The sliding clamp tethers the endonuclease domain of MutL to DNA

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ABSTRACT

The sliding clamp enhances polymerase processivity and coordinates DNA replication with other critical DNA processing events including translesion synthesis, Okazaki fragment maturation and DNA repair. The relative binding affinity of the sliding clamp for its partners determines how these processes are orchestrated and is essential to ensure the correct processing of newly replicated DNA. However, while stable clamp interactions have been extensively studied; dynamic interactions mediated by the sliding clamp remain poorly understood. Here, we characterize the interaction between the bacterial sliding clamp (β-clamp) and one of its weak-binding partners, the DNA mismatch repair protein MutL. Disruption of this interaction causes a mild mutator phenotype in Escherichia coli, but completely abrogates mismatch repair activity in Bacillus subtilis. We stabilize the MutL-β interaction by engineering two cysteine residues at variable positions of the interface. Using disulfide bridge crosslinking, we have stabilized the E. coli and B. subtilis MutL-β complexes and have characterized their structures using small angle X-ray scattering. We find that the MutL-β interaction greatly stimulates the endonuclease activity of B. subtilis MutL and supports this activity even in the absence of the N-terminal region of the protein.

INTRODUCTION

Protein–protein and protein–nucleic acid interactions regulate virtually every cellular transaction. However, we only have a fragmented understanding of these regulatory processes due to the intrinsic challenges associated with studying short-lived interactions. Most well characterized protein–protein complexes form stable, high-affinity interactions ($K_D < 10^{-6}$ M). Weak-affinity ($K_D > 10^{-4}$ M) and transient interactions are equally important in the regulation of many cellular pathways (1), but they are poorly understood. Weak and transient interactions are especially difficult to study for proteins that coordinate multiple processes, because they interact with many binding partners often using a common interface. Allostery and conformational malleability are defining aspects of the hierarchy of these interactions (1,2), however most structural biology approaches fail to provide this type of information.

The sliding β-clamp (β) and its eukaryotic counterpart (PCNA) are a paradigm for proteins coordinating multiple interactions using a common binding site. They were first identified as processivity factors that tether the replicative polymerase to DNA during DNA replication. However, they also play critical roles coordinating DNA replication with other key cellular functions including Okazaki fragment maturation, polymerase switching during lesion bypass, DNA repair and DNA transposition (3–6). Sliding β-clamps form ring-shaped structures that are conserved at the structural, but not sequence, level. Their central cavity locally, clamp-binding partners contain a conserved linear motif known as PIP box (PCNA-interacting protein box) or β-binding motif that binds the hydrophobic groove of the clamp. β-binding motifs are not as well conserved as PIP boxes and, hence, β-clamp binding partners are difficult to identify (9). The sequence variability of this binding
motif has been correlated to binding affinity, thereby allowing clamps to mediate both weak and strong interactions (10–14).

One of the repair pathways orchestrated by the sliding β-clamp is DNA mismatch repair, a conserved post-replicative repair pathway that corrects replication errors introduced by DNA polymerase. The initiation of the mismatch repair response in Escherichia coli requires the coordinated action of three proteins: MutS, MutL and MutH. MutS recognizes base mismatches and small insertion or deletion loops and, once bound to the mismatch, it recruits MutL in an ATP-dependent manner (15,16). Together, MutS and MutL activate the MutH endonuclease that nicks the newly synthesized strand at a nearby hemimethylated GATC site (16–18). While the DNA mismatch repair pathway is evolutionary conserved, MutH is only found in a subset of gamma-proteobacteria including E. coli. In organisms lacking MutH, MutL homologs harbour a weak endonuclease activity (19–24).

Bacterial MutL consists of two structurally conserved domains connected by a flexible linker (25). The N-terminal domain (NTD) supports DNA binding and ATPase activity (26,27). The C-terminal domain (CTD) mediates protein dimerization and harbours a metal binding motif associated to the endonuclease activity and a conserved β-binding motif (10,19–24,26–28).

Beyond, its role at the DNA synthesis step, the sliding β-clamp plays critical roles at earlier steps of the mismatch repair process. It interacts with MutS and recruits it to the mismatched sites (29,30). The bacterial β-clamp also interacts weakly, yet specifically, with the two structured domains of MutL (10,29), however the role of this interaction remains unclear. Mutation of the β-binding motif in the endonuclease domain of Bacillus subtilis MutL abrogates mismatch repair activity (24), whereas mutation of the same motif in E. coli MutL only causes a mild mutator phenotype (10,29), suggesting that this interaction is more important in organisms that lack the MutH endonuclease. PCNA stimulates the endonuclease activity of human MutLo and, due to its loading orientation, helps determine the strand that MutLo nicks (31). However, the direct interaction between PCNA and the endonuclease domain of MutLo has not been demonstrated biochemically.

In order to understand how the β-clamp regulates the activity of MutL in organisms containing or lacking MutH, we devised a strategy to stabilize the MutL-β clamp interaction using E. coli and B. subtilis proteins and analyzed the structure and function of these complexes. We find that binding to the β-clamp has a marginal effect on the activities of E. coli MutL, but it greatly stimulates the endonuclease activity of B. subtilis MutL. The interaction promotes endonuclease activity of B. subtilis MutL even in the absence of the ATPase domain of the protein, presumably by bypassing the DNA binding defect of the endonuclease domain of B. subtilis MutL. Based on these results, we propose a model to describe the role of the sliding β-clamp on the activation of MutL endonuclease activity of MutL.

MATERIALS AND METHODS

Cloning of the MutL and β-clamp cysteine variants

The expression plasmids encoding E. coli β-clamp and MutL were kind gifts from Dr Michael O’Donnell and Dr Wei Yang, respectively. Variants of both proteins, where surface exposed cysteines had been replaced by serines, were generated by overlap PCR. E. coli β (pAG 8769; residues 1–367) harboring mutations C260S, C333S, L366S and C367 was subcloned into a modified pET15b vector lacking the histidine tag. E. coli MutL (pAG 8814; residues 1–615), and its C-terminal domain MutL CTD (pAG 8768; residues 431–615), including mutations C61S, C446S, and C588S were subcloned in pET15b using Ndel and BamHI. Variants of E. coli MutL (eMutL*; pAG 8824) and MutL CTD (eMutL CTD*; pAG 8772) lacking the β-binding motif (482QPLLIP → 482ASAAA) were generated by overlap PCR. Variants of B. subtilis MutL and β lacking surface exposed cysteine residues were generated similarly. B. subtilis β (pAG 8807; residues 1–380) including the Ser379–Cys380 dipeptide at the extreme C-terminus of the protein was subcloned into a modified pET15b vector including a TEV-removable histidine tag. B. subtilis MutL (pAG 8842; residues 1–627), and its C-terminal domain MutL CTD (pAG 8803; residues 433–627), including mutations C61S, C446S, E485C and C531S were subcloned in the pProExHTa vector. An inactive variant of MutL CTD including two additional point mutations (C573S and C604S), MutL CTDI (pAG 8927; residues 433–627), was generated by site-directed mutagenesis. Variants of B. subtilis MutL (bMutL*; pAG 8908), MutL CTD (bMutL CTD*; pAG 8909) and MutL CTD (bMutL CTD*; pAG 8929) lacking the β-binding motif (482QEMIVP → 482AEMAAP) were generated by overlap PCR. All mutants were verified by DNA sequencing (MOBIX, McMaster University).

Characterization of the MutL and β-clamp cysteine variants

E. coli and B. subtilis β and MutL cysteine-modified variants were over-produced and purified as described previ-
ously (10,24,27,28) and eluted from a size exclusion chromatography column at retention volumes similar to their native counterparts (Supplementary Figure S1). All purified cysteine-variants were monodisperse in solution as measured using a plasmid shuffle assay (33) (Table 1). Briefly, E. coli strains were transformed with the incompatible plasmids pACM, pACM* (VB100) and pACM* (VB101) strains were used for subsequent genetic analyses (Table 2). The frequency of spontaneous mutation of rpoB to rifampicin resistance (RifR) of strains VB100 and VB101 was measured (35), and 95% confidence limits were calculated as described previously (36). Mutation rates were calculated using FALCOR, a Mu-Sandri-Sarkar Maximum Likelihood Estimator (37). The Mann–Whitney U test (http://www.socscistatistics.com/tests/mannwhitney/Default.aspx) was used to determine whether mutation rate differences were statistically significant. Steady state levels of β-clamp proteins were measured using Western blot analysis (33). Doubling times for strains VB100 and VB101 grown at 37°C in liquid LB medium were calculated from linear portions of growth curves.

**B. subtilis** strains, bacteriology and western blotting for analysis of cysteine-variants of *B. subtilis* MutL and β in *vivo*

Each *B. subtilis* strain was created by first amplifying the mutant allele from plasmids (pAG 8807, pAG 8842). For JRR20, the mutant dnaN allele was placed into the pBGSC6 plasmid containing a chloramphenicol resistance marker and integrated into the native dnaN locus via single crossover. JRR21 was built by placing the mutant mutL allele into the pMiniMad plasmid with upstream and downstream sequence and integrating at the native mutL locus via double crossover. JRR28 was created by purifying genomic DNA from JRR20 and transforming the genomic DNA into competent JRR21 cells and selecting for chloramphenicol resistance. PB112 was made by placing upstream and downstream mutL sequence into pMiniMad and removing the gene via double crossover at the native locus. All strains were verified via DNA sequence analysis and are listed in Supplementary Table S3.

**Analysis of the function of the cysteine-variant of E. coli β in vivo**

The ability of *E. coli* β to support viability was measured using a plasmid shuffle assay (33) (Table 1). Briefly, strain MS201 lacks a functional β-clamp gene (*dnaN*), due to a frameshift mutation (*dnaN*−1FS). Viability of strain MS201 relies on the ampicillin resistant plasmid pAMP* dnaN*+, which expresses physiological levels of the wild type β-clamp (34). MS201 bearing pAMP* dnaN*+ was transformed with the incompatible and chloramphenicol resistant pACM (negative control), pACM* dnaN*+ (εβWT) or pACMβCys (εβCys) plasmids, which contain the same origin of replication as pAMP* dnaN*+. Twenty randomly selected transformants were passaged ~100 generations in Luria-Bertani (LB) media supplemented with 40 µg/ml chloramphenicol. The frequency of pAMP* dnaN*+ retention was measured by patching cells onto agar plates supplemented with either 150 µg/ml ampicillin (to score for pAMP* dnaN*+) or 40 µg/ml chloramphenicol (control). The presence of the dnaN−1FS alleles, and lack of the pAMP* dnaN*+ plasmid, in strains bearing pACM* dnaN*+ or pACMβCys was verified by diagnostic PCR and nucleotide sequence analysis of the chromosomal dnaN locus, as well as plasmid mini-prep and nucleotide sequence analysis, as described previously (33).

Single isolates of the εβWT (VB100) and εβCys (VB101) strains were used for subsequent genetic analyses (Table 2). The frequency of spontaneous mutation of rpoB to rifampicin resistance (RifR) of strains VB100 and VB101 was measured (35), and 95% confidence limits were calculated as described previously (36). Mutation rates were calculated using FALCOR, a Mu-Sandri-Sarkar Maximum Likelihood Estimator (37). The Mann–Whitney U test (http://www.socscistatistics.com/tests/mannwhitney/Default.aspx) was used to determine whether mutation rate differences were statistically significant. Steady state levels of β-clamp proteins were measured using Western blot analysis (33). Doubling times for strains VB100 and VB101 grown at 37°C in liquid LB medium were calculated from linear portions of growth curves.

**Table 1. Ability of εβCys to support *E. coli* viability**

| Transforming plasmid<sup>a</sup> | β-Clamp protein | Frequency of pAMP* dnaN*+ retention<sup>b</sup> | *E. coli* viability<sup>c</sup> | β-Clamp expression levels<sup>d</sup> | Doubling time (min)<sup>e</sup> |
|---|---|---|---|---|---|
| pACM | None (negative control) | 20/20 (100%) | – | 0.93 ± 0.08 (P = 0.26) | 37 ± 1.5 |
| pACM* dnaN*+ | εβWT (positive control) | 2/20 (10%) | + | 0.95 ± 0.13 (P = 0.56) | 42 ± 2.1 (P = 0.03) |
| pACMβCys | εβCys | 2/20 (10%) | + | 0.95 ± 0.13 (P = 0.56) | 42 ± 2.1 (P = 0.03) |

<sup>a</sup>Strain MS201 bearing plasmid pAMP* dnaN*+ was transformed with the incompatible plasmids pACM, pACM* dnaN*+ or pACMβCys, as indicated.

<sup>b</sup>The frequencies of pAMP* dnaN*+ retention in 20 randomly selected pACM, pACM* dnaN*+ or pACMβCys transformants following ~100 generations are indicated. Representative clones lacking pAMP* dnaN*+ were characterized further to verify they contained both the chromosomally-encoded dnaN−1FS as well as the indicated plasmid-encoded dnaN allele.

<sup>c</sup>Viability refers to the ability of pACM, pACM* dnaN*+ or pACMβCys to support growth of *E. coli* in the absence of pAMP* dnaN*+.

<sup>d</sup>Values represent the average of triplicates ± one standard deviation, and are expressed relative to the level observed in the isogenic strain RW118. P values were calculated relative to the isogenic parent strain RW118 using the Student’s t-test.

<sup>e</sup>Results shown are the average of three independent determinations ± one standard deviation. The P value for the strain bearing pACMβCys was calculated relative to the pACM* dnaN*+ strain using the Student’s t-test.
Growth curve analysis was done by growing each strain in culture in 3 ml of LB liquid and grown to an OD₆₀₀ of between 0.4 and 0.6. Each culture was then back diluted to an OD₆₀₀ of 0.05 and five 200 μl aliquots were distributed into wells of a sterile 96-well plate for each strain. This plate was incubated at 37 °C, shaking overnight and the absorbance at 600 nm was taken every 0.5 h by an Omega plate reader. The standard deviation between the five aliquots was calculated and plotted.

Western blotting was performed by growing each strain to an OD₆₀₀ of between 0.8 and 1. Cell number was then normalized to 1 ml of OD₆₀₀ equal to 1 for each strain and cells were pelleted by centrifugation for 2 min at 14 000 rcf. The supernatant was aspirated and cells were resuspended in 50 μl of lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 50 mM EDTA, 1 × protease inhibitor cocktail (Thermoscientific), 10 mg/ml lysozyme). Cells were then incubated at 37 °C for 30 min then left on ice for 10 min. The cell lysate was then centrifuged at 14 000 rcf for 5 min to remove cell debris. 50 μl of 10% SDS, and loading dye to 1 × were then added and samples were heated to 100 °C for 10 min before loading onto an 8% SDS-PAGE. The protein was transferred onto a nitrocellulose membrane and blocked for 30 min in TBST and visualized using an IR dye imager.

To form the MutL-β and MutLₐClD-β complexes, β was incubated with either MutL or MutLₐClD at a 1:1 ratio to a final concentration of 20 μM. The samples (1–2 ml) were dialyzed against 11 of dialysis buffer A (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM DTT, 5% glycerol for E. coli proteins; or 20 mM Tris pH 7.6, 150 mM KCl, 10 mM DTT, 10% glycerol for B. subtilis proteins) for 2 h at 4 °C. The mixture was transferred into dialysis buffer B (same as A but with 5 mM DTT) for 1 h, followed by 1 h in dialysis buffer C (without DTT). The sample was then left overnight in dialysis buffer C. Complex formation was monitored over time by resolving the samples on 11% (E. coli MutL-β and B. subtilis MutLₐClD-β) or 15% (E. coli MutLₐClD-β) denaturing gels stained with Coomassie Brilliant Blue. Prior to forming the E. coli MutL–β complex, full-length MutL (43 kDa) was pre-incubated in the absence and presence of 2 mM AMPNP (Sigma) for 1 h at room temperature followed by an overnight incubation at 4 °C. Association of the N-terminal domains of E. coli MutL was monitored as previously described (26,28).

SAXS data collection and analysis

E. coli MutLₐClD-β samples were resolved over a Superdex-200 (GE Healthcare) size exclusion chromatography column. B. subtilis MutLₐClD-β samples were treated with 120 μM methyl methanethiosulfonate (MMTS) for 10 min at 22 °C to sulfenylate unreacted thiol groups and resolved over a Mono Q 5/50 GL (GE Healthcare) ionic exchange column equilibrated with buffer D (20 mM Tris pH 7.6, 150 mM KCl, 0.5 mM EDTA, 1 mM MMTS, and 10% glycerol). B. subtilis MutLₐClD-β samples were exchanged into buffer D using a 100 kDa MWCO concentrator. Sample homogeneity was confirmed by dynamic light scattering (Malvern Instruments). Samples (35 μl) were spun at 15 700 x g for 10 min and scattering data was collected on a Rigaku BioSAXS-1000 instrument at 10 °C. Consecutive scans of 10, 30, 60 and/or 180 min were collected over a range of protein concentrations (μM): 47–186 μM; eMutLₐClD-β (Day 1): 1.8–2.0 μM; eMutLₐClD-β (Day 2): 9.5–123 μM; bβ: 27–73 μM; bMutLₐClD-β: 88–218 μM; bMutLₐClD-β: 22–37 μM (Day 2)). SAXSLab 3.0.0r1 (Rigaku) was used to generate the scattering curves. Comparing 10-min exposures collected before and after data col-

**Table 2. Ability of E. coli βCys to support mismatch repair function in vivo**

| Strain | β-Clamp protein | Spontaneous mutation frequency (RifR) | Spontaneous mutation rate (RifR) |
|--------|----------------|-------------------------------------|----------------------------------|
| VB100  | eβWT          | 1.79 × 10⁻⁹ (≤8.40 × 10⁻¹⁰ – 4.76 × 10⁻⁹) | 1.28 × 10⁻⁸                      |
| VB101  | eβCys         | 1.68 × 10⁻⁹ (8.40 × 10⁻¹⁰ – 3.36 × 10⁻⁹) | 1.57 × 10⁻⁸ (P = 0.79)           |

a Strains VB100 and VB101 are representative plasmid shuffle isolates (derivatives of MS201 bearing the Camβ plasmids) and express physiological levels of either wild type β-clamp (VB100) or βCys (VB101) as the sole clamp protein.

b Median value from 14 (VB100) or 15 (VB101) independent cultures; 95% confidence intervals are in parentheses.

c Spontaneous RifR mutation rates were calculated from respective frequencies using the web tool FALCOR. The p value for strain VB101 was calculated relative to strain VB100 using the Mann-Whitney U test.
lection and resolving the samples on SDS-polyacrylamide gels before and after data collection confirmed sample integrity during data collection. The 1D scattering profile pairs were identical for all samples. Data quality was assessed by comparing scattering curves over a range of protein concentrations and exposure times using the ATSAS 2.6.0 program suite (Supplementary Figures S3–S5) (40).

Mismatch-independent MutL endonuclease assays were performed as described previously (31) with minor modifications. The linear DNA substrate (200 base pairs) was end-labeled with either 32P end-labeled 5′-P-d(CGGCAACAATTAATAGACTGGAGGCG) and 32P 5′-P-d(CGGCAACATGTTAGACTGGAGGCG). DNA (5 nM) was incubated with E. coli MutL and MutL-β complex from 40 to 320 nM in reaction buffer (20 mM Tris pH 8.0, 90 mM KCl, 15% glycerol). The E. coli MutL-β complex used for these assays was prepared by freezing a mixture of eMutL and eβ after incubation for 24 h in the absence of reducing agents (this is equivalent to the ‘Day 1’ sample used on the SAXS analysis). To confirm that the E. coli MutL–β complex binds DNA, the complex was incubated with DNA at 22°C for 10 min followed by 30 min on ice. Samples (15 μl) were resolved on 4.5% tris–borate–EDTA gels and visualized using the Typhoon Trio+ (GE Healthcare). All experiments were performed in triplicate.

Helicase assays were performed as described previously (28) with minor modifications. The UvrD expression vector (pWY 1365) was a kind gift from Dr Wei Yang. The 250 bp substrate described above was nicked near the center using Nb.BsrDI (New England Biolabs). The nicked DNA substrate (5 nM) was pre-incubated with increasing amounts of either E. coli MutL or MutL-β (5–80 nM) for 20 min at 22°C in reaction buffer (20 mM Tris pH 7.5, 50 mM NaCl, 3 mM MgCl2, 0.1 mg/ml BSA, 0.05 mg/ml BSA), 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.5 mM EDTA, 10% glycerol). Following a 1-h incubation at 37°C, the reaction was stopped with 25 mM EDTA and 1 mg/ml proteinase K (55°C for 20 min). Two times loading dye (90% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.5 mM EDTA, 10% glycerol) was added and reaction mixtures were resolved on 8% polyacrylamide (8 M urea) gels in 0.5× tris–borate–EDTA buffer. Gels were visualized using the Typhoon Trio+ (GE Healthcare). All experiments were performed in triplicate and error bars represent the standard deviation of the mean.

RESULTS AND DISCUSSION

Cysteine-modified variants of MutL and β are functional in vivo

We have previously shown that the conserved QXX[L/I]XP motif (48) in E. coli and 487 QEMVIP in B. subtilis found in the dimerization domain of MutL is a genuine β-clamp binding motif (10). However, the complex between MutL and β is weak and, thus, difficult to study using structural biology techniques. To stabilize the complex, we exploited the presence of a naturally occurring cysteine in E. coli MutL (Cys480), located immediately upstream to the β-binding motif. This residue is not conserved in other MutL homologues (48), suggesting that it is likely dispensable for MutL function or its interaction with β. Furthermore, mutation of this residue does not affect DNA mismatch repair activity in vitro or in vivo (49). B. subtilis MutL has a glutamate residue at the equivalent position (Glu485). Based on the lack of conservation, and the fact that E. coli MutL tolerates a cysteine residue at this position, we generated a
variant of *B. subtilis* MutL where a cysteine residue replaced Glu485.

In the crystal structure of the *E. coli* β-clamp bound to the β-binding peptide derived from DNA polymerase II, the C-terminal residue of the β-clamp (Leu366) is less than 10 Å away from the N-terminal of the polymerase II peptide (Figure 1). Therefore, we added a cysteine at the C-terminus of the *E. coli* β-clamp (Cys367) to promote the formation of a disulfide bridge with Cys480 found in MutL. We could not predict how far the C-terminus of *B. subtilis* β would be from Cys485 in *B. subtilis* MutL. Therefore, we engineered a cysteine at the C-terminus of *B. subtilis* β (Cys380) preceded by an additional residue (Ser379) to provide enough flexibility to this C-terminal extension and enhance the interaction with Cys485. Serine residues replaced other surface-exposed cysteine residues in MutL and the β-clamp to minimize the formation of unspecific complexes.

Kosinski *et al.* had previously shown that the single-cysteine variant of *E. coli* MutL (L<sup>380C</sup>) retains normal DNA mismatch repair activity *in vivo* (49). We also wanted to ensure that the cysteine-modified variants of *B. subtilis* MutL, as well as *E. coli* and *B. subtilis* β, retained normal function *in vivo*. The β-clamp plays a critical role in DNA replication, therefore we used a β-clamp plasmid shuffle assay to investigate the function of *E. coli* β *in vivo*. This assay measures the ability of a plasmid expressing physiological levels of wild type or cysteine-modified β to support viability of an *E. coli* strain harboring a frame shift in the endogenous β gene (*dmaN*) (33). The plasmid expressing cysteine-modified *E. coli* β was as efficient as wild-type β at replacing pACYC<sub>dmaN</sub><sup>N</sup>, indicating the cysteine-modified variant of β was functional in replication (Table 1).

For subsequent analysis, we selected representative isolates of both the wild-type β (VB100) and cysteine-modified β (VB101) shuffle strains. Based on Western blotting with anti-β antibodies, cysteine-modified β was expressed at physiological levels (Table 1). The doubling time of the cysteine-modified β strain was slightly slower than that of the isogenic wild type strain (42 min versus 37 min; see Table 1), indicating the modification conferred a modest growth defect. To ensure that this variant of β supported DNA mismatch repair activity *in vivo*, we measured both the frequency and the rate of spontaneous mutation of *rpoB* to rifampicin resistance (Rif<sup>R</sup>) for the wild type β and cysteine-modified β strains. Based on a previous study (10), disruption of the β-clamp-MutL interaction resulted in a 12- to 43-fold increase in spontaneous mutation rate in Rif<sup>R</sup>. Both the wild type β and β<sup>Cys</sup> strains displayed similar mutation frequencies and rates (Table 2), indicating that cysteine-modified β supports wild type mismatch repair function *in vivo*.

Having shown that the cysteine-modified variants are active in *E. coli*, we performed similar experiments in *B. subtilis* to determine if the MutL and β-clamp variants were also functional in *B. subtilis*. The cysteine-modified variant of *mutL* and *dmaN* were integrated into their native chromosomal location to replace the wild type allele as described (see Materials and Methods). We performed a Western blot to probe for protein accumulation *in vivo* and found that cysteine-modified MutL and β-clamp accumulated to levels similar to the wild type protein using DnaX as a loading control (Figure 2A). Analysis of the growth curve showed that the single cysteine-modified MutL and β-clamp strains, and double mutant strain grew the same as the wild type control strain PY79 (Figure 2B). We then asked if the MutL and β-clamp variants were able to participate in mismatch repair *in vivo*. We assayed mutation rate by fluctuation analysis as an indicator for mismatch repair (38). We found that the cysteine variants of MutL and β-clamp, as well as the double mutant, conferred mutation rates within error of the wild type control strain (Figure 2C). In contrast, the ΔmutL control showed a 25-fold increase in mutation rate, which is similar to our previous measurements for defects in the mismatch repair pathway (38,50).

Given that the cysteine-modified variants of MutL and the β-clamp were fully active *in vivo*, all subsequent experiments described in this manuscript were performed with these variants of MutL and β. Henceforth, for the remainder of the manuscript MutL, MutL<sup>CTD</sup> and β refer to the cysteine-modified variants of the proteins unless explicitly specified.

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**Table 3.** SAXS data-collection and scattering-derived parameters for MutL<sup>CTD</sup>-β

| Data-collection parameters | cMutL<sup>CTD</sup>-cβ | bMutL<sup>CTD</sup>-bβ |
|---------------------------|------------------------|------------------------|
| Exposure time (min)        | 180                    | 180                    |
| Concentration (µM)         | 2                      | 38.5                   |
| Structural parameters      |                        |                        |
| I₀ (cm<sup>−1</sup>) [from Guinier] | 0.1 ± 0.0               | 1.3 ± 0.0               |
| R₀ (Å) [from Guinier]      | 39.4 ± 2.1             | 35.1 ± 0.2             |
| I₀ (cm<sup>−1</sup>) [from P( vegetation)] | 0.1 ± 0.0               | 1.3 ± 0.0               |
| R₀ (Å) [from P( vegetation)] | 36.3 ± 0.4             | 35.3 ± 0.1             |
| D<sub>max</sub> (Å)        | 104                    | 101                    |
| Experimental MW [from Q( vegetation)] | 152,490 Da             | 143,778 Da             |
| Calculated MW              | 122,371 Da             | 122,371 Da             |
| Ab initio analysis         | GASBOR                 | GASBOR                 |
| X <sup>2</sup> of ab initio models | 1.0                    | 1.2–1.3                |

*MW determined using ScÅtter (47).*
Figure 2. MutL and β-clamp cysteine variants are functional in B. subtilis. (A) Immunoblot analysis of the soluble fraction of B. subtilis cell lysate. The soluble fraction was probed for the presence of MutL and β-clamp (DnaN) cysteine variants. DnaX is a loading control. (B) Growth curves for wild type (PY79), null mutL (ΔmutL), MutL and β-clamp cysteine variants (mutLCys and dnaNCys), and double mutant (mutLCys, dnaNCys) B. subtilis strains. (C) Mutation rate analysis of wild type (PY79), null mutL (ΔmutL), MutL and β-clamp cysteine variants (mutLCys and dnaNCys), and double mutant (mutLCys, dnaNCys) B. subtilis strains.

The C-terminal domain of MutL forms a specific complex with β

The cysteine-variants of E. coli and B. subtilis MutL and β were monodisperse in solution and eluted from a size exclusion chromatography column at retention volumes comparable to their native counterparts (Supplementary Figure S1). Furthermore, differential scanning fluorimetry revealed that all MutL and β variants, with the exception E. coli MutL CTD that had a melting temperature 14°C lower than native MutL CTD, had similar thermal stability to their wild-type counterparts (Supplementary Figure S1). Therefore, we presumed that the cysteine mutations not only preserved the function of MutL and β, but also their native conformations.

The C-terminal domain of E. coli MutL (eMutL CTD) was incubated with E. coli β-clamp (eβ) in the absence of reducing agents to promote the formation of a disulfide bridge when the two proteins interact. We resolved the reaction mixtures in SDS-polyacrylamide gels and found that a new species of ~60 kDa immediately appeared (Figure 3A). This new species had a molecular weight consistent with the presence of a monomer of each protein and it accumulated at the same rate as free eMutL CTD and eβ disappeared. Furthermore, it dissociated in the presence of reducing agent, indicating that a disulfide bridge mediated its formation. To determine whether the eMutL CTD-eβ interaction was specific, we assembled the complex using a variant of eMutL CTD (eMutL CTD*) lacking the β-binding motif (10). Incubation of eMutL CTD* with eβ resulted in the accumulation of two new species of molecular weights ~60 and ~80 kDa in a denaturing polyacrylamide gel (Figure 3A). However, there was a significant delay in the accumulation of the 60 kDa species suggesting the β-binding motif enhances the formation of this species (Figure 3A). We reasoned that the 80 kDa molecular weight species could either be caused by the association of two eβ molecules or the interaction of a partially exposed cysteine (Cys180) present in eβ with an additional eMutL CTD* molecule. We favour the latter, because the relative amounts of free eMutL CTD and eMutL CTD* with respect to free eβ seem to suggest the formation of higher order complexes with eMutL CTD* (Figure 3A). Interestingly, the 80 kDa species does not form when the β-binding motif of MutL is present, reinforcing the idea that the formation of a specific complex prevails over non-specific crosslinked products.

Incubation of an inactive variant of C-terminal domain of B. subtilis MutL (bMutL CTD) with B. subtilis β-clamp (bβ) also yielded a major species at around 75 kDa, consistent with the interaction of one MutL and one β monomer (Figure 3B). However, the formation of this species was not as efficient as in the case of E. coli and longer incubations were required to accumulate significant amounts of this crosslinked product. In contrast to E. coli, formation of the 75 kDa species was almost completely abrogated when
we assembled the \textit{B. subtilis} MutL\textsuperscript{CTD1–}\beta complex with a variant of MutL lacking the \beta-binding motif (bMutL\textsuperscript{CTD1}). The different behaviour of the \textit{E. coli} and \textit{B. subtilis} proteins seemed to suggest that the MutL-\beta complexes are different depending on whether MutL has endonuclease activity; therefore, we decided to characterize both complexes further.

\subsection*{Structural characterization of the MutL\textsuperscript{CTD–}\beta complex}

An intriguing difference between the \textit{B. subtilis} and \textit{E. coli} crosslinking experiments was the relatively slow formation of the \textit{B. subtilis} MutL\textsuperscript{CTD1–}\beta complex (Figure 3). \textit{E. coli} MutL\textsuperscript{CTD–}\beta complex starts accumulating as soon as reducing agents are removed from the buffer and two days later there are no free MutL\textsuperscript{CTD} or \beta-clamp left in the solution (Figure 3A). Conversely, a significant amount of free \textit{B. subtilis} MutL\textsuperscript{CTD1} and \beta remain in solution even after three days without reducing agents (Figure 3B). We entertained the possibility that the \textit{E. coli} proteins could form two distinct complexes, whereas the \textit{B. subtilis} proteins could not. Therefore, we decided to characterize \textit{E. coli} MutL\textsuperscript{CTD–}\beta complex at two time points, herein referred to as ‘Day 1’ and ‘Day 2’ (see the third and fourth panels in Figure 3A). Conversely, we analyzed the \textit{B. subtilis} MutL\textsuperscript{CTD1–}\beta complex only at the later time point to maximize the amount of complex present in solution (Figure 3B, fourth panel).

We used small angle X-ray scattering (SAXS), a technique where scattering data is collected in solution, thereby facilitating the analysis of samples at different time points. Using dynamic light scattering, we confirmed that the \textit{E. coli} and \textit{B. subtilis} MutL\textsuperscript{CTD} and \beta proteins were monodisperse (Supplementary Figure S1). Then, we collected scattering data of the individual proteins at a range of concentrations. None of the samples had concentration-dependent interparticle interactions (Supplementary Table S1 and Figure S3). Furthermore, the scattering curves for MutL\textsuperscript{CTD} and \beta were also similar to the theoretical scattering profiles derived from the crystal structures of MutL\textsuperscript{CTD} (PDB ID: 1XMZ and 3KDK) and \beta-clamp (PDB ID: 1MM1 and 4RT6). The discrepancy between the theoretical and experimental scattering curves resulted in \(\chi^2\) values of 1.1 (eMutL\textsuperscript{CTD}), 1.0 (bMutL\textsuperscript{CTD}), 1.3 (e\beta) and 1.5 (b\beta) (Supplementary Figures S4 and S5), compare black lines to the experimental scattering curves. Accordingly, the pair-distance distribution functions were indicative of toroidal (e\beta and b\beta) and elongated (eMutL\textsuperscript{CTD} and bMutL\textsuperscript{CTD1}) particles (Figures 4 and 5 and Supplementary Figures S4 and S5).

We then incubated eMutL\textsuperscript{CTD} and e\beta in the absence of reducing agents for either one (‘Day 1’) or two (‘Day 2’) days, resolved the mixtures by size exclusion chromatography and collected scattering data for both samples (Figure 4C and D and Table 3). The samples showed no signs of protein aggregation and the Kratky plots indicated the presence of globular structure (Supplementary Figure S4). They had similar pair-distance distribution functions and their estimated molecular weights were consistent with a dimer of MutL\textsuperscript{CTD} interacting with one dimer of \beta (Figure 4 and Table 3). We generated twenty independent \textit{ab initio} models for each sample and clustered them based on their normalized spatial discrepancy using DAMCLUST (45). We identified four different clusters for the sample at ‘Day 1’ and five different clusters for the sample at ‘Day 2’. However, in both cases one of the clusters was significantly more populated than the rest. Ten out of twenty models of ‘Day 1’ and eleven out of twenty models for ‘Day 2’ belonged to a single cluster. Therefore, we based our analysis on the representative model from the most populated cluster (Figure 4C-D).

The representative model for ‘Day 1’ forms a ring-shaped structure with a handle that resembles a hollow curling stone (Figure 4C). The toroidal moiety of the model is closely related to the SAXS model of e\beta and the ‘handle’ has similar shape and dimensions to the SAXS model of eMutL\textsuperscript{CTD} (Figure 4C). The \beta-binding motifs are found at both ends of the eMutL\textsuperscript{CTD} dimer, therefore the ‘Day 1’ \textit{E. coli} MutL\textsuperscript{CTD–}\beta complex seems to adopt a structure where only one protomer of the MutL dimer is bound to the \beta ring (Figure 4C). Conversely, on the representative model for ‘Day 2’ both ends of eMutL\textsuperscript{CTD} seem to be interacting with \beta (Figure 4D). The ‘handle’ of the model has collapsed on top the \beta moiety, suggesting that the two \beta-binding motifs of \textit{E. coli} MutL\textsuperscript{CTD} are bound to e\beta (Figure 4D). The lack of free \textit{E. coli} MutL\textsuperscript{CTD} and \beta after two days of incubation also supports the idea that both subunits of eMutL\textsuperscript{CTD} are bound to the e\beta ring (Figure 3A, fourth panel). The differences between the two models, however, should be interpreted with caution. The samples for ‘Day 1’ were collected...
Figure 4. Structural models of the E. coli MutL$^{CTD}$–$\beta$ complexes. (A) Ab initio bead model of E. coli MutL$^{CTD}$ (eLC; orange) shown alongside its crystal structure (PDB ID: 1X9Z). (B) Orthogonal views of the refined ab initio model of the E. coli $\beta$-clamp (e$\beta$; gray) compared to its crystal structure (PDB ID: 1MMI). (C and D) Orthogonal views of the representative models for each of the most populated clusters of eMutL$^{CTD}$-e$\beta$ at ‘Day 1’ (C) and ‘Day 2’ (D). The moieties presumed to represent MutL$^{CTD}$ and $\beta$ are highlighted in orange and grey. The overall dimensions of the bead models and crystal structures are indicated for reference.

at much lower concentration than those for ‘Day 2’ and, therefore, the differences could also result from the different signal-to-noise ratio of the two scattering curves.

To understand the formation of the MutL-$\beta$ complex further, we decided to perform the same experiment with B. subtilis MutL$^{CTD}$ and $\beta$. We incubated the proteins in the absence of reducing agents for two days and collected scattering data (Figure 3B and Table 3). The samples showed no signs of protein aggregation and were folded (Supplementary Figure S5). The B. subtilis MutL$^{CTD}$–$\beta$ complex had similar pair-distance distribution function to the E. coli MutL$^{CTD}$–$\beta$ samples (Supplementary Figures S4 and S5). The estimated molecular weight (137 kDa) is comparable to the calculated molecular weight (136 kDa) of the bMutL$^{CTD}$–$\beta$ complex at a 1:1 ratio (Table 3). We generated 20 independent ab initio models and produced a refined ab initio model using DAMMIN (43). The general features of the B. subtilis MutL$^{CTD}$–$\beta$ complex resembled more closely the ‘Day 1’ than the ‘Day 2’ E. coli MutL$^{CTD}$–$\beta$ complex, thereby suggesting that the B. subtilis MutL dimer cannot bind the two protomers of the $\beta$ ring simultaneously (Figure 5). The presence of free B. subtilis MutL$^{CTD}$ and $\beta$ in the crosslinking experiments, even after three days of incubation, also supports this interpretation (Figure 3B, fifth panel).

Binding partners of the sliding $\beta$-clamp typically bind a single cleft on the ring (34,51–53). Therefore, the complex with a single protomer of the MutL dimer bound to the $\beta$ ring may represent the functional form of the MutL-$\beta$ complex (Figures 4 and 5), with the complex where both protomers of MutL are bound to $\beta$ being an artifact caused by the presence of a reactive cysteine at the other end of the dimer. This explanation is appealing because B. subtilis MutL$^{CTD}$ only forms one complex with $\beta$ (Figure 5). Furthermore, PCNA (the eukaryotic counterpart of $\beta$) stimulates the endonuclease activity of eukaryotic MutLo, which only have a single endonuclease site per dimer, implicitly suggesting that only one of the protomers needs to interact with the sliding clamp. However, we cannot rule out the possibility that both E. coli MutL-$\beta$ complexes are indeed functional in vivo.

Full-length MutL binds a single cleft of the $\beta$-clamp

MutL undergoes a large conformational change upon ATP binding (20,26,28,54,55). Furthermore, ATP-binding greatly stimulates the endonuclease activity of bacterial and eukaryotic MutL (21,22,24). It is possible that the presence of the N-terminal domains of MutL or additional mismatch repair factors favour the formation of one of the complexes with $\beta$. Therefore, we repeated the crosslinking experiments with full-length E. coli MutL (eMutL). The cysteine-modified variant of E. coli MutL undergoes the characteristic nucleotide-dependent conformational change when pu-
Figure 5. Structural model of the *B. subtilis* MutL<sup>CTD</sup>–β complex. (A) *Ab initio* bead model of *B. subtilis* MutL<sup>CTD</sup> (bLCI, orange) shown alongside its crystal structure (PDB ID: 3KDK). (B) Orthogonal views of the most representative *ab initio* model of the *B. subtilis* β-clamp (ββ; grey) compared to its crystal structure (PDB ID: 4RT6). (C and D) Orthogonal views of the refined model for the bMutL<sup>CTD</sup>–ββ at ‘Day 2’. The moieties presumed to represent MutL<sup>CTD</sup> and β are highlighted in orange and grey, respectively. The overall dimensions of the bead models and crystal structures are indicated for reference.

rifed over a size exclusion chromatography column (Supplementary Figure S6A).

To test whether eMutL favors the singly- or doubly-bound form of the MutL-β complex, we incubated it with equimolar amounts of eβ in the absence of reducing agents to promote cysteine-mediated crosslinking of the complex. In good agreement with previous experiments using the C-terminal domain of MutL, a new species consistent with the formation of a 1:1 complex readily appeared and accumulated over time (Figure 6). However, this new species stopped accumulating after ‘Day 1’ and a significant amount of free MutL and β remained in solution (Figure 6), thereby suggesting that MutL only binds to one cleft of the β-clamp ring.

Complex formation was significantly impaired when we used a variant of MutL lacking the β-binding motif (MutL*<sup>482QPLL→ASAAA</sup>). Mirroring the results obtained with MutL<sup>CTD*</sup>, incubation of MutL* with β resulted in the formation of two new species of molecular weights ~130 and ~180 kDa in a denaturing polyacrylamide gel (compare Figures 3A and 6). These two products were sensitive to the presence of reducing agent, indicating that disulfide bridges mediate these interactions. The lower molecular weight species is consistent with the presence of one monomer of eMutL and one monomer of eβ and it can presumably form because Cys480 (MutL) and Cys367 (β) can partially react even in the absence of the β-binding motif. The higher molecular weight species is consistent with the presence of two monomers of eMutL and one monomer of eβ (180.7 kDa). The eβ variant has a partially exposed cysteine (Cys180) that could mediate the interaction with the second eMutL monomer. It is worth noting that Cys180 (β) does not mediate the interaction with a second monomer of eMutL unless the β-binding motif has been disrupted, suggesting that the conformation of the 130 kDa species formed with eMutL* may not be identical to that formed with eMutL (Figure 6).

The presence of the N-terminal domain of MutL bias the interaction to a singly bound MutL-β complex and this effect is independent of the presence of nucleotide (Supplementary Figure S6B), indicating that the nucleotide-induced conformational change of MutL is not required to form a specific complex with β.

### Functional implications of the MutL interaction with the β-clamp

We have previously shown that disruption of the β-binding motif found in MutL causes a severe mismatch repair defect in organisms lacking MutH, but only a moderate defect in those encoding a *mutH* gene (10,24). Mismatch recognition by MutS and MutS-dependent activation of MutL are common steps in MutH-dependent and MutH-independent mismatch repair pathways, suggesting that the *E. coli* β-
DNA substrates (Supplementary Figure S7A). Since both
interaction between MutL and the interac-
plain why disruption of the
donuclease activity of human MutL
stimulates the unwinding activity of UvrD when the MutL-
tary Figure S7A). However, we only observed a minimal in-
crease in UvrD unwinding activity when the MutL-β complex
replaced MutL and only when using long (>250 bp)
DNA substrates (Supplementary Figure S7A). Since both
MutL and MutL-β had similar ATPase and DNA binding
activities (Supplementary Figure S7B-C), the interaction
between MutL and β seems to be responsible for the en-
hanced stimulation of UvrD. The marginal effect would explain
why disruption of the β-binding motif in E. coli MutL only causes a weak mutator phenotype (10).

The functional implications of this interaction in B. sub-
tilis are starkly different. Human PCNA stimulates the endonuclease activity of human MutLα (21,22). Therefore, we
tested whether B. subtilis β could stimulate the nicking ac-
tivity of MutL on a linear substrate. To see the effect of
β, we set the experiment at concentrations of bMutL that barely had nicking activity (Figure 7A). Addition of sto-
chiometric amounts of B. subtilis β greatly stimulated the nicking activity of MutL, an effect that was dependent on the availability of divalent metal ions (Figure 7A). The effect of the β-clamp was also dependent on the physical interaction between MutL and β because the endonuclease activity of bMutL* was not stimulated by bβ (Figure 7B). Interestingly, bβ stimulated the endonuclease activity of bMutL to a greater extent when it was mixed with MutL rather than crosslinked to MutL (Figure 7C). The difference may be attributed to the restricted flexibility of the crosslinked MutL-β complex, or the fact that the crosslinked complex may be a mixture of specific and non-specific complexes, thereby reducing the effective concentration of functional MutL-β complex. While the latter is likely a contributing factor based on the presence of unspecific products when the crosslinking experiments were done with the active cysteine variant of B. subtilis MutLCTD (compare Figure 3B and Supplementary Figure S8), the former is probably important because it has been shown that DNA must access the central cavity of β to exhibit binding (7), and the permanent presence of MutL bound to β may prevent DNA access to the central cavity. It is also tempting to speculate that the transient nature of the MutL-β interaction could provide an integral regulatory mechanism to prevent excessive nicking by MutL. However, this idea awaits validation.

We have previously shown that the endonuclease domain of B. subtilis MutL (bMutLCTD) does not have endonucle-
ase activity because this region of the protein does not bind DNA (24). We presumed that β stimulates the nicking activity of MutL because it threads DNA onto the endonuclease site and, hence, predicted that the interaction with β may bypass the DNA-binding defect of the endonuclease domain. To probe this idea, we tested whether bβ stimulated the endonuclease activity of bMutLCTD and found that it, indeed, stimulated the nicking activity of bMutLCTD, but not that of the bMutLCTD* variant (Figure 7D). Interestingly, an 8-fold excess of bMutLCTD was required to observe comparable nicking activity to bMutL suggesting a role for the N-terminal domain in the endonuclease activity (Figure 7B and D, compare lane 4 in both gels). DNA bound at the N-terminus (26,57,58) likely enhances catalysis by increasing the frequency of MutL bound to DNA while the β-clamp may specifically feed DNA into its active site. Indeed, modelling the bMutLCTD-β complex using the solution scattering data and the X-ray data from bMutLCTD (PDB 3KDK) and bβ (PDB 4TR6) generated a model where the proximal endonuclease active site of MutLCTD is aligned with the central cavity of β (Supple-
mentary Figure S9). Therefore, it is not surprising that mu-
tations abrogating the MutL-β interaction result in strong mutator phenotypes in B. subtilis (24).

CONCLUSIONS

Characterization of the B. subtilis MutL endonuclease re-
vals that the β-binding motif in the endonuclease domain of MutL facilitates the β-dependent activation of MutL. The endonuclease domain of MutL is sufficient for cataly-
most binding partners interact with the sliding β-clamp using the same molecular determinants, this approach can be easily translated to study the roles of the sliding β-clamp in other cellular processes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Authors Contributions: M.C.P., A.G., V.M.P.B., M.D.S., J.R.R. and L.A.S. designed the experiments and analyzed data. M.C.P., J.C., V.M.P.B. and J.R.R. performed the experiments and prepared figures. M.C.P., A.G., M.D.S. and L.A.S. wrote the manuscript.

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Authors Contributions: M.C.P., A.G., V.M.P.B., M.D.S., J.R.R. and L.A.S. designed the experiments and analyzed data. M.C.P., J.C., V.M.P.B. and J.R.R. performed the experiments and prepared figures. M.C.P., A.G., M.D.S. and L.A.S. wrote the manuscript.

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