Selfish maintenance by EcoRII restriction-modification system is enhanced by a mutation in its modification enzyme

Satona Ohno

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Supervisor Ichizo Kobayashi

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ABSTRACT

Several Type II restriction-modification gene complexes can force their maintenance on their host bacterial cells by killing cells that have lost them through restriction attack on their chromosome. Such ability to cause post-segregational host killing is a feature of classical plasmid addiction modules encoding a stable toxin and an unstable antitoxin. The gene loss leads to preferential decay of the antitoxin and allows toxin expression/ action. In this study, we demonstrated post-segregational killing by wild-type EcoRII gene complex. We also analyzed its mutant form that causes host cell death upon shift to a higher temperature. Our sequence analysis and site-directed mutagenesis revealed that this temperature-sensitive phenotype is conferred by a single amino acid substitution, T239C = L80P, at the N-terminal region of the modification enzyme. Upon shift to the higher temperature *in vivo*, this mutant enzyme lost methylation activity. However, a mutant enzyme deleted for the N-terminal region showed methylation at the higher temperature as well as the lower temperature. The point-mutant EcoRII gene complex showed a stronger activity in plasmid maintenance than the wild-type EcoRII. These results demonstrate that the selfish maintenance of EcoRII system can be modulated by sequence in a dispensable region of its

modification enzyme. This finding is in accord with the selfish gene concept for the Type II restriction-modification systems.

INTRODUCTION

Post-segregational systems and instability of their antitoxin

Post-segregational killing by an addiction module of the proteic toxin-antitoxin system was first identified as a maintenance mechanism by plasmids and has been suggested to play an important role in cell death and survival (5, 7, 15, 19). The toxin-antitoxin system is generally composed of two adjacent genes, one for toxin protein and the other for antitoxin protein. The toxin inhibits cell growth or, in some cases, kills cells by inhibiting important cellular processes such as translation. The cognate antitoxin counteracts the toxin action through direct interaction. The toxin is stable whereas the cognate antitoxin is metabolically unstable because of degradation by a specific protease. Such differential stability was shown to be critically important for the post-segregational killing. When cells fail to retain the addiction module, degradation of the unstable antitoxin leads to imbalance between the toxin concentration and the antitoxin concentration. This imbalance would result in release of the toxin to attack its cellular target.

The instability of the antitoxin is also the central feature of a second type of post-segregational killing: anti-sense RNA-mediated systems (6). An antisense RNA

of the toxin gene serves as an antitoxin by inhibiting translation of the toxin gene. Loss of the toxin/ antitoxin gene leads to preferential decay of the antisense RNA, which then allows toxin expression.

Post-segregational killing by Type II restriction modification systems

5 A third type of post-segregational killing systems consists of Type II restriction modification systems (28), (18), (20). A Type II restriction-modification (RM) gene system is typically composed of a gene encoding a DNA endonuclease (restriction enzyme) (R), which cleaves double-stranded DNA at a specific recognition sequence, and a gene encoding a DNA methyltransferase (modification enzyme) (M), which

10 methylates the same recognition sequence to protect DNA from the cleavage. It has been generally believed that RM systems provide effective defense against invading foreign DNAs such as phage genomes and plasmids and have been selected and maintained during evolution for this benefit they provide to the host bacteria (cellular defense hypothesis).

15 We found that a plasmid carrying a Type II RM gene complex resists loss through post-segregational host killing (28) These include PaeR7I, EcoRI, EcoRV and SsoII (1, 23, 28, 29). Chromosomal RM gene complexes also resisted replacement by an allelic DNA (11, 34). The killing takes place because the restriction enzyme cleaves

the host chromosome at unmodified recognition sites that the modification enzyme fails to protect after the genes of RM are lost in the cells (10).

Type II RM as selfish elements

Analyses of various prokaryotic genomes provided evidence that RM genes can move between prokaryotic genomes (18, 21). Several RM gene systems are often linked with mobile genetic elements such as plasmids, viruses, transposons and integrons. Comparison of closely related bacterial genomes also suggests that, at times, RM genes themselves behave as mobile elements and cause genome rearrangements (1, 30).

A hypothesis was proposed by our laboratory that Type II RM gene systems represent selfish genetic elements: they can maintain themselves and increase their copy number even when they do not confer any advantageous phenotype on their host cells (selfish gene hypothesis) (18, 20). The selfish gene concept was given a strong support when a RM gene complex on a chromosome was found to multiply in tandem in a manner dependent on functional restriction gene (34). Furthermore Type II RM systems show some similarity to viruses in their regulation of gene expression (37, 39)(29, 42). When they enter a new host, they have to establish themselves in the host without excessive killing of the host cells. After establishment, Type II RM systems are expected to tightly regulate their gene expression to maintain constant cellular levels of

restriction enzyme and modification enzyme to prevent attack on the host until critical events such as the gene loss happens to trigger the attack.

Comparison of Type II RM systems and toxin-antitoxin systems

In a Type II RM system, the restriction enzyme play the role of a toxin while the modification enzyme play the role of an antitoxin. Rather than interacting directly with the toxin or inhibiting its expression, the antitoxin protects the target of the toxin, which consists of numerous copies of their recognition sequence along the chromosome. After gene loss, dilution of the antitoxin would lead to exposure of target sites along the newly replicated chromosomes to lethal attack by the remaining restriction enzyme. If this antitoxin metabolically unstable as in the other types of post-segregational killing, the host killing would be accelerated from the rate caused solely by dilution. A recent test of this idea in our laboratory, however, revealed that M.EcoRI is as stable as R.EcoRI *in vivo* (16).

Competition between RM systems for recognition sequence

Post-segregational host killing by RM systems may provide an explanation for individual specificity and collective diversity of their sequence recognition (23), which has been explained by frequency-dependent selection by invading genetic elements. When two RM systems within one host recognize the same sequence, only one of them

can force their maintenance through post-segregational killing: loss of one RM would not lead to chromosomal attack by its restriction enzyme because modification enzyme of the other RM would protect the recognitions sequence. This prediction was verified in laboratory experiments in the absence of any invading elements (23). Such competition between RM systems for a recognition sequence may have accelerated the evolution of specificity and diversity in their sequence recognition.

In a similar way, post-segregational killing by an RM system would be prevented by a solitary methyltransferase that recognizes the same sequence. This was demonstrated for EcoRII RM system, a Type II system with a popular recognition sequence of 5' CCW (= A or T) GG, and Dcm, a solitary methyltransferase found in the chromosome of *Escherichia coli* and related bacteria (41). Dcm was hypothesized to have evolved to serve as a molecular vaccine against parasitism by EcoRII isoschizomers.

Unusual behavior of EcoRII system

In the above and a related work (1) we realized the unique feature of host killing by EcoRII RM gene complex. When a bacterial strain carrying a plasmid with temperature-sensitive replication machinery and with insertion of an addiction module including Type II RM gene complex is shifted to a non-permissive temperature, there is stop of increase of viable cell counts as well as plasmid-carrying cell counts because

a cell remains viable as long as it carries the plasmid. In such experiments, however, EcoRII gene complex showed immediate and severe decrease of cell viability instead of growth arrest. We named this phenomenon postdisturbance cell killing as opposed to post-segregational cell killing.

Present study

In the present article, we found that this killing involves a mutation in the EcoRII modification enzyme gene. Our analysis demonstrated that the selfish maintenance of EcoRII system can be modulated by sequence in a dispensable region of its modification enzyme. This finding is in accord with the selfish gene concept for the Type II RM systems.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage and media

The new nomenclature for restriction modification enzymes was followed (33).

The *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Betrtani (LB) medium (27). When required, antibiotics were added at the following concentrations: ampicillin (Amp), 50 μg/ml; tetracycline (Tet), 15 μg/ml; chloramphenicol (Cml), 25 μg/ml; oxacillin, 50 μg/ml.

pOS28 and pOS30 were constructed as follows. The *ecoRIIRM* were amplified from plasmid N3 using the primers RII1 and RII2 (41), which contain an XmaI site. The amplified fragment was digested with XmaI and inserted into the corresponding site of pUC19 or pHSG415 (12), and transformed into *E. coli* strain DH5.

pOS34 and pOS39 were constructed from pOS28 and pOS30, respectively, by HindIII digestion, recovery of the larger fragment from agarose gel electrophoresis, and its self-ligation.

pOS22 and pOS48 were constructed from pNY30 (41) by site-directed mutagenesis using the following primers: IKOS31 (5'-GACGTTAGCCGAAGAGGAACTTC*TACGTA*AAATGCTTCCGGAAGCGC-3') and IKOS32 (5'-GCGCTTCCGGAAGCATTT*TACGTA*GAAGTTCCTCTTCGGCTAACGTC-3')

for pOS22; IKOS50 (5’-CTGACGTTAGCCGAAGAGGAACTTCTACGAAAAATGCTTCCGGAAGCGC-3’) and IKOS50-r (5’-GCGCTTCCGGAAGCATTTTTCGTAGAAGTTCCTCTTCGGCTAACGTCAG-3’) for pOS48. The italicized letters indicate SnaBI site introduced as a marker, while the underline letters indicate the mutant base pair. The amplified fragment was digested with DpnI and then transformed into *E. coli* strain DH5.

pOS41 was constructed from pNY30, while pOS43 and pOS45 were constructed from pOS28 as follows. The *ecoRIIM* was amplified from pNY30 and pOS28 using the following primers: IKOS46 (5’-GG*GAATTC*ATGTCTGAATTTGAATTAC-3’) and IKOS47 (5’-GG*AGATCT*TCAGATTCGTTCAACCTT-3’) for pOS41 and pOS43, IKOS47 and IKOS48 (5'-GG*GAATTC*CTTCCGGAAGCG) for pOS45. The italic letters indicate introduced EcoRI and BglII sites. The amplified fragment was digested with EcoRI and BglII, ligated with the larger fragment of pFLAG2 (Sigma) digested with EcoRI and BglII, and transformed into *E. coli* strain DH5. The *ecoRIIM* gene, its T239C mutant or its N83 deletion mutant was inserted into pFLAG2 (Sigma) so that each gene is fused with FLAG tag under control of an IPTG inducible promoter.

pOS51 and pOS54 was constructed from pNY41 and pNY31 (41), respectively, by site-directed mutagenesis using the primers IKOS50 and IKOS50-r (see above). pOS57 was constructed from pNY31 by site-directed mutagenesis using the following primers: IKOS49 (5'-GCTTGATGAGCAAGGGGGGACTGTAAGGAAGTAAATATTTGGGTATG-3') and IKOS49-r (5'-CATACCCAAATATTTACTTCCTTACAGTCCCCCCTTGCTCATCAAGC-3'). The underlines indicate the mutant base pair.

All transformation to construct plasmid was performed by RbCl method (9). All plasmid were prepared by GenElute Plasmid Miniprep kit (Sigma).

During these plasmids construction, EcoRII restriction/ modification activity was confirmed using lambda phage infection. EcoRII restriction activity *in vivo* was measured by determining efficiency of plaque formation (EOP) of unmodified and *dcm*-modified *lambda vir* phage (laboratory collection) on the *E. coli* strain to be tested.

EcoRII modification activity *in vivo* was assessed by growing *lambda vir* phage on *E. coli* strain carrying a plasmid to be tested and then determined efficient of plaque formation of this modified phage on *E. coli* strain carrying pNY30.

Measurement of temperature-sensitivity of bacterial growth

A bacterial strain carrying one of the *ecoRIIRM* plasmids were grown at 30°C in LB liquid medium with selective antibiotics until OD660 of the culture reached 0.5. The culture was diluted and spread on an LB agar plate with and without the antibiotics.
5 The plates were immediately placed at 30°C or at 42°C for overnight incubation for colony counting.

Post-segregational killing by temperature shift

A bacterial strain carrying the *ecoRIIRM* plasmid with temperature-sensitive replication machinery was grown with aeration at 30°C in LB liquid medium with the
10 selective antibiotics until OD660 of the culture reached 0.5. Then, the cells were collected by centrifugation, suspended in LB liquid medium without the antibiotics, and grown at 42°C. The cells were diluted whenever OD660 of the culture reached 0.6. The total cell number was counted under a microscope. The number of viable cells is defined as the number of colony forming units on LB agar plate without the
15 antibiotics at 30°C. The number of cells carrying the *ecoRIIRM* plasmid is defined as the number of colony forming units on LB agar plate with the antibiotics at 30°C.

Detection of M.EcoRII-mediated plasmid methylation *in vivo*

Strains carrying pOS41 and pOS43, respectively, were grown with aeration at 30°C in

LB liquid medium with the selective antibiotics (Amp) and 1mM IPTG until OD660 of the culture reached 0.5. Then Cml (final, 25 μg/ml) was added to inhibit protein synthesis (2), and the incubation temperature was shifted to 42°C. The cells were sampled at 2, 3 and 4 hours later. The control cultures were grown at the same
5 temperature (30°C or 42°C) before and after addition of Cml. One fifth of plasmid DNA recovered from 1.5 ml culture was treated with 2.5 U of EcoRII restriction enzyme (Wako) for 1 hour at 37°C in 15 μl.

Strain carrying the pOS45 was grown with aeration at 30°C in LB liquid medium with the selective antibiotics (Amp) and 0.4% glucose until OD660 of the culture reached
10 0.5. Then 1mM IPTG was added, and then the cultivation was continued at 30°C or 42°C for 2 more hours. The plasmid was recovered and 100ng of plasmid molecule was treated with 2.5 U of EcoRII restriction enzyme (Wako) for 1 hour at 37°C.

Measurement of plasmid maintenance

All the cultivation procedures were carried out at 30°C. A single colony on LB agar
15 with the selective antibiotics (Cml) was suspended in 5 ml of LB liquid medium with the antibiotics and grown overnight with aeration. The saturated culture was diluted (to obtain approximately 10^3 cells/plate) and spread onto LB agar with or without the antibiotics. The overnight culture was diluted 10^{-5} in LB without antibiotic for

overnight growth. This cycle of sampling, dilution and overnight growth was repeated. The generation number was calculated from the viable cell count. The detailed procedure was described elsewhere (41).

RESULTS

Mutation in M.EcoRII causing temperature sensitivity in host cell growth

We have characterized EcoRII RM system before. However, it shows drastic postsegregational killing under the same experiment above. Furthermore, even if it was connected to temperature resistant plasmids like pUC19, still significantly cell death occurred at higher temperature.

We obtained the temperature sensitive mutant of M.EcoRII. The colony forming unit of *E. coli* strain GM31 carrying pNY30 (pUC19 carrying *ecoRIIR* (T402C) *M* (T239C)) was lower than that of *r-* strain at 42°C (Table .2). Even though the replication of this plasmid is not temperature sensitive.

This phenomenon was also observed in *E. coli* BNH670 (*delta dcm*) carrying pNY30 (pUC19 carrying *ecoRIIR* (T402C) *M* (T239C)) incubated at 42°C. Thus, this temperature sensitivity came from the mutation in EcoRII RM gene complex.

To decide the cause for this temperature sensitive phenotype, we sequenced *ecoRIIRM*. In the result, two mutations were found that might have been produced during cloning by PCR. One mutation was found in the *ecoRIIM*: T239C with amino acid substitution L80P (Fig. 1). One is another mutation T402C in *r* gene was found T402C, but it is without amino acid substitution change (Ile134) or significant

codon-usage change. The codon-usage of Ile in *E. coli* is ATT: 0.47, ATC: 0.46 (Codon usage database: http://www.kazusa.or.jp/codon/index.html). The other mutation in the *ecoRIIM* is T239C with amino acid substitution L80P (Fig. 1).

To investigate whether the cause of cell death at 42°C is T239C (L80P) mutation in
5 *ecoRIIM* or the T402C mutation in *ecoRIIR*, we replaced these two mutations with wild type sequence in *ecoRIIR* or *ecoRIIM* by site-directed mutagenesis. The wild-type sequence was referred to these articles (32, 38). Then examined the their colony formation unit. The *E. coli* strain GM31 carrying pOS22 (pUC19 carrying *ecoRIIR* (T402C) *M*) can grow at 42°C. However *E. coli* strain GM31 carrying
10 pOS48 (pUC19 carrying *ecoRIIRM* (T239C)) failed to grow at 42°C (Table. 2). We also observed the *E. coli* strain GM31 carrying the wild-type EcoRII RM was successfully to grow at 42°C.

These results indicated that the T239C (L80P) mutation in *ecoRIIM* is responsible for cell death at 42°C

15 **Post-segregational killing by wild-type EcoRII RM gene complex.**

To investigate whether EcoRII RM gene complex shows postsegregational killing, we performed the temperature shift experiment in the liquid media. The EcoRII RM gene complex was inserted into a plasmid that possesses a temperature- sensitive

replication initiator in order to analyze the effect of loss of the gene complex during

cell growth.

The inhibition of plasmid replication following the shift of EcoRII *r*$^+$ and *r*$^-$ strains'

cultures to the non-permissive temperature for plasmid replication stopped the increase

5 of plasmid-carrying cells on both the strains. The temperature shift attenuated the

increase in viable cell counts for the r$^+$ culture but not for the r$^-$ control. The increase

in the total cell number as estimated under a microscope was also eventually stopped in

the r$^+$ culture (Fig. 3B3A). These results were obtained with BNH670 (*delta dcm*).

Our preliminary experiments suggested that post-segregational killing is weaker, if any,

10 with GM31 (*dcm-6*) (data not shown).

Then to obtain an evidence of for the death of individual cells, we observed cell

morphology with DAPI (4', 6'-diamino-2-phenylindole) staining following loss of the

RM plasmid (10). The growth of the EcoRII R$^-$M$^+$ strain was slower than R+M+

strain. The filamentated cells appeared 6-8h after the temperature shift (Fig. 3C). The

15 filamentation was in *r*$^+$ dependent manner. A small but significant fraction of the cells

appeared anucleated in the *r*$^+$ cells.

Furthermore to obtain the evidence of the individual cell death, the chromosomal DNA

in these cells was analyzed directly by agarose electrophoresis. As in our earlier

work, the small DNA passed the well of 0.7% agarose gel, but large DNA such as intact chromosomal DNA are trapped in the well (23, 28). Absence of DNA signals in the wells were taken as the evidence of chromosomal degradation. In the r^+ strain, the DNA in the wells was gradually lost after the temperature shift (data not shown).
5 These features correspond to those observed in classical postsegreagional killing systems for by other RM gene complexes on plasmids (19). These results indicate that EcoRII RM gene complex mediate the postsegregational killing.

In vivo **methylation by wild type, L80P and Δ N83 M.EcoRII**

In order to investigate whether mutant M.EcoRII methylates CCWGG sequence at
10 30°C or 42°C, we performed *in vivo* methylation assay using pOS41 (pFLAG2 carrying *ecoRIIM* (T239C)) and pOS43 (pFLAG2 carrying wild-type *ecoRIIM*). The *E. coli* strain BNH670 carrying pOS41 or pOS43 were cultured with 1mM IPTG at 30°C until OD660 reached to 0.5, then added Cml (25 μg/ml) and shift to 42°C. The Cml inhibit the protein *de novo* synthesis and lead cell growth inhibition, however
15 plasmid of the ColE1 origin continues to replicate in the presence of Cml (14). The M.EcoRII is not produced no longer but new plasmids replicate and accumulate in the cell at 42°C. The plasmid molecules were recovered and digested with EcoRII restriction enzyme to evaluate the ability of methylation of M.EcoRII at 42°C.

Furthermore we constructed the deletion mutant of M.EcoRII. These 83 amino acids at the N-terminus are not essential for the function was already reported (3). Whereat we investigate whether the region of 83 amino acids at the N-terminus are essential for the function at 42°C or not, we constructed the pFLAG2 carrying M.EcoRII deficient 83

5 amino acids at the N-terminus (M.EcoRII (Δ N83)). And experimented same as described before. This plasmid was methylated at both 30°C and 42°C (Fig. 4B). These results revealed that the 83 amino acids at the N-terminus were not essential for the function at 42°C.

These results suggest that the T239C (L80P) mutation was not inhibit the any functions

10 of this region, it may regulate expression and/or activity of EcoRII M negatively.

The result with N83 deletion mutant suggests that this region is dispensable for methylation reaction. In other words, the L80P sequence in this otherwise dispensable region conferred an *ad hoc* phenotype of temperature sensitivity.

L80P mutation in M.EcoRII enhances plasmid maintenance

15 Insertion of a Type II RM gene complex into a plasmid leads to its stable maintenance in the cell because of the post-segregational killing (1, 23, 28, 29). We found that a plasmid carrying *ecoRIIRM* (T239C) was more stable than an isogenic plasmid carrying *ecoRIIRM* (Fig. 4). These results with p15A replication machinery were

confirmed with pSC101 replication machinery (date not shown). This stronger maintenance by the mutant corresponds to its stronger post-segregational killing.

DISCUSSION

Mutant M.EcoRII

We identified a mutant of EcoRII RM gene complex that makes host growth temperature sensitive. The L80P /T239C mutation in the modification enzyme/gene (Fig. 1) was shown to be responsible for this phenotype. The mutant enzyme is temperature-sensitive in that it loses the capacity to methylate replicating DNA after the temperature shift (Fig. 3). We concluded the cell death at the high temperature by the mutant EcoRII is caused by insufficient methylation of the host genome and its exposure to lethal attack by the restriction enzyme.

Post-segregational killing by wild-type EcoRII RM

After loss of the wild-type EcoRII RM gene complex, many cells became elongated and lost their nuclei (Fig. 2B). This clearly demonstrates postsegregational killing is caused by EcoRII RM gene complex. This is the typical feature of Type II restriction modification gene complex (10). Previous reports in our laboratory suggested postsegregational killing by EcoRII RM gene complex (1, 41), however it is the first time to demonstrate postsegregational killing by wild-type EcoRII RM gene complex.

Mechanisms of temperature sensitivity of the mutant and growth at 42°C

We carried out *in vivo* methylation assay (Figure 3) except without addiction of Cml

with the wild-type M.EcoRII, its L80P mutant, its mutant deleted for the N-terminal 83 amino acids. The delta N mutant methylated the plasmid but M.EcoRII (L80P) failed to do so at 42°C (data not shown). These results imply that the loss of the methylation activity of M.EcoRII (L80P) at 42°C is cannot be explained by a simple loss of some function of the N-terminal region necessary for the methylation activity. Possible mechanisms to explain the decreased the methylation ability of M.EcoRII (L80P) are i) the mutant works as a super repressor of itself, or ii) the M.EcoRII (L80P) is more sensitive to protease(s) than wild-type, or iii) this mutant is more unstable than wild-type, or iv) the mutant inhibits the function of another domain(s).

The accumulating studies about the function of the N-terminal region of M.EcoRII led us propose to the possibility of a super repressor. The position of L80P mutation is located in the N-terminal domain of M.EcoRII. The 83 amino acids at N-terminus contain no conserved motif of m5C methylases, and not essential for the modification activity. (3, 40). The M.EcoRII regulates the expression of itself through binding to an inverted repeat on the promoter region of the modification gene, and the N-terminal region is essential for the regulation (39, 40). The predicted secondary structure of M.EcoRII N-terminal region suggested that this region has a HTH, and it is about 20 residues uptream from L80P mutation (17). The mutation might affect the binding

activity of M.EcoRII to regulatory cis-element of itself, lead to more severe autoregulation than wild type, followed by decreased methylation at 42 °C. However, the dominant negative experiment revealed that the phenotype of M.EcoRII (L80P) was not dominant (data not shown). These results suggest that the mechanism of the

5 decreased the mehtylation ability of M.EcoRII (L80) is more protein stability (ii, iii, iv) than behaving as a super repressor (i).

However the results of Western-blotting using anti-FLAG antibody revealed that the wild-type and delta N83 M.EcoRII were detected under IPTG induced condition at 30°C and 42°C (data not shown). However, the M.EcoRII (L80P) was not detected at

10 30°C or 42°C under the same condition. These results suggest that M.EcoRII (L80P) is less stable than wild-type protein.

We tested the possibility of any genetic interaction between the M.EcoRII (L80P) and proteases. Whether cell death at 42°C by *ecoRIIRM* (T239C) gene complex is rescued in protease defective cells (*clpP, clpA, clpX* or *Lon*) was examined, because

15 some antitoxins are degraded cellular proteases such as Lon and ClpXP as described before (4). However we could not find improved growth in any strains (data not shown). The decreased expression of the mutant EcoRII might be caused by other mechanism, although a possibility that the degradation is caused by some other

protease or some proteases target M.EcoRII is not excluded.

In this study revealed that M.EcoRII (L80P) protein lost the ability of methylation and this is the temperature-sensitive mutant, however the mutant protein decreased or inhibit own functional domain(s) at 42°C was not made clearly.

5 **Correlation with plasmid maintenance**

The L80P mutant EcoRII RM gene complex showed stronger capacity of maintenance. The weaker methylase activity (antitoxin) appears to correlate with increase of maintenance of plasmid stability. It is the novel observation there is a correlation the activity difference and plasmid stability by RM system. In the case of the
10 toxin-antitoxin systems, as for example RelBE, the antitoxin RelB is degraded by Lon protease. The plasmid stabilization by RelBE is abolished in lon deficient cells (8). In other words, when antitoxin is stable plasmid stabilization is reduced, in contrast, if antitoxin is unstable, plasmid stabilization is enhanced. Same phenomena were observed in another toxin-antitoxin systems (Pem/ParD, Phd/Doc) (24, 43). The
15 post-segregational killing by the large virulence plasmid of Shigella is stronger at higher temperature (35).

The several antitoxins are unstable in the cell because of their degradation by cellular proteases (4). This causes strong cell death after gene segregation. This is the

well-controlled strategy of toxin-antitoxin systems. If the antitoxin was strong, the post-segregational killing is decreased. And if the antitoxin was very weak, the cell can't survive because of the effect of the toxin. In this study the toxin-antitoxin have evolved and maintained with under the perfect balance. Taken together, these results are comparable with the toxin-antitoxin concept of restriction-modification systems.

The 83 amino acids at N-terminus are not essential for catalytic function of M.EcoRII at 37°C (3) and at 42°C (Fig. 3). However, the mutant deleted for them showed increased K_m for cofactor and for DNA and decreased methylation activity (3).

We would like to suggest that the N-terminal domain might serve as a regulator of post-segregational killing. Because a part of this N-terminal domain binds to the promoter of its gene for negative autoregulation (39),. N-terminal domain of M.EcoRII might accerelate post-segregational killing by sensing loss of this promoter and of the ecoRII gene complex by causing susceptibility to proteases. This region might bind to the vicinity of the recognition sequence to help it sense DNA. When M.EcoRII recognizes loss of DNA, this could decrease the methylation activity and accelerate the cell death. These processes are as in SOS induction of prophage.

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TABLES

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Source	Reference
E. coli			
GM30	*F*$^-$ *lambda*$^-$ *thr-1 araC14 leuB6 fhuA31 lacY1 tsx-78 glnV44 galK2 galT22 hisG4 rpsL136 xylA5 mtl-1 thi-1*	M. Marinus	(26)
GM31	*F*$^-$ *lambda*$^-$ *thr-1 araC14 leuB6 fhuA31 lacY1 tsx-78 glnV44 galK2 galT22 dcm-6 hisG4 rpsL136 xylA5 mtl-1 thi-1*	M. Marinus	(26)
BNH670	=CC221 (= Δ *(supD-dcm-fla) zee3129*::Tn10)	G. Macintyre	(25)
Plasmids			
pNY30	pUC19 carrying *ecoRIIR* (T402C) *M* (T239C), *Amp*^{r}	Y. Naito	(41)
pNY31	pUC19 carrying *ecoRIIR* (T402C) *M* (T239C), *Amp*^{r}	Y. Naito	(41)
pOS22	pUC19 carrying *ecoRIIR* (T402C) *M*, *Amp*^{r}	This study	
pOS48	pUC19 carrying *ecoRIIR*$^-$*M* (T239C), *Amp*^{r}	This study	
pOS28	pUC19 carrying wild-type *ecoRIIRM*, *Amp*^{r}	This study	
N3	Natural plasmid carrying *ecoRIIRM*, *Tet*^{r}	S. Hattman	(13)
pOS34	pUC19 carrying *ecoRIIR*$^-$*M* (T239C), *Amp*^{r}	This study	
pOS36	pUC19 carrying *ecoRIIR*$^-$*M*, *Amp*	This study	
pFLAG2	Expression vector, ColE1, *Amp*	Sigma	
pOS41	pFLAG2 carrying *ecoRIIM* (T239C), *Amp*^{r}	This study	
pOS43	pFLAG2 carrying wild-type *ecoRIIM*, *Amp*^{r}	This study	

pOS45	pFLAG2 carrying *ecoRIIM* (Δ N83), *Amp*^{r}	This study	
pHSG415	Vector derived from pSC101, temperature-sensitive for replication, *Amp*^{r}, *Kan*^{r}, *Cml*^{r}	J. Kato	(12)
pOS30	pHSG415 carrying wild-type *ecoRIIRM*, *Cml*^{r}	This study	
pOS39	pHSG415 carrying *ecoRIIR*$^{-}$*M*, *Amp*^{r}, *Cml*^{r}	This study	
pNY31	pACYC184 carrying *ecoRIIR* (T402C)*M* (T239C), *Amp*, *Cml*^{r}	Y. Naito	(41)
pNY41	pACYC184 carrying *ecoRIIR*$^{-}$*M* (T239C), *Cml*^{r}	Y. Naito	(41)
pOS51	pACYC184 carrying *ecoRIIR*$^{-}$*M*, *Cml*^{r}	This study	
pOS54	pACYC184 carrying *ecoRIIRM* (T239C), *Cml*^{r}	This study	
pOS57	pACYC184 carrying wild-type *ecoRIIRM*, *Cml*^{r}	This study	

Table 2. Temperature-sensitivity of bacterial growth conferred by mutant EcoRII gene complexes

Bacterial strain [plasmid]	genotype			Colony forming units at 42°C/ colony forming units at 30°C[a]	
	dcm	*r*	*m*	with Amp[b]	without Amp[b]
GM31 [pUC19]	*dcm-6*	(-)	(-)	(= 1)	(= 1)
GM31 [pNY31]	*dcm-6*	-	T239C	0.91 ± 0.20	1.16 ± 0.51
GM31 [pOS28]	*dcm-6*	+	+	0.85 ± 0.29	1.13 ± 0.35
GM31 [pOS48]	*dcm-6*	+	T239C	6.6E-07 ± 2.4E-07	3.7E-06 ± 2.8E06
GM31 [pOS22]	*dcm-6*	T402C	+	0.63 ± 0.13	0.82 ± 0.20
GM31 [pNY30]	*dcm-6*	T402C	T239C	6.2E-07 ± 2.8E-07	3.8E-06 ± 1.7E-06
BNH670 [pUC19]	*Δ dcm*	(-)	(-)	(= 1)	(= 1)
BNH670 [pOS28]	*Δ dcm*	+	+	1.45 ± 0.44	1.19 ± 0.18
BNH670 [pOS36]	*Δ dcm*	-	+	2.91 ± 1.05	0.95 ± 0.08
BNH670 [pNY30]	*Δ dcm*	T402C	T239C	5.1E-07 ± 3.5E-07	2.1E-06 ± 1.2E-06

[a]*E. coli* strains grown at 30 °C with antibiotic selection were spread on LB agar with and without antibiotics and incubated overnight at 30°C or 42°C for colony counting.

5 [b]Their ratio was normalized with that for the vector pUC19 in each strain. Duplicate measurement was carried out independently three times. Their average is shown with standard deviation.

FIGURELEGENDS

Fig. 1. EcoRII modification enzyme and its gene with the L80P (T239C) mutation.

Conserved motifs are designated by Roman numerals (22, 31). I: AdoMet binding; II, III: supports AdoMet binding; IV: part of catalytic site; V: supports AdoMet binding; VI: hold target cytosine in active site; VII: supports AdoMet binding; VIII: holds target cytosine in active site; IX, X: align DNA binding regions with active site (36). The thermosensitive mutation is shown.

Fig. 2. Post-segregational killing by EcoRII RM gene complex.

A. Growth inhibition following loss of the EcoRII RM gene complex. The *E. coli* strain BNH670 (Δ *dcm*) carrying pOS30 (pHSG415 (temperature-sensitive for replication) with inserted wild-type *ecoRIIRM*, Cml^r) or pOS39 (its *r*-negative version) was grown at 30°C in LB medium until OD660 of the culture reached 0.5 with antibiotic selective for the plasmid (Cml), shifted to 42°C and incubated without antibiotic selection. The total cells were counted under a microscope. The viable cells were counted on LB agar without the antibiotics at 30°C. The plasmid-carrying cells were counted on LB agar with the antibiotics at 30°C. **B. Cell shape.** The above cells were harvested at the indicated time intervals after the temperature shift and then fixed for staining with DAPI.

Fig. 3. Temperature-sensitive methylation activity *in vivo*.

A. Loss of methylase activity under a block to protein synthesis.

E. coli strain BNH670 (*Δ dcm*) carrying a plasmid with *ecoRIIM* (wild type) or *ecoRIIM* (T239C) were grown in LB medium with antibiotic selective for the plasmid (Amp) and IPTG at 30°C until OD660 of the culture reached 0.5. Cml was added for inhibition of protein synthesis and cell growth, and the cells were incubated at 42°C. Plasmid DNA prepared at the indicated time interval was treated with EcoRII for electrophoresis through 0.7% agarose gel. In the control cultures, growth was either at 30°C throughout or at 42°C throughout.

B. Temperature-dependent methylation activity of M.EcoRII Δ N83 mutant.

E. coli strain BNH670 (*Δ dcm*) carrying a plasmid with *ecoRIIM* (Δ N83) was grown at 30°C or 42°C. Its modification enzyme was induced with IPTG. Plasmid molecules were prepared from the cells, treated with EcoRII and run through agarose (0.7%).

Fig. 4. Mutation (T239C) in the modification enzyme enhances maintenance of EcoRII RM gene complex.

The *E. coli* strain BNH670 carrying pOS51 (pACYC184, wild-type *ecoRIIR*$^{-}$*M*, *Cml*^{r}), pOS54 (pACYC184, *ecoRIIRM* (T239C), *Cml*^{r}) or pOS57 (pACYC184, wild-type *ecoRIIRM, Cml*^{r}) were grown at 30°C in LB medium with antibiotics selective for the

plasmid (Cml). The culture was sampled for plasmid-carrying cell counts (on LB agar with Cml at 30°C) and viable cell counts (on LB agar without Cml at 30°C) and diluted 100000-fold into LB medium without the antibiotics for overnight incubation at 30°C. Their ratio of the plasmid-carrying cell counts to the viable cell counts was plotted
5 against the generation number, which was calculated from the viable cell counts.

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5

FIGURES

Fig1

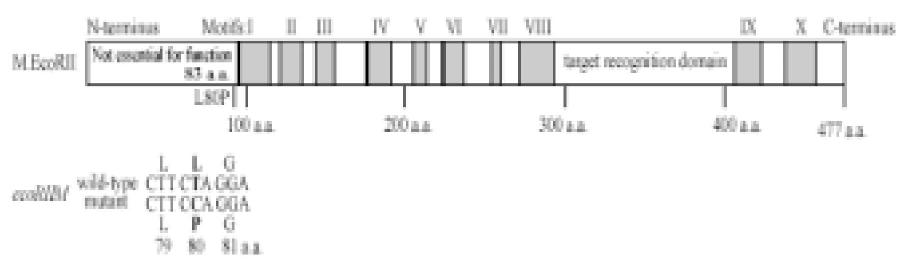

Fig2

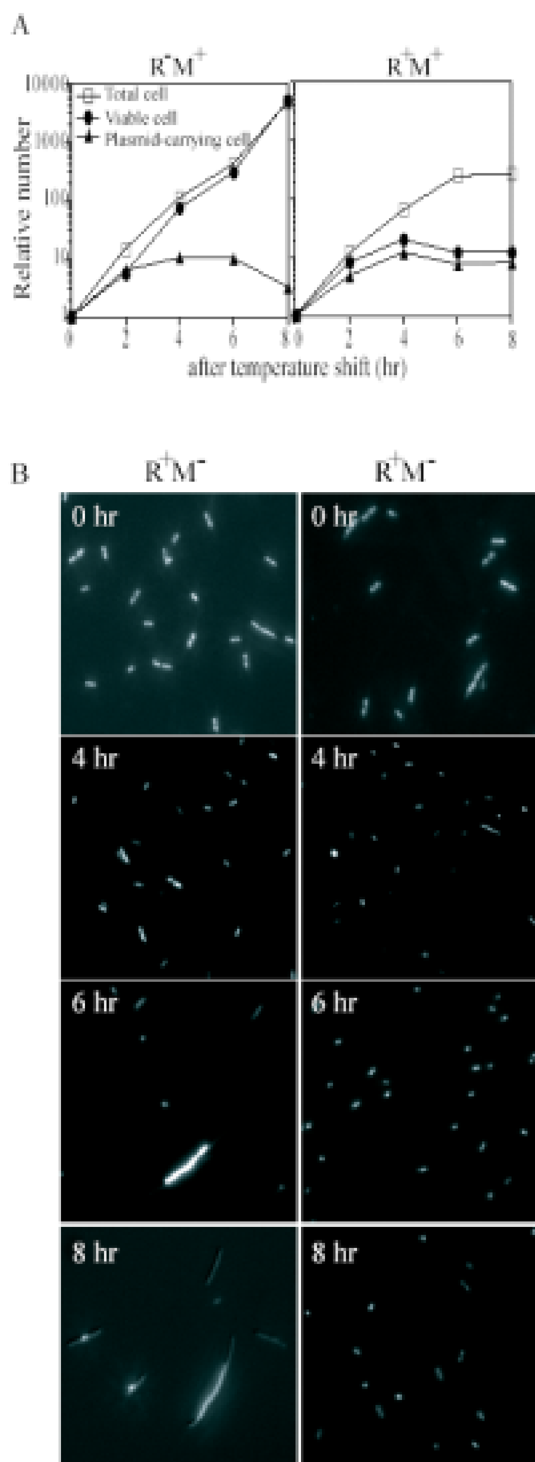

Fig3

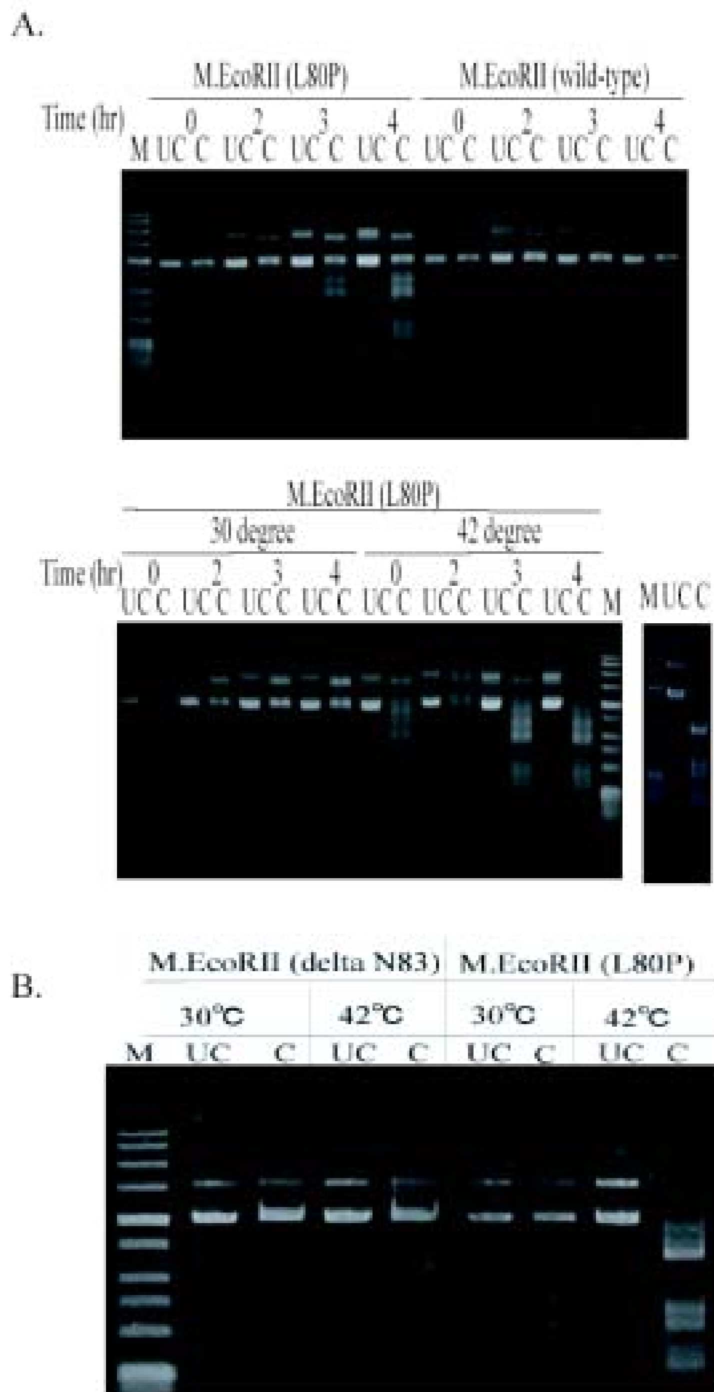

Fig4