The importance of repairing stalled replication forks

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The bacterial SOS response to unusual levels of DNA damage has been recognized and studied for several decades. Pathways for re-establishing inactivated replication forks under normal growth conditions have received far less attention. In bacteria growing aerobically in the absence of SOS-inducing conditions, many replication forks encounter DNA damage, leading to inactivation. The pathways for fork reactivation involve the homologous recombination systems, are nonmutagenic, and integrate almost every aspect of DNA metabolism. On a frequency-of-use basis, these pathways represent the main function of bacterial DNA recombination systems, as well as the main function of a number of other enzymatic systems that are associated with replication and site-specific recombination.

In bacterial cells, replication forks often encounter template DNA damage that can inactivate the fork. This problem is not restricted to situations in which cells are stressed by ultraviolet irradiation or other damaging treatments. Instead, replication forks are routinely inactivated under normal aerobic growth conditions, where SOS is not induced and some SOS functions are not present. Here we briefly review the pathways for the reactivation of these replication forks as framed in proposals from a number of laboratories based on more than a decade of work[1–24]. The main conclusions of this intensive research are that (1) most, if not all, of the replication forks initiating at the bacterial origin, *oriC*, encounter DNA damage under normal growth conditions; (2) many of these encounters inactivate the replication forks; (3) reactivation of the fork requires DNA recombination functions, a system for the origin-independent restart of replication and additional enzymes to reverse potentially detrimental side products of recombination; and (4) the pathways for reactivating the fork under normal growth conditions are nonmutagenic. In effect, the reactivation of replication forks represents a major housekeeping function in bacteria. Here we focus attention on the nonmutagenic pathways for replication fork reactivation and the enzymes involved in them, as well as highlighting the important contribution this process makes to cellular DNA metabolism during normal bacterial growth.

Figure 1 presents a few of the current ideas for replication fork demise and nonmutagenic reactivation, although we acknowledge at the outset that many of the molecular details of these pathways remain to be elucidated. The replication forks originating at *oriC* include the DNA polymerase III holoenzyme and a primosome consisting of DnaB and DnaG. An encounter with DNA damage results in an enzymatic train wreck that we refer to as either replication fork demise or inactivation. This is a working definition, underscoring that replication fork progression has been arrested. It is not yet known to what extent the replication machinery disassembles in these situations, and the molecular consequences may be as varied as the types of damage encountered.

The two main possibilities for fork demise that we consider are, first, if a strand break is encountered, a double-strand break will be generated. And, second, if an unrepaired DNA lesion is encountered, the replication fork halts and a DNA gap will be created. In broad strokes, the events required to reactivate the replication fork in both cases involve recombination (by at least two major pathways), replication restart, completion of elongation of nascent chains and resolution of any dimeric chromosomal products that result from recombination (Fig. 2). The elaborate molecular requirements for reactivating a replication fork imply a high degree of coordination between recombination and replication functions,

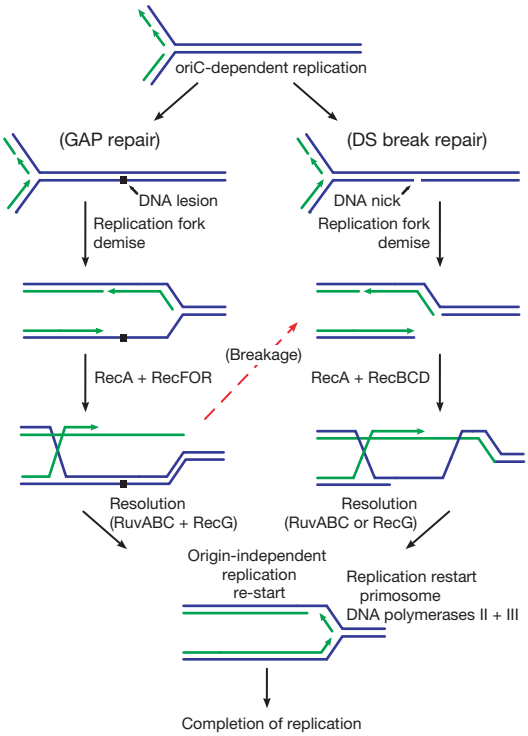

Figure 1 Some potential pathways for the nonmutagenic re-establishment of inactivated replication forks in bacteria. The pathways shown illustrate two of the important situations during normal cell growth that may result in replication fork demise, encounter with a DNA lesion or a DNA strand break. Reactivation involves the two main homologous genetic recombination pathways. The processes shown are broadly based on some published studies and discussions at recent national meetings; however, many of the details shown are speculative. The configurations of DNA strands shown in the intermediates are neither representative of all the proposals for fork reactivation nor intended to represent anyone's ideas of the most likely paths.

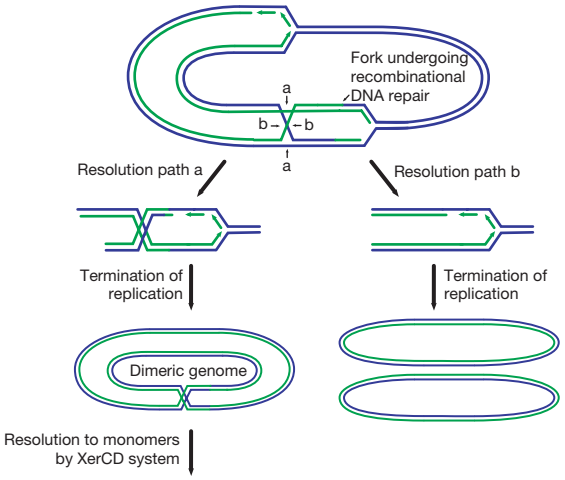

Figure 2 Creation and resolution of contiguous chromosomal dimers as a byproduct of the recombination required for re-establishment of inactivated replication forks. If Holliday junctions are created upstream from the replication fork during the repair process, their resolution can result in the creation of chromosomal dimers that must be resolved by the XerCD site-specific recombination system.

although the biochemical interactions between the replication and recombination systems remain mostly unexplored.

The entire process may involve more than two dozen proteins (Table 1), and only small parts have been reconstituted *in vitro*. Notably, the process includes important functions for several enzymatic systems whose functions have been, until recently, obscure. These include (1) the replication restart primosome, a complex of seven proteins discovered during investigation of the replication of bacteriophage ϕX174 DNA *in vitro*[25,26], but later found to be unnecessary for *oriC*-dependent replication[22]; (2) DNA polymerase II, discovered almost 30 years ago, but until recently without a significant identified function in bacterial DNA metabolism[18]; (3) the XerCD site-specific recombination system[17]; and (4) the RecF, O and R proteins, for which the associated recombination pathways have generally not been apparent unless other pathways are mutationally altered or removed[27].

The general concepts of bacterial replication fork demise and reactivation are not new, but have evolved during the work of multiple laboratories over a period of nearly 35 years. The first suggestion that a replication fork might be inactivated at the site of a strand break came in 1966 (ref. 1). On the basis of studies of phage lambda DNA replication, Shalka[2] later argued that pre-existing nicks in the parental template can lead to replication fork demise, and also that replication forks might be reactivated by a recombinational process. A link between recombination and DNA repair was evident in the phenotypes of the first recombination mutants[28,29]. Some still-viable proposals for the recombinational repair of DNA gaps and double-strand breaks after the demise of a replication fork are decades old[2,3]. An early model for the integration of recombination and replication was provided by the work of Mosig, Alberts and their colleagues on the bacteriophage T4 system[4,5]. Indeed, the recombination-dependent and nonmutagenic initiation of replication is needed for repair of inactivated replication forks and has a major role in the life cycle of bacteriophages such as T4 (ref. 16). The first recognition that replication fork demise (and a need for reactivation) might be commonplace in bacteria under normal growth conditions arose from studies of the phenotypes of *priA* mutants in 1991 (refs 16, 30).

More recently, Kuzminov[7] consolidated many disparate results regarding *Escherichia coli* replication and recombination into a clear model for reactivating bacterial replication forks that had been inactivated upon an encounter with a template strand break, and argued that the main role of *E. coli* recombination proteins is to reconstitute inactivated forks. Kuzminov[31] also argued that stalled replication forks are subject to an active breakage process (followed by reactivation). Michel and colleagues[14] provided experimental evidence for this directed breakage event, as well as insight to the frequency at which double-strand breaks occur under normal growth conditions. The work of Kogoma and colleagues also played a key role in the development of these ideas. Building on extensive studies of the recombination-dependent replication observed during the SOS response[8,10], Kogoma[10] pointed out that these processes could provide a general pathway to reactivate replication forks. Studies elucidating the function of the PriA protein[6,11,32], other primosomal components[9,33,34] and the XerCD site-specific recombination system[15,17] have been critical in developing the idea that replication fork reactivation is a housekeeping function of bacterial cells that operates at high frequency under normal growth conditions. More complete accounts of these works

Table 1 Proteins that may participate in replication fork reactivation

Protein*	Main activity	Role in fork reaction
RecA	Strand pairing/exchange	Formation of joint molecules
RecBCD	Chi site-modulated nuclease	Initiation of repair at double-strand breaks
RecF	Binds single- and double-stranded DNA	Limits extension of RecA filament
RecO	Binds SSB-coated DNA	Facilitates RecA loading to SSB-coated DNA
RecR	Binds SSB-coated DNA	Facilitates RecA loading to SSB-coated DNA
RuvAB	Branch migration DNA helicase	Resolution of joint molecules
RecG	Branch migration DNA helicase	Modulate structure of stalled fork?
RuvC	Holliday junction endonuclease	Resolution of joint molecules
RusA	Holliday junction endonuclease	Resolution of joint molecules
PriA	3′ → 5′ DNA helicase, binds bent DNA	Initiates replication fork assembly
PriB	Facilitates complex formation between PriA and DnaT	Replication fork assembly
PriC	Primosome assembly	Replication fork assembly
DnaT	Primosome assembly	Replication fork assembly
DnaB	5′ → 3′ DNA helicase	Replication fork helicase
DnaC	Binds and loads DnaB to DNA	Replication fork assembly
DnaG	Primase	Okazaki fragment primase
Rep	3′ → 5′ DNA helicase	Processing of stalled fork?
DNA polymerase III holoenzyme	DNA polymerase	Replicative polymerase
DNA polymerase II	DNA polymerase	Replication-restart at template lesions
DNA polymerase I	DNA polymerase, 5′ → 3′ exonuclease	Gap sealing
DNA ligase	Ligase	Gap sealing
SSB	Single-stranded DNA-binding protein	Coats single-stranded DNA
XerCD	Site-specific resolvase	Resolves dimeric chromosomes

*This listing does not include proteins that are known or suspected to be involved in replication for which a biochemical function has not been elucidated.

and additional syntheses can be found in a number of recent reviews[10,22–24,35].

Replication fork reactivation is required often

Quantitative estimates for the frequency of replication fork demise and reactivation are gradually becoming available. These have been reviewed[23,36] and will only be summarized here. Chromosomal dimers are found in bacterial cells under normal growth conditions. Although these dimers might sometimes arise for other reasons, the vast majority of them almost certainly result from the recombinational repair events required to reactivate replication forks[15,37] (Fig. 2). Studies by Kuempel and co-workers[15,37] on the frequency of chromosomal dimer resolution by the XerCD recombinase thus provides a useful lower limit for the frequency of replication fork reactivation. Under normal growth conditions, about 15% of bacterial chromosomes undergo recombination required for reactivation of a replication fork to generate a crossover leading to formation of a contiguous chromosomal dimer. If resolution of Holliday junction recombination creates crossovers 50% of the time, this measurement only detects half of the fork reactivation events. The real frequency of fork reactivation by these pathways might be different if the Holliday junction resolution is skewed to favour either crossovers or non-crossovers.

Further estimates come from studies of mutants affecting enzymes involved in fork reactivation. Elimination of RecBCD results in the appearance of unrepaired double-strand breaks, presumably arising from inactivation and cleavage of replication forks, in 15–20% of the cells under normal growth conditions[13,14]. Many other studies suggest that fork reactivation is yet more frequent. Up to 50% of cultured *recA* cells are dead and a substantial number of chromosomes have been lost[38]. Viability of cells lacking PriA function is even lower[9,22]. Both *recA* and *priA* null mutants become inviable when paired with mutations in a number of other recombination functions.

As reviewed elsewhere[10,22–24,35], the most prominent phenotypic effects of *rec* and *pri* mutants are closely linked to DNA damage under conditions in which cells are actively replicating DNA. The requirements for the *rec* and *pri* functions in reactivating replication forks provide an internally consistent explanation for the phenotypes of cells lacking one or more of these functions. At present, there are no alternative hypotheses that can explain the demonstrated importance of these genes to cell viability under normal growth conditions *in vivo*.

Replication fork reactivation encompasses redundant pathways that adapt to whatever DNA structure is presented to the cell at the site of an inactivated replication fork, with some of these outlined in Fig. 1. Because of this redundancy, the effects of some single mutations inactivating a component of these pathways are deceptively modest. However, bacterial cells are inviable if all avenues for replication fork reactivation are removed (by mutation of required genes), and this in turn implies that most replication forks must be reactivated at some point during their journey from *oriC* to the terminus. Certain proteins such as RecA and PriA seem to be required for the main reactivation pathways. However, the lack of complete inviability seen for individual mutations suggests that not all pathways require RecA, PriA and/or RuvC. For example, preliminary results suggest that a PriA-independent pathway for replication fork reactivation might involve PriC and the Rep helicase, because the *priA priC* (S. J. S., unpublished data) and *priA rep*[14] double mutants are both inviable. On the other hand, the *priC rep* double mutant is viable (S. J. S., unpublished data).

The evident prevalence of replication fork demise and reactivation may require a readjustment of the commonly cited rate of replication fork propagation of 1,000 nucleotides per second that has been based on the time required for completion of one round of chromosomal replication[39]. Replication fork reactivation clearly takes time. Estimates extrapolated from the reconstitution of small parts of these systems *in vitro*, as well as observations *in vivo*, range from 15–50 minutes[40–42]. Thus, it would seem that the fork has to be able to move faster than the previously calculated estimate.

Relationship to other systems

The concept of replication fork demise and reactivation under normal growth conditions owes much to studies in other areas. Work on bacterial recombination and unusual modes of replication has largely focused either on the SOS response or on bacterial conjugation, where important phenomena are amplified.

Some of the effects of replication fork demise can be more readily studied when all replication is synchronously halted, as occurs when artificially elevated levels of DNA damage lead to the induction of the SOS system[43]. However, the resulting data are complicated by the induction of new pathways for replication that appear to be unique to the SOS response. SOS includes a number of downstream processes, such as cell-cycle arrest induced by SulA and the specialized mutagenic repair brought about by DNA polymerases IV (DinB) and V ($UmuD'_2C$)-mediated replication fork bypass[44–46]. These can be considered extreme measures that evolved to maximize cell survival under conditions where many cells are destroyed. SulA, DinB and UmuD′C are functions present at significant levels only when SOS is induced. UmuD is present in cells under normal growth conditions (mostly in the unactivated form), but UmuC is not detectable[47]. The nonmutagenic pathways for replication fork reactivation almost certainly operate during SOS, particularly at early stages before the full induction of the mutagenic paths. However, the presence of SOS-specific avenues for replication can make it difficult for studies under SOS conditions to illuminate the nonmutagenic pathways featured under normal growth conditions.

Not all of the SOS-specific replication processes are inherently mutagenic. As demonstrated primarily by Kogoma and colleagues[8,10], a form of replication, called inducible stable DNA replication (iSDR), which requires neither ongoing protein synthesis nor a functional *oriC*, is also induced during the SOS response. iSDR requires recombination functions as well as the replication restart primosome. To categorize the manner in which replication was initiating during iSDR, Kogoma and colleagues[10] coined the term RDR, for recombination-dependent replication, defining it as "homologous recombination function-dependent replication triggered by a duplex DNA end." RDR can be observed in many contexts, including bacterial conjugation, the replication of bacteriophage T4, unusual modes of replication during SOS and other processes. iSDR itself is a specialized cellular function and initiates only at unique origins of replication, of which there are two major ones, *iriM1* and *oriM2*, and requires a hypothetical SOS-induced endonuclease to make the initiating double-strand break[8,10]. iSDR also does not process inactivated replication forks at the site of their demise; rather, it effects rescue by reinitiating synthesis of the entire chromosome. However, the study of iSDR and its requirements has contributed to evidence for fork reactivation pathways that are also utilized under normal growth conditions.

The work on conjugation has been critical in defining recombination pathways and discovering recombination functions. In particular, the RecBCD pathway for recombination, representing one of the principal avenues for reactivation of replication forks (Fig. 1), was mainly defined in studies of conjugation-associated recombination. In normal bacterial populations, however, conjugation events are typically separated by tens of thousands of cell generations.

Both conjugation and SOS are special situations that illuminate, but do not accurately reflect, the normal condition in bacterial cells. The nonmutagenic pathways for reactivating replication forks under normal growth deserve increased experimental attention. If replication fork reactivation is required in virtually every cell in every cell generation, then it is straightforward to conclude that the

processes outlined in Fig. 1 represent the main function of all of the enzymes listed here and in Table 1, with the exception of DNA polymerase III. Thus, the nonmutagenic re-establishment of inactivated replication forks is an essential housekeeping function. It includes all pathways of recombination (with or without double-strand breaks), replication restart and resolution of any chromosomal dimers that result through XerCD-mediated site-specific recombination.

It is important to note that reactivation of the replication fork does not necessarily require the repair of the lesion that caused its initial demise. Strand breaks will be repaired as a consequence of the recombination processes (Fig. 1), but lesions may simply be left behind in regions of now double-stranded DNA. They can subsequently be repaired by the excision repair or other repair systems, or they may cause additional problems during the next replication cycle. These repair systems may also be integrated into the fork reactivation pathways, but the extent of such integration (if any) remains to be determined.

Enzymatic conundrums in DNA metabolism resolved

The nonmutagenic reactivation of replication forks provide a *raison d'être* for a number of enzymatic systems that were previously considered enigmatic. Studies of the initiation of bacterial replication at *oriC* left no evident role for PriA and several associated proteins in what had been defined as the ϕX174-type primosome[22,48]. Nevertheless, cells deficient in PriA function are only marginally viable, and possess only one-fiftieth and one-eightieth of the wild-type capacity for recombination and the repair of ultraviolet-damaged DNA, respectively[30,49]. This fact can be readily explained by the role of PriA in replication fork reactivation[33]. Because the role of the ϕX174-type primosome in the cell has now been clarified, it has been proposed[35] that it be referred to as the "replication restart primosome" (Fig. 1).

Xer site-specific recombination functions to resolve chromosomal dimers, and the importance of this resolution is readily understood within the context of replication fork reactivation. Several studies suggest that the XerCD system is well integrated into DNA metabolism and the cell cycle[50,51].

Recently, the 'orphan' DNA polymerase II has also found an important function *in vivo*, with a pivotal role in pathways for nonmutagenic initiation of replication restart during SOS, and probably in normal cells as well[18]. Here it appears that the restart primosome is required to initiate Okazaki fragments in the pol II replication restart pathway (M. F. G., unpublished data) and that pol II is then replaced subsequently by a reassembled replication complex containing pol III (ref. 18).

The recombination functions of the RecF pathway, particularly RecF, O and R, are required for conjugational recombination only in the absence of both RecBCD (required for recombinational repair of double-strand breaks) and SbcBC functions[27] and hence have also seemed superfluous. Within the context of replication fork reactivation, however, there is ample evidence that the RecBCD and RecFOR pathways of recombination are of similar importance to the cell[15,23,52].

Analogues in other systems?

The problems faced by *E. coli* in completing replication of its genome are probably faced by all organisms. Do similar processes exist in other bacteria, viral systems and even eukaryotic cells?

The use of a single replication origin and terminus region in bacterial systems would seem to place a premium on reactivation pathways to deal with forks that don't make the traverse of the entire genome successfully. Thus, perhaps the safest prediction is that many, if not all, bacteria will have similar systems. Key components such as RecA, XerCD and PriA are highly conserved among bacterial species. Interestingly, however, the complexity of the multiple fork reactivation pathways may vary. Many bacteria lack obvious PriB and PriC homologues. We have already noted the importance of recombination-dependent replication for the life cycles of bacteriophage such as T4, as well as for replication fork reactivation.

Multiple origins, the myriad layers of control on initiation and the existence of multiple checkpoint systems make prognostication of the existence of fork reactivation systems in eukaryotes more problematic. Nevertheless, several groups have recently presented evidence that portions of eukaryotic chromosomes can be replicated by a recombination-dependent mechanism that may reflect nonmutagenic replication fork reactivation. In *Saccharomyces cerevisiae*, an internal chromosome break can be repaired by a process that very probably involves the establishment of a new replication fork through recombination with an intact homologue[53]. The new replication fork than traverses the length of the chromosome out to the telomere[54,55]. In addition, the successful replication of chromosomal ends in the absence of functional telomerase is dependent on recombination proteins[56,57]. A surprising and very elegant study[58] has recently shown that the normal structure of telomeres in higher eukaryotic cells involves a protein-mediated, folded-back D-loop, with the distal tip of the chromosome invading an internal repeat of the same sequence; this is precisely the structure that initiates replication in recombination-dependent replication pathways that depend on double-strand breaks. It has also been suggested that recombination-directed replication can account for nonreciprocal chromosome translocations[59,60]. Another study has provided a first look at the effects of encounters with a DNA lesion by an SV40 replication fork[61].

Questions

Research to date has only scratched the surface of the causes of replication fork demise and the pathways of subsequent reactivation. Although replication can clearly be halted by DNA damage, there is little information concerning the molecular events associated with the demise of a replication fork. What are the specific types of damage present as a function of growth conditions? Does either the type of DNA lesion or its location (that is, leading- or lagging-strand template) dictate the pathway of replication fork reactivation taken? What does an inactivated replication fork look like? Are all of the twenty or so proteins at the replication fork lost, requiring reassembly of an entirely new complex? Does the restart primosome stay together until chromosome replication is completed, or is it replaced by the DnaB–DnaG primosome used at *oriC*? What transpires at the recombination–replication interfaces? Reconstitution of even one of the pathways for fork reactivation promises to be a major enzymological challenge.

In eukaryotic cells, DNA damage can induce one of several cell-cycle checkpoints. Irradiated cells can, for example, either experience a lengthening of S phase or be delayed at the G2/M boundary until the damage is repaired. A recent proposal suggests that a similar checkpoint, mediated by the SOS-inducible *umuDC* gene products, exists in *E. coli*[62]. Fork reactivation under normal growth conditions in bacterial cells may require a checkpoint system as well (J. McCool and S. J. S., unpublished data).

Replication forks have a range limited by DNA damage. If any one of the events outlined in Fig. 1 fails to take place, the affected cell will either die or undergo an aberrant cell-division event. This potential catastrophe may have provided the selective pressure needed for the evolution of homologous recombination systems and other enzymatic components needed for fork reactivation, a critical step paving the way for the evolution of organisms with larger genomes. □

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