

Mismatch repair causes the dynamic release of an essential DNA polymerase from the replication fork

Andrew D. Klocko, Jeremy W. Schroeder, Brian W. Walsh, Justin S. Lenhart, Margery L. Evans and Lyle A. Simmons*

Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA.

Summary

Mismatch repair (MMR) corrects DNA polymerase errors occurring during genome replication. MMR is critical for genome maintenance, and its loss increases mutation rates several hundred fold. Recent work has shown that the interaction between the mismatch recognition protein MutS and the replication processivity clamp is important for MMR in *Bacillus subtilis*. To further understand how MMR is coupled to DNA replication, we examined the subcellular localization of MMR and DNA replication proteins fused to green fluorescent protein (GFP) in live cells, following an increase in DNA replication errors. We demonstrate that foci of the essential DNA polymerase DnaE–GFP decrease following mismatch incorporation and that loss of DnaE–GFP foci requires MutS. Furthermore, we show that MutS and MutL bind DnaE *in vitro*, suggesting that DnaE is coupled to repair. We also found that DnaE–GFP foci decrease *in vivo* following a DNA damage-independent arrest of DNA synthesis showing that loss of DnaE–GFP foci is caused by perturbations to DNA replication. We propose that MutS directly contacts the DNA replication machinery, causing a dynamic change in the organization of DnaE at the replication fork during MMR. Our results establish a striking and intimate connection between MMR and the replicating DNA polymerase complex *in vivo*.

Introduction

Prokaryotes and eukaryotes have evolved a series of conserved pathways dedicated to DNA repair ensuring genomic preservation (for review see Friedberg *et al.*, 2006). One pathway that shares exquisite conservation

across all domains of life is DNA mismatch repair (MMR) (for review see Eisen, 1998; Schofield and Hsieh, 2003; Kunkel and Erie, 2005; Modrich, 2006). MMR contributes to a variety of cellular pathways, including anti-recombination in *Escherichia coli* (Rayssiguier *et al.*, 1989) and DNA damage checkpoint activation in eukaryotes (Hickman and Samson, 1999; 2004; Yoshioka *et al.*, 2006). The most well-studied role for MMR is in the correction of DNA replication errors, manifested as mismatches, insertions and deletion loops (for review see Schofield and Hsieh, 2003; Kunkel and Erie, 2005; Li, 2008). In both bacteria and eukaryotes, deletion of the highly conserved MMR genes *mutS* and *mutL* results in a several hundred fold increase in mutation frequency (Cox *et al.*, 1972; Fishel *et al.*, 1993; Hamilton *et al.*, 1995; Ginetti *et al.*, 1996). In humans, germline MMR defects predispose people to several different cancers, including hereditary non-polyposis colorectal cancer (i.e. Lynch syndrome), Turcot syndrome and several other sporadic cancers (Hamilton *et al.*, 1995; Nystrom-Lahti *et al.*, 2002; Peltomaki, 2005). Furthermore, in pathogenic bacteria loss of MMR contributes to an increase in the occurrence of multidrug resistant strains found in hospital settings (e.g. Denamur *et al.*, 2002; Chopra *et al.*, 2003). These studies underscore the critical importance of MMR to several aspects of human health.

The Gram-negative bacterium *E. coli* has been the most well understood bacterial MMR system to date. In *E. coli*, the sensor protein MutS recognizes a mismatch and initiates MMR by recruiting the linker protein MutL (Schofield *et al.*, 2001). MutL co-ordinates the action of the nicking endonuclease MutH (Hall and Matson, 1999; Ahrends *et al.*, 2006), which senses the methylation state of the DNA at d(GATC) sequences and subsequently nicks the unmethylated strand representing the nascent DNA. MutL and MutH together co-ordinate the loading of the DNA helicase, UvrD at the incised nick (Hall *et al.*, 1998; Mechanic *et al.*, 2000). UvrD, fuelled by ATP hydrolysis, separates the mismatch-bearing strand for degradation by one of several exonucleases (Viswanathan *et al.*, 2001). Once the mismatch containing strand is degraded, DNA pol III holoenzyme is recruited through an unknown mechanism to resynthesize the gapped region (Lahue *et al.*, 1989). Although many of the steps of *E. coli* MMR are conserved, DNA methylation and endonuclease cleavage

Accepted 6 September, 2011. *For correspondence. E-mail: lasimm@umich.edu; Tel. (+1) 734 647 2016; Fax (+1) 734 647 0884.

by MutH are mostly restricted to *E. coli* and its very close relatives (Eisen, 1998; Eisen and Hanawalt, 1999; Culligan *et al.*, 2000). It is well accepted that most bacteria and all eukaryotes lack the methylation-directed repair pathway characteristic of *E. coli*.

It has been shown in *E. coli* that subunits of DNA polymerase III holoenzyme (pol III) interact with MutS and MutL (Lopez de Saro and O'Donnell, 2001; Lopez de Saro *et al.*, 2006; Li *et al.*, 2008; Pluciennik *et al.*, 2009). MutS and MutL bind to the DNA replication processivity factor, β clamp and several components of the clamp loader complex (i.e. γ complex) (Lopez de Saro and O'Donnell, 2001; Lopez de Saro *et al.*, 2006; Li *et al.*, 2008; Pluciennik *et al.*, 2009). MutL binding to β clamp is dependent on single-stranded DNA (Lopez de Saro *et al.*, 2006) and mutation of the β clamp binding site on MutL increases the spontaneous mutation frequency, supporting a functional role for interaction between these two proteins during MMR *in vivo* (Lopez de Saro *et al.*, 2006). Other work shows that *E. coli* MutL binds to clamp loader proteins γ , δ and δ' (Li *et al.*, 2008). In addition, MutS and MutL have been shown to bind to pol III core and the γ complex (Pluciennik *et al.*, 2009). Within pol III core, MutS and MutL interact with the catalytic subunit α (DnaE) (Pluciennik *et al.*, 2009). Thus, in *E. coli*, MutS and MutL bind to multiple subunits of DNA polymerase III, suggesting that these pathways might be physically linked *in vivo*. Although each of these binding events appears relatively strong, it is not clear how interaction between MMR proteins and components of DNA polymerase III contribute to the mismatch correction pathway *in vitro* or *in vivo*.

Cell biological experiments in the Gram-positive bacterium *Bacillus subtilis* have shown that foci of MutS fused to yellow fluorescent protein (YFP) colocalize or overlap with the replisome protein DnaX fused to cyan fluorescent protein (CFP) in ~48% of cells in response to the mismatch-inducing base-analogue 2-aminopurine (2-AP) (Smith *et al.*, 2001). The authors concluded that mismatches are detected at the site of DNA synthesis and that MutS may move away from the replisome following repair complex assembly and continued DNA synthesis (Smith *et al.*, 2001). These results support the hypothesis that mismatch detection is coupled to the DNA replication machinery in *B. subtilis* and that the site of DNA synthesis is the preferred location for repair (Smith *et al.*, 2001). Experiments using MutS and MutL fused to green fluorescent protein (GFP) have shown that MutS–GFP and MutL–GFP requires ongoing DNA replication for organization into foci *in vivo* (Smith *et al.*, 2001). Moreover, it was shown that MutS interacts with β clamp in *B. subtilis*, and that this interaction is critical for MutS–GFP focus formation and efficient MMR *in vivo* (Simmons *et al.*, 2008a; Dupes *et al.*, 2010). These observations further support the idea that mismatch recognition and/or repair

are targeted to the site of DNA replication in *B. subtilis*. It is not clear however, if the replication machinery is altered or influenced during the repair process or if other DNA replication proteins in addition to β clamp are involved in this process.

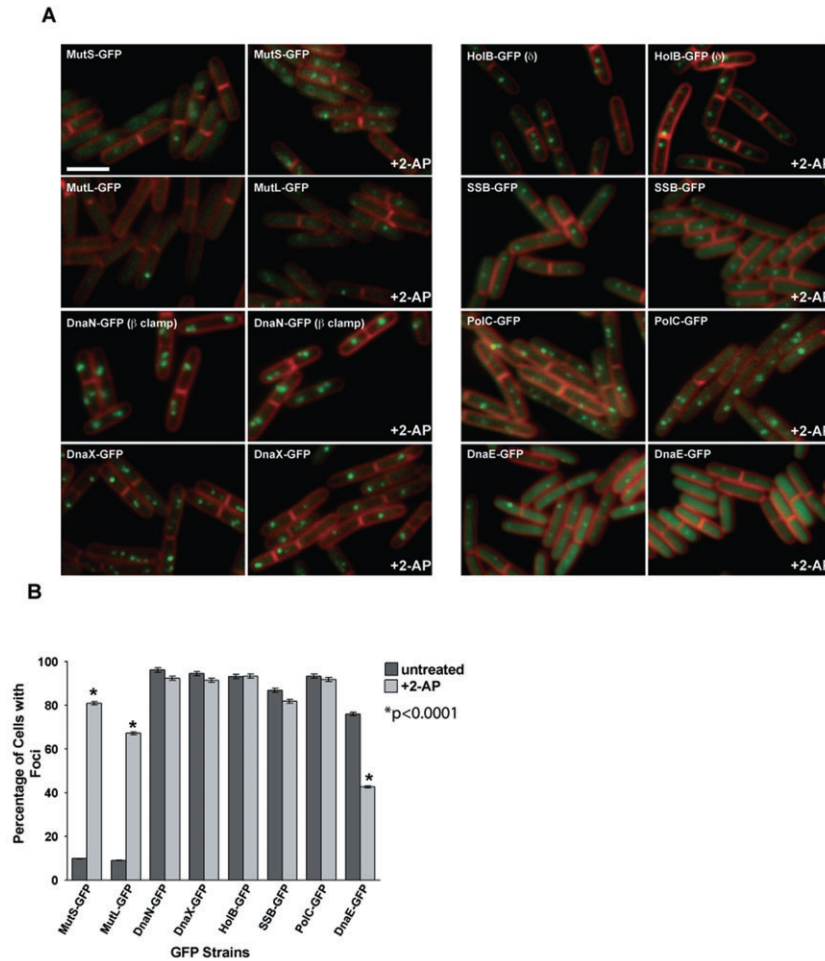
Here, we examined the subcellular localization of fluorescent fusions to six DNA replication proteins during MMR *in vivo*. We found that foci of the essential DNA polymerase DnaE–GFP decreased following the introduction of mismatches with 2-AP or with a proofreading deficient replicative DNA polymerase (*polC mut-1*) allele. We show that the loss of DnaE–GFP foci depends on the presence of MMR proteins, specifically MutS. Further investigation showed that a DNA damage-independent block to DNA synthesis caused DnaE–GFP foci to decrease in *B. subtilis* and that this effect was independent of the MMR pathway. These results suggest that loss of DnaE–GFP foci is an indicator of perturbations to DNA replication in *B. subtilis*. In addition, protein-blotting experiments show that MutS and MutL bind DnaE, which supports the hypothesis that the MMR proteins are physically coupled to the DNA replication machinery. It is hypothesized that in DNA methylation-independent MMR systems including human and *S. cerevisiae*, the MMR proteins utilize strand discontinuities to direct the repair process *in vitro* (Genschel *et al.*, 2002; Genschel and Modrich, 2003; 2006; Dzantiev *et al.*, 2004; Kadyrov *et al.*, 2006; Kadyrov *et al.*, 2007). It is tempting, therefore, to consider that a physical connection between MMR and the DNA replication machinery would provide a mechanism for the repair machinery to target strand termini, thereby aiding in the recognition of the mismatch-containing strand in organisms that lack a methylation directed signal, including *B. subtilis*.

Results

DnaE–GFP foci decrease following mismatch formation

In *B. subtilis*, the DNA replication machinery forms foci at distinct subcellular positions characterized as midcell or future midcell positions (Lemon and Grossman, 1998; Migocki *et al.*, 2004; Berkmen and Grossman, 2006). Previous analysis of the subcellular position of the replication protein DnaX–GFP (τ) showed no change in focus position or the percentage of cells with foci following challenge with the mismatch-inducing agent 2-AP (Smith *et al.*, 2001). It is not clear if the subcellular position of DnaX–GFP is representative of the other replication proteins during MMR in *B. subtilis*.

To address whether addition of 2-AP alters the subcellular localization of other components of the DNA replication machinery, we individually analysed the percentage of cells with foci bearing fusions to the replication sliding



clamp, β clamp (DnaN-GFP), the polymerase dimerization and clamp loading component [DnaX-GFP (τ)], a component of the clamp loader complex [HolB-GFP (δ)], DNA single strand binding protein (SSB-GFP, also known as SsbA-GFP in *B. subtilis*), the leading and lagging strand DNA polymerase (PolC-GFP), and the essential lagging strand primer maturation DNA polymerase (DnaE-GFP), in addition to MutS-GFP and MutL-GFP (Fig. 1). Cells expressing each GFP fusion protein were scored for focus formation untreated or with 2-AP added to the growth medium. For β clamp, DnaX, δ , PolC, MutS and MutL, each fusion allele was integrated at its normal locus in the *B. subtilis* chromosome, placing expression of each fusion protein under control of its native promoters (Lemon and Grossman, 1998; Smith *et al.*, 2001; Berkmen and Grossman, 2006; Simmons *et al.*, 2008a) (see Table S1 for a list of strains). The *ssb-gfp* and *dnaE-gfp* fusion alleles were in merodiploid strains, where each was expressed from an ectopic locus with the native allele intact as described (Dervyn *et al.*, 2001; Berkmen and Grossman, 2006). The *ssb-gfp* allele was under control of its native promoters at *lacA* and expression of the *dnaE*-

gfp allele was controlled by a xylose inducible promoter from the *amyE* locus (Dervyn *et al.*, 2001; Berkmen and Grossman, 2006). We also integrated *dnaE-gfp* or *dnaE* bearing a monomeric *gfp* variant (*dnaE-mgfp*) under control of its native promoter as the only copy of *dnaE* in the cell. The cells were viable and DnaE-GFP foci were observed with both fusion proteins; however, the foci that formed were weak and difficult to characterize (data not shown). For this reason, we chose to study the xylose inducible *dnaE-gfp* allele with the native *dnaE* gene intact as described previously (Dervyn *et al.*, 2001; Costes *et al.*, 2010).

The MMR proteins MutS-GFP and MutL-GFP formed foci at primarily midcell positions and focus formation was induced by the presence of 2-AP, confirming previous observations (Fig. 1 and Table 1) (Smith *et al.*, 2001; Simmons *et al.*, 2008a; Dupes *et al.*, 2010). Scoring of β clamp-GFP, DnaX-GFP, PolC-GFP, HolB-GFP and SSB-GFP foci showed virtually no change when we compared the percentage of untreated cells with foci to the percentage of cells with foci following challenge with 2-AP (Fig. 1 and Table 1). We did, however, notice a striking

Fig. 1. DnaE-GFP foci decrease following mismatch insertion.

A. Representative images of MMR proteins MutS-GFP and MutL-GFP and replication proteins DnaN-GFP (β clamp), DnaX-GFP, HolB-GFP (δ), SSB-GFP, PolC-GFP and DnaE-GFP are shown untreated and following 2-AP challenge. In each case, the panel on the left is untreated and the panel on the right shows cells challenged with 2-AP. The membrane is stained with the vital membrane stain FM4-64. The bar indicates 3 μ m.

B. Scoring of the percentage of cells with foci untreated (dark grey bars) and following 2-AP treatment (light grey bars). The error bars reflect the 95% confidence interval. The asterisk indicates the results are significant with $P < 0.0001$. The bar graph represents a summary of the complete data set presented in Table 1.

Table 1. Percentage of cells with MMR and replisome foci following 2-AP challenge.

Fusion	2AP	No. of cells	Percentage of cells with <i>n</i> foci					Average no. of foci per cell
			0	1	2	3	>4	
MutS–GFP	–	1447	90.1	6.8	3.1	0	0	0.13
	+	1589	19.0	18.8	33.1	19.2	9.8	1.82
MutL–GFP	–	1592	90.9	9.1	0	0	0	0.09
	+	2065	32.7	45.6	22.4	2.7	0.5	0.97
DnaN–GFP (β clamp)	–	858	3.8	28.1	39.3	15.6	13.2	2.06
	+	1072	7.6	23.8	45.0	16.4	7.2	1.92
DnaX–GFP (τ)	–	1889	5.4	28.3	45.5	9.2	11.6	1.93
	+	2045	8.4	41.1	39.8	5.3	5.3	1.58
HoIB–GFP (δ)	–	767	6.8	19.8	39.2	18.0	16.2	2.17
	+	847	6.6	22.0	34.0	18.2	19.1	2.21
SSB–GFP	–	1057	13.1	34.0	42.8	5.8	4.4	1.55
	+	1569	18.1	35.4	37.7	6.2	2.5	1.40
PolC–GFP	–	1283	6.6	37.2	37.1	9.4	9.7	1.78
	+	1524	8.1	33.3	41.3	9.1	8.3	1.76
DnaE–GFP	–	2912	23.9	51.9	19.3	4.6	0.4	1.06
	+	4131	57.4	38.2	4.2	0.2	0	0.47

Strains were grown in S7₅₀ minimal medium with 0.2% D-glucose except for strain AK74 harbouring the *amyE::P_{xyI} dnaE–gfp* allele. AK74 was grown in 1% L-arabinose to allow for expression with the addition of 0.125% D-xylose. The numbers reported are from at least three independent experiments.

loss in the percentage of cells with DnaE–GFP foci in the presence of 2-AP when compared with the untreated control (Fig. 1 and Table 1). DnaE–GFP in the absence of 2-AP formed foci in ~76% of cells ($n = 2912$). Following 2-AP challenge, DnaE–GFP foci were only observed in ~43% of cells ($n = 4131$) (Fig. 1 and Table 2, $P < 0.0001$). Qualitatively, in cells that maintained foci, the fluorescence of DnaE–GFP foci in 2-AP treated cells were decreased and, in many cases, barely detectable (Fig. 1). Because 2-AP is a base analogue and it does not form a true mismatch, we assayed for DnaE–GFP foci in a strain that contained the *mut-1* allele of *polC* (referred to here as *polCexo*[–]), which is defective in proofreading (Sanjanwala and Ganesan, 1991). This allele confers a substantial increase in mutation frequency due to an increase in DNA replication errors (Sanjanwala and Ganesan, 1991). Analysis of DnaE–GFP foci in the *polCexo*[–] showed a

significant decrease in focus formation as compared with an isogenic *polC*⁺ strain (Fig. S1). The loss of DnaE–GFP foci in the *polCexo*[–] strain was similar to what we observed following 2-AP addition, with $P < 0.0001$ (compare Fig. 1 and Fig. S1). Thus, we observe both a quantitative and qualitative reduction in DnaE–GFP foci following the addition of 2-AP to the growth medium or in cells lacking a proofreading proficient *polC* allele. Therefore, we conclude that mismatches cause a decrease in the percentage of cells with DnaE–GFP foci.

A decrease in DnaE–GFP foci could be directly caused by mismatches or could be a general response to DNA damage or incorporation of DNA replication errors. To address this concern, we challenged cells with mitomycin C (MMC) and monitored DnaE–GFP foci (Fig. 2). MMC forms a mono adduct at the N² position of guanine as well as inter- and intra-strand cross-links (for review see

Table 2. Percentage of cells with DnaE–GFP foci.

Strain (% xylose)	2-AP	No. of cells	Percentage of cells with <i>n</i> foci					Average no. of foci per cell
			0	1	2	3	>4	
DnaE–GFP (0.125%)	–	1082	15.9	52.9	23.2	7.0	1.0	1.24
	+	1003	47.0	41.3	9.7	1.6	0.3	0.67
DnaE–GFP (0.025%)	–	962	36.4	43.7	17.2	2.6	0.2	0.87
	+	1414	56.3	37.0	6.4	0.4	0	0.51
DnaE–GFP (0.005%)	–	1040	54.6	34.4	9.3	1.5	0	0.58
	+	1671	80.3	18.4	1.3	0	0	0.21

Strains were grown in defined S7₅₀ minimal medium with 1% arabinose with the amount of xylose indicated in parentheses. Cultures were split, followed by the addition of 2-AP (600 $\mu\text{g ml}^{-1}$) to one of the cultures for 1 h prior to imaging (*Experimental procedures*).

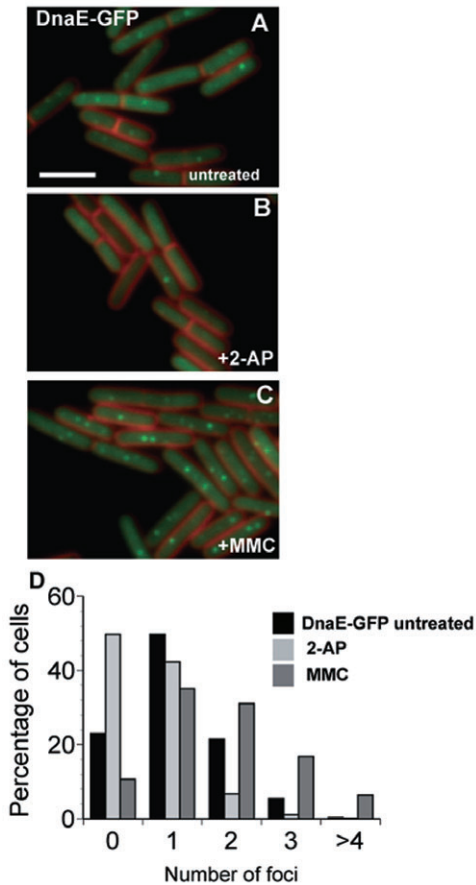


Fig. 2. The DNA damaging agent mitomycin C increases the percentage of cells with DnaE-GFP foci. A–C. Shown are cells (A) untreated, (B) challenged with 2-AP ($600 \mu\text{g ml}^{-1}$) and (C) challenged with mitomycin C (MMC) (200 ng ml^{-1}). The white bar indicates $3 \mu\text{m}$. D. The percentage of cells with 0, 1, 2, 3 and >4 DnaE-GFP foci per cell following the indicated growth condition are presented (the following number of cells were scored: untreated $n = 1277$; +2-AP $n = 980$; +MMC $n = 881$).

Dronkert and Kanaar, 2001). We found that the percentage of cells with DnaE-GFP foci did not decrease following MMC challenge (Fig. 2). To the contrary, we measured an increase in the percentage of cells with DnaE-GFP foci following MMC challenge (Fig. 2, compare B and C). Thus, we found that loss of DnaE-GFP foci is not a widespread signal for DNA perturbations in *B. subtilis*.

As discussed above, the *dnaE-gfp* fusion allele was ectopically expressed from a xylose inducible promoter (P_{xyI}) (Dervyn *et al.*, 2001; Costes *et al.*, 2010). We examined the level of DnaE-GFP expression relative to native DnaE and found that with 0.125% xylose the level of DnaE-GFP was comparable to the level of native DnaE *in vivo* (Fig. S2). To determine if the level of DnaE-GFP protein *in vivo* contributed to the decrease in localization following 2-AP challenge, we scored the percentage of cells with DnaE-GFP foci grown in medium containing

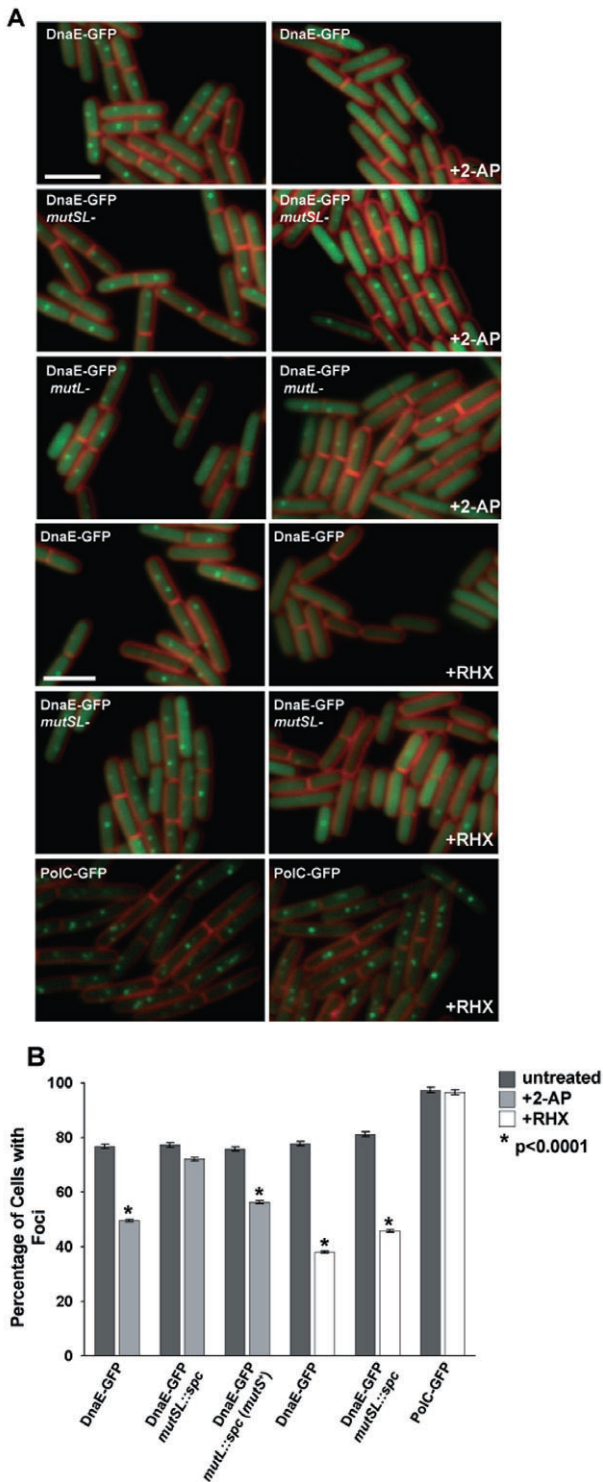
three different amounts of the inducer (xylose, at 0.125%, 0.025% and 0.005%) in the presence or absence of 2-AP. We found that the percentage of untreated cells with DnaE-GFP foci did decrease as the amount of xylose in the medium was reduced (Fig. S3, Table 2). However, when cells at each xylose amount were examined following challenge with 2-AP, we observed a significant reduction in the percentage of cells with DnaE-GFP foci (Fig. S3; $P < 0.0001$ for each xylose concentration). We performed an immunoblot to detect DnaE-GFP levels *in vivo* with antibodies against the GFP moiety. We found that the levels of DnaE-GFP foci were indeed reduced as the amount of xylose was reduced (Fig. S3C). We did not detect release of GFP from DnaE by proteolysis *in vivo*, as judged by immunoblotting (data not shown). Furthermore, at each percentage of xylose tested, the level of DnaE-GFP protein was unaffected by the addition of 2-AP. As a control, we analysed β clamp (DnaN) levels *in vivo* and found that the level of β clamp was unchanged by the amount of xylose, or by the addition of 2-AP (Fig. S3C). Thus, although the percentage of cells with DnaE-GFP foci is influenced by the amount of xylose added, 2-AP addition causes DnaE-GFP foci to decrease at each level of xylose we examined.

Complementary to these studies, we found that a strain expressing *dnaE-mgfp* from its native locus was also decreased for focus formation when 2-AP was added to the growth medium. In this experiment $\sim 45\%$ ($n = 201$) of untreated cells showed DnaE-mGFP foci, while only $\sim 15\%$ ($n = 325$) of cells showed DnaE-mGFP foci following 2-AP challenge, with $P < 0.0001$ (data not shown). Taken together, under every experimental condition we examined, including the use of different fusions to DnaE and different levels of DnaE-GFP in the cell, 2-AP challenge caused a significant loss of DnaE-GFP foci *in vivo*.

Loss of DnaE-GFP foci requires the mismatch sensor protein MutS

Our results indicate that 2-AP challenge causes DnaE-GFP foci to decrease *in vivo*. To understand if loss of DnaE-GFP foci is dependent on the MMR pathway, we scored DnaE-GFP foci following 2-AP treatment in a strain disrupted for the *mutS* and *mutL* genes (Fig. 3 and Table 3). We found that in cells disrupted for both *mutS* and *mutL* (*mutSL::spc*), DnaE-GFP foci were maintained following 2-AP addition (Fig. 3 and Table 3) and the foci in these cells qualitatively resembled untreated cells (Fig. 3). This experiment supports the hypothesis that a functional MMR pathway is required to observe a decrease of DnaE-GFP foci *in vivo* (Fig. 3 and Table 3).

To distinguish the component of the MMR pathway responsible for a decrease in DnaE-GFP foci, we scored DnaE-GFP in a strain lacking the *mutL* gene and express-



ing *mutS* in the presence and absence of 2-AP. Addition of 2-AP to this strain caused a decrease in DnaE–GFP foci, compared with untreated cells, with $P < 0.0001$ (Fig. 3 and Table 3). This result suggests that MutS is necessary for loss of DnaE–GFP foci following challenge with 2-AP.

Fig. 3. Loss of DnaE–GFP foci in response to DNA synthesis perturbations caused by mismatches requires MutS and MutL.

A. Representative epi-fluorescence micrographs of DnaE–GFP in genetic backgrounds disrupted for *mutSL::spc* and *mutL::spc*, and either untreated (left column) or challenged with 2-AP or arginine hydroxamate (RHX) (right column). The membrane is stained with FM4-64 and the bar indicates 3 μ m.

B. A bar graph quantifying the percentage of cells with DnaE–GFP foci under the conditions indicated in the panel is shown. The bar graph represents a summary of the complete data set shown in Table 3. The asterisk indicates $P < 0.0001$ between treated and untreated samples. Error bars indicate the 95% confidence interval.

In an effort to further demonstrate that loss of DnaE–GFP foci in response to 2-AP treatment was *mutS* dependent, we tested the effect of 2-AP on DnaE–GFP foci in a strain disrupted for the *mutSL* operon, with *mutL* expression restored from an ectopic locus located at *lacA* (Fig. 4). In this strain, *mutS* is not expressed (Fig. 4A), and *mutL* expression is driven by IPTG at a concentration of 200 μ M restoring MutL protein to wild-type levels (Fig. 4A). Also in this strain, DnaE–GFP expression is driven by xylose at a concentration of 0.125% as described above. Upon addition of 2-AP to cultures lacking *mutS* and expressing MutL and DnaE–GFP, we found virtually no change in DnaE–GFP foci in cells untreated or challenged with 2-AP (Fig. 4B and C). With these data, we conclude that loss of DnaE–GFP foci upon treatment with 2-AP is dependent on MutS (see *Discussion*).

DNA replication arrest causes decrease of DnaE–GFP foci

To investigate if loss of DnaE–GFP foci is a consequence of inhibition of DNA replication, we used an established DNA damage-independent method to arrest DNA synthesis in *B. subtilis* (Wang *et al.*, 2007). Challenge of *B. subtilis* cells with the amino acid analogue arginine hydroxamate (RHX) causes amino acid starvation and induces the stringent response (Wang *et al.*, 2007). The stringent response produces the small signalling molecules ppGpp and pppGpp [collectively referred to as (p)ppGpp] (for review see Paul *et al.*, 2004; Srivatsan and Wang, 2008) both of which bind and inhibit primase (DnaG), causing a rapid arrest in DNA synthesis. Using this method, nucleotide incorporation becomes undetectable within minutes of RHX addition (Wang *et al.*, 2007 and data not shown).

To determine if replication fork arrest results in a decrease in DnaE–GFP foci, we induced stringent response arrest of DNA replication following the addition of RHX. Indeed, we found that DnaE–GFP foci decreased in cells challenged with RHX (Fig. 3A and Table 3), and that the stringent response-induced decrease of DnaE–GFP foci was more severe than the response we

Table 3. Percentage of cells with DnaE–GFP foci in strains containing or lacking mismatch repair genes, grown in the presence or absence of 2-aminopurine (2-AP) or arginine hydroxamate (RHX).

Relevant characteristics	Additive	No. of cells	Percentage of cells with <i>n</i> foci					Average no. of foci per cell
			0	1	2	3	>4	
DnaE–GFP	–	1460	23.2	48.8	21.8	5.5	0.6	1.12
	2-AP	1710	50.4	37.0	11.2	1.1	0.1	0.64
DnaE–GFP, <i>mutSL::spc</i>	–	1889	22.8	52.1	20.0	4.7	0.4	1.08
	2-AP	1356	28.0	52.5	16.5	2.8	0.2	0.95
DnaE–GFP, <i>mutL::spc</i> (<i>mutS</i> ⁺)	–	1082	24.3	48.9	20.0	6.0	0.7	1.10
	2-AP	2239	43.6	46.9	7.5	1.7	0.3	0.68
DnaE–GFP	–	1187	22.3	52.6	20.0	4.6	0.6	1.09
	RHX	1273	62.0	31.4	5.9	0.7	0	0.45
DnaE–GFP, <i>mutSL::spc</i>	–	928	18.9	56.6	21.6	2.5	0.6	1.09
	RHX	872	54.2	36.1	8.3	1.3	0.1	0.57
PolC–GFP	–	1051	2.7	20.2	45.2	13.6	18.4	2.25
	RHX	1141	3.5	33.2	42.0	10.3	11.0	1.92

Cells were grown in defined S7₅₀ minimal medium with 1% arabinose and 0.125% xylose where DnaE–GFP was imaged and in 0.2% glucose when PolC–GFP was imaged. 2-AP was used at a final concentration of 600 µg ml⁻¹ and RHX was used at a final concentration of 0.5 mg ml⁻¹. Cells were imaged following treatment for 1 h with RHX or 2-AP.

observed with 2-AP (compare +2-AP and +RHX panels in Fig. 3A and the bar graph in Fig. 3B).

A strain of *B. subtilis* lacking the MMR proteins MutS and MutL (*mutSL::spc*) still shows a decrease in DnaE–GFP foci in the presence of RHX, identical to the decrease of DnaE–GFP foci observed in a strain containing MutS and MutL (Fig. 3). Therefore, the RHX effect on DnaE–GFP foci is independent of the MMR pathway, and is indicative of a general perturbation of the DNA replication fork that results in loss of DnaE–GFP foci *in vivo*.

The specificity of DnaE–GFP focus loss was evident when we compared the foci of a strain harbouring PolC–GFP in the presence and absence of RHX. We scored PolC–GFP foci and found that the percentage of cells with PolC–GFP foci was nearly identical in cells that were growing normally and in cells that experienced a stringent response arrest of DNA replication (Fig. 3). Therefore, only DnaE–GFP shows a dramatic loss of foci, indicating that DnaE undergoes a strong change following perturbations to DNA synthesis. This DNA replication disruption appears to cause a more striking effect than the perturbation exerted by MMR. However, it is clear that a disruption to DNA replication, such as that caused by mismatches, results in a MMR-dependent decrease to DnaE–GFP foci *in vivo*.

Protein levels of DnaE, MutL, MutS and β clamp (DnaN) are unchanged following 2-AP challenge

We have shown that the percentage of cells with DnaE–GFP foci decreases in response to replication errors (Figs 1–4). One explanation for the loss of DnaE–GFP foci is that the MMR proteins, or DnaE, were degraded.

Therefore, we performed immunoblots to determine if the levels of DnaE–GFP were altered following 2-AP challenge in both the presence and absence of the MMR gene products. Using monoclonal antibodies against GFP, we found that DnaE–GFP levels in cells growing normally or following 2-AP challenge were unchanged (Fig. 5A). Furthermore, DnaE–GFP levels were unaffected by the presence or absence of the MMR proteins MutS and MutL (Fig. 5A). Additionally, we also did not detect proteolytic release of GFP following 2-AP challenge (Fig. 5B), nor did we detect an increase in the DnaE protein pool, which could result from degradation of the GFP moiety (Fig. S2). Using antibodies generated against MutS, MutL and β clamp (DnaN), we found that the levels of each of these proteins were unchanged in cells challenged with 2-AP. As controls, we show that MutS and MutL were not detected in whole-cell extracts prepared from cells disrupted for their respective genes (Fig. 5A). With these data, we conclude that the levels of MMR proteins, DnaE, and β clamp were unchanged following 2-AP challenge, and that the loss of DnaE–GFP foci does not result from degradation of DnaE, proteolytic release of the GFP moiety from DnaE or degradation of GFP from DnaE.

Replication fork integrity is maintained during mismatch-induced perturbations to DNA replication

One explanation for the loss of DnaE–GFP foci upon 2-AP addition to growth medium is that the introduction of mismatches could destabilize the replication fork, causing fork collapse and subsequent changes to the subcellular localization of DnaE. To investigate the integrity of the replication fork during MMR, we analysed the recruitment

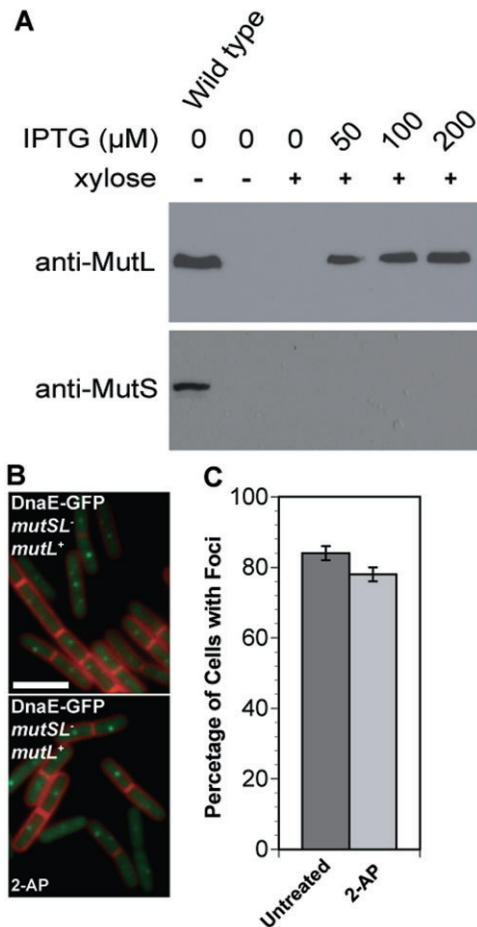


Fig. 4. MutS and 2-AP are necessary and sufficient to cause loss of DnaE–GFP foci.

A. Shown are immunoblots of whole-cell extracts for strain JWS68 (relevant genotype: *amyE::P_{xy}dnaE-gfp; mutSL::kan; lacA::P_{spac}mutL*) bearing a *mutSL* deletion with *mutL* expression restored to wild-type levels from the *lacA* locus. The first lane is an extract from PY79, while the remaining lanes are extracts probed for MutL from strain JWS68 with the indicated amount of IPTG. For xylose, the (+) symbol designates 0.125% xylose was added.

B. Representative images of DnaE–GFP are shown in the presence or absence of 2-AP with *mutL* expression restored from the *lacA* locus. The white bar indicates 3 μm.

C. The bar graph quantifies the percentage of cells with DnaE–GFP foci untreated or following challenge with 2-AP. The number of cells scored for the untreated sample were as follows: untreated ($n = 1154$) and 2-AP ($n = 1251$). The error bars indicate the 95% confidence interval.

of RecA–GFP to the replication fork by fluorescence microscopy (Simmons *et al.*, 2007; 2009). In *B. subtilis*, RecA–GFP has been shown to form foci following replication fork stress (Simmons *et al.*, 2007; Bernard *et al.*, 2010). In addition, Wang and co-workers showed that stringent response arrest of DNA synthesis does not stimulate recruitment of RecA–GFP into foci *in vivo* even though DNA synthesis rapidly arrests (Wang *et al.*, 2007). We examined the response of cells to 2-AP, and stringent

arrest with RHX, and found no difference in the percentage of cells with RecA–GFP foci when compared with untreated cells (Fig. 6). By contrast, the addition of the DNA-damaging agent MMC had a significant effect on the recruitment of RecA–GFP into foci; the percentage of cells with RecA–GFP foci was significantly higher when challenged with MMC, compared with unchallenged cells (Fig. 6, $P < 0.0001$). The percentage of cells with RecA–GFP foci compared between the untreated and 2-AP or RHX challenged groups was not significant (Fig. 6). Therefore, perturbations in DNA replication caused by mismatches or amino acid starvation fail to recruit RecA–GFP, indicating a distinct correlation between conditions causing a decrease of DnaE–GFP but do not promote recruitment of RecA–GFP into foci. We suggest that loss of DnaE–GFP foci does not cause excess ssDNA to accumulate, which would have been indicative of replication fork stress exerted by MMR.

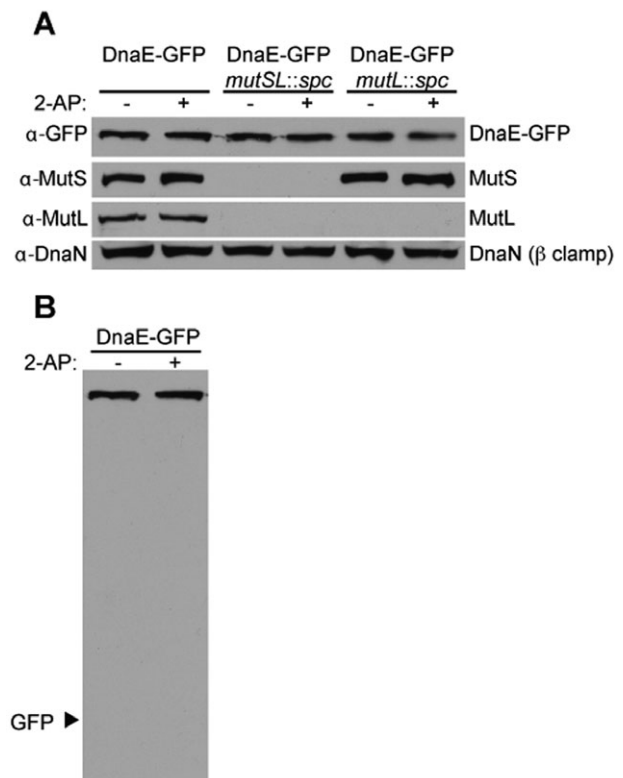


Fig. 5. Cellular levels of replication and MMR proteins are unchanged following 2-AP challenge.

A. Shown is an immunoblot of DnaE–GFP, MutS, MutL and DnaN (β clamp). The relevant genetic markers for the strains prepared for immunoblot analysis are indicated and cells treated with 2-AP are also indicated. Cultures with 2-AP were challenged for 1 h with 600 μg ml⁻¹ in LB medium. Each blot is representative of multiple independent experiments.

B. Shown is the full immunoblot of DnaE from the first two lanes of (A) with the expected position of proteolytically released GFP indicated.

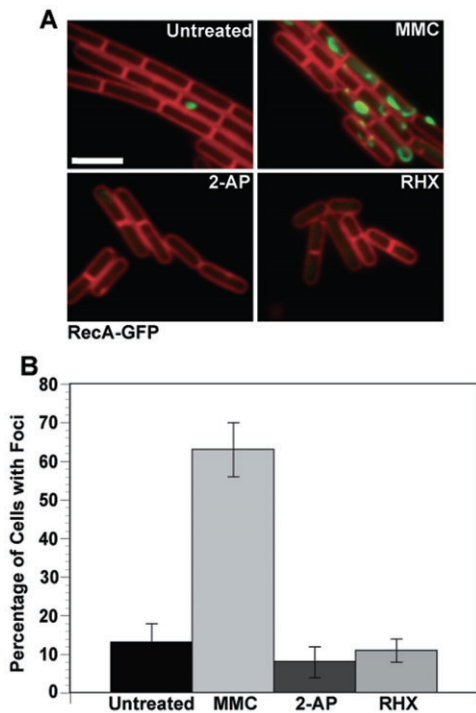


Fig. 6. Replication fork integrity is maintained during MMR. A. Shown are representative epi-fluorescence micrographs of RecA-GFP foci in cells untreated; or challenged with MMC (100 ng ml^{-1}); 2-AP ($600 \mu\text{g ml}^{-1}$); or arginine hydroxamate (RHX). RecA-GFP foci are shown in green and the cell membrane is shown in red and was visualized using the vital membrane stain FM4-64. The white bar indicates $3 \mu\text{m}$. B. A bar graph showing the percentage of cells with RecA-GFP foci following the indicated conditions. For each condition, the following numbers of cell were scored: untreated ($n = 201$), MMC ($n = 168$), 2-AP ($n = 210$) and RHX ($n = 349$). These data were analysed using a two-tailed z-test for significance between the untreated and treated groups. While the difference between untreated, +2-AP and +RHX is insignificant, the +MMC bar has a significant difference from the other three ($P < 0.0001$). Error bars reflect the 95% confidence interval.

MutS and MutL directly bind DnaE in vitro

We hypothesized that the mechanism resulting in loss of DnaE-GFP foci could be caused by a direct interaction between MMR proteins and the DNA replication machinery. Given that DnaE-GFP foci decreased in response to mismatches, we asked if MutS and MutL bound DnaE *in vitro*. To this end, we cloned, overexpressed and purified MutS, MutL, β clamp and DnaE using an N-terminal poly-histidine tag that was cleaved following purification. We also purified His₆-SSB for comparison (see *Experimental procedures*). All five proteins were purified to near homogeneity and were judged as $> 95\%$ pure by SDS-PAGE (data not shown). Using these purified proteins, we affinity purified rabbit polyclonal antibodies that had been generated against MutS, MutL and DnaE (see *Experimental procedures*). To test the specificity of the affinity-purified antibodies, we spotted 40 pmol of each DNA replication

protein, as well as BSA as a negative control, on a nitrocellulose membrane, followed by probing the membrane with one of three affinity-purified polyclonal antibodies against DnaE, MutL or MutS. Anti-DnaE and anti-MutS antibodies were highly specific and detected only DnaE and MutS proteins respectively (Fig. 7A). For anti-MutL, we observed very slight cross-reactivity with high levels of purified MutS, but we did not observe any cross-reactivity with the remaining proteins examined (Fig. 7A). Thus, the purified antibodies against MutS, MutL and DnaE are specific and were used in the experiments that follow.

To determine if MutS and MutL directly bind DnaE *in vitro*, we performed an immunodot blot (far-western) analysis. In this experiment, we spotted increasing amounts (2.5–40 pmol) of the indicated 'bait' proteins on a nitrocellulose membrane. The membrane was then probed with the indicated 'prey' protein, to allow for binding between the bait and prey proteins. Next, the membrane was probed with antibodies specific for the 'prey' protein to determine the retention of the 'prey' protein on the membrane and thus reveal interactions between the proteins examined.

We initially performed an immunodot blot experiment where DnaE and MutS were immobilized on the membrane, followed by probing with MutL. Strikingly, we found that MutL bound DnaE and MutS even down to the lowest amount of bait protein examined (2.5 pmol) (Fig. 7B). As a control, we found that MutL did not bind BSA and interestingly showed some binding to His₆-SSB (Fig. 7B). We performed the reciprocal experiment, probing for retention of MutS on a membrane through interaction with DnaE and MutL. We found that MutS also bound DnaE and MutL with a comparable level of retention, while MutS did not bind BSA or His₆-SSB at the protein concentrations tested (Fig. 7C). We did, however, observe some interaction between MutS and His₆-SSB when we probed the membrane with a higher concentration of MutS (data not shown).

We applied MutS and MutL to a nitrocellulose membrane to compare the retention of DnaE by each of these MMR proteins. We found that both MutS and MutL bound DnaE, and interestingly, MutS retained more DnaE than MutL (Fig. 7D). We interpret these results to mean that MutS may have a stronger affinity for DnaE than does MutL. As a positive control, we detected a weak interaction between DnaE and β clamp, as described (Simmons *et al.*, 2008a). We also found that DnaE bound His₆-SSB, suggesting a strong interaction exists between these two DNA replication proteins (Fig. 7D). Our finding that DnaE binds SSB has also been recently reported, and this work showed that DnaE binding to SSB is mediated through the C-terminal region of SSB (Costes *et al.*, 2010). We conclude that the MMR proteins MutS and MutL directly interact with the essential DNA polymerase DnaE.

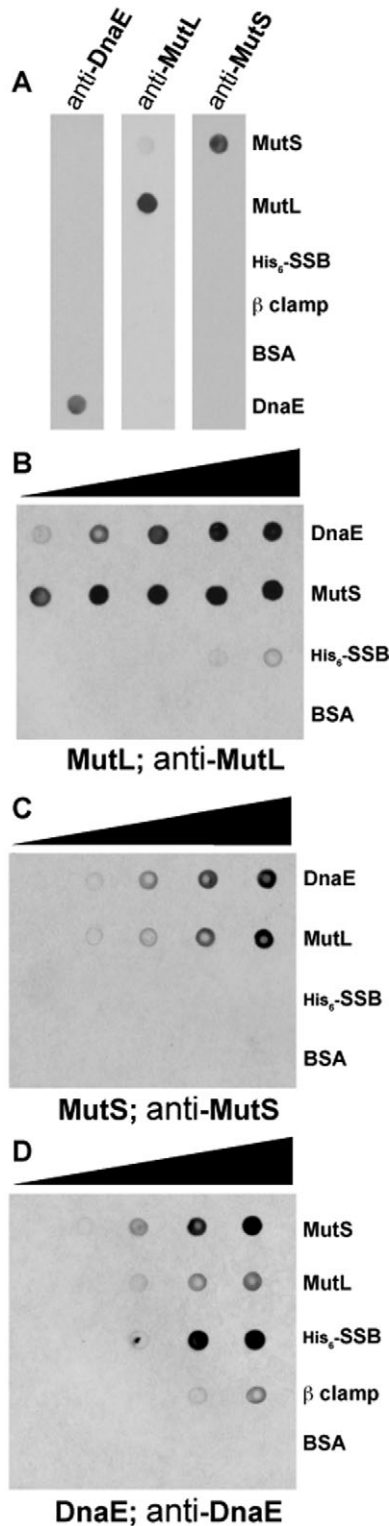


Fig. 7. MutS and MutL directly bind DnaE.

A. Specificity test of the polyclonal antibodies against DnaE, MutL and MutS. Forty picomoles of each protein was spotted onto a nitrocellulose membrane and probed with the indicated antibodies. B–D. Immunodot blot binding analysis of the indicated proteins. Each protein was applied to a nitrocellulose membrane, following incubation with (B) 400 nM MutL; (C) 400 nM MutS; and (D) 400 nM DnaE, each membrane was then probed with the indicated polyclonal antibody against the indicated prey protein as described in *Experimental procedures*.

Discussion

Here, we have fused GFP to components of the *B. subtilis* DNA replication machinery to investigate the effects of MMR on the subcellular organization of the DNA replication complex. We found that incorporation of mismatches induces loss of DnaE–GFP foci and that loss is dependent on the mismatch sensor MutS. Our analysis of six replication proteins indicates that the other five DNA replication proteins we examined were unaffected by 2-AP challenge, given that the percentage of cells with foci was unchanged between the conditions tested. It is striking that DnaE–GFP was the only replication protein altered following mismatch formation. For years, it has been known in *B. subtilis* and in human cells that MutS homologues colocalize with the DNA replication machinery *in vivo* (Kleczkowska *et al.*, 2001; Smith *et al.*, 2001). Earlier studies in both organisms have centred on understanding the connection between MMR proteins and the replication sliding clamps: β clamp and PCNA in *B. subtilis* and human cells respectively (Kleczkowska *et al.*, 2001; Iyer *et al.*, 2008; Simmons *et al.*, 2008a; Pluciennik *et al.*, 2010). Replication sliding clamps are known to bind MutS homologues in both systems and are known to have roles in regulating protein traffic at sites of DNA synthesis (for review see Lopez de Saro *et al.*, 2004; Sutton, 2009). Until now, it has been unclear whether the DNA replication machinery itself is affected by MMR. Our results are perhaps the most striking example showing an intimate connection between the DNA replication machinery and the MMR pathway, as we found that MutS causes loss of DNA polymerase DnaE–GFP following mismatch formation *in vivo*.

Our results raise the question of why foci of DnaE–GFP are altered during repair. It is possible that the biological function of DnaE at a replication fork makes it susceptible to focus loss following perturbation to DNA replication. For example, the slower rate of DNA synthesis by DnaE (Sanders *et al.*, 2010), or the hypothesis that the turnover of DnaE at replication forks is higher than other proteins, given that it switches with PolC during lagging strand synthesis (Sanders *et al.*, 2010) could make DnaE more susceptible to a decrease in localization in response to DNA replication fork perturbations. Another reason that DnaE–GFP focus formation could be influenced is due to

the fact that DnaE is involved in lagging strand maturation (Sanders *et al.*, 2010), and MMR shows preference for repairing mismatches in the lagging strand of *S. cerevisiae* and *E. coli* (Fijalkowska *et al.*, 1998; Pavlov *et al.*, 2003). Although the overall mechanism causing a decrease in DnaE–GFP foci is not clear, loss of DnaE–GFP foci is signalled following mismatch detection by MutS. Since *dnaE* is essential and required for replication, we investigated the possibility that nascent strand synthesis was altered during MMR. We did measure a slight decrease in nascent strand synthesis using ³H-thymidine incorporation *in vivo*, but these results overall were not conclusive (data not shown). However, we cannot exclude the possibility that a brief MMR-dependent pause occurs in DNA synthesis that cannot be readily measured in ³H incorporation experiments.

It was recently shown that the C-terminus of SSB is involved in the recruitment of several proteins to the DNA replication machinery in *B. subtilis*, including DnaE (Costes *et al.*, 2010). Strains bearing a truncated version of the SSB C-terminus ($\Delta 35$ or $\Delta 6$) grow slowly but are viable, yet DnaE–GFP foci are not observed in these cells (Costes *et al.*, 2010). Since SSB interacts with a host of proteins involved in DNA metabolism, it was not determined if the slow growth phenotype results from the action of an individual protein or a protein complex. Moreover, ectopic expression of *dnaE* alone did not rescue the slow growth phenotype (Costes *et al.*, 2010). Together, these data suggest that the assembly of DnaE into a focus is not required for viability, although it could very well contribute to a normal growth rate in *B. subtilis*. One possibility is that MutS binding to DnaE prevents DnaE from interacting with SSB resulting in loss of DnaE–GFP foci.

Wang and co-workers have shown that amino acid starvation arrests DNA replication in *B. subtilis* by inhibiting primase (DnaG) (Wang *et al.*, 2007). Amino acid starvation is a known method that causes DNA damage-independent perturbation to DNA replication (Wang *et al.*, 2007). We found that stringent arrest of DNA replication indeed induced a loss of DnaE–GFP foci (Fig. 3). In contrast, another replication protein, the predominant DNA polymerase PolC, was unaffected by treatment with RHX (Fig. 3). These RHX results suggest that DnaE–GFP focus formation is a sensitive single cell assay for DNA damage-independent perturbations to DNA replication, based on our observations that mismatches and RHX arrest of DNA synthesis cause a loss in DnaE–GFP foci.

We speculate that following mismatch detection, MutS binds β clamp, thereby decreasing the amount of β clamp available for DnaE. The reduction of available β clamp could subsequently decrease the processivity of DnaE-dependent DNA synthesis (Sanders *et al.*, 2010), resulting in a general perturbation to DNA replication and loss of

DnaE–GFP foci. It has been shown that binding of MutS to β clamp is critical for repair and for the formation of MutS–GFP foci in response to mismatches *in vivo* (Simmons *et al.*, 2008a; Dupes *et al.*, 2010). We propose that following mismatch detection, MutS occupies the general protein binding site on β clamp, which would in turn prevent or reduce binding of DnaE to this site on β clamp and cause the loss of DnaE–GFP foci. This hypothesis is being tested through our attempts to isolate mutant forms of MutS that bind DnaE and are unable to bind β clamp. So far, we have yet to isolate such a MutS mutant with these characteristics. As mentioned above, another possibility is that binding of MutS to DnaE prevents interaction between DnaE and the C-terminal region of SSB, which would decrease the accumulation of DnaE at sites of DNA replication as described (Costes *et al.*, 2010).

We also show that MutL binds DnaE *in vitro*, yet we show that the *mutL* gene in the absence of *mutS* is unable to cause a loss of DnaE–GFP foci in response to 2-AP. MutL is hypothesized to have a role in recruitment of a DNA polymerase for DNA synthesis following removal of the mismatch-containing strand. A possible role for the interaction we uncovered between MutL and DnaE could be for directing DnaE for resynthesis of the DNA during repair. We speculate that this interaction does not occur during initial mismatch detection and MMR activation as MutS and 2-AP are required for loss of DnaE–GFP foci *in vivo*.

Bacillus subtilis uses a methylation-independent MMR mechanism like most bacteria and all eukaryotic systems (Eisen, 1998; Eisen and Hanawalt, 1999; Culligan *et al.*, 2000). The mechanism that *B. subtilis* uses to identify the mismatch containing strand from the template strand is still unknown. It has been shown, however, that *B. subtilis* MutL is an endonuclease and the structure of the endonuclease domain has been solved (Pillon *et al.*, 2010; 2011). It has also been shown that integrity of the endonuclease active site is required for MMR *in vivo* and the endonuclease containing domain binds to β clamp (Pillon *et al.*, 2010; 2011). It is generally accepted that DNA strand discontinuities, including a nick, or a gap may be used to direct the repair machinery to the nascent strand in organisms that lack a methyl-directed signal (e.g. see Lacks *et al.*, 1982; Bruni *et al.*, 1988; Holmes *et al.*, 1990; Genschel *et al.*, 2002; Genschel and Modrich, 2003; Dzantiev *et al.*, 2004 for review; Schofield and Hsieh, 2003; Kunkel and Erie, 2005; Hsieh and Yamane, 2008; Larrea *et al.*, 2010). Furthermore, recent work suggests that endonuclease containing MutL proteins are capable of nicking a strand with a terminus (Kadyrov *et al.*, 2007; Pluciennik *et al.*, 2010). If strand discontinuities do indeed establish identification of the correct strand, it is not clear how the MMR machinery is targeted to strand termini at a replication fork.

It has been suggested that interactions between MutS homologues and their cognate processivity clamps could provide such a function (Flores-Rozas *et al.*, 2000; Kleczkowska *et al.*, 2001; Lopez de Saro and O'Donnell, 2001; Lee and Alani, 2006; Lopez de Saro *et al.*, 2006; Simmons *et al.*, 2008a; Dupes *et al.*, 2010). This suggestion comes from the numerous published manuscripts showing that MutS and MutL homologues bind processivity clamps (e.g. Flores-Rozas *et al.*, 2000; Kleczkowska *et al.*, 2001; Lee and Alani, 2006; Simmons *et al.*, 2008a; Dupes *et al.*, 2010) and the observation that processivity clamps are loaded at strand termini (see Jeruzalmi *et al.*, 2001; Georgescu *et al.*, 2008 for review; Johnson and O'Donnell, 2005). Many studies on the physical connections between MMR and DNA replication proteins have centred on the processivity clamps in a variety of organisms.

The results we present here provide striking evidence for a perturbation to the replisome following mismatch detection by MutS *in vivo* and direct interaction between the mismatch repair proteins and the essential DNA polymerase DnaE. Collectively, these data establish an intimate connection between MMR and the replication fork, and we suggest that MMR is integrated and actively engaged with the DNA replication complex to a greater extent than has previously been appreciated.

Experimental procedures

Bacteriological methods

The *B. subtilis* strains used in this study are described in Table S1. Strains used here were constructed using standard procedures (Hardwood and Cutting, 1990); resulting progeny were grown on plates containing appropriate antibiotics correlating to the specific alleles to be transferred. Antibiotics were used at the following final concentrations: 100 µg ml⁻¹ spectinomycin (*spc*); 5 µg ml⁻¹ kanamycin (*kan*); 5 µg ml⁻¹ chloramphenicol (*cat*); 5 µg ml⁻¹ tetracycline (*tet*); 0.5 µg ml⁻¹ erythromycin as described (Hardwood and Cutting, 1990; Simmons *et al.*, 2008a,b; Klocko *et al.*, 2010).

Live cell microscopy

Cells were prepared for live imaging essentially as described (Klocko *et al.*, 2010). Briefly, strains were grown at 30°C in 1× S7₅₀ media supplemented with 0.2% glucose, except DnaE–GFP, which required 1% arabinose and xylose (typically 0.125%, except the concentrations indicated in Fig. S3). Cells were grown to an optical density at 600 nm (OD₆₀₀) of ~0.3–0.4. Cultures were split, and 2-aminopurine was added to one culture to a final concentration of 600 µg ml⁻¹. Cells were allowed to grow for an additional hour to form mismatches. The hour time point for visualization of DnaE–GFP foci was used because 2-AP incorporation into DNA is inefficient (Goodman *et al.*, 1977; Watanabe and Goodman, 1981; Grafstrom *et al.*, 1988) and because MutS–GFP and

MutL–GFP have been shown to respond and form foci in approximately ~50% of cells following 1 h of 2-AP treatment as described (Smith *et al.*, 2001; Simmons *et al.*, 2008a; Dupes *et al.*, 2010; Klocko *et al.*, 2010). Following incubation, 300 µL aliquots of cultures were incubated with the vital membrane stain FM4-64 and cells were allowed to settle on microscope slides containing 1% agarose pads prepared with 1× Spizizens salts (Simmons *et al.*, 2008a; 2009). Experiments performed with JWS68 were carried out as described, with 0.125% xylose and 200 µM IPTG in the culture medium to drive expression of *mutL* and *dnaE–gfp*. Cells were imaged using an Olympus BX61 microscope equipped with a Hamamatsu ORCAR2 CCD cooled camera and a Lumen 200 arc metal light source (Prior). An Olympus 100× oil immersion 1.45 NA TIRFM objective lens was used as described (Klocko *et al.*, 2010). For detection of GFP and FM4-64 the following filter sets were used: FITC excitation 460–500, emission 510–560; TRITC excitation 510–560, emission 572–648. Images were captured and processed using Slidebook 4.2 (Advanced Imaging Software) and Photoshop (Adobe). Foci were scored using the software ImageJ (<http://rsbweb.nih.gov/ij/>), and quantification graphs were made using Sigma-Plot (Systat) or Deltagraph 5 (Redrock Software). Images were assembled into figures using Illustrator (Adobe). Imaging for each strain was performed at least twice.

Immunoblotting

Immunodetection of MMR and DNA replication was performed as described (Simmons *et al.*, 2003; 2007; Rokop *et al.*, 2004). Briefly, 10 ml cultures of DnaE–GFP cells were grown at 30°C in 1× S7₅₀ media supplemented with 1% arabinose and xylose (grown identically to imaging conditions) to an optical density of OD₆₀₀ ~0.3–0.4, whereupon 2-aminopurine was added to 600 µg ml⁻¹ final concentration. Cells were grown an additional hour at 30°C and 1.5 optical density 600 nm units of cells were removed, concentrated by centrifugation, frozen in liquid N₂ and stored at –80°C. Whole-cell lysates were prepared as described (Rokop *et al.*, 2004). Lysates corresponding to an equivalent number of cells were separated in a 6% denaturing SDS-PAGE followed by transfer to a nitrocellulose membrane (Whatman) as described (Simmons *et al.*, 2003). Membranes were probed with the indicated 1° Antibody (rabbit, typically 1:1000 dilution), and goat anti-rabbit HRP conjugated secondary antibody (Pierce, typically 1:10 000 dilution) in 5% Milk + 1× TBST [1× Tris buffered saline + 0.02% Tween20 (Sigma)]. Membranes were then exposed to film (BioExpress), images scanned into Photoshop (Adobe), and figures assembled in Illustrator (Adobe).

For the immunoblots shown in Fig. 4, a single colony was inoculated into Luria–Bertani (LB) with spectinomycin, erythromycin and kanamycin, and grown to mid-log phase at 37°C, at which time they were diluted to OD₆₀₀ = 0.05 in LB supplemented with the indicated amounts of IPTG and xylose. Cells were harvested at OD₆₀₀ = 1, at which point 1.5 OD₆₀₀ units of cells were centrifuged for use in immunoblots. Blots were then performed as above, with the following exceptions: secondary antibody was goat anti-rabbit-HRP at a 1:2000 dilution, and antibody binding and washing steps were performed in 5% milk in PBS-T [1× phosphate-buffered saline (150 mM NaCl) with 0.02% Tween-20].

Plasmid construction for protein expression

A derivative of pET28a, pET28aPB, encoding a PreScission protease cleavage site, was used for the overexpression of MutS, MutL, β clamp, DnaE and SSB. Following protease cleavage, amino acids GPGS remain at the N-terminus of each protein as described (Schwartz *et al.*, 1999a,b,c).

pBW18 was constructed by placing *ssb* (also known as *ssbA*) into plasmid pET28aPB by ligation following digestion with BamHI and XhoI. The primers used to amplify the 516 bp of *ssb* are as follows: forward, 5'-cgc gga tcc atg ctt aac cga gtt gta tta gtc gga aga; reverse 5'-ccg ctc gag cta gaa tgg aag atc atc atc cga gat gtc aat.

pBW1 was constructed by placing *dnaN* into plasmid pET28aPB by ligation following digestion with BamHI and XhoI. The primers used to amplify the 1134 bp of *dnaN* are as follows: forward, 5'-cgc gga tcc atg aaa ttc acg att caa aaa gat cgt ctt; reverse, 5'-ccg ctc gag cta ata ggt tct gac agg aag gat aag ctg tac.

pBW25 was constructed by placing *dnaE* into plasmid pET28aPB by ligation following digestion with BamHI and XhoI. The primers used to amplify the 3345 bp of *dnaE* are as follows: forward, 5'-cgc gga tcc atg tct ttt gtt cac ctg caa gtg cat agc; reverse, 5'-ccg ctc gag cta cca ctg ttt taa aac gac gtt ttt ttg acc.

pLS126 was generated for overexpression of *mutL*. The *mutL* gene was PCR amplified followed by digestion with BamHI and XhoI and ligation into pET28aPB. The following primers were used for amplification of the *mutL* gene: forward, 5'-cgc gga tcc gtg gca aaa gtc atc caa ctg tca gat gag; reverse, 5'-ccg ctc gag tta cat cac gcg ttt gaa cat ctt tc cat ctc ata.

pLS128 was constructed for overexpression of *mutS*. The *mutS* gene was PCR amplified followed by digestion with BamHI and XhoI and ligation into plasmid pET28aPB following digestion of pET28aPB with the same restriction enzymes. The following primers were used for amplification of the *mutS* gene: forward 5'-cgc gga tcc atg gcc ggt tat acg cct atg ata cag caa; reverse 5'-ccg ctc gag tta atg taa ttt ctt ttg cag ctt gta cat ttc.

pJS33 was constructed for the purpose of inserting *mutL* under control of the *Pspac* at the *lacA* locus via double cross-over integration. The *mutL* coding region was PCR amplified and digested with SphI and Sall and inserted between the same sites of pA-spac (Hartl *et al.*, 2001). The resulting plasmid, pJS33, was then used to transform *B. subtilis* and followed by verification of double cross-over integration by diagnostic PCR amplification of the *lacA* locus (data not shown). Primers used to amplify *mutL* were as follows: forward, 5'-gtg gtc gac taa gga ggt ata cat gtg gca aaa gtc atc caa c; reverse, 5'-gtg gca tgc tta tta cat cac gcg ttt gaa cat c.

All constructs were sequenced prior to use (University of Michigan core sequencing facility).

Protein purification

Following cloning of MutS, MutL, DnaE and β clamp into pET28aPB (fusing an N-terminal His₆ tag), these proteins were overexpressed in *E. coli* BL21_{DE3} cells using standard procedures. Cell pellets containing overexpressed proteins

were resuspended in cell lysis buffer [50 mM Tris-HCl (pH 7.6), 10% sucrose, 200 mM NaCl, 20 mM SpCl₃] and lysed by French press at 4°C. The lysates were then clarified by centrifugation. All subsequent steps were conducted at 4°C. Equilibrated Ni²⁺-agarose beads (Qiagen, Hilden, Germany) were incubated with the prepared supernatant for 1 h and mixed by rotation. The supernatant was drained in a Poly-Prep chromatography column (Bio-Rad, Hercules, CA, USA) washed with Ni²⁺-wash buffer [Tris-HCl (pH 7.6), 150 mM imidazole, 0.5 M NaCl and 15% glycerol], and eluted with elution buffer [Tris-HCl (pH 6.0), 400 mM imidazole, 0.5 M NaCl and 15% glycerol]. MutS and β clamp were dialysed for 16 h in 20 mM Tris-HCl (pH 7.6), 15% glycerol, 60 mM NaCl, and 1 mM DTT. DnaE was dialysed for 16 h in 20 mM Tris-HCl (pH 7.6), 20% glycerol, 200 mM NaCl and 1 mM DTT. MutL was dialysed for 16 h in 20 mM Tris-HCl (pH 7.6), 15% glycerol, 300 mM NaCl and 1 mM DTT. The N-terminal His-tag was removed with PreScission protease (2 units per 100 μ g fusion protein) (GE Healthcare, UK) during dialysis. Protein solutions were then applied to a Ni²⁺-agarose column (prepared as stated earlier), and the flow through of proteins lacking the polyhistidine tag was collected. All proteins were quantified using extinction coefficients derived at ExPASy proteomics server (<http://expasy.org/>) using a 50-bio UV visible spectrophotometer (Varian, Palo Alto, CA) and purity was verified as > 95% using SDS-PAGE (data not shown). Proteins were subsequently aliquoted into usable amounts, flash frozen with liquid N₂ and stored at -80°C prior to use.

Affinity purification of antibodies

All primary serum was prepared through Covance (Denver, PA, USA), and was raised against the indicated antigen that was purified as described above. To affinity purify the DnaE, MutS and MutL antiserum further, 100 μ g of purified protein (untagged) was electrophoresed per lane on a 6% SDS-PAGE. Proteins were then transferred to nitrocellulose (Whatman, Dassel, Germany) using standard procedures (Simmons *et al.*, 2003). The membrane was stained with PonceauS and the portion representing the targeted protein was excised and blocked in PBS + 5% milk for 20 min, and then rinsed with PBS. The membrane was then incubated in 500 μ L of antisera for 1 h at room temperature and then rinsed with PBS. The targeted antibodies were removed from the membrane with a strip solution [5 mM glycine, 150 mM NaCl (pH 2.4)] and then neutralized with 1 M NaPO₄ at pH 8.0.

Immunodot blotting

For immunodot blotting, 16 μ M protein stocks were diluted 1:2 into immunoblotting binding buffer (50 mM Tris, pH 8.0; 300 mM NaCl; 10% glycerol) five times, giving protein concentration solutions of 8.0, 4.0, 2.0 and 1.0 μ M. Protein dilutions (2.5 μ L) were then blotted onto nitrocellulose membranes (Whatman), giving the indicated protein amounts (40 pmol to 2.5 pmol). Following blocking of the membrane in 5% Milk + 1 \times TBST (20 mM Tris-HCl pH 7.6, NaCl 300 mM, 0.1% Tween 20), the indicated prey protein (400 nM final concentration) was incubated with the membrane in 5% milk with binding buffer (50 mM Tris, pH 8.0; 300 mM NaCl; 10%

glycerol) overnight at 4°C. Membranes were washed 3× for at least 5 min with wash buffer [4 mM KH₂PO₄, 16 mM Na₂HPO₄ (pH 7.6), 300 mM NaCl], and subsequently probed with affinity purified 1° antibody (rabbit) (1:2 000) and goat anti-rabbit HRP conjugated secondary antibody (1:2000) (Pierce) identically to immunoblotting. Control blots in Fig. 7A were performed in an identical manner, except that only bait protein was used and the prey protein step was omitted to determine the cross-reactivity of the antiserum with the other proteins used in the assay.

Statistical analysis

Bar charts are presented with error bars representing the 95% confidence interval, and statistical significance was determined using a two-tailed z-test, as described (Utts and Heckard, 2006).

Acknowledgements

We thank Drs Etienne Dervyn, S. Dusko Ehrlich (Génétique Microbienne, Institut National de la Recherche Agronomique, Jouy en Josas, France), and Alan Grossman for strains (Massachusetts Institute of Technology). We thank Drs Amy Klocko and Eric Nielsen (University of Michigan) for anti-GFP antibodies (Abcam). We thank Drs Jim Bardwell, Janine Maddock, Robert Bender, Stefan Walter, and Matthew Chapman (University of Michigan) for use of equipment and reagents. We are indebted to Heather Schroeder for help with statistical analysis and we thank Kaleena Crafton for initiating this work. We also thank members of the Simmons lab for comments on the manuscript. This study was supported by start-up funds from the College of Literature, Science and Arts, from the Department of Molecular, Cellular, and Developmental Biology at the University of Michigan. This work was also supported by a grant from the Wendy Will Case Cancer Fund and grant MCB1050948 from the National Science Foundation to L.A.S.

References

- Ahrends, R., Kosinski, J., Kirsch, D., Manelyte, L., Giron-Monzon, L., Hummerich, L., *et al.* (2006) Identifying an interaction site between MutH and the C-terminal domain of MutL by crosslinking, affinity purification, chemical coding and mass spectrometry. *Nucleic Acids Res* **34**: 3169–3180.
- Berkmen, M.B., and Grossman, A.D. (2006) Spatial and temporal organization of the *Bacillus subtilis* replication cycle. *Mol Microbiol* **62**: 57–71.
- Bernard, R., Marquis, K.A., and Rudner, D.Z. (2010) Nucleoid occlusion prevents cell division during replication fork arrest in *Bacillus subtilis*. *Mol Microbiol* **78**: 866–882.
- Bruni, R., Martin, D., and Jiricny, J. (1988) d(GATC) sequences influence *Escherichia coli* mismatch repair in a distance-dependent manner from positions both upstream and downstream of the mismatch. *Nucleic Acids Res* **16**: 4875–4890.
- Chopra, I., O'Neill, A.J., and Miller, K. (2003) The role of mutators in the emergence of antibiotic-resistant bacteria. *Drug Resist Updat* **6**: 137–145.
- Costes, A., Lecointe, F., McGovern, S., Quevillon-Cheruel, S., and Polard, P. (2010) The C-terminal domain of the bacterial SSB protein acts as a DNA maintenance hub at active chromosome replication forks. *PLoS Genet* **6**: e1001238.
- Cox, E.C., Degnen, G.E., and Scheppe, M.L. (1972) Mutator gene studies in *Escherichia coli*: the *mutS* gene. *Genetics* **72**: 551–567.
- Culligan, K.M., Meyer-Gauen, G., Lyons-Weiler, J., and Hays, J.B. (2000) Evolutionary origin, diversification and specialization of eukaryotic MutS homolog mismatch repair proteins. *Nucleic Acids Res* **28**: 463–471.
- Denamur, E., Bonacorsi, S., Giraud, A., Duriez, P., Hilali, F., Amorin, C., *et al.* (2002) High frequency of mutator strains among human uropathogenic *Escherichia coli* isolates. *J Bacteriol* **184**: 605–609.
- Dervyn, E., Suski, C., Daniel, R., Bruand, C., Chapuis, J., Errington, J., *et al.* (2001) Two essential DNA polymerases at the bacterial replication fork. *Science* **294**: 1716–1719.
- Dronkert, M.L., and Kanaar, R. (2001) Repair of DNA inter-strand cross-links. *Mutat Res* **486**: 217–247.
- Dupes, N.M., Walsh, B.W., Klocko, A.D., Lenhart, J.S., Peterson, H.L., Gessert, D.A., *et al.* (2010) Mutations in the *Bacillus subtilis* beta clamp that separate its roles in DNA replication from mismatch repair. *J Bacteriol* **192**: 3452–3463.
- Dzantiev, L., Constantin, N., Genschel, J., Iyer, R.R., Burgers, P.M., and Modrich, P. (2004) A defined human system that supports bidirectional mismatch-provoked excision. *Mol Cell* **15**: 31–41.
- Eisen, J.A. (1998) A phylogenomic study of the MutS family of proteins. *Nucleic Acids Res* **26**: 4291–4300.
- Eisen, J.A., and Hanawalt, P.C. (1999) A phylogenomic study of DNA repair genes, proteins, and processes. *Mutat Res* **435**: 171–213.
- Fijalkowska, I.J., Jonczyk, P., Tkaczyk, M.M., Bialoskorska, M., and Schaaper, R.M. (1998) Unequal fidelity of leading strand and lagging strand DNA replication on the *Escherichia coli* chromosome. *Proc Natl Acad Sci USA* **95**: 10020–10025.
- Fishel, R., Lescoe, M.K., Rao, M.R.S., Copeland, N.G., Jenkins, N.A., Garber, J., *et al.* (1993) The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis cancer. *Cell* **75**: 1027–1038.
- Flores-Rozas, H., Clark, D., and Kolodner, R.D. (2000) Proliferating cell nuclear antigen and Msh2p-Msh6p interact to form an active mispair recognition complex. *Nat Genet* **26**: 375–378.
- Friedberg, E.C., Walker, G.C., Siede, W., Wood, R.D., Schultz, R.A., and Ellenberger, T. (2006) *DNA Repair and Mutagenesis*, 2nd edn. Washington, DC: American Society for Microbiology, pp. 389–433.
- Genschel, J., and Modrich, P. (2003) Mechanism of 5'-directed excision in human mismatch repair. *Mol Cell* **12**: 1077–1086.
- Genschel, J., and Modrich, P. (2006) Analysis of the excision step in human DNA mismatch repair. *Methods Enzymol* **408**: 273–284.
- Genschel, J., Bazemore, L.R., and Modrich, P. (2002) Human exonuclease I is required for 5' and 3' mismatch repair. *J Biol Chem* **277**: 13302–13311.

- Georgescu, R.E., Kim, S.S., Yurieva, O., Kuriyan, J., Kong, X.P., and O'Donnell, M. (2008) Structure of a sliding clamp on DNA. *Cell* **132**: 43–54.
- Ginetti, F., Perego, M., Albertini, A.M., and Galizzi, A. (1996) *Bacillus subtilis* *mutS mutL* operon: identification, nucleotide sequence and mutagenesis. *Microbiology* **142** (Pt 8): 2021–2029.
- Goodman, M.F., Hopkins, R., and Gore, W.C. (1977) 2-Aminopurine-induced mutagenesis in T4 bacteriophage: a model relating mutation frequency to 2-aminopurine incorporation in DNA. *Proc Natl Acad Sci USA* **74**: 4806–4810.
- Grafstrom, R.H., Amsterdam, A., and Zachariasewycz, K. (1988) *In vivo* studies of repair of 2-aminopurine in *Escherichia coli*. *J Bacteriol* **170**: 3485–3492.
- Hall, M.C., and Matson, S.W. (1999) The *Escherichia coli* MutL protein physically interacts with MutH and stimulates the MutH-associated endonuclease activity. *J Biol Chem* **274**: 1306–1312.
- Hall, M.C., Jordan, J.R., and Matson, S.W. (1998) Evidence for a physical interaction between the *Escherichia coli* methyl-directed mismatch repair proteins MutL and UvrD. *EMBO J* **17**: 1535–1541.
- Hamilton, S.R., Liu, B., Parsons, R.E., Papadopoulos, N., Jen, J., Powell, S.M., et al. (1995) The molecular basis of Turcot's syndrome. *N Engl J Med* **332**: 839–847.
- Hardwood, C.R., and Cutting, S.M. (1990) *Molecular Biological Methods for Bacillus*. Chichester: John Wiley & Sons, pp. 27–446.
- Hartl, B., Wehrl, W., Wiegert, T., Homuth, G., and Schumann, W. (2001) Development of a new integration site within the *Bacillus subtilis* chromosome and construction of compatible expression cassettes. *J Bacteriol* **183**: 2696–2699.
- Hickman, M.J., and Samson, L.D. (1999) Role of DNA mismatch repair and p53 in signaling induction of apoptosis by alkylating agents. *Proc Natl Acad Sci USA* **96**: 10764–10769.
- Hickman, M.J., and Samson, L.D. (2004) Apoptotic signaling in response to a single type of DNA lesion, O(6)-methylguanine. *Mol Cell* **14**: 105–116.
- Holmes, J., Jr, Clark, S., and Modrich, P. (1990) Strand-specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines. *Proc Natl Acad Sci USA* **87**: 5837–5841.
- Hsieh, P., and Yamane, K. (2008) DNA mismatch repair: molecular mechanism, cancer, and ageing. *Mech Ageing Dev* **129**: 391–407.
- Iyer, R.R., Pohlhaus, T.J., Chen, S., Hura, G.L., Dzantiev, L., Beese, L.S., and Modrich, P. (2008) The MutSalphaproliferating cell nuclear antigen interaction in human DNA mismatch repair. *J Biol Chem* **283**: 13310–13319.
- Jeruzalmi, D., O'Donnell, M., and Kuriyan, J. (2001) Crystal structure of the processivity clamp loader gamma (gamma) complex of *E. coli* DNA polymerase III. *Cell* **106**: 429–441.
- Johnson, A., and O'Donnell, M. (2005) Cellular DNA replicases: components and dynamics at the replication fork. *Annu Rev Biochem* **74**: 283–315.
- Kadyrov, F.A., Dzantiev, L., Constantin, N., and Modrich, P. (2006) Endonucleolytic function of MutLalpha in human mismatch repair. *Cell* **126**: 297–308.
- Kadyrov, F.A., Holmes, S.F., Arana, M.E., Lukianova, O.A., O'Donnell, M., Kunkel, T.A., and Modrich, P. (2007) *Saccharomyces cerevisiae* MutLalpha is a mismatch repair endonuclease. *J Biol Chem* **282**: 37181–37190.
- Kleczkowska, H.E., Marra, G., Lettieri, T., and Jiricny, J. (2001) hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. *Genes Dev* **15**: 724–736.
- Klocko, A.D., Crafton, K.M., Walsh, B.W., Lenhart, J.S., and Simmons, L.A. (2010) Imaging mismatch repair and cellular responses to DNA damage in *Bacillus subtilis*. *J Vis Exp* **36**: 1–4.
- Kunkel, T.A., and Erie, D.A. (2005) DNA mismatch repair. *Annu Rev Biochem* **74**: 681–710.
- Lacks, S.A., Dunn, J.J., and Greenberg, B. (1982) Identification of base mismatches recognized by the heteroduplex-DNA-repair system of *Streptococcus pneumoniae*. *Cell* **31**: 327–336.
- Lahue, R.S., Au, K.G., and Modrich, P. (1989) DNA mismatch correction in a defined system. *Science* **245**: 160–164.
- Larrea, A.A., Lujan, S.A., and Kunkel, T.A. (2010) SnapShot: DNA mismatch repair. *Cell* **141**: 730 e731.
- Lee, S.D., and Alani, E. (2006) Analysis of interactions between mismatch repair initiation factors and the replication processivity factor PCNA. *J Mol Biol* **355**: 175–184.
- Lemon, K.P., and Grossman, A.D. (1998) Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science* **282**: 1516–1519.
- Li, F., Liu, Q., Chen, Y.Y., Yu, Z.N., Zhang, Z.P., Zhou, Y.F., et al. (2008) *Escherichia coli* mismatch repair protein MutL interacts with the clamp loader subunits of DNA polymerase III. *Mutat Res* **637**: 101–110.
- Li, G.M. (2008) Mechanisms and functions of DNA mismatch repair. *Cell Res* **18**: 85–98.
- Lopez de Saro, F., Georgescu, R.E., Leu, F., and O'Donnell, M. (2004) Protein trafficking on sliding clamps. *Philos Trans R Soc Lond B Biol Sci* **359**: 25–30.
- Lopez de Saro, F.J., and O'Donnell, M. (2001) Interaction of the beta sliding clamp with MutS, ligase, and DNA polymerase I. *Proc Natl Acad Sci USA* **98**: 8376–8380.
- Lopez de Saro, F.J., Marinus, M.G., Modrich, P., and O'Donnell, M. (2006) The beta sliding clamp binds to multiple sites within MutL and MutS. *J Biol Chem* **281**: 14340–14349.
- Mechanic, L.E., Frankel, B.A., and Matson, S.W. (2000) *Escherichia coli* MutL loads DNA helicase II onto DNA. *J Biol Chem* **275**: 38337–38346.
- Migocki, M.D., Lewis, P.J., Wake, R.G., and Harry, E.J. (2004) The midcell replication factory in *Bacillus subtilis* is highly mobile: implications for coordinating chromosome replication with other cell cycle events. *Mol Microbiol* **54**: 452–463.
- Modrich, P. (2006) Mechanisms in eukaryotic mismatch repair. *J Biol Chem* **281**: 30305–30309.
- Nystrom-Lahti, M., Perrera, C., Raschle, M., Panyushkina-Seiler, E., Marra, G., Curci, A., et al. (2002) Functional analysis of MLH1 mutations linked to hereditary nonpolyposis colon cancer. *Genes Chromosomes Cancer* **33**: 160–167.
- Paul, B.J., Ross, W., Gaal, T., and Gourse, R.L. (2004) rRNA transcription in *Escherichia coli*. *Annu Rev Genet* **38**: 749–770.
- Pavlov, Y.I., Mian, I.M., and Kunkel, T.A. (2003) Evidence for

- preferential mismatch repair of lagging strand DNA replication errors in yeast. *Curr Biol* **13**: 744–748.
- Peltomaki, P. (2005) Lynch syndrome genes. *Fam Cancer* **4**: 227–232.
- Pillon, M.C., Lorenowicz, J.J., Uckelmann, M., Klocko, A.D., Mitchell, R.R., Chung, Y.S., *et al.* (2010) Structure of the endonuclease domain of MutL: unlicensed to cut. *Mol Cell* **39**: 145–151.
- Pillon, M.C., Miller, J.H., and Guarne, A. (2011) The endonuclease domain of MutL interacts with the beta sliding clamp. *DNA Repair (Amst)* **10**: 87–93.
- Pluciennik, A., Burdett, V., Lukianova, O., O'Donnell, M., and Modrich, P. (2009) Involvement of the beta clamp in methyl-directed mismatch repair in vitro. *J Biol Chem* **284**: 32782–32791.
- Pluciennik, A., Dzantiev, L., Iyer, R.R., Constantin, N., Kadyrov, F.A., and Modrich, P. (2010) PCNA function in the activation and strand direction of MutLalpha endonuclease in mismatch repair. *Proc Natl Acad Sci USA* **107**: 16066–16071.
- Rayssiguier, C., Thaler, D.S., and Radman, M. (1989) The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. *Nature* **342**: 396–401.
- Rokop, M.E., Auchtung, J.M., and Grossman, A.D. (2004) Control of DNA replication initiation by recruitment of an essential initiation protein to the membrane of *Bacillus subtilis*. *Mol Microbiol* **52**: 1757–1767.
- Sanders, G.M., Dallmann, H.G., and McHenry, C.S. (2010) Reconstitution of the *B. subtilis* replisome with 13 proteins including two distinct replicases. *Mol Cell* **37**: 273–281.
- Sanjanwala, B., and Ganesan, A.T. (1991) Genetic structure and domains of DNA polymerase III of *Bacillus subtilis*. *Mol Gen Genet* **226**: 467–472.
- Schofield, M.J., and Hsieh, P. (2003) DNA mismatch repair: molecular mechanisms and biological function. *Annu Rev Microbiol* **57**: 579–608.
- Schofield, M.J., Nayak, S., Scott, T.H., Du, C., and Hsieh, P. (2001) Interaction of *Escherichia coli* MutS and MutL at a DNA mismatch. *J Biol Chem* **276**: 28291–28299.
- Schwartz, T., Lowenhaupt, K., Kim, Y.G., Li, L., Brown, B.A., 2nd, Herbert, A., and Rich, A. (1999a) Proteolytic dissection of Zab, the Z-DNA-binding domain of human ADAR1. *J Biol Chem* **274**: 2899–2906.
- Schwartz, T., Rould, M.A., Lowenhaupt, K., Herbert, A., and Rich, A. (1999b) Crystal structure of the Zalpha domain of the human editing enzyme ADAR1 bound to left-handed Z-DNA. *Science* **284**: 1841–1845.
- Schwartz, T., Shafer, K., Lowenhaupt, K., Hanlon, E., Herbert, A., and Rich, A. (1999c) Crystallization and preliminary studies of the DNA-binding domain Za from ADAR1 complexed to left-handed DNA. *Acta Crystallogr D Biol Crystallogr* **55**: 1362–1364.
- Simmons, L.A., Felczak, M., and Kaguni, J.M. (2003) DnaA Protein of *Escherichia coli*: oligomerization at the *E. coli* chromosomal origin is required for initiation and involves specific N-terminal amino acids. *Mol Microbiol* **49**: 849–858.
- Simmons, L.A., Grossman, A.D., and Walker, G.C. (2007) Replication is required for the RecA localization response to DNA damage in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **104**: 1360–1365.
- Simmons, L.A., Davies, B.W., Grossman, A.D., and Walker, G.C. (2008a) Beta clamp directs localization of mismatch repair in *Bacillus subtilis*. *Mol Cell* **29**: 291–301.
- Simmons, L.A., Grossman, A.D., and Walker, G.C. (2008b) Clp and Lon proteases occupy distinct subcellular positions in *Bacillus subtilis*. *J Bacteriol* **190**: 6758–6768.
- Simmons, L.A., Goranov, A.I., Kobayashi, H., Davies, B.W., Yuan, D.S., Grossman, A.D., and Walker, G.C. (2009) Comparison of responses to double-strand breaks between *Escherichia coli* and *Bacillus subtilis* reveals different requirements for SOS induction. *J Bacteriol* **191**: 1152–1161.
- Smith, B.T., Grossman, A.D., and Walker, G.C. (2001) Visualization of mismatch repair in bacterial cells. *Mol Cell* **8**: 1197–1206.
- Srivatsan, A., and Wang, J.D. (2008) Control of bacterial transcription, translation and replication by (p)ppGpp. *Curr Opin Microbiol* **11**: 100–105.
- Sutton, M.D. (2009) Coordinating DNA polymerase traffic during high and low fidelity synthesis. *Biochim Biophys Acta* **1804**: 1167–1179.
- Utts, J.M., and Heckard, R.F. (2006) *Mind on Statistics*. Belmont, CA: Thomson Brooks/Cole.
- Viswanathan, M., Burdett, V., Baitinger, C., Modrich, P., and Lovett, S.T. (2001) Redundant exonuclease involvement in *Escherichia coli* methyl-directed mismatch repair. *J Biol Chem* **276**: 31053–31058.
- Wang, J.D., Sanders, G.M., and Grossman, A.D. (2007) Nutritional control of elongation of DNA replication by (p)ppGpp. *Cell* **128**: 865–875.
- Watanabe, S.M., and Goodman, M.F. (1981) On the molecular basis of transition mutations: frequencies of forming 2-aminopurine.cytosine and adenine.cytosine base mispairs in vitro. *Proc Natl Acad Sci USA* **78**: 2864–2868.
- Yoshioka, K., Yoshioka, Y., and Hsieh, P. (2006) ATR kinase activation mediated by MutSalpha and MutLalpha in response to cytotoxic O6-methylguanine adducts. *Mol Cell* **22**: 501–510.

Supporting information

Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.