Contribution of RecFOR machinery of homologous recombination to cell survival after loss of a restriction–modification gene complex

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Loss of a type II restriction–modification (RM) gene complex, such as EcoRI, from a bacterial cell leads to death of its descendent cells through attack by residual restriction enzymes on undermethylated target sites of newly synthesized chromosomes. Through such post-segregational host killing, these gene complexes impose their maintenance on their host cells. This finding led to the rediscovery of type II RM systems as selfish mobile elements. The host prokaryote cells were found to cope with such attacks through a variety of means. The RecBCD pathway of homologous recombination in *Escherichia coli* repairs the lethal lesions on the chromosome, whilst it destroys restricted non-self DNA. *recBCD* homologues, however, appear very limited in distribution among bacterial genomes, whereas homologues of the RecFOR proteins, responsible for another pathway, are widespread in eubacteria, just like the RM systems. In the present work, therefore, we examined the possible contribution of the RecFOR pathway to cell survival after loss of an RM gene complex. A *recF* mutation reduced survival in an otherwise *rec*-positive background and, more severely, in a *recBC sbcBC* background. We also found that its effect is prominent in the presence of specific non-null mutant forms of the RecBCD enzyme: the resistance to killing seen with *recC1002*, *recC1004*, *recC2145* and *recB2154* is severely reduced to the level of a null *recBC* allele when combined with a *recF*, *recO* or *recR* mutant allele. Such resistance was also dependent on RecJ and RecQ functions. UV resistance of these non-null *recBCD* mutants is also reduced by *recF*, *recJ* or *recQ* mutation. These results demonstrate that the RecFOR pathway of recombination can contribute greatly to resistance to RM-mediated host killing, depending on the genetic background.

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INTRODUCTION

This work describes one aspect of interaction between a restriction–modification (RM) system (Roberts *et al.*, 2007) and its host bacterial cells. Type II RM systems consist of a restriction enzyme that cleaves DNA at or near a specific sequence and a cognate modification enzyme that methylates the same sequence to protect it from the restriction cleavage. The genes encoding these two enzyme activities are usually tightly linked and form an RM gene complex. The widespread occurrence of RM systems throughout prokaryotes has been explained by cellular

defence: RM systems will attack incoming DNAs that are not methylated properly to protect bacterial cells.

A contrasting concept originated from the discovery of host attack by RM systems (Kusano *et al.*, 1995; Naito *et al.*, 1995). When an RM gene complex is lost from a cell, its descendants will contain fewer and fewer molecules of the modification and restriction enzymes. Eventually, the capacity of the modification enzyme to protect many sites in newly generated chromosomes becomes inadequate, so that those unmethylated sites are exposed to lethal attack by any of the remaining restriction-enzyme molecules (Handa & Kobayashi, 1999; Handa *et al.*, 2000; Kusano *et al.*, 1995; Naito *et al.*, 1995). Such host killing, designated post-segregational cell killing or genetic addiction (Kobayashi, 2004a), provides an advantage to the RM gene complex and to any linked stretch of DNA in

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Abbreviations: DSB, double-strand break; RM, restriction–modification.

competitive exclusion (Handa *et al.*, 2001; Naito *et al.*, 1995; Sadykov *et al.*, 2003). A theoretical work revealed conditions for evolution of such host-killing gene complexes (Mochizuki *et al.*, 2006).

Post-segregational cell-killing activity suggests that these RM gene complexes can behave as selfish mobile genetic elements, just like transposons and virus genomes (Kobayashi, 2001, 2004b). By now, there are numerous lines of evidence for their mobility and association with genome rearrangements (Kobayashi, 2001, 2004b). In fact, RM gene complexes can self-multiply (Sadykov *et al.*, 2003) and they may have a life cycle similar to that of virus genomes in naturally competent prokaryotes (Kobayashi, 2002). Just like the temperate bacteriophages, RM gene complexes have specific regulatory systems for establishment, maintenance and host killing (Karyagina *et al.*, 1997; Nakayama & Kobayashi, 1998; Tao *et al.*, 1991).

Bacteriophages and plasmids have developed various means to defend themselves from attack by RM systems (Tock & Dryden, 2005). Bacteriophage-mediated homologous recombination can repair restriction breaks (Handa *et al.*, 2005; Takahashi & Kobayashi, 1990); the host bacteria also have similar mechanisms. For example, a solitary methyl-transferase (not paired with a restriction endonuclease) defends a genome from attack by an RM system by recognizing and methylating the same target sequence (Ohno *et al.*, 2008; Takahashi *et al.*, 2002). The RecBCD/RecA recombination machinery of *Escherichia coli* helps cells to survive the attack by repairing broken chromosomes after loss of an RM gene complex (Handa *et al.*, 2000).

The RecBCD recombination/repair machinery plays a central role in dealing with chromosomal DNA double-strand breaks (DSBs) (Kowalczykowski *et al.*, 1994; Kuzminov, 1999). It degrades double-stranded DNA (dsDNA) from a blunt or nearly blunt end, but its destruction activity is switched to a 5' single-stranded DNA (ssDNA) exonuclease when it encounters a specific DNA sequence called Chi (5'-GCTGGTGG-3') (Bianco & Kowalczykowski, 1997). Then, a final product of a 3' ssDNA tail terminated at Chi serves as a substrate for RecA-mediated pairing with a homologous DNA (Churchill & Kowalczykowski, 2000; Spies & Kowalczykowski, 2006). The RecBCD enzyme destroys, through complete degradation, restricted non-self DNA lacking the Chi sequence (Handa *et al.*, 2000; Simmon & Lederberg, 1972).

Several non-null alleles of *recB* and *recC* do not respond to the Chi sequence, but retain other activities to a varying extent (Amundsen *et al.*, 1990; Holbeck & Smith, 1992; Schultz *et al.*, 1983). One group of them, called 'star' (*), which includes *recC1001*, *recC1002*, *recC1003* and *recC1004*, showed a partial deficiency in conjugational recombination and UV repair in a *recF*⁻ background (Schultz *et al.*, 1983). Another group of non-null, Chi-insensitive alleles called class II, which includes *recC2145*, *recB2154* and *recB2155*, was reported to be recombination-deficient in a *recF*⁻ background (Amundsen *et al.*, 1990).

Defects of *recB* or *recC* null mutants in some recombination assays are suppressed by alleles of *sbcB*, which encodes a 3'-end-specific ssDNA exonuclease called exonuclease I, and of *sbcC* (Lloyd & Buckman, 1985; Thoms *et al.*, 2008). These mutations activate an alternative recombinational pathway, designated the RecFOR (or RecF) pathway for the genes involved (Kushner *et al.*, 1971; Lloyd & Buckman, 1985). The RecFOR machinery is active in the absence of the *sbcB* allele in some other recombination assays (Lloyd & Low, 1996). Synergistic effects of *recBC* and *recFOR* mutations for UV survival have been reported previously (Horii & Clark, 1973; Lloyd *et al.*, 1988). Recently, functional cooperation between the two machineries was demonstrated for *recB1080*, a nuclease-negative mutant allele: the recombination proficiency of the RecB1080CD machinery depends on the RecF pathway (Amundsen *et al.*, 2000; Amundsen & Smith, 2003; Ivancic-Bace *et al.*, 2003, 2005; Jockovich & Myers, 2001).

Although we demonstrated that RecBCD resists RM-mediated post-segregational killing (Handa *et al.*, 2001), *recBCD* homologues are very limited in distribution among eubacterial phylogenetic groups (Rocha *et al.*, 2005). In contrast, homologues of RecFOR-related proteins are found in almost all groups of eubacteria (Rocha *et al.*, 2005), just like the RM systems. Functional analogues of RecFOR proteins are present in essentially all groups of organisms (Beernink & Morrical, 1999; Sung & Klein, 2006). Therefore, whether resistance to RM systems through homologous recombination is a general phenomenon in the prokaryotes has remained unanswered.

In this work, we asked whether RecFOR-mediated recombination can contribute to cell survival after post-segregational attack by an RM system in *E. coli*. To conveniently detect its contribution, we used several non-null *recBCD* mutants. Our results clearly demonstrated RecFOR-mediated resistance to the threat posed by the RM systems.

METHODS

Bacterial strains, bacteriophages and plasmids. All bacterial strains used here are derivatives of *E. coli* K-12 and are listed in Table 1. For transduction, P1vir (a bacteriophage P1 strain with virulent phenotype; laboratory collection) was used. Both bacteriophage λ strains LIK916 [*Bam*10 Δ B *int*⁻ Δ (*red-gam*) *imm*21 *nin*5 *shn*60] and LIK950 [*Bam*10 Δ B *int*::Chi⁺ *C157* Δ (*red-gam*) *imm*21 *nin*5 *shn*60] are defective for their own recombination function (*red*) and were described previously (Handa *et al.*, 1997). In LIK950, a small PCR fragment (5'-GGATCCTTAACAAAATCGCAATGCTTGGAACTGAGAAGACAGCGGAAGCTGTGGCGTTGATAAGTCGCAGATCAGCTGGTGGGAAGAGGGACTGGATTCCAAAAGTTCTCAATGCTGCTTGCTGTTCTTGAATGGGGGGTCGTTGACGACGACATGGCTCGATTGGATCC-3') [the Chi sequence is shown in bold, the mutant base (T) is underlined and BamHI sites are italicized] carrying Chi⁺ *C157* was inserted into a BamHI site of the *int* gene. λ vir (a bacteriophage λ strain with virulent phenotype; laboratory collection) was used to test restriction activity of bacterial strains.

Plasmids pIK172 [pSC101^{ts} replicon harbouring EcoRI r⁺m⁺, ampicillin-resistance (Amp) and chloramphenicol-resistance (Cml)]

Table 1. Bacterial strains

Strain	Other name	Genotype	Construction	References and source
BIK788	AB1157	<i>thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 supE44</i>		Bachmann (1987)
BIK752	JC7623	As BIK788, but <i>recB21 recC22 sbcB15 sbcC201</i>		Kushner <i>et al.</i> (1971); Lloyd & Buckman (1985)
BIK749	JC8111	As BIK752, but <i>recF143</i>		Horii & Clark (1973)
BIK796	V66	F ⁻ λ^- <i>rac^- argA21 hisG4 met rpsL31 galK2 xyl-5 recF143</i>		Schultz <i>et al.</i> (1983)
BIK800	NK5992	F ⁻ λ^- IN (<i>rrnD-rrnE</i>)1 <i>argA81::Tn10</i>		A. Taylor, Fred Hutchinson Cancer Research Center, Seattle, WA, USA
BIK806	NK5992	As BIK788, but <i>recD::Tn10</i>		Takahashi <i>et al.</i> (1993)
BIK1399	AM265	As BIK788, but <i>recB21 recC22 sbcA23 recR::mini-Tn10kan</i>		Takahashi <i>et al.</i> (1993)
BIK1272	V69	As BIK796, but <i>recC1002</i>		Schultz <i>et al.</i> (1983)
BIK1273	V71	As BIK796, but <i>recC1003</i>		Schultz <i>et al.</i> (1983)
BIK1274	V72	As BIK796, but <i>recC1004</i>		Schultz <i>et al.</i> (1983)
BIK1275	V73	As BIK796, but <i>recC1001</i>		Schultz <i>et al.</i> (1983)
BIK1276	A211	W3110 <i>lacZ²⁰ Y^{const} gyrB⁺ recF⁺ zic::Tn10</i>		A. Miura, Institute of Medical Science, University of Tokyo, Tokyo, Japan
BIK1282		As BIK1273, but <i>recF⁺ zic::Tn10</i>		Handa <i>et al.</i> (1997)
BIK1284		As BIK1274, but <i>recF⁺ zic::Tn10</i>		Handa <i>et al.</i> (1997)
BIK1286		As BIK1275, but <i>recF⁺ zic::Tn10</i>	P1 (BIK1276) to BIK1275	This work
BIK1288		As BIK796, but <i>recF⁺ zic::Tn10</i>		Handa <i>et al.</i> (1997)
BIK1290		As BIK1272, but <i>recF⁺ zic::Tn10</i>	P1 (BIK1276) to BIK1272	This work
BIK1910	V1296	As BIK796, but <i>recC2145</i>		Amundsen <i>et al.</i> (1990)
BIK1911	V1360	As BIK796, but <i>recB2154</i>		Amundsen <i>et al.</i> (1990)
BIK2411	V68	As BIK796, but <i>recC73</i>		Schultz <i>et al.</i> (1983)
BIK2445		As BIK1910, but <i>recF⁺ zic::Tn10</i>		Handa <i>et al.</i> (1997)
BIK2446		As BIK1911, but <i>recF⁺ zic::Tn10</i>		Handa <i>et al.</i> (1997)
BNH701		As BIK796, but <i>recD::Tn10</i>	P1 (BIK806) to BIK796	This work
BNH702		As BIK1288, but <i>recR::mini-Tn10kan</i>	P1 (BIK1399) to BIK1288	This work
BNH704		As BIK2445, but <i>recR::mini-Tn10kan</i>	P1 (BIK1399) to BIK2445	This work
BNH705		As BIK2446, but <i>recR::mini-Tn10kan</i>	P1 (BIK1399) to BIK2446	This work
BNH707		As BIK1288, but <i>recO::Tn5</i>	P1 (BIK1192) to BIK1288	This work
BNH708		As BIK1290, but <i>recO::Tn5</i>	P1 (BIK1192) to BIK1290	This work
BNH709		As BIK2445, but <i>recO::Tn5</i>	P1 (BIK1192) to BIK2445	This work
BNH710		As BIK2446, but <i>recO::Tn5</i>	P1 (BIK1192) to BIK2446	This work
BNH713		As BIK1288, but Tc ^s	Selected on fusaric acid plate (Bochner <i>et al.</i> , 1980)	This work
BNH728		As BNH713, but <i>recD::Tn10</i>	P1 (BIK806) to BNH713	This work
BNH732		As BIK2411, but <i>argA81::Tn10</i>	P1 (BIK800) to BIK2411	This work
BNH738		As BNH713, but <i>recC73 argA81::Tn10</i>	P1 (BNH732) to BNH713	This work
BIK2680		As BIK788, but <i>recQ1803::Tn3</i>		Handa & Kobayashi (2003)
BIK5248	STL126	As BIK788, but <i>recB21 recC22 sbcA23 recJ2003::Tn10-9</i>		S. T. Lovett; Lovett & Suter (1995); Viswanathan & Lovett (1998)
BIK5290		As BIK1288, but <i>recQ1803::Tn3</i>	P1 (BIK2680) to BIK1288	This work
BIK5292		As BIK1290, but <i>recQ1803::Tn3</i>	P1 (BIK2680) to BIK1290	This work
BIK5296		As BIK2446, but <i>recQ1803::Tn3</i>	P1 (BIK2680) to BIK2446	This work
BIK5274		As BIK1288, but <i>recJ2003::Tn10-9</i>	P1 (BIK5248) to BIK1288	This work
BIK5276		As BIK1290, but <i>recJ2003::Tn10-9</i>	P1 (BIK5248) to BIK1290	This work
BIK5278		As BIK2446, but <i>recJ2003::Tn10-9</i>	P1 (BIK5248) to BIK2446	This work

genes] and pIK173 (restriction-negative version of pIK172) are derivatives of pHSG415 (Hashimoto-Gotoh *et al.*, 1981) and were described previously (Kusano *et al.*, 1995; Naito *et al.*, 1995).

Media, DNA preparation and transformation. *E. coli* cells were grown in L broth (Sambrook *et al.*, 1989) to which 50 μ g ampicillin ml⁻¹ and 200 μ g meticcillin ml⁻¹ were added for plasmid selection.

Plasmid DNA was prepared by a published method (Sambrook *et al.*, 1989). Plasmids were introduced into *E. coli* cells by electroporation with a Gene Pulser (Bio-Rad) at 2.5 kV, 25 mF and 200 Ω .

Measurement of plaque size of phage λ . A fresh, single colony of a bacterial strain on L agar was suspended in λ tryptone broth (Handa *et al.*, 1997) for overnight growth at 37 °C with shaking. The cells were diluted 50-fold in λ tryptone broth and grown to mid-exponential phase at 37 °C with shaking. A 0.1 ml aliquot of these cells was mixed with 2.5 ml melted and pre-warmed 0.6% top agar and poured onto a λ polypeptone agar plate [1.0% polypeptone (Wako), 0.5% NaCl, 1.2% agar]. Ten minutes later, λ phages with or without Chi sequence were spotted onto the lawn of cells in serial dilutions. The plates were incubated at 37 °C overnight. The plaque size of bacteriophage λ depends on medium composition, concentration and depth of agar, moisture, age of agar plate and location within a plate, as well as phage and host genotypes. To minimize variation from these effects, experiments were carried out with two independent bacterial cultures with plates prepared on the same day as the experiments. After overnight incubation, the plates were scanned by a scanner (model F917500; Canon) connected to a computer. The plaques for measurement were chosen at two different positions on a plate. The image was analysed with Illustrator 10.0.3 software (Adobe) at 1200% enlargement. Diameter of ten plaques for each strain was measured with a pointing tool. These values were calibrated by the values for a ruler scanned in the same manner.

Chi-recognition phenotype of non-null *recBCD* alleles in a *recFOR*⁺ background. Because the non-null RecBCD mutants used here, *recC1001*, *recC1002*, *recC1003*, *recC1004*, *recC2145* and *recB2154*, were originally isolated and characterized in a *recF*[−] background (Amundsen *et al.*, 1990; Schultz *et al.*, 1983), the *recF*⁺ allele was restored to the mutant strains (Methods; Table 1). These mutant strains were then examined for the Chi-recognition phenotype in the phage-plaque assay (Table 2). The rationale behind this assay is as follows. In wild-type *E. coli* cells, λ phage with defects in its recombination function (*red* gene product) and in an inhibitor of the RecBCD enzyme (*gam* gene product) makes smaller plaques, because the RecBCD enzyme degrades the phage genome from the end. However, if the phage carries an appropriately oriented Chi sequence, it forms larger plaques. This is explained by the formation of λ genome concatemers, substrates of packaging into progeny phage particles, by homologous recombination or rolling-circle replication (Lam *et al.*, 1974). Plaques produced by a strain with a *recBC* null allele, such as *recC73*, are larger than those produced by a *recBC*⁺ strain, because it lacks exonuclease activity of the RecBCD enzyme.

These *recF*⁺ versions still showed the Chi-recognition deficiency characteristic of their *recBCD* alleles (Table 2). In the *recBC*⁺ host, the plaque size of Chi⁺ phage was much larger than that of Chi⁰ phage. All of the six non-null mutant alleles analysed gave indistinguishable ($P > 0.05$, *t*-test, one-sided) plaque sizes for phages with and without Chi (Table 2). The *recF*⁺ function resulted in significantly ($P < 0.05$) enlarged Chi⁺ plaques in all of the non-null alleles examined except for *recC1002* ($P = 0.16$). The plaque sizes of the star (*) class (*recC1001*, *recC1002*, *recC1003* and *recC1004*) were similar to those reported previously (Schultz *et al.*, 1983). Those of class II (*recC2145* and *recB2154*) were slightly different (Amundsen *et al.*, 1990). These authors reported that *recC2145* gives smaller plaques than *recB2154*. We do not know whether the difference is due to variation in the genetic backgrounds. We did not pursue this further.

Cell death following loss of the EcoRI gene complex on a thermosensitive plasmid. We performed three assays to examine cell death after loss of a thermosensitive plasmid carrying the EcoRI RM gene complex. In the first assay, *E. coli* strains carrying pIK172 (pSC101^{ts}, EcoRI r⁺m⁺, Amp^r) or pIK173 (its r[−] version) were aerated at 30 °C in L broth (with appropriate antibiotics to select the plasmid) and grown to an OD₆₆₀ of 0.3. Portions of the culture were spread onto two L agar plates without antibiotics. One of the plates was incubated at 37 °C overnight and the other at 30 °C.

The second assay measured cell viability. For a culture with an OD₆₆₀ of approximately 0.3, the number of cells was counted under a microscope. They were diluted and spread on non-selective agar plates for overnight incubation at 37 °C for colony counting.

The third method was observation in a liquid culture. After the cells reached an OD₆₆₀ of 0.3, the antibiotics were diluted (5-fold for r⁺ cells and 10-fold for r[−] cells) and the culture temperature was shifted to 42 °C. The culture was then diluted when its OD₆₆₀ reached 0.3. The total number of cells was counted under a microscope. Viable cells and plasmid-carrying cells were defined as colony formers after an overnight incubation at 30 °C on L agar plates without and with selective antibiotics, respectively. Viability was calculated as viable cell count/total cell count.

UV-sensitivity measurement. Exponential-phase cultures in L broth were diluted in M9 minimal medium (Miller, 1992) and spread onto L agar plates. The plates were irradiated with UV light (Toshiba GL10) at 52 cm from the bottom of the plate. The dose was measured at UV-C (254 nm) by UV dosimeter. Colonies were counted after their incubation at 37 °C for 20 h in the dark.

Table 2. Chi activity as measured by λ phage plaque size in various *recBCD* mutants in *recF*^{+/−} backgrounds

<i>recBCD</i> genotype	Bacterial <i>recF</i> ⁺ strain	Plaque size*		Bacterial <i>recF</i> [−] strain	Plaque size*	
		Chi ⁰ phage	Chi ⁺ phage		Chi ⁰ phage	Chi ⁺ phage
+	BIK1288	0.36 ± 0.07	0.85 ± 0.11	BIK796	0.35 ± 0.08	0.79 ± 0.12
<i>recC73</i>	BNH738	1.1 ± 0.2	1.1 ± 0.1	BIK2411	0.99 ± 0.14	0.92 ± 0.11
<i>recC1001</i>	BIK1286	0.75 ± 0.23	0.67 ± 0.14	BIK1275	0.53 ± 0.06	0.56 ± 0.05
<i>recC1002</i>	BIK1290	0.60 ± 0.16	0.64 ± 0.21	BIK1272	0.53 ± 0.11	0.56 ± 0.11
<i>recC1003</i>	BIK1282	0.41 ± 0.08	0.36 ± 0.06	BIK1273	0.30 ± 0.10	0.29 ± 0.08
<i>recC1004</i> †	BIK1284	0.24 ± 0.06	0.28 ± 0.07	BIK1274	0.20 ± 0.05	0.19 ± 0.05
<i>recC2145</i> †	BIK2445	0.21 ± 0.04	0.24 ± 0.07	BIK1910	0.22 ± 0.04	0.19 ± 0.02
<i>recB2154</i>	BIK2446	0.62 ± 0.22	0.64 ± 0.25	BIK1911	0.33 ± 0.09	0.33 ± 0.07

*Diameter, mean ± SD (mm) ($n = 10$).

†The *recC1004* and *recC2145* mutants gave very tiny plaques in either a *recF*⁺ or a *recF*[−] background.

RESULTS

Effect of a *recF* mutation on cell death after loss of the EcoRI RM gene complex

Loss of a type II RM gene complex causes cell death via chromosomal breakage by residual levels of cellular restriction endonuclease (Kusano *et al.*, 1995; Naito *et al.*, 1995). To assess the effects of host mutations on cell death, we have applied a system in which an RM (EcoRI) gene complex was connected with a plasmid with a thermosensitive replication machinery (Hashimoto-Gotoh *et al.*, 1981). After a block to plasmid replication by temperature upshift, the descendent cells form smaller colonies or no visible colonies, depending on the strength of post-segregational killing. For example, a single null mutation in the *recB* or *recC* gene (such as *recC73*)

showed dramatic post-segregational killing, giving almost no viable colonies on agar at a semi-permissive temperature (37 °C) (Fig. 1a, the left photograph, compare the left r^+m^+ strain with the right r^-m^+ control strain in the same plate) as reported previously (Handa *et al.*, 2000). The viable cell counts of the r^+m^+ cells dropped dramatically (Fig. 1b), whereas the r^-m^+ version showed only a moderate decrease in viability.

Single mutations in *recFOR* genes had no detectable effect in a *recBC*⁺ background in our plate assay (Fig. 1a, middle photograph; Fig. 2a, compare the bottom part of a plate between the first and second columns). However, colony counting revealed that the RecF function is also important ($P < 0.05$ in a *t*-test; Fig. 1b, fifth column versus first column). Because a *recC* single mutation already results in a severe decrease in this assay (Fig. 1a, b, 3rd column)

Fig. 1. Cell death caused by loss of the EcoRI RM gene complex. (a) Bacterial cells carrying the EcoRI r^+m^+ gene complex or its r^-m^+ derivative on a thermosensitive plasmid were grown to exponential phase with selective antibiotics at 30 °C, streaked on L agar without the selective antibiotics and then incubated overnight at 37 °C. r^+m^+ , Bacteria losing the r^+m^+ plasmid; r^-m^+ , bacteria losing the r^-m^+ plasmid. (b) Viability after loss of the RM gene complex on a thermosensitive plasmid. Total indicates cell counts under a microscope before colony assay; 37 °C viable indicates colony counts after overnight incubation at 37 °C without plasmid-selecting antibiotics. Results of independent measurements are presented. Bars are means of the plots. (c) Viability in a RecF pathway-activated strain, *recBC sbcBC*, and its *recF* derivative. The data are presented as in (b). (d) Viability in a *recD* strain and its *recF* derivative. The data are presented as in (b).

Fig. 2. RecFOR function, in the presence of Chi recognition-defective mutations, protects cells from killing after loss of the EcoRI gene complex. (a) Bacterial cells carrying the EcoRI *r*⁺*m*⁺ gene complex or its *r*⁻*m*⁺ derivative on a thermosensitive plasmid replicon were grown with selective antibiotics to exponential phase at 30 °C, streaked on L agar without the selective antibiotics and then incubated overnight at 37 °C. *r*⁺*m*⁺, Bacteria losing the *r*⁺*m*⁺ plasmid; *r*⁻*m*⁺, bacteria losing the *r*⁻*m*⁺ plasmid. (b) Viability after loss of the RM gene complex on a thermosensitive plasmid. Total indicates cell counts under a microscope before colony assay; 37 °C viable indicates colony counts after overnight incubation at 37 °C without plasmid-selecting antibiotics. Results of independent measurements are presented. Bars are means of the plots. All except one allele (*recC2145*; $P=0.29$ in a *t*-test) showed a significant difference ($P<0.05$) between the *recF*⁺ and *recF*⁻ genetic backgrounds.

(Handa *et al.*, 2000), we were unable to detect a more severe defect, if any, by addition of the *recF143* mutation to this genetic background in these assays ($P=0.12$ in a *t*-test; Fig. 1b, seventh column versus third column). On the other hand, the *recC* mutation showed a significant difference when added to the *recF* mutation ($P<0.05$ in a *t*-test; Fig. 1b, seventh column versus fifth column). As expected, the *recF* mutation led to dramatic cell killing in RecFOR recombination-activated cells, i.e. in the *sbcBC* background ($P<0.05$

in a *t*-test; Fig. 1c), but we carried out further analyses in the absence of the *sbcBC* mutations for simplicity.

Several *recD* mutations of the RecBCD enzyme impair its nuclease activity, but not its recombination capacity (Lovett *et al.*, 1988). As expected, post-segregational killing in a *recD*-negative mutant was not so severe and was not affected significantly by the *recF* mutation ($P=0.17$ in a *t*-test; Fig. 1a, right; Fig. 1d).

The RecFOR recombination machinery contributes considerably to resistance to RM-mediated post-segregational killing in the presence of several non-null *recBCD* alleles

Several non-null alleles of *recBC* genes do not give as severe a recombination deficiency as the null alleles. Some of them lack Chi-recognition capacity, but retain helicase activity and nuclease activity to a varying extent (Amundsen *et al.*, 1990; Arnold *et al.*, 1998; Schultz *et al.*, 1983). We switched to analysing the effect of *recFOR* mutations in the presence of several non-null *recBCD* alleles, hoping to detect and analyse their effects in a simpler way.

In our plate assay for post-segregational killing, cells carrying the EcoRI RM gene complex on a thermosensitive plasmid formed smaller and fewer colonies if the plate was incubated at a semi-permissive temperature (37 °C) than control cells carrying the r^- plasmid [Fig. 2a, first row, first column (*recFOR*⁺), lower part (*recBCD*⁺), compare the right and left in the same plate]. In the presence of non-null *recBCD* alleles *recC1002*, *recC2145* or *recB2154*, smaller and fewer colonies appeared (Fig. 2a, first column, top left of each plate). The killing was less strong than with the null *recBCD* allele (Fig. 1a, left plate). However, when one of the components (*recF*, *recO* or *recR*) in the RecFOR pathway was missing by a mutation, colonies with these non-null *recBCD* mutations were dramatically reduced in number (Fig. 2a, columns 2–4).

The viability assay also indicated that a *recF* mutation reduces viability when combined with non-null *recBCD* alleles (Fig. 2b). A decrease in viability of approximately two orders of magnitude (for *recC1002*; $P < 0.05$ in a *t*-test) or approximately one order of magnitude (*recB2154*; $P < 0.05$ in a *t*-test) was observed. Additionally, the *recC1004* mutation showed a similar (approx. one order of magnitude; $P < 0.05$ in a *t*-test) reduction in viability by the *recF* mutation (Fig. 2b). The viability level reached by two of the double mutants (*recC1002 recF* and *recB2154 recF*) was close to the level reached by the *recBC*-null *recF* strain (Fig. 1b).

Taken together, these results suggest strongly that some functional cooperation of the RecFOR machinery and each of these three non-null RecBCD mutant (*recC1002*, *recC1004*, *recB2154*) enzymes can contribute greatly to cell survival after loss of the RM gene complex.

This effect was examined more closely for the *recC1002* allele by following the cell-death process in liquid culture after temperature shift to a non-permissive temperature of 42 °C to block plasmid replication more tightly (Fig. 3, right). The temperature shift slowed down the increase of the r^+ cells in viable cell counts compared with the r^- control (Fig. 3, top left), as reported previously for other strains (Handa *et al.*, 2000; Kusano *et al.*, 1995; Naito *et al.*, 1995). The difference between the r^+ and the r^- strains in the viable cell count was statistically significant ($P = 0.0003$, 0.0076 and 0.0178 for 4, 6 and 8 h, respectively, in four measurements). The *recF143* mutation strongly inhibited

Fig. 3. Effect of a *recF* mutation on cell growth after loss of the EcoRI gene complex in a *recC1002* background. Bacterial cells carrying the EcoRI r^+m^+ gene complex or its r^-m^+ derivative on a thermosensitive plasmid replicon were grown with selective antibiotics to exponential phase at 30 °C, then shifted to 42 °C and grown in the absence of selective antibiotics. The number of viable cells was calculated by counting colonies formed on L agar without selective antibiotics after overnight incubation at 30 °C. The number at time 0 was set to unity. Viability was calculated as [viable cell count (left panel)/total number of cells counted under a microscope]. The number of plasmid-carrying cells was calculated by counting colonies formed on L agar with selective antibiotics after overnight incubation at 30 °C. The number at time 0 was set to unity. ○ indicates cells losing the r^+m^+ plasmid; ● indicates cells losing the r^-m^+ plasmid. Measurements were carried out in duplicate and essentially the same results were obtained in two independent experiments.

the increase in viable cell counts of the r^+ cells (Fig. 3, bottom left). It appeared to delay recovery in viable cell counts. The difference between the ratio (viable cell count for r^+)/(viable cell count for r^-) for the *recF*⁺ strain and the ratio for the *recF*[−] strain was highly statistically significant ($P = 0.0001$, 0.0011, 0.0099 and 0.0063 for 2, 4, 6 and 8 h, respectively, in four measurements). This is confirmed by a plot of viability, which is defined here as the viable cell count divided by the total cell count as determined under a microscope (Fig. 3, middle). The difference between the ratio (viability for r^+)/(viability for r^-) for the *recF*⁺ strain and the ratio for the *recF*[−] strain was statistically significant at 4, 6 and 8 h ($P = 0.022$, 0.007 and 0.032, respectively).

RecQ and RecJ contribute to resistance to RM-mediated post-segregational killing in the presence of the non-null *recBCD* alleles

Because RecFOR-mediated recombination requires RecQ helicase and RecJ nuclease functions (Lovett & Clark, 1984;

Nakayama *et al.*, 1984), we examined whether the cooperation of the RecFOR machinery and the non-null mutant RecBCD machinery requires RecQ and RecJ functions. Introducing a *recJ* or a *recQ* mutation into these four non-null *recBC* alleles caused severe cell killing similar to that seen when *recFOR* mutations were introduced after loss of the EcoRI RM plasmid (Fig. 4a, b and data not shown). In the individual viable cell-count experiments (Fig. 4b), we carried out statistical analysis, and a significant difference was observed between *rec*⁺ and either *recC1002* or *recB2154* for *recQ* or *recJ* mutation ($P < 0.05$ in a *t*-test). No significant difference was observed between *recC1002* and *recB2154* mutations in the presence of a *recQ* or *recJ* mutation ($P = 0.43$ or 0.35 , respectively). Therefore, both RecQ and RecJ appear to play an important role in repair of the DNA lesions induced by RM loss in the presence of these non-null *recBCD* alleles.

Comparison with resistance to UV

In order to compare the possible cooperation of the above non-null *recBC* mutants and the *recF* function in the resistance to damage caused by RM systems with that in the resistance to another type of DNA damage, we measured cell survival after UV irradiation in the various mutants (Fig. 5a). The non-null *recBCD* alleles *recC1002*, *recC2145* and *recB2154* showed more resistance than the *recC73* null allele in the presence of a functional *recF* gene (Fig. 5a, left). The partial resistance by *recC1002* was reported previously (Schultz *et al.*, 1983). This resistance was, however, reduced dramatically when combined with the *recF* mutation (Fig. 5a, right). The level was indistinguishable from that of the *recBC*-null *recF* mutant for *recC2145* and *recB2154*. The level for *recC1002* was higher than the level found for *recC1004* (Handa & Kowalczykowski, 2007; Schultz *et al.*, 1983).

Synergistic UV sensitivity by a *recBC*-null mutation with a *recF* mutation was demonstrated previously (Hori & Clark, 1973; Lloyd *et al.*, 1988). However, the present results indicate some sort of RecBCD–RecF interaction specific to the non-null *recBCD* alleles (*recC2145* and *recB2154*) that is completely dependent on RecF function. The nature of functional cooperation between the non-null RecBCD machinery and the RecF machinery will be discussed later.

Interestingly, UV survival of the RecQ/RecJ mutants under a non-null *recBCD* allele was distinguishable from post-segregational killing by loss of the RM complex (Fig. 5b). The *recJ* mutation increased sensitivity in the presence of the *recC1002* or *recC2154* allele to the level of the null *recBC* allele, just as the *recF* mutation did (Fig. 5b; cf. Fig. 5a, right). However, the *recQ* mutation caused a less severe increase in sensitivity. These results indicate that the RecQ and RecJ functions contribute to cell survival following DNA damage in the presence of these non-null *recBCD*

Fig. 4. Effect of *recQ* and *recJ* mutations in Chi recognition-defective mutants on cell growth after loss of the EcoRI gene complex. (a) Bacterial strains carrying the EcoRI *r*⁺*m*⁺ gene complex or its *r*⁻*m*⁺ derivative on a thermosensitive plasmid replicon were grown with selective antibiotics to exponential phase at 30 °C, streaked on L agar without selective antibiotics and then incubated overnight at 37 °C. *r*⁺*m*⁺, Bacteria losing the *r*⁺*m*⁺ plasmid; *r*⁻*m*⁺, bacteria losing the *r*⁻*m*⁺ plasmid. (b) Viability after loss of the RM gene complex on a thermosensitive plasmid. Total indicates cell counts under a microscope before colony assay; 37 °C viable indicates colony counts after overnight incubation at 37 °C without plasmid-selecting antibiotics. Results of independent measurements are presented. Bars are means of the plots.

alleles. Furthermore, they suggest that the requirements for RecQ and RecJ vary according to the cause of DNA damage (DNA restriction or UV).

Fig. 5. UV sensitivity of various *rec* mutants. (a) Wild-type, non-null and null *recBCD* alleles in the absence (left) or presence (right) of the *recF* mutation. (b) Effect of *recQ* and *recJ* mutations on two non-null *recBC* alleles. Results of two independent experiments are presented.

DISCUSSION

Contribution of RecF function to resistance to post-segregational killing by RM systems

A *recF*-defective mutation in the presence of functional RecBCD machinery reduced cell viability after the EcoRI RM system was lost from the cell (Fig. 1a, b). The effect of the *recF* mutation was even larger when the cells were defective for RecBCD machinery and carried suppressor mutations (*sbcBC*) that activated the RecFOR machinery (Fig. 1c). These results demonstrate that the RecFOR machinery can contribute to cell survival following the loss of an RM system. The RecFOR machinery probably repairs lethal DNA lesions, even if the RecBCD machinery is missing.

Involvement of the RecFOR machinery in resistance to RM-mediated post-segregational killing has broad implications, because RecFOR homologues have been identified in almost all groups of eubacterial genomes, whereas RecBCD

homologues are limited to only a few groups of eubacteria (Rocha *et al.*, 2005). RM gene homologues are also found throughout prokaryotic genomes (Roberts *et al.*, 2007). We may imagine that RecFOR-like homologous-recombination machinery modulates host attack by these organisms and, by extrapolation, similar selfish DNases of broad distribution (Fukuda *et al.*, 2008).

The parallel reductions of viable cell count in the restriction-negative controls indicate that such a defensive role of RecFOR machinery extends to spontaneous DNA damage.

Functional cooperation between RecF function and RecBCD function

Partial resistance to RM-mediated killing was observed with four non-null mutants of the RecBCD enzyme (Figs 2a, b, 3). The *recF*, *recO* and *recR* genes for the RecFOR machinery contribute greatly to the resistance in at least three of these genetic backgrounds (Fig. 2b). Essentially the same results were obtained for cell survival after UV irradiation (Fig. 5a). Therefore, we conclude that the RecFOR machinery and the RecBCD machinery, at least with these mutations, can engage in some functional cooperation to repair some DNA lesions after RM loss.

Although RecBCD homologues are limited to few groups of eubacteria (Rocha *et al.*, 2005), each of them recognizes a unique sequence, a putative identity sequence of a group of genomes (Chedin & Kowalczykowski, 2002). RecBCD enzymes and their functional analogues may play the role of exonuclease-based self-recognition systems, just as the RM systems play the role of endonuclease-based self-recognition systems (Handa *et al.*, 2000). The diversity of RecBCD analogues (Amundsen *et al.*, 2008) may correspond to the diversity of restriction enzymes (Chedin *et al.*, 2006; Orłowski & Bujnicki, 2008; Roberts *et al.*, 2007). Thus, it is possible that *recBCD* analogues remain undetectable by homology searching in many prokaryotic genomes. Several bacteria other than *E. coli* carry RecBCD-like and RecFOR-like pathways (e.g. Criss *et al.*, 2005; Hill *et al.*, 2007). *recFOR* homologues in a genome might have functional cooperation with its RecBCD analogue in the fight against RM systems, as we demonstrated here.

The wild-type RecBCD machine of *E. coli* will repair a restricted chromosomal DNA carrying a Chi sequence, but destroy a restricted foreign DNA lacking a Chi sequence (Handa *et al.*, 2000; Simmon & Lederberg, 1972). The bacteriophage-mediated homologous-recombination machinery, on the other hand, repairs restricted phage genomes and other incoming DNAs (Handa & Kobayashi, 2005; Takahashi & Kobayashi, 1990). Cooperation of the RecFOR machinery and RecBCD machinery may be viewed in such a context. The conflicts and collaborations among the RM systems, the multiple cellular recombination machineries and the phage recombination machineries might have driven their co-evolution at the level of

molecular mechanisms and shaped the genomes (Kobayashi, 2004b).

Some hints about underlying molecular mechanisms

What do our present phenomenological analyses tell us about the underlying molecular mechanisms of RecFOR-mediated survival after RM loss in the absence and presence of the RecBCD enzyme? The simplest interpretation of RecFOR-mediated survival is that RecFOR-mediated homologous recombination repairs chromosomal DSBs made by the restriction enzyme. This has been demonstrated *in vivo* (Handa & Kobayashi, 2003; Ivancic-Bace *et al.*, 2003; Seigneur *et al.*, 2000; Takahashi *et al.*, 1992) and *in vitro* (Handa *et al.*, 2009). The RecFOR machinery can act on a single-stranded tail generated at the ends (Handa & Kobayashi, 2003; Kuzminov, 1999). The homologous recombination stimulated by DNA ends is non-conservative in that it generates only one recombinant DNA out of two parental DNAs, at least in a *recBC sbcBC* background (Takahashi *et al.*, 1992). Such non-conservative recombination should be able to reconstruct a continuous chromosome out of two daughter chromosomes, each with a restriction break.

In addition, the RecFOR machinery can act on a single-strand gap, as suggested strongly to occur *in vivo* (Courcelle *et al.*, 2003; Kuzminov, 1999) and demonstrated *in vitro* (Bork *et al.*, 2001; Morimatsu & Kowalczykowski, 2003). At least for a mutant restriction enzyme, single-strand breaks are proposed to represent lethal damage, probably through their conversion into DSBs upon encounter with a replication fork (Muir *et al.*, 1997). RecFOR action on a single-strand gap could prevent such formation of lethal DSBs in our experiments.

More specifically, what could be the mechanisms underlying the apparent functional cooperation between the RecFOR machinery and the mutant RecBCD machinery? The functional cooperation described in the present work appears different from the synergism defined by a *recBC* null mutation and a *recF* mutation (Horii & Clark, 1973; Lloyd *et al.*, 1988). The UV experiments indicate that the functional cooperation for survival is specific to the non-null alleles *recC2145*, *recB2154* and *recC1002* (Fig. 5a) and that it is completely (*recC2145*, *recB2154*) or substantially (*recC1002*) dependent on RecF function. A similar reduction to the null-*recBC* mutant level by *recF* mutation was found for four non-null *recBC* alleles (*recC1002*, *recC1004*, *recC2145* and *recB2154*) in resistance to RM-mediated killing (Figs 1b, 2a, b), whilst for two more non-null alleles, *recC1001* and *recC1003*, the effect of the *recF* mutation seemed to be absent in the plate assay (data not shown). This could be due to the higher recombination in *recC1001* and *recC1003* mutants (Schultz *et al.*, 1983). Because the effect of the *recF*-null mutation varies among *recBCD* alleles, it is possible that the modes of cooperation and, therefore, the role of RecF function vary among the different *recBCD* alleles.

The cooperation could be entirely indirect. For example, if there is some threshold level in the number of instances of lethal damage (such as DSBs) that a cell can tolerate, independent action of two repair systems would appear synergistic. The cooperation could possibly involve gene expression. For example, the SOS response may be induced by RecBCD action and the genes related to the RecF pathway could be induced. Their functional cooperation could also result from their sequential action on a DSB. If so, the present data, which include involvement of RecJ nuclease and RecQ helicase (Figs 4, 5b), may impose some limits on possible molecular mechanisms.

The wild-type RecBCD enzyme degrades dsDNA from the end, with unequal exonucleotic degradation for 5' and 3' strands. Until it recognizes a Chi sequence, the 3' strand is degraded more thoroughly (Anderson & Kowalczykowski, 1997). Its destruction activity is switched to a 5' ssDNA exonuclease when it encounters Chi. The non-null mutant RecBCD enzymes used in this work can, to a varying extent, digest DNA from a DSB, but cannot be altered by Chi (Amundsen *et al.*, 1990; Schultz *et al.*, 1983). It would leave mostly 5' overhangs. The action of RecJ, a 5'-end-specific ssDNA exonuclease, there would generate dsDNA with a 3' overhang, which may, in turn, recruit RecFOR proteins (Handa *et al.*, 2009; Morimatsu & Kowalczykowski, 2003). Resection of the 5' strand is a process essential to the initiation of homologous recombination (Gravel *et al.*, 2008; Mimitou & Symington, 2008; Nimonkar *et al.*, 2008; Zhu *et al.*, 2008). An alternative possibility that RecJ acts at a later step (Corrette-Bennett & Lovett, 1995) cannot be excluded.

Another possible role of the mutant RecBCD enzyme is suggested from the role of an *sbcB* mutation in activating the RecFOR pathway (Fig. 1c). The recombinogenic 3' ssDNA may be degraded by exonuclease I (the *sbcB* gene product). The non-null RecBCD enzymes might protect the 3' ssDNA from such degradation and save them for the RecFOR machinery.

RecQ helicase may supplement the partial helicase defect, if any, of the mutant RecBCD enzymes and/or help with the RecJ action described above. RecQ could function at later steps (Harmon & Kowalczykowski, 1998; Veaute *et al.*, 2003). The less stringent requirement for RecQ in UV survival could be due to a difference in lethal DNA damage and/or the presence of redundant helicases, such as UvrD, Rep and HelD, for UV repair (Lovett & Suttera, 1995; Mendonca *et al.*, 1993). The requirement for RecQ helicase distinguishes the present hypothetical pathway from the RecBCD–RecFOR hybrid pathway proposed for the *recB1080* allele, which encodes a nuclease-defective RecBCD enzyme (Amundsen & Smith, 2003; Ivancic-Bace *et al.*, 2005).

Homologous recombination in the *recD*-negative mutant, which is also exonuclease-defective, depends on RecJ, but not on RecF or RecQ (Lloyd *et al.*, 1988; Lovett *et al.*, 1988). Resistance to RM-mediated killing in the *recD*-

negative mutant was found to be independent of RecF function (Fig. 1a, d). In the case of the *recB1080* allele, a linked *recD*⁻ mutation recovered recombination independently of RecF (Amundsen *et al.*, 2000).

Conclusion

In conclusion, the RecFOR machinery, abundant in the eubacterial world, can fight against host attack by an RM system. Its contribution can be large in certain genetic backgrounds. This result will form a solid basis for understanding the biology of RM systems, especially their interactions with their host bacteria and their effects on the genome evolution.

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