R1 replication maps on a small *PstI* fragment (the F_1 fragment) very near the replication origin (10). (The *PstI* F_1 fragment can be distinguished from the F_2 fragment [Fig. 1] by the presence of a *SalI* site in the former fragment. The *cop* gene discussed here is located on the *PstI* F_1 fragment.) This function acts in *trans* and is involved in incompatibility as well as copy number control. It was, therefore, of interest to test the function of the analogous gene from the runaway mutant. The strains and plasmids used are listed in Table 1.

First, we cloned the different *PstI* fragments from the miniplasmid pKN402 on the cloning vehicle pBR322. Each of these chimeric plasmids was transformed to strain LC343*int500* at 30°C, and the resulting strains were tested for growth at 42°C. Strain LC343*int500* has the *dnaA46* mutation and is integratively suppressed by the insertion into the chromosome of a small plasmid (wild-type copy number) derived from plasmid R1. Thus, at temperatures above 42°C the chromosome replication is initiated and controlled from the integrated plasmid. As described recently (10), this strain can be used to identify R1 replication control functions carried by plasmid vectors.

All plasmids harboring the *cop* gene (*PstI* F_1 fragment) from pKN402, and only these, inhibited growth of LC343*int500* at 42°C. This effect was not dependent on the orientation of the inserted F_1 fragment. One such example is plasmid pKN244, which consists of plasmid pBR322 with the F_1 fragment from plasmid pKN402 inserted in the β-lactamase gene of pBR322. Total DNA synthesis in strain LC343*int500* harboring plasmid pKN244 was analyzed at 30°C and after a temperature shift to 42°C. As shown in Fig. 2A, total DNA synthesis was strongly repressed at 42°C, and the shape of the curve was indistinguishable from what has been observed for analogous plasmids derived from the wild-type R1 plasmid (10). Figure 2B shows a similar experiment using the small runaway plasmid pKN402A; also, in this case, switch-off of chromosome replication is observed at the high temperature.

These experiments clearly show that the temperature-dependent runaway plasmids do express the negative *trans*-acting function from the *cop* gene even at the temperature at which

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replication of these plasmids is usually uncontrolled.

Incompatibility is at least in part caused by the *cop* gene (17). The incompatibility phenotype of the runaway plasmids was analyzed in two ways. (i) The chimeric plasmid pKN244 was transformed to strain C600 harboring plasmid R1*drd-19*. After selection at 42°C for either the incoming plasmid only or for both plasmids, the segregation of plasmid R1*drd-19* was examined and found to be as fast as previously shown for wild-type *PstI* F_1 fragments (10) (data not shown). (ii) We have observed that in strain CR34 the small runaway plasmid pKN402A was considerably amplified at 42°C, but its replication was somehow limited such that runaway replication did not occur at this temperature (Table 2). Thus, the incompatibility phenotype of pKN402A could be analyzed in this strain at both 30 and 42°C. The runaway plasmid was introduced by transformation into CR34(R1*drd-19K1*) (at 30°C), and, after selection for both plasmids and subsequent growth without selection pressure, the plasmid content was analyzed (resistance phenotypes of single-cell colonies) at 30 and 42°C. As shown in Table 2, the runaway

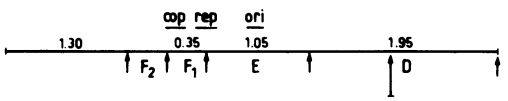

FIG. 1. *Physical and genetic map of plasmid pKN402. The locations of restriction enzyme sites and replication genes refer to previously published data (10, 11, 14). The identification and mapping of the PstI F_2 fragment was recently done by Uhlin (unpublished data) and by Werner Goebel (personal communication). The molecular masses of the PstI fragments are indicated and expressed in megadaltons. The gene symbols cop, rep, and ori stand for copy number control, replication factor, and origin of replication, respectively. Arrow, PstI site; barred arrow, EcoRI site.*

TABLE 1. *Bacterial strains and plasmids*

Strain or plasmid	Parent	Genotype[a]	Reference
E. coli strain			
C600		*thr leu thi*	(1)
CR34	C600	*thr leu thi thyA dra*	
LC343*int-500*	LC343	*leu thy pro lac dnaA46 aphA*$^+$	(4, 10)
Plasmid			
R1*drd-19*		*tra*$^+$ *fin*$^+$ *bla*$^+$ *cat*$^+$ *aphA*$^+$ *sul*$^+$ *aadA*$^+$	(9)
R1*drd-19K1*		*aphA*$^+$ *tra*$^+$ *fin*$^+$	(3)
pKN402[b]			(18)
pKN402A[b]		*bla*$^+$	(16)
pBR322	pMB1	*tet*$^+$ *bla*$^+$	(5)
pKN244	pBR322	*tet*$^+$ *inc* (FII)	This paper

[a] Gene symbols are according to Bachmann et al. (2) and Novick et al. (13).

[b] Temperature-dependent runaway-mutant derivatives of plasmid R1*drd-19*.

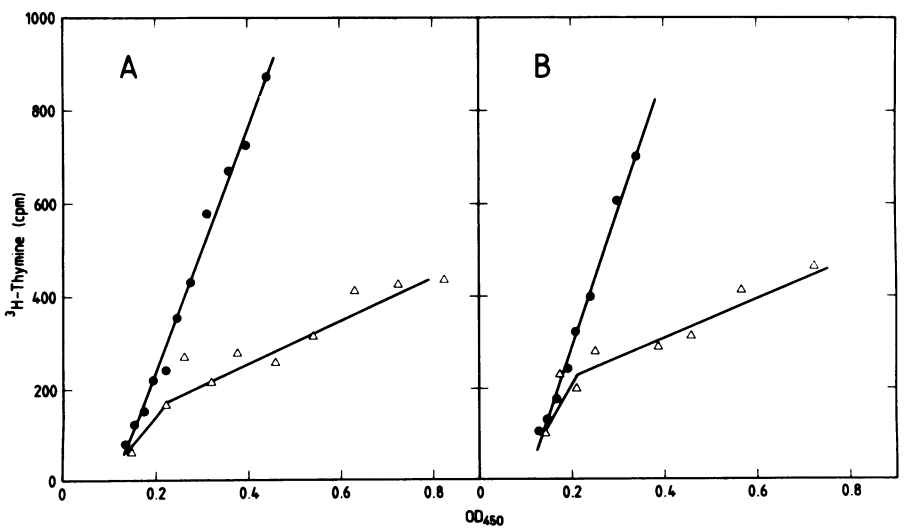

FIG. 2. *Total DNA synthesis in the strains LC343int500(pKN244) (A) and LC343int500(pKN402A) (B) at 30°C (●) and after a temperature shift to 42°C (△). Cultures of the strains were grown exponentially in LB medium supplied with 10 μg of thymine per ml. At an optical density at 450 nm of 0.1, [^{3}H]thymine was added (5 μCi/ml, 30 Ci/mmol), and half the culture was transferred to 42°C while the other half was kept at 30°C. From both the 30°C cultures and the 42°C cultures, 0.1-ml samples were taken to 5% trichloroacetic acid, and growth was monitored spectrophotometrically in a Zeiss PMQ3 spectrophotometer. Incorporation of [^{3}H]thymine was analyzed after filtration and extensive washing with ice-cold trichloroacetic acid as described recently (10).*

TABLE 2. *Incompatibility properties of runaway plasmids in CR34*

Plasmid[a]	Temp (°C)	Fraction of cells[b] retaining:		
		R1*drd-19K1* only	pKN402A only	Both
R1*drd-19K1* + pKN402A	30	0	0.53	0.47
R1*drd-19K1* + pKN402A	42	0	0.89	0.11

[a] The copy numbers of the plasmids in strain CR34 were determined as single-cell resistances to benzylpenicillin. It was found that the resistance level of plasmid R1*drd-19* was 100 μg/ml, i.e., the same as normally found in *Escherichia coli* strains. For plasmid pKN402A the resistance level was 200 to 300 μg/ml at 30°C and 1,500 to 2,000 μg/ml at 42°C.

[b] Colonies growing on plates selecting for both plasmids (LA [11] plus ampicillin and kanamycin) were spread on LA plates without antibiotics. Single colonies on these were spread again on LA plates, and 100 single colonies were eventually tested for the presence of the plasmids by their resistance phenotype.

mutant was preferentially retained at both temperatures, and this tendency is expressed more at the high temperature. (This dominance of the mutant plasmid in an incompatibility test was also observed for the large runaway-replication plasmid pKN400 [not shown].) As discussed previously (17), such a phenotype is not expected for a mutant with simply an inactive replication inhibitor.

The data presented here suggest that uncontrolled plasmid replication (runaway replication) is not necessarily the result of inactivation of the *cop* gene product of the *PstI* F_1 fragment. In fact, we have found no indication that this function is mutated at all in the runaway-replication mutant (this paper) or in the parental temperature-dependent copy mutant pKN301 (Molin, unpublished data). Thus, these particular temperature-dependent copy mutants per se do not provide evidence for the involvement of a negative control element in replication (6–8, 12, 15). Furthermore, a recessive copy mutant of plasmid R1 was recently found to lack inhibitory activity from the *cop* gene (10), although the mutant plasmid does not express runaway behavior but has a stable, non-conditionally elevated copy number (17). Evidently the inhibitory activity from the *PstI* F_1 fragment is not the only regulatory function in the copy number control. At present we do not know which functions are mutated to produce runaway replication, but the observations indicate that more than one gene product is involved in replication control of plasmid R1.

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