DNA Restriction-Modification Systems Mediate Plasmid Maintenance

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Two plasmid-carried restriction-modification (R-M) systems, EcoRI (from pMB1 of Escherichia coli) and Bsp6I (from pXH13 of Bacillus sp. strain RFL6), enhance plasmid segregational stability in E. coli and Bacillus subtilis, respectively. Inactivation of the endonuclease or the presence of the methylase in trans abolishes the stabilizing activity of the R-M systems. We propose that R-M systems mediate plasmid segregational stability by postsegregational killing of plasmid-free cells. Plasmid-encoded methyltransferase modifies host DNA and thus prevents its digestion by the restriction endonuclease. Plasmid loss entails degradation and/or dilution of the methylase during cell growth and appearance of unmethylated sites in the chromosome. Double-strand breaks, introduced at these sites by the endonuclease, eventually cause the death of the plasmid-free cells. Contribution to plasmid stability is a previously unrecognized biological role of the R-M systems.

The stable inheritance of plasmids in a bacterial population is ensured by different types of maintenance systems, the components of which are encoded either by the plasmid itself or by the chromosome of the host (33). Well-studied examples include the site-specific recombination systems, which resolve plasmid multimers, such as res-cre of prophage P1 and cer-xer of plasmid ColE1, and active partition systems, such as par of prophage P1 and sop of plasmid F (33).

A different class of systems is based on the mechanism termed postsegregational killing (8, 17), and accounts for killing or reducing the growth of plasmid-free cells. These systems comprise two components: a relatively stable toxin and an unstable “antidote”. In the case of plasmid loss, rapid decay of the antidote allows the action of the toxin. A well-characterized example is that of the hok-sok system of plasmid R1. Synthesis of Hok protein leads to damage of the cell membrane and, eventually, cell death. Translation of long-lived hok mRNA is prevented by the unstable antisense sok RNA. As a result, the hok gene product is expressed only in the cells which have lost R1 during cell division (8, 30). Plasmid F also encodes a system of a different type, the toxic protein CcdB, which efficiently traps gyrase. The antidote, CcdA protein, not only prevents the gyrase poisoning activity of CcdB but also reverses its effect on gyrase (1). Similarly, the poison-antipoison components of the pem system of R100 are also proteins (31). Postsegregational killing by such systems efficiently ensures the retention of plasmids in the population. For example, the hok/sok system, when introduced into unstable pBR322 or p15 derivatives, decreases the appearance of plasmid-free cells by a factor of $10^3$ to $10^5$ (7). Two components of these systems, one causing cell death and the other protecting from the killing, recall another large family of proteins, the restriction-modification (R-M) systems.

R-M systems, which occur primarily in bacteria, are classified into three main groups, according to their components and cofactor requirements (34). The best-studied are type II systems, which contain two protein activities. One, DNA methyltransferase, catalyzes the transfer of the methyl group from S-adenosylmethionine to one of the nucleotides within a recognition site on both strands of DNA. The other, endonuclease, catalyzes the double-strand cleavage of unmethylated DNA at the same recognition site. Methylation of the correct nucleotide on at least one strand of the recognition sequence protects from cleavage (23, 34, 35). About 200 different type II R-M specificities are now known (24), over 100 have been cloned, many have been sequenced (35), and the crystal structures of several endonucleases, methylases, and their complexes with DNA have been determined (14, 23). The biological function of these systems is generally thought to be the protection of the bacterial cells from incoming phage and plasmid DNA, although their involvement in recombination has also been reported (3, 6, 26, 27). Here, we present data showing that type II R-M systems may increase plasmid stability, possibly by postsegregational killing of plasmid-free cells. This reveals a previously unrecognized biological role of the R-M systems.

MATERIALS AND METHODS

Bacterial strains. Escherichia coli K-12 strain MC1061 (F-, araD139 Δ[araE–lac]7696 galE11 galK16 Δ[lac]+74 rpsL hsdR2 mcrA mcrB1) (32) and Bacillus subtilis SB302 (tpcC2 tryR1 hsdH2 araR2; laboratory collection) were used.

Plasmids. For testing of R-M systems in E. coli, we used pAM34, a pBR322 derivative with its replication primer RNA under control of the lac promoter-operator (9). This plasmid replicates only when IPTG (isopropyl-β-D-thiogalactopyranoside) is added in the medium. The Sfl-Smal fragment, encoding the adaQ (SpeI) gene of pAM34 was replaced by the HaeIII-SalI fragment of pMB1 (2), carrying the EcoRI R-M system, to yield pJJC1418 (Fig. 1). Digestion with PstI, end filling, and ligation led to the EcoRI restriction endonuclease-deficient pJJC1428. Similarly, the EcoRI-PstI fragment of pBsp6IRM2LA, carrying the genes of the Bsp6I R-M system (18), was ligated to the EcoRI-PstI fragment of pAM34 to yield pJJC1415. Removal of the NruI site led to its endonuclease-deficient derivative pJJC1420. Later, the SalI site was used to fuse pJJC1418, pJJC1428, pJJC1415, and pJJC1420 to pL253 (28), a derivative of the broad-host-range plasmid pMMB1 (13), a procedure which resulted in the hybrid plasmids pJJC1445, pJJC1446, pJJC1441, and pJJC1442, respectively (Fig. 1). These hybrid plasmids, because of the replicon of pMMB1, can be maintained in B. subtilis.

pEcoRI-M was kindly provided by V. Sikinsy. This plasmid carries the EcoRI methylase gene on the HindIII-HindIII fragment of pMB1 inserted in the HindIII site of pACYC184 (25).

General methods. Standard molecular genetic methods (19) were employed. Growth conditions for bacteria and phages, concentrations of growth factors and antibiotics, and transformation procedures are described in reference 20. Restriction enzymes, ligase, and T4 DNA polymerase were used according to the instructions of the manufacturer.

Restriction assay. The activity of restriction endonucleases was measured in vivo, by determining efficiency of plating of the unmodified phage λvir (for E. coli) or SPF1 (for B. subtilis).

Modification assay. The presence of the methyltransferase activity was tested...
in vitro as the protection of a plasmid, carrying R-M genes, from digestion by the EcoRI or BsoFI (isoschizomer of Bsp6I) endonuclease. The same procedure was used to determine the presence of methylase activity in strains carrying pJJC1418 and pJJC1428; just chromosomal DNA was used as a substrate for digestion with the EcoRI endonuclease, because the plasmids do not have EcoRI recognition sites.

**Determination of the proportion of plasmid-containing cells.** *E. coli* strains carrying an R-M system on a plasmid were grown overnight in Luria-Bertani (LB) broth with IPTG (0.5 mM) and ampicillin (60 μg/ml). The cultures were diluted 10^6 times into LB broth with IPTG (0.5 mM). Samples were plated on LB agar plates with IPTG (0.5 mM) with and without ampicillin. The ratio of the number of colonies appearing on LB agar and on ampicillin-containing plates is taken as the proportion of plasmid-containing cells in the culture. After 20 generations of growth, cells were diluted and the proportion of plasmid-containing cells was determined again. At the end of the experiment Amp^R colonies were tested for the presence of methyltransferase and endonuclease activities and plasmid DNA from them was digested with several restriction endonucleases to establish that the plasmids did not undergo major rearrangements.

A *B. subtilis* strain, carrying an R-M system on a plasmid, was pregrown in LB broth with erythromycin (3 μg/ml) for 6 h and then diluted in LB broth without an antibiotic. Samples were plated on LB agar plates with and without erythromycin to determine the proportion of plasmid-containing cells. As in the experiments with *E. coli*, the culture was periodically diluted and the proportion of plasmid-containing cells was determined.

**RESULTS AND DISCUSSION**

To test the effect of R-M systems on plasmid stability, we used plasmid pAM34 (9), which is unstable, since it was lost from 97% of the cells after 100 generations of growth in LB broth supplemented with 0.5 mM IPTG. A segment of pMB1 (2) carrying the EcoRI restriction endonuclease and methylase genes was inserted into pAM34, and the stability of the resulting plasmid, pJJC1418, was determined (Fig. 1). Plasmid-free cells began to appear only after 60 generations, and the plasmid was present in 95% of the cells after 100 generations (Fig. 2A). This stabilization was due to the endonuclease, since an isogenic plasmid, pJJC1428, that carries a mutation in the endonuclease gene (Fig. 1) was lost in the 98.5% of the population after 100 generations (Fig. 2A).

When an additional copy of the EcoRI methylase gene was present in trans, on the compatible plasmid pEcoRIM, pJJC1418 was not stably maintained in the population despite the active endonuclease gene (Fig. 2A). This suggests that plasmid stability is mediated by the endonuclease cleavage of the nonmethylated recognition sites, which appear upon plasmid loss.

The stabilization effect of the other R-M system, Bsp6I, was only slight, since the losses of plasmids pJJC1415 (Bsp6I R' M') and pJJC1420 (Bsp6I R' M') were similar, with a somewhat higher maintenance of the plasmid containing the active endonuclease during the first 40 generations (Fig. 2B). This was not due to the failure to express the nuclease gene in *E. coli* or the inactivation of the nuclease gene during growth, as judged by the restriction of *λ*ir phage. Strains carrying pJJC1415 restricted *λ*ir by a factor of 10^4, whereas those that carried pJJC1418 restricted by a factor of 10^2. The more efficient restriction may be due to the larger number of Bsp6I recognition sites than the number of EcoRI sites in phage DNA.

To test the effect of R-M systems in a host other than *E. coli*, we constructed hybrid plasmids carrying the pAMβ1 origin of replication (Fig. 1), which can replicate in *B. subtilis*. In this host, the EcoRI system did not increase stability of the vector, since plasmid pJJC1445 and its endonuclease-deficient derivative pJJC1446 were lost from more than 99% of the population after 36 generations (Fig. 3A). In contrast, the Bsp6I R-M system on pJJC1441 ensured the presence of the plasmid in 100% of the cells when the culture was grown for 80
generations, whereas its endonuclease-deficient counterpart pJJC1442 was present in less than 0.1% of the cells after 45 generations (Fig. 3B). Results presented here suggest that the EcoRI R-M system in E. coli and the Bsp61 system in B. subtilis act as plasmid maintenance systems. Their efficiency, especially in B. subtilis, is equivalent to that of the hok/sok system (7). On the basis of the enzymatic activities of the two components of the R-M systems, restriction endonuclease and methyltransferase, functions that are well understood, we suggest that the R-M systems can contribute to plasmid stability by postsegregational killing of plasmid-free bacteria. In plasmid-containing cells all recognition sites in DNA, GAAATTC for EcoRI (11) and GCNGC for Bsp61 (18), are methylated because of the methylase activity and are thus protected from cleavage by the cognate restriction endonuclease, which is also present and active in the cell. If the plasmid is lost, both components of the R-M system, endonuclease and methylase, remain in the cell but are not produced de novo. During growth they are both “diluted”, and possibly also degraded, in the daughter cells while the amount of their target is simultaneously increasing. After a certain time the intracellular concentration of the R-M enzymes decreases to a level at which there is not enough methylase to completely modify all the sites. At this moment nonmethylated sites, the targets for the restriction endonuclease, appear. The endonuclease introduces double-strand breaks which, if not repaired, cause destruction of the chromosome and eventual death of the cell. The ability of an intracellular restriction endonuclease to digest DNA and cause cell death was shown in a temperature-sensitive mutant of the EcoRI endonuclease (12) and by heat inactivation of the temperature-sensitive methylase in a cloned HpaII R-M system (16). To verify this, we tested the effect of massive plasmid loss, caused by replication blockade, on growth of E. coli. pJJC1428 (EcoRI R' M') had no effect on growth of the culture in the absence of IPTG. However, blockage of replication of pJJC1418 (EcoRI R' M') caused transient cessation of growth of the culture (data not shown).

The model presented requires that a certain ratio of endonuclease and methylase intracellular activities be attained. The restriction endonuclease has to be still active enough to introduce a sufficient number of double-strand breaks when unmethylated sites appear in the host DNA. It was reported that E. coli can repair no more than two or three double-strand breaks (21), while in the chromosome of E. coli there are at least 632 EcoRI recognition sites (15). In B. subtilis this number exceeds 20,000 for Bsp61. This indicates that much lower levels of endonuclease activity than methyltransferase activity are required for postsegregational killing. Hemimethylated DNA, which is present in the host immediately after replication, is resistant to double-strand cleavage by most of the restriction endonucleases, but some endonucleases are reported to introduce a single-strand nick, which can be repaired by DNA ligase. In these cases relative levels of methylase, endonuclease, and ligase become important (23).

Homologous but not heterologous systems stabilized plasmids in E. coli and B. subtilis. This “host specificity” could be due to different means of regulation of expression in E. coli and B. subtilis. It was reported that the plasmid carrying the BamHI R-M system shows a higher level of endonuclease activity in the homologous host B. subtilis than in E. coli (5). Also, stabilities of R-M enzymes could be different in the two hosts, and more rapid decay of the endonuclease would let the cells survive plasmid loss. Differences in the ability to repair double-strand breaks also have to be considered. Further work would be required to understand the host specificity in more detail.

How general could the role of R-M systems in plasmid stabilization be? In natural strain isolates, some R-M systems were found to be encoded by small plasmids (EcoRI [2] and PvuII [4]) as well as by large plasmids (PaeRI [29] and EcoRII [10]). It is possible that these systems can participate in ensuring the presence of plasmids in the bacterial population. This function can be extended to two other types of R-M systems, since some members of type 1 R-M systems, like StyR1241 and EcoP15 (type III), are also encoded by plasmids (3). The type III R-M system, EcoP1, encoded by prophage P1 (3), could also contribute to prophage stability. These examples indicate that R-M systems might stabilize a number of plasmids. It is possible that R-M systems were acquired by these plasmids from the host chromosome, for a mutual benefit of the plasmid and the host, since the former would be maintained more stably and the latter may be protected from incoming DNA to a higher extent, due to enhanced expression of the R-M genes from a multicopy element.

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FIG. 3. Effect of R-M systems on plasmid stability in B. subtilis SB202. (A) Experiment with the EcoRI R-M system. Squares, pJJC1445 (R' M'); circles, pJJC1446 (R' M'). (B) Experiment with the Bsp61 R-M system. Squares, pJJC1441 (R' M'); circles, pJJC1442 (R' M').
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ADDENDUM

Observations and conclusions essentially analogous to those reported here were reported independently after the manuscript was submitted for publication (22, 36).

REFERENCES


