Stationary-state mutagenesis in *Escherichia coli*: a model

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Abstract

Stationary-phase mutagenesis in nondividing *E. coli* cells exposed to a nonlethal stress was, a few years ago, claimed to be a likely case of a Lamarckian mechanism capable of producing exclusively useful mutations in a directed manner. After a heated debate over the last decade it now appears to involve a Darwinian mechanism that generates a transient state of hypermutagenesis, operating on a large number of sites spread over the entire genome, at least in a proportion of the resting cells. Most of the studies that clarified this position were on the reversion of a frameshift mutation present in a *lacI–lacZ* fusion in *E. coli* strain FC40. Several groups have extensively examined both the sequence changes associated with these reversions and the underlying genetic requirements. On the basis of our studies on the genomic sequence analysis, we recently proposed a model to explain the specific changes associated with the reversion hotspots. Here we propose a more detailed version of this model that also takes into account the observed genetic requirements of stationary-state mutagenesis. Briefly, G:T/U mismatches produced at methylatable cytosines are preferentially repaired in nondividing cells by the very short patch mismatch repair (VSPMR) mechanism which is itself mutagenic and can produce mutations in very short stretches located in the immediate vicinity of these cytosine methylation sites. This mechanism requires a homologous or homeologous strand invasion step and an error-prone DNA synthesis step and is dependent on RecA, RecBCD and a DNA polymerase. The process is initiated near sequences recognized by Dcm and Vsr enzymes and further stimulated if these sequences are a part of CHI or CHI-like sequences, but a double-strand-break-dependent recombination mediated by the RecBCD pathways proposed by others seems to be nonessential. The strand transfer step is proposed to depend on RecA, RuvA, RuvB and RuvC and is opposed by RecG and MutS. The model also gives interesting insights into the evolution of the *E. coli* genome.

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Introduction

Following a lead given by an earlier work of Shapiro (1984), Cairns *et al.* (1988) claimed that *E. coli* cells may possess mechanisms that enable them to produce exclusively growth promoting mutations (also called adaptive mutations) when, and only when, they are exposed to a growth-blocking nonlethal stress. Due to its Lamarckian connotations this paper raised a heated debate in the scientific community. A large number of papers, either supporting or opposing these claims, appeared in quick succession (see Foster 1993, 1999a; Bhattacharjee and Mahajan 1998; Lombardo and Rosenberg 1999 for reviews and references). The debate has now essentially been concluded in favour of the neo-Darwinistic dogma (but, see Hall 1999). However, this debate has not been totally fruitless. It has thrown up evidence for the existence of specialized mutagenic processes with pronounced activity in stationary-state cells. Though these processes are not strictly Lamarckian in the sense of producing exclusively useful (or adaptive) mutations (Foster 1997; Torkelson *et al.* 1997), careful elucidation of their nature is essential for finally settling the controversy between Darwinism and the Lamarckian possibilities of generating exclusively growth-promoting mutations in nondividing cells. These mechanisms seem to provide an important tool of evolvability to organisms exposed to variable environment and appear to contribute significantly

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to the genomic evolution (Bhattacharjee and Mahajan 1998; Mahajan *et al.* 1998). Therefore, their elucidation should help in explaining the composition and organization of genomic sequences, data for which are accumulating rapidly. Further, these mechanisms may also be useful in understanding important biological phenomena like cancer, which involve the production of fast-growing mutants in nondividing or slowly dividing cell populations (Modrich and Lahue 1996), and somatic hypermutations responsible for the generation of immunoglobulin variability (Cascalho *et al.* 1998).

The best evidence for a specific mutagenic mechanism being hyperactive in stationary-state cells comes from studies with the *E. coli* K-12 strain FC40, though it is not confined to this strain or organism alone (Jayaraman 1992; Prival and Cebula 1992, 1996; Foster and Cairns 1998; Massey *et al.* 1999). FC40 carries an F′ episome harbouring a *lacI33–lacZ* fusion that also has a +1 frameshift mutation in *lacZ*, making the host strain Lac$^-$ (Cairns and Foster 1991). Lac$^+$ revertants can arise in either exponentially growing or stationary-state FC40 cells but the genetic requirements of the mutagenesis and the sequence changes associated with the reversion are distinctly different in the two cases (Foster and Trimarchi 1994; Rosenberg *et al.* 1994). We recently proposed that the sequence spectrum of the stationary-state mutants can be explained by a model, which postulates that the very short patch mismatch repair (VSPMR) pathway involved in the repair of T:G/U mismatches produced at sites of cytosine methylation (Radman 1988) is hyperactive and mutagenic in stationary-state cells (Bhattacharjee and Mahajan 1998). We also suggested that this hyperactivity was due to lower levels in the stationary-state cells of the proteins, especially MutS, involved in the methylation-dependent long patch mismatch repair pathway (LPMR; also called methylation-dependent mismatch repair, MMR, or methyl-directed mismatch repair system, MRS) (Modrich 1991; Brégeon *et al.* 1999). Computer analysis of the entire *E. coli* genomic sequence confirmed several predictions of this hypothesis and further suggested that in the neighbourhood of the recombinogenic octamer CHI (GCTGGTGG), and other related octamers which contain the cytosine methylation pentamer DCMIII (GCTGG.CCAGC), this mutagenic mechanism may have enhanced activity (Mahajan *et al.* 1998). Here we present a more detailed version of our model, specifying various steps of the VSPMR mechanism and the enzymes/proteins involved in these steps. This version is suggested by a detailed examination of the known effects of mutations in various DNA metabolism genes on stationary-state mutagenesis (SSM) in FC40 and other *E. coli* strains. Before presenting the model, we briefly summarize the relevant results.

Important genetic characteristics of stationary-state mutagenesis in FC40

(i) Stationary-state mutagenesis (SSM) in FC40 is prevented by mutational inactivation of *recA* or *recB*, two genes essential for homologous recombination involving linear duplex DNA substrates like in conjugation or generalized transduction (Mahajan 1988; Foster *et al.* 1996; Harris *et al.* 1994, 1996).

(ii) It uses a pathway other than that used by the normal SOS repair, which is also dependent on RecA, and produces mutations by an error-prone DNA replication step (Walker 1985; Cairns and Foster 1991; Foster *et al.* 1996).

(iii) It is dependent on RuvAB, a helicase involved in extending strand transfer, and RvuC, an endonuclease involved in terminating strand exchange, and is countered by RecG, a helicase involved in strand exchange between homologous DNA molecules but whose absence has little effect on the yield of recombinants in conjugation crosses with wild-type recipients (Whitby and Lloyd 1995; Foster *et al.* 1996; Harris *et al.* 1996; West 1996).

(iv) Null mutations in *mutS* or *mutL* enhance SSM while MutS overproduction decreases it (Boe 1990; Foster and Cairns 1992; Jayaraman 1992; Foster *et al.* 1996). Stationary-state cells show reduced MutS and MutH but not MutL and reduced MMR activity; the latter reduction can be countered by overproduction of MutS or MutH but not by overproducing MutL (Foster *et al.* 1995; Feng *et al.* 1996; Harris *et al.* 1997b; Tsui *et al.* 1997; Brégeon *et al.* 1999).

(v) Overproduction of Vsr endonuclease increases the SSM rate about 100-fold in wild-type cells but not in *mutL* derivatives (Foster and Rosche 1998).

(vi) SSM is a genome-wide activity operating not only on the *lacI33–lacZ* fusion but also on other genes present on F′ in FC40, on the chromosome or on nonconjugative plasmids such as pBR322 (Foster 1997; Torkelson *et al.* 1997); significantly, stationary-state mutations in other genes also show the predominance of −1 frameshifts characteristic of the *lacI*$^+$*Z* hotspots (Torkelson *et al.* 1997).

(vii) CHI seems to stimulate adaptive mutagenesis in stationary phase by an activity different from its classical RecA,RecBCD-dependent recombinogenic activity (Mahajan *et al.* 1998).

(viii) SSM frequency is much higher when the target locus is on F′ than on the chromosome and is dependent on episome transfer functions, although transfer *per se* is not needed for this process (Foster and Trimarchi 1995; Galitski and Roth 1995; Peters and Benson 1995; Radicella *et al.* 1995).

(ix) SSM is modulated in a complex manner by mutations in different DNA polymerase genes: proofreading-deficient DNA PolII increases SSM while an antimutator mutation in PolIII decreases it (Fijalkowska *et al*. 1993; Escarceller *et al.* 1994; Foster *et al.* 1995; Longerich *et al.* 1995; Harris *et al.* 1997a; Rangarajan *et al.* 1997).

(x) The characteristic mutational spectrum of the *lacI33–lacZ* reversion to Lac$^+$ in stationary state is reproduced in growing cells harbouring *mutS* or *mutL* mutations (Longerich *et al.* 1995).

The groups of Foster and Rosenberg have recently proposed models for stationary-state mutagenesis which

seem to account for many of the above characteristics (Foster and Rosche 1999a; Lombardo and Rosenberg 1999). Both these groups essentially propose that these mutations are produced during DNA replication associated with RecA–RecBCD-dependent repair of double-strand breaks (DSBs) generated by collapse of an F′ replication fork when it meets a nick generated at *oriT* by a site-specific endonuclease associated with conjugative transfer. According to both groups lower levels of the activity of the MutSLH-dependent MMR in stationary-state cells (Feng *et al.* 1996; Harris *et al.* 1997b; Tsui *et al.* 1997; Brégeon *et al.* 1999) are responsible for the higher rate of mutation fixation in these cells. The two groups also agree that the opposite effects of RuvAB and RecG on stationary-state mutagenesis are due to their opposite effects on the polarity of movement of the four-stranded Holliday junction created during the DSB repair (DSBR), but they differ in the details of the actions of these two proteins. In the model of Foster and Rosche RuvAB moves the Holliday junction towards the 3′ end of the first invading strand, and RecG towards the 5′ end, while Lombardo and Rosenberg suggest opposite roles for these two enzymes.

While these models can explain many of the genetic characteristics of SSM they suffer from several infirmities including the following: (i) They do not even attempt to explain the peculiar sequence spectrum of the SSM in FC40, where some nucleotides are hotspots of mutagenesis while mutations at other similar sites make much smaller contributions. (ii) They assume that SSM has a recombination step similar to that included in the RecBCD pathway of conjugational recombination, where the RecBCD enzyme is generally believed to be loaded on to DNA from a double-stranded end and uses its nuclease and helicase activities to generate an invasive 3′ single-strand end. However, the effect of mutations in the strand transfer genes (*ruvA, ruvB, ruvC* and *recG*) on SSM is very different from their effect on conjugational recombination by the RecBCD pathway. Mutational inactivation of any of these genes only marginally reduces RecBCD recombination while the yield of stationary-state mutations is drastically reduced by *ruvA*, *ruvB* and *ruvC* inactivation and significantly enhanced by *recG* inactivation. Further *ruvA recG* and *ruvB recG* double mutants drastically reduce recombination by the RecBCD pathway but stimulate stationary-state mutagenesis (Foster *et al.* 1996; Harris *et al.* 1996). (iii) Since stationary-state cells experience mutations all over the genome (Foster 1997; Torkelson *et al.* 1997) the above models would require genome-wide availability of replication forks, implying that these mutations arise only in cells with replicating genomes. However, there is good evidence to show that they can arise in nondividing cells (Reddy and Gowrishankar 1997; McEnzie *et al.* 1998). (iv) Strains harbouring a defective *mutS* gene show enhanced SSM (Boe 1990; Foster and Cairns 1992; Jayaraman 1992). This, according to the above models, implies that in spite of the low level/activity of MutS the residual MMR/LPMR activity in *mutS*$^+$ stationary-state cells repairs most of the new mutations produced during DSBR. This would mean that MMR, which requires hemimethylated GATC in the neighbourhood of the mismatch to be corrected, is active everywhere in wild-type stationary-state cells. This again requires extensive DNA synthesis, which is unlikely in energy-depleted resting cells. (v) These models are silent on the role of Vsr whose overproduction enhances SSM (Foster and Rosche 1998).

The model and discussion

In figure 1 we present details of a model that does not suffer from the above difficulties. The detailed postulates of the model are given in the legend to the figure. The following points are noteworthy.

(i) According to this model, stationary-state mutations occur, at least preferentially, near the sites of methylatable cytosine. Since such sites are scattered all over the genome the mutagenesis would be a genome-wide phenomenon, but in view of the short range of the repair tracts (see below) the probability of the occurrence of mutation may be very different at different points on the genome and should depend on their distance from the nearest methylatable cytosine. This can explain both the existence of mutational hotspots and the failure to recover mutations in certain nonselected genes (Foster and Cairns 1992).

(ii) This model recognizes three functions of MutS, namely (a) competitive inhibition of Vsr binding to G:T/U mismatches, (b) inhibition of the RecA-mediated strand transfer, and (c) initiation of MMR by recognizing a mismatch. Of these the first two may be more important in keeping down the frequency of VSPMR-dependent mutations while the third may be important for keeping down all mutations. The presence of mutational hotspots near DCM sites (Bhattacharjee and Mahajan 1998; Mahajan *et al.* 1998) in stationary-state cells where the MutS levels are low (Feng *et al.* 1996; Tsui *et al.* 1997), or in *mutS* mutants (Foster and Cairns 1992; Jayaraman 1992; Longerich *et al.* 1995) is consistent with this idea. This model does not require a major role for MMR in nonreplicating cells, which are unlikely to harbour much hemimethylated DNA. It may be mentioned here that Foster (1999b) has also questioned the idea that adaptive mutations are due to a decline in the mismatch repair. Harris *et al.* (1999) contested this but they failed to recognize that MutS depletion could enhance SSM independently of its effect on MMR. We believe that the MMR activity may have only a minimum effect on SSM, largely by correcting some of the mismatches generated by VSPMR.

(iii) While the model postulates a RecA-mediated strand transfer step, which is helped by RuvAB and RuvC and is countered by RecG (and MutS), it does not require a DSB-dependent recombination step usually postulated to account for the RecBCD dependence of the process. Instead, we

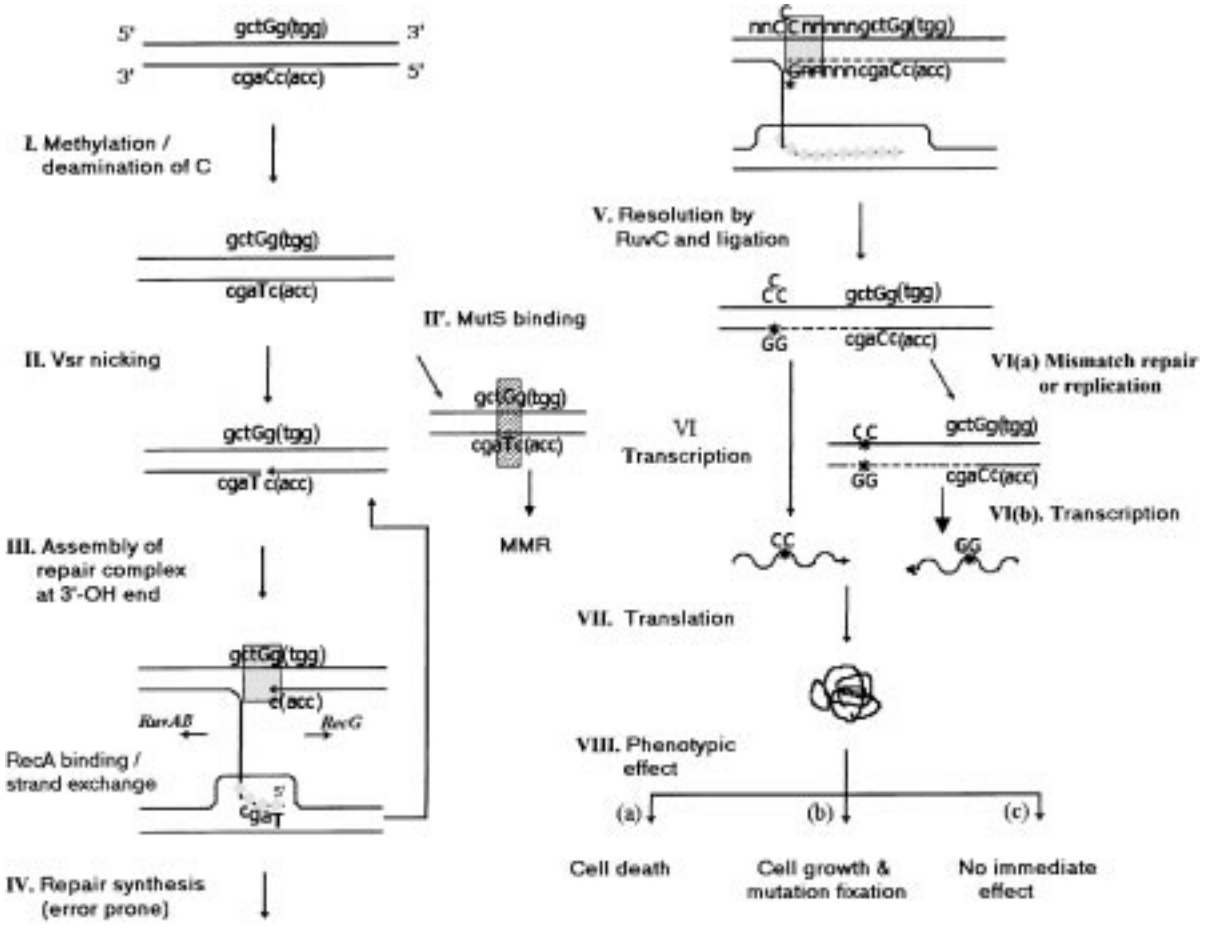

Figure 1. Schematics showing the roles of various proteins/enzymes in generating mutations in the neighbourhood of methylatable cytosines. I. Methylation/deamination of C: G:T/U mismatches are generated at the site of a methylatable cytosine (DCM sequences) by deamination of 5 meC/C. GCTGG.CCAGC, the most efficient pentamer for stationary-state mutagenesis, is shown here. TGG.CCA in parentheses indicates that CHI-like sequence contexts can stimulate error-prone repair in their neighbourhood. II. Vsr nicking: Vsr recognizes these mismatches and nicks the T-containing strand 5′ to T which is the first step in initiation of VSPMR. II′. MutS, the first protein of the error-free MMR process, can also recognize and bind to the G:T mismatch in competition with Vsr. Therefore the error-prone VSPMR is initiated more frequently when MutS concentration is low or Vsr concentration is high. III. Assembly of the repair complex and strand invasion: A complex consisting of a DNA polymerase, RecA, RecBCD, and other enzymes such as DNA ligase is assembled at the 3′−OH end generated in step II. RecA-dependent invasion of a homologous genomic site by the T/U-containing 5′-strand initiates the formation of a heteroduplex and a D-loop. In this three-stranded structure the helicase action of RuvAB would extend the heteroduplex while RecG would tend to reverse the strand transfer process. MutS may also interfere in the heteroduplex extension. IV. Repair synthesis: The repair complex, while reversing the C → T transition, creates fresh mutations in the neighbourhood. DNA polymerase slippage when the repair complex encounters a tract of mononucleotide repeats leading to deletion (or addition) of a nucleotide would be a major mechanism of these mutations. The length of the mutagenic repair tract is short (approx. 10–15 nt), primarily because the RecA-mediated heteroduplex formation cannot extend into the heterologous region. The length of the repair tract, and hence the range of mutagenesis, may be extended if a second copy of the homologous sequences is available, as in the case of a multicopy plasmid. V. Resolution by RuvC and ligase: RuvC terminates the extension of a heteroduplex by nicking the invading T-containing strand. The 5′ end thus generated is ligated to the newly synthesized repair tract. Some of the errors thus generated, namely those produced on the coding strand, would require an additional mismatch correction step to transfer the mutation to the transcribed strand. VI. Transcription: Transcription of the mutated gene, either directly if the mutation is produced on the transcribed strand of the given gene (which will happen if the methylatable cytosine lies on this strand), or (VIa and VIb) after a mismatch correction step that transfers the mutation to the transcribed strand, produces a variant mRNA. VII. Translation: Translation of this mRNA yields a variant protein. VIII. Phenotypic effect: (a) If the new mutation inactivates some essential gene it may be lethal to the cell; (b) if it reverts the original growth-blocking mutation or at least reverses the effect of the original mutation it may promote growth of the host cell; (c) if it is neutral or affects a nonessential gene it may have no significant immediate effect.

postulate that RecBCD acts as a part of a multiprotein repair complex, which has a DNA polymerase, DNA ligase, RecA and some unknown proteins as its other components. As a part of this complex RecBCD is postulated not to require a double-stranded end for entering DNA substrates. The complex may be assembled at or enter DNA at a single-stranded nick or gap. There is evidence for the existence of such a complex in *E. coli* (Martin 1997; Mahajan *et al.* 1998). It is postulated here that this complex carries out a short-range, error-prone repair synthesis primed by the 3′OH generated by the Vsr-induced nick 5′ to T/U at the site of a G:T/U mismatch (Hennecke *et al.* 1991). The repair synthesis displaces the strand containing the mismatched T at its 5′ end. This strand is coated by RecA and invades a homologous/homeologous complementary sequence elsewhere on the genome to initiate strand transfer which is helped by RuvAB and is countered by MutS and RecG. High level of RecG reverses this process and releases the invading strand permitting it to interact with its original partner, thus interfering with the mutagenic repair. On the other hand, absence of active RecG stabilizes the heteroduplex and helps the error-prone repair synthesis. These inferences are consistent with the results of Whitby and Lloyd (1995) who showed that in a three-strand reaction RecG reverses the RecA-mediated strand transfer with $5' \rightarrow 3'$ polarity.

(iv) The point beyond which the heteroduplex formation cannot proceed owing to nonhomology between the invading and the displaced sequences determines the length of the mutagenic repair tract. At this point the strand exchange intermediate would stall until RuvC resolves it. Normally this point should not go beyond about 10 nucleotides in *E. coli* but a longer range may be possible when a completely homologous target is available, for instance, in the case of multiple-copy plasmids such as pBR322 derivatives. The spectrum of mutations produced in the Tet^R locus on pBR322 (Foster 1997; Torkelson *et al.* 1997) and the Rif^R mutations studied by Rangarajan *et al.* (1997) showed that most of these mutations were in the neighbourhood (within 15 nucleotides) of a DCM-like site.

(v) According to this model transfer replication of F′ is not directly involved in enhancing the mutagenic repair but it may facilitate stabilization and extension of the heteroduplex by the relaxing action of the nick produced at *oriT*. Another possibility is that it provides homologous targets for anchoring of the displaced strand during the repair synthesis (see (iv) above).

(vi) The main function of RecBCD in the repair complex seems to be to stimulate the polymerase activity probably by enhancing the binding of the 3′−OH end to the template strand. The presence of the RecD subunit does not seem to be essential for this purpose (Foster and Rosche 1999b). The enhanced recovery of mutants in *recD* cells can be understood if it is postulated that in such cells double-strand breaks are less likely to be lethal owing to the absence of the degradative action of ExoV. Such DSBs are likely to be generated when Vsr produces nearby nicks on opposite strands containing two methylatable cytosines, either within a single canonical DCM site or in two nearby DCM sites (located on opposite strands).

(vii) This model can explain the reported features of the effects of different DNA polymerase mutations in the following way. Most of the mutagenic (i.e. error-prone) repair of G:T/U mismatches may be mediated by a PolI-containing complex (Martin 1997). PolII may also frequently participate in the repair complex but this repair may be usually error free. PolB deletion may make most of the repair target accessible to PolI, thus enhancing the overall error rate. PolB Exo^- may either produce mutations on its own or is unable to compete with PolI for the 3′−OH end. PolIII appears not to participate frequently in this VSPMR. However, DnaE915 may compete better with PolI and PolII. The relative contribution of the three enzymes may be different on the chromosome than on F′.

(viii) The above model could predict that an excess of Vsr should stimulate VSPMR and therefore enhances SSM, as seen by Foster and Rosche (1998). Their failure to see this enhancement in *mutL* cells can be understood in view of the observation that MutL stimulates the binding of Vsr to the heteroduplex DNA (Karin *et al.* 1998).

(ix) The nonrecombinogenic activity of CHI involved in stimulating SSM (Mahajan *et al.* 1998) may be provided by the affinity of RecA for GCTGG, and that of RecBCD for CHI octamer. This may help in assembling the repair complex, thereby stimulating the error-prone repair synthesis. It should also be noted that the overabundance of DCM and CHI-like sequences on the *E. coli* chromosome ensures that a homologous partner for the displaced T/U-containing strand will be easily available without the need of general genome-wide replication. However, the presence of a fully homologous site either due to local amplification or multicopy nature of a plasmid may help.

Concluding remarks

The model presented here differs from the other models in several important aspects, namely (a) it does not require a DSB-dependent recombination; (b) it does not require general genome-wide replication; (c) it considers the error-prone VSPMR, and not the diminished MMR, as the main source of hypermutagenesis in stationary-state cells; (d) it is not dependent on *oriT* as the site of nicking; (e) it uses a strand transfer step but only to keep the displaced strand away to avoid interference with repair replication; (f) it can explain the peculiar spectrum as well as the genome-wide occurrence of SSM; (g) it implicates CHI-like (overabundant) sequences as better instigators of hypermutagenesis in their neighbourhood.

It has been suggested that the recombinogenic activity of CHI is responsible for its overrepresentation on the genome

(Kuzminov 1995; Blattner *et al.* 1997; Tracy *et al.* 1997; Colbert *et al.* 1998). According to our analysis of the genomic sequence, in most of the highly abundant octamers (including CHI) the overabundance was strongly dependent on the presence of a methylatable cytosine (Mahajan *et al.* 1998). The CHI context seems to stimulate the adaptive advantage conferred by these potential Dcm substrates by activating the error-prone VSPMR. The much shorter range (approx. 15 bp) of this repair mechanism compared to that of recombination stimulation by CHI (approx. 10 kb) (McMilin *et al.* 1974) supports the distinct nature of these two activities of CHI. The recognition of CHI-like sequences by RecBCD or a multiprotein complex containing it may use the single-strand nicks or gaps for loading on to DNA without requiring a double-strand break which is the case for the recombinogenic action of CHI.

Finally, the temptation to simplistically equate specific genes and enzymes with the phenomena or the metabolic process in which they were first implicated should be resisted. For instance, the mere dependence of a process on RecA and RecBCD, which have multiple activities, is no guarantee that it involves a DSB-dependent recombination step. Similarly, the enhancement of stationary-state mutagenesis by the deficiency of active MutL/S proteins or decrease in this mutagenesis by the overproduction of these proteins does not necessarily imply that these proteins are actively involved in a mismatch correction process in the cell. Nor does the discovery of CHI as a recombination-stimulating octamer dictate that all the effects of this sequence must have a recombination step. Nature seems to be much too opportunistic to accept such constraints.

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