In early reactions of DNA mismatch repair, MutS recognizes mismatched bases and activates MutL endonuclease to incise the error-containing strand of the duplex. DNA sliding clamp is responsible for directing the MutL-dependent nicking to the newly synthesized/error-containing strand. In *Bacillus subtilis* MutL, the β-clamp-interacting motif (β motif) of the C-terminal domain (CTD) is essential for both in vitro direct interaction with β-clamp and in vivo repair activity. A large cluster of negatively charged residues on the *B. subtilis* MutL CTD prevents nonspecific DNA binding until β clamp interaction neutralizes the negative charge. We found that there are some bacterial phyla whose MutL endonucleases lack the β motif. For example, the region corresponding to the β motif is completely missing in *Aquifex aeolicus* MutL, and critical amino acid residues in the β motif are not conserved in *Thermus thermophilus* MutL. We then revealed the 1.35 Å-resolution crystal structure of *A. aeolicus* MutL CTD, which lacks the β motif but retains the metal-binding site for the endonuclease activity. Importantly, there was no negatively charged cluster on its surface. It was confirmed that CTDs of β motif-lacking MutLs, *A. aeolicus* MutL and *T. thermophilus* MutL, efficiently incise DNA even in the absence of β-clamp and that β-clamp shows no detectable enhancing effect on their activity. In contrast, CTD of *Streptococcus mutans*, a β motif-containing MutL, required β-clamp for the digestion of DNA. We propose that MutL endonucleases are divided into three subfamilies on the basis of their structural features and dependence on β-clamp.

DNA mismatch repair (MMR) recognizes and corrects mismatched bases that are generated by errors in DNA replication.

MutS initiates MMR by recognizing mismatched bases and recruits a nicking endonuclease that incises the mismatched duplex (1, 2, 9–11). Then, DNA helicase and single-stranded DNA-specific exonuclease removes the error-containing region. The resultant single-stranded DNA region is filled by DNA polymerase and DNA ligase. MMR needs to direct the nicking endonuclease activity to the error-containing strand of the mismatched duplex (1, 12–14). However, mismatch itself has no signal to discriminate which strand is incorrect.

In *Escherichia coli*, MutS, in cooperation with MutL, activates MutH endonuclease, which incises a GATC site. After DNA replication and before a site-specific DNA methylase works, hemi-methylated GATC sites exist, then MutH specifically incises the strand containing unmethylated GATC site, i.e. newly synthesized strand. Thus, *E. coli* MMR reads the absence of methylation as the strand discrimination signal. However, MutH endonuclease is only conserved in a part of γ-proteobacteria, and the other species adopt another strand discrimination signal.

Modrich and co-workers (15, 16) established that eukaryotic MutL homologues provide the nicking endonuclease activity required for MMR. In eukaryotes, the three distinguishable MutL homologues are known, MutLα, MutLβ, and MutLγ, which are heterodimers comprised of MLH1-PMS2 (Mlh1-Pms1 in yeast), MLH1-PMS1, and MLH1-MLH3, respectively. MutLα and MutLβ are involved mainly in MMR, whereas the major role of MutLγ is in meiotic DNA recombination. It has been clarified that PMS2 and MLH3 subunits contain a catalytic site for the nicking endonuclease activity, and the MLH1 subunit partially contributes to form the site (17, 18). It has also been revealed that the majority of eubacteria possess a homodimeric MutL, which shows significant amino acid sequence similarity to eukaryotic PMS2 (Pms1 in yeast) and harbors a nicking endonuclease activity essential for MMR (19–22).

MutL endonucleases have two domains: the N-terminal ATPase and the C-terminal endonuclease domains (15, 16, 23–25). Crystallographic analyses on the C-terminal domain (CTD) of eukaryotic and bacterial MutL endonuclease have
shown that the domain is composed of regulatory and dimerization subdomains (11, 26–28). The dimerization subdomain contains the metal-binding site essential for the endonuclease activity (29), in which two zinc ions are coordinated by the residues from highly conserved motifs: DQHEXXE, ACR, and CPHGRP (11, 15, 26, 30). These motifs are conserved in MutL from the majority of organisms except for limited species in γ-proteobacteria (12, 15, 16, 19–21, 31).

The in vitro reconstituted system using eukaryotic nuclear extracts has revealed that strand discontinuities in the substrate serve as a signal to direct the MutL homologue-dependent incision to the discontinuous strand (1, 2, 32, 33). It has been thought that the termini of the Okazaki fragment and the leading strand are utilized as the discrimination signal, indicating the daughter strand. Recently, accumulating evidences indicate that a nick or gap generated during repair processes of oxidative damage and mis-incorporated ribonucleotide in DNA also serves as a signal for the newly synthesized strand (34–36).

It also has been clarified that PCNA, the DNA sliding clamp, conveys the strand discrimination signal to MutL endonuclease through a direct interaction (10, 15, 37). PCNA binds to the 3′-terminus of the primed sites in DNA. Because PCNA has two nonequivalent faces and binds to a strand break with a specific orientation, the direct interaction between PCNA and MutL CTD may facilitate the asymmetric binding of the mismatched duplex.

The strand discrimination strategy in bacterial MMR is thought to be similar to that in eukaryotic one. *Bacillus subtilis* MutL (BsMutL) endonuclease is also interacted and stimulated by a bacterial counterpart of PCNA, β-clamp (β subunit of DNA polymerase III holoenzyme) (38). β-Clamp-interacting motifs are found in a variety of bacterial proteins that are involved in DNA replication and repair (39–41). Pillon et al. (26) has shown that the regulatory subdomain of BsMutL CTD contains the β-clamp-interacting motif (β motif) as QEMIVP. They confirmed that the β motif is responsible for the in vitro interaction with β-clamp and that mutation in the motif abolishes the MMR activity in vivo (26, 38, 42). To explain the enhancing effect of β-clamp interaction, an attractive model has been proposed in which a cluster of negatively charged residues in MutL CTD prevents the DNA binding and β-clamp neutralizes the negative charge to enable DNA binding (26).

Interestingly, MutL endonucleases from Aquificae phylum lack the regulatory subdomain (see Fig. 1). In addition, MutL endonucleases from Deinococcus-Thermus phyla have no obvious β-clamp-interacting motifs, although they retain the regulatory subdomain (see Fig. 1). We previously reported a partial in vitro reconstituted MMR system by using proteins from *Thermus thermophilus*, where incision by MutL was mismatch-, MutS-, and ATP-dependent (43). However, *T. thermophilus* MutL incised both strands of the duplex despite the existence of strand discontinuity, β-clamp, and clamp loader. In summary, in the partial reconstituted system of *T. thermophilus* MMR, although the mismatch specificity is reconstituted, the strand discrimination is not yet. Therefore, it should be carefully investigated whether or not the strand discrimination mechanism is universal among the MutL endonuclease–possessing species.

In this study we characterized the structures and functions of β motif-containing and -lacking CTds of MutL endonucleases. As the result, it was revealed that there were β-clamp-dependent and -independent MutL endonucleases. We propose that bacterial MutL endonucleases can be divided into three subfamilies: subfamily I, II, and III. Subfamily I MutL endonucleases are characterized by the β motif, negatively chargedcluster on the surface of their CTds and requirement of β-clamp for their endonuclease activity. Subfamily II and III MutL endonucleases lack the β motif and negatively charged cluster on their CTds, and β-clamp is not essential for their endonuclease activities. Subfamily III MutLs are distinguishable from subfamily II MutLs by their complete missing of the regulatory subdomains in their CTds. Our results provide a possibility that bacterial species possessing subfamily II and III MutLs adopt a β-clamp-independent mechanism for directing the MutL endonuclease activity to the error-containing strand.

**Results and Discussion**

**Primary Structure Analysis—**BsMutL CTD includes the β motif QEMIVP (QXX(L/I)XP) at its regulatory subdomain (26). It is reported that the glutamine to an alanine or the isoleucine to an aspartate mutations in the motif significantly increased the mutation rate of *B. subtilis* to the same extent with that caused by the disruption of nucleotide motif (26, 38). On the other hand, the replacement of the proline residue in the motif with an alanine did not show any influence on the in vivo mutation rate (26, 38).

We found that MutL CTds from Aquificae and Deinococcus-Thermus phyla seemed to contain imperfect or no amino acid sequence corresponding to the β motif QXX(L/I)XP (Fig. 1A). Subsequently, we performed exhaustive analysis searching for the MutL lacking the β motif using BLAST search (44) and ClustalW alignment program (45). As a result, the complete loss of the regulatory subdomain was found only in MutL from the Aquificae phylum. On the other hand, MutL proteins without the glutamine and leucine/isoleucine residues of the β motif were found not only in Deinococcus-Thermus but also in Thermotoga, Cyanobacteria, and Nitrospira phyla (Fig. 1B). Thus, a significant proportion of the bacterial species seemed to be equipped with MutL endonuclease lacking the β motif in its CTD.

In this study, for convenience, we divided MutL endonucleases into three subfamilies. Subfamily I MutLs contain the β motif, QXX(L/I)XP, in their CTds, whereas subfamily II MutLs lack the critical glutamine and leucine/isoleucine residues of the motif. Subfamily III MutLs are only found in Aquificae phylum and lack the β-clamp-interacting regulatory subdomain of CTD.

**Overall Structure of aqMutL CTD—**The crystal structure of aqMutL CTD was determined by cadmium single-wavelength anomalous dispersion phasing, which was refined to 1.35 Å with R < 1.0% and Rfree values of 14.0 and 18.9%, respectively (Fig. 2A, Table 1). The tertiary structure of aqMutL CTD showed significant similarity to that of the dimerization subdomain in BsMutL CTD and *S. cerevisiae* MutLo CTD (Fig. 2, B and C). The superposition of the structures was performed by using Dali pairwise comparison program (46). The root mean square deviations from aqMutL CTD to
MutL Endonucleases without β-Clamp-interacting Motif

**FIGURE 1.** The CTD of MutL endonuclease. A, a schematic representation of the domain structures of MutL endonucleases. MutL endonuclease is composed of the N-terminal ATPase and C-terminal endonuclease domain. BsMutL CTD is further divided into dimerization and regulatory subdomains. The dimerization subdomain includes conserved sequence motifs required for the endonuclease activity and zinc binding. ttMutL CTD retains the regulatory subdomain. The glutamine and leucine/isoleucine residues critical for β-clamp interaction are not conserved in ttMutL CTD. aqMutL lacks the region corresponding to the regulatory subdomain. B, amino acid sequence alignment around the β-motif in MutL endonucleases. Sequences were aligned by ClustalW and visualized by GeneDoc software. Asterisks indicate the metal binding motif essential for the endonuclease activity. The underline indicates the β-motif-interacting motif, QXX(L/I)XP. The phylum names are described in the parenthesis after the species names. Numbers on the left of the sequences indicate the distances from the protein N termini.

**FIGURE 2.** The crystal structure of aqMutL CTD. A, overall structure of aqMutL CTD monomer. The locations of conserved motifs are indicated. All graphic representations of crystal structures in this paper were prepared using PyMol software. B, superimposition of aqMutL CTD and BsMutL CTD (PDB ID 3DKK). C, superimposition of aqMutL CTD and the CTD of PMS1 subunit of S. cerevisiae MutL (PDB ID 4E4W). D, dimeric structures of aqMutL CTD (upper) and BsMutL CTD (lower). One subunit of the two is shown in color. E, the α1 helix of aqMutL CTD (blue) is directly connected to α2 helix via a short turn indicated by a blue arrow. On the other hand, α1 helix of BsMutL CTD (gray) is connected to regulatory subdomain. F, the α1 and α2 helices of aqMutL CTD connected by a helix-turn-helix-like local structure contain the conserved DQHA(X)E(X)E and ACR motifs (underlined residues in the motifs are labeled in the panel). G, the endonuclease assay of wild type and R372A mutant of aqMutL. The 573-bp dsDNA was incubated with the proteins at 60 °C for 1 h, and the incised products were separated by polyacrylamide gel electrophoresis under denaturing conditions as described in “Experimental Procedures.” H, the ratio of digested DNA signals to all DNA signals in G were quantified. The initial rates were calculated and plotted against protein concentrations. The experiments were repeated three times, and the average values are shown with S.D.
Importantly, each of the regulatory subdomain is specific for MutL CTD that lacks the regulatory subdomain. Therefore, the helix-turn-helix-like local structure of the α1 helix by an extremely short turn, which resembles the helix-turn-helix motif of DNA-binding proteins (Fig. 2, E and F). On the other hand, α1 helix of BsMutL is followed by its regulatory subdomain. Therefore, the helix-turn-helix-like local structure is specific for MutL CTD that lacks the regulatory subdomain. Importantly, each of the α1 and α2 helices contains the well conserved sequence motifs DQHAX_4E and ACR, respectively. The catalytic role of DQHAX_4E and ACR motifs for the endonuclease activity has been clarified by a number of previous studies (15, 19, 21, 26, 33). As shown later, we also found that simultaneous replacements of Asp-351 and Glu-357 of the X_4E motif for the endonuclease activity. Thus, the helix-turn-helix-like local structure (α1 and α2 helices) of aqMutL CTD maintained the three-dimensional positions of the conserved residues essential for the endonuclease activity.

### TABLE 1

| Parameters          | Native | Cd-SAD |
|---------------------|--------|--------|
| Data collection*   |        |        |
| Beamline            | SPRing 8 BL38B1 | SPRing 8 BL38B1 |
| Detector            | Rayonix MX225HE | Rayonix MX225HE |
| Wavelength (Å)      | 1.000  | 1.500  |
| Exposure time (s)   | 1.0    | 1.0    |
| Camera distance (mm) | 100   | 100    |
| Oscillation angle (°) | 0.5  | 0.5    |
| Oscillation range (°) | 180  | 360    |
| Space group         | P4_32,2 | P4_32,2 |
| Cell dimensions a, b, c (Å) | 35.3, 35.3, 161.5 | 35.3, 35.3, 161.4 |
| Resolution (Å)      | 24.7-1.35 (1.40-1.35) | 24.7-1.67 (1.73-1.67) |
| Rmerge (%)          | 5.6 (84.3) | 6.9 (27.1) |
| l/a                | 59.8 (2.7) | 92.5 (6.3) |
| dRmerge (%)         | 82.1 | 97.4 |
| Completeness (%)    | 99.8 (100) | 99.0 (92.4) |
| Redundancy          | 13.2 (13.5) | 21.5 (8.2) |

* One crystal was used for each data set. Values in parentheses are for the highest resolution shell.

![FIGURE 3. Metal-binding site of aqMutL CTD.](image)

As expected from the sequence analysis (Fig. 1), the crystal structure confirmed the lack of the regulatory subdomain in aqMutL CTD (Fig. 2D). The α1 helix is connected directly to α2 helix by an extremely short turn, which resembles the helix-turn-helix motif of DNA-binding proteins (Fig. 2, E and F). On the other hand, α1 helix of BsMutL is followed by its regulatory subdomain. Therefore, the helix-turn-helix-like local structure is specific for MutL CTD that lacks the regulatory subdomain. Importantly, each of the α1 and α2 helices contains the well conserved sequence motifs DQHAX_4E and ACR, respectively. The catalytic role of DQHAX_4E motif for the endonuclease activity has been clarified by a number of previous studies (15, 19, 21, 26, 33). As shown later, we also found that simultaneous replacements of Asp-351 and Glu-357 of the X_4E motif for the endonuclease activity. Thus, the helix-turn-helix-like local structure (α1 and α2 helices) of aqMutL CTD maintained the three-dimensional positions of the conserved residues essential for the endonuclease activity.

**Three Metal Ions Were Found around the Nuclease Catalytic Site**—The endonuclease activity of MutL required divalent metal ions such as manganese, cobalt, and nickel ions (19, 21, 23, 26). Although zinc ion alone was not sufficient for exertion of the endonuclease activity, co-existence of zinc and manganese ions is effective on the N-terminal domain-dependent stimulation of the CTD in aqMutL (23). These results suggest that MutL binds zinc and other divalent ions simultaneously and zinc ion has a regulatory effect on the activity.

The previously reported crystal structures of CTDs of BsMutL (26) and *S. cerevisiae* MutLa (28) contained two zinc ions at the metal-binding site composed of DQHAX_4E, ACR, and CPHGRP motifs. In this study the crystal of aqMutL CTD was grown in the buffer containing cadmium ions, and as shown in Fig. 3A, three cadmium ions were found at the metal-binding site. The locations of the cadmium ions were confirmed in the anomalous Fourier difference map. The cadmium ion in the M1 position is coordinated by side chains of Glu-357, Cys-402, and His-404 from DQHA liganded by His-353, Glu-357, and Cys-371 side chains from *S. cerevisiae* PMS1 CTD. Two zinc ions and amino acid side chains from *S. cerevisiae* PMS1 CTD are shown in gray.
Among the three cadmium ions, the positions M1 and M2 were exactly the same as the previously reported positions of zinc ions in BsMutL CTD (Fig. 3B) and S. cerevisiae MutL/H9251 CTD (Fig. 3C), which is consistent with the fact that aqMutL possesses an endonuclease activity like other MutL endonucleases. The other position M3 is unique for our structure. Cadmium ion is known to behave quite similarly to zinc ion; therefore, the observed M3 position of the cadmium ion may represent a novel zinc-binding site in aqMutL. Alternatively, it is possible that the position M3 is the binding site for manganese ion that is critical for the endonuclease activity. Mizushima et al. (47) have revealed that, using NMR spectroscopy, some of zinc-coordinating residues also participate in the binding of manganese ion. However, we cannot exclude the possibility that the position M3 does not reflect the physiological metal-binding state because our crystallization buffer used here contained an unphysiological concentration (50 mM) of cadmium ion.

The Endonuclease Activities of MutL in the Absence of β-Clamp—We found that aqMutL, a subfamily III MutL endonuclease, has no obvious cluster of negatively charged residues on the surface of CTD (Fig. 4B). In BsMutL, a subfamily I MutL endonuclease, a negatively charged region is found on the surface of the regulatory and dimerization subdomains of CTD (Fig. 4B, lower panel). It has been proposed that the negative charge prevents binding of the DNA phosphate backbone and that the interaction of β-clamp masks the charge to allow DNA binding (26, 38, 42). Intriguingly, it was found that the majority of residues forming the negatively charged cluster in BsMutL CTD were conserved in subfamily I MutL endonucleases but not in subfamilies II and III MutLs, that lack the β-clamp-interacting motif. The predicted structures of T. thermophilus MutL (ttMutL) CTD (subfamily II) and Streptococcus mutans MutL (smMutL) CTD (subfamily I) are shown in Fig. 4, C and D, respectively, which illustrate that the presence or absence of the negatively-charged cluster is correlated with the presence or absence of the β motif, QXX(L/I)XP.

These findings led us to a hypothesis that the CTDs from subfamily II and III MutL endonucleases incise substrate DNA efficiently even in the absence of β-clamp. To test this, we compared the endonuclease activities of MutL CTDs using smMutL CTD, ttMutL CTD, and aqMutL CTD concentrations. Triangles in G and H represent the results of D364A ttMutL CTD and D351A/E357Q aqMutL CTD, respectively. Experiments were repeated three times, and the average values are shown. Bars indicate S.D.
As shown in Fig. 4, E–H, both aqMutL CTD (subfamily III) and ttMutL CTD (subfamily II) efficiently incised the plasmid DNA to generate open circular form. Replacements of Asp and Glu residues in the DQHX,EX,E diminished the endonuclease activities of both aqMutL CTD and ttMutL CTD (Fig. 4, G and H), showing that we detected the bona fide nicking activities of the proteins, not a contaminating nuclease activity. On the other hand, smMutL CTD (subfamily I) exhibited no endonuclease activity in the absence of β-clamp (Fig. 4F), which is consistent with a previous report about the BsMutL CTD (subfamily I) (26). The reaction period was elongated from 30 min to 3 h; however, the nicking activity of smMutL CTD was still concealed (data not shown). The lack of detectable activity is not due to denaturation of the protein, because we, as shown later, confirmed that smMutL CTD incises DNA in the presence of β-clamp. These results strongly support our hypothesis that CTDs from subfamilies II and III MutL endonucleases are active on their own, whereas subfamily I MutL CTD is not, probably because its negatively charged region prevents DNA binding.

**β-Clamp Had No Effect on the Endonuclease Activity of aqMutL and ttMutL.**—Previously, Pillon et al. (38) showed that, in BsMutL, the endonuclease activity of CTD against linear double-stranded DNA (dsDNA) was strongly activated by direct interaction with β-clamp. As mentioned above, although the catalytic dimerization subdomain of MutL CTD is highly conserved among all subfamilies of MutLs, CTDs of subfamilies II and III MutLs lack the β-motif. In addition, the CTDs of subfamilies II and III seem to have no cluster of negatively charged side chains that is expected to be essential for β-clamp-dependent regulation of the endonuclease activity. Then, we examined whether or not β-clamp stimulates the endonuclease activities of ttMutL (subfamily II) and aqMutL (subfamily III).

The assay was performed using a linear dsDNA as the substrate, as it is known that the termini of dsDNA enable the β-clamp to bind dsDNA without clamp loader (48). The 573-bp dsDNA was amplified, purified, and incubated with MutL proteins. The endonuclease activity of MutL CTDs against linear dsDNA is extremely weak; therefore, we used full-length MutL proteins for this assay. The reaction products were separated by polyacrylamide gel electrophoresis under denaturing conditions, and the initial rate for the reaction was determined.

First, as a positive control, we examined the activity of subfamily I, smMutL, with Streptococcus mutans β-clamp. smMutL contains the β motif QQLVP in its CTD (Fig. 1A). In the absence of β-clamp, smMutL digested the substrate dsDNA at an extremely weak intensity, and initial rates did not saturate under the examined conditions (Fig. 5A). The addition of S. mutans β-clamp significantly increased the intensity, and initial rates reached a plateau. It is also confirmed that S. mutans β-clamp exhibits a clear enhancing effect on the nuclease activity of smMutL CTD (data not shown), strongly indicating that the enhancing effect is based on the interaction between MutL CTD and β-clamp. The dependence of smMutL endonuclease activity on the β-clamp is in good agreement with the previous study on BsMutL (38), a subfamily I MutL.

Next, we tested the dependence of ttMutL and aqMutL endonuclease activities on β-clamp. We confirmed that the T. thermophilus and A. aeolicus β-clamp possess binding activities against linear dsDNA in the absence of clamp loader (data not shown), which is consistent with previous report on E. coli β-clamp (48). MutL endonucleases from Deinococcus-Thermus phylum retain the regulatory subdomain in their CTDs; nevertheless, they have no sequence satisfying the criteria for the clamp-interacting motif. Our previous study showed that the endonuclease activity of ttMutL was indeed required for in vivo MMR activity but that β-clamp and clamp-loader were not able to direct the ttMutL-dependent incision to the discontinuous strand in the in vitro reconstituted system (43). As shown in Fig. 5, B and C, (left and middle panels), β-clamp showed no positive influence on the activity of ttMutL and aqMutL. The experiments with lower and higher substrate concentrations (Fig. 5, B and C, right panel) showed decreased and increased reaction rates, respectively. This excludes the possibility that saturation observed in the middle panel was caused by high MutL:substrate ratios, masking the effect of β-clamp. Thus, the endonuclease activities of both ttMutL and aqMutL were not stimulated by β-clamp. The independence of ttMutL and aqMutL on β-clamp is consistent with the fact that ttMutL and aqMutL CTDs lack the β-motif.

It has been reported that bacterial MutL proteins contain a β-clamp-interacting site also at its N-terminal ATPase domain and that the N-terminal domain interacts with β-clamp in an ATP-dependent manner (40, 41). The LF motif in the N-terminal ATPase domain of E. coli MutL was verified to be essential for the interaction with β-clamp. We found that the LF motif of N-terminal domain is highly conserved among MutL endonucleases from almost all phyla including Aquifaeae and Deinococcus-Thermus. Therefore, experiments were performed in the presence of ATP; however, we failed to detect any enhancing effect of β-clamp on the endonuclease activities of full-length aqMutL and ttMutL (data not shown). In conclusion, the endonuclease activity of subfamily II and III MutL was independent of β-clamp.

**MutL Proteins without the β Motif in Their CTDs Have Short or No Interdomain Linkers between the N- and C-terminal Domains.**—We found that the existence and non-existence of the β motif are highly correlated with the length of the interdomain linker region between the N-terminal and C-terminal domains of bacterial MutL proteins (Fig. 6). When the MutL protein contains the β motif in its CTD, the protein has a longer interdomain linker and vice versa. In eukaryotes, a possible role of the flexible interdomain linker is implicated in bypassing nucleosome structures (49). However, to date, the physiological function of the interdomain linker region of bacterial MutL is totally unclear. The linker may be required for the β-clamp-dependent discrimination of the newly synthesized strand. In any case, the co-existence of imperfect clamp-interacting motif and the shorter interdomain linker would be utilized as a simple hallmark for the β-clamp-independent MutL endonuclease.

**Conclusions**

In this study we divided bacterial MutLs into three subfamilies, I, II, and III. Subfamily I MutL endonucleases are homologous to BsMutL and smMutL. They are characterized by the existence of the β motif QXX(L/I)XP, the negatively charged clus-
MutL Endonucleases without β-Clamp-interacting Motif

A

Without β-clamp With β-clamp (5 μM)

[sMutL] (μM) 0 0.8 1.5 3 6 12 0 0.8 1.5 3 6 12

Withtype + 5 μM β-clamp D474A/E480Q + 5 μM β-clamp

B

Without β-clamp With β-clamp (5 μM)

[ttMutL] (μM) 0 0.5 1 2 3 4 0 0.5 1 2 3 4

Withtype + 5 μM β-clamp Wildtype D364A

C

Without β-clamp With β-clamp (5 μM)

[aqMutL] (μM) 0 0.5 1 2 3 4 0 0.5 1 2 3 4

Withtype + 5 μM β-clamp Wildtype D351A/E357Q

FIGURE 5. Effect of β-clamp on the endonuclease activity of MutL proteins. A, the substrate 573-bp linear dsDNA (0.57 μM) was reacted with full-length smMutL in the presence (5 μM) or absence of S. mutans β-clamp at 37 °C for 5 h. The reactions were also performed with different concentrations of smMutL for 3 h and the ratios of digested DNA signals to unreacted DNA signals were quantified. Then, initial rates were calculated and plotted against concentrations of smMutL (right panel). Diamonds, circles, and triangles indicate reactions of wild type ttMutL, wild type smMutL with β-clamp, and D474A/E480Q smMutL with β-clamp, respectively. B, the substrate dsDNA (0.57 μM) was incubated with various concentrations of full-length ttMutL in the presence of 0, 5, or 10 μM T. thermophilus β-clamp at 55 °C for 3 h. The reactions were also performed for 1 h, and initial rates were calculated and plotted against protein concentrations in the middle panel. Diamonds, circles, squares, and triangles indicate the profiles of wild type ttMutL, wild type ttMutL with 5 μM β-clamp, wild type ttMutL with 10 μM β-clamp, and D364A ttMutL, respectively. The reactions by wild type ttMutL were also examined using 0.14 or 2.2 μM substrate dsDNA (right panel). The activity of the full-length aqMutL against 0.57 μM dsDNA was examined in the presence of 0, 5, or 10 μM A. aeolicus β-clamp at 60 °C for 3 h. The reactions were also performed for 1 h, and initial rates were calculated and plotted against protein concentrations in the right panel. Diamonds, circles, squares, and triangles indicate reactions of wild type ttMutL, wild type aqMutL with 5 μM β-clamp, wild type ttMutL with 10 μM β-clamp, and D351A/E357Q ttMutL, respectively. The reactions by wild type aqMutL were also examined using 0.14 or 2.2 μM substrate dsDNA (right panel).

FIGURE 6. Interdomain linkers of bacterial MutL endonucleases. A, a schematic representation of the interdomain linker region of MutL endonucleases. B, comparison of amino acid residue content in the interdomain linker region. The amino acid sequences of MutL endonucleases were aligned within each phylum (each sheet for one phylum in supplemental Table S1) in which one can confirm the residue numbers of the interdomain linker regions.

The existence of β-clamp-independent MutL endonucleases implies that some bacterial species adopt a β-clamp-independent strand discrimination strategy for MMR. This notion is also supported by previously reported in vitro partial reconstitution system of T. thermophilus MMR, where β-clamp has no effect on the strand incision (43). To unveil the mechanism, biochemical properties of clamp-independent MutL endonuclease should be further examined by experiments, such as investigating the nucleotide-sequence specificity, binding partner, and response to ATP. In addition, experiments to test the MMR activity using cell extracts would be beneficial.
onto DNA, although strand-discrimination mechanism of *E. coli* MMR does not rely on β-clamp (41). This interaction is suspected to be required for excision step in MMR. Because subfamily II and III MutL endonucleases retain a β-clamp-interacting motif, LF, at their N-terminal ATPase domains just like *E. coli* MutL, it may be possible that they are loaded onto DNA by β-clamp at some step during MMR.

**Experimental Procedures**

**Plasmid Construction**—DNA fragments coding the full-length and the CTD (residues 325–425) of aqMutL were generated by PCR using *A. aeolicus* VF5 genomic DNA as a template. The forward and reverse primers used for the amplification of the full-length and the CTD fragments were 5’-ATATCATATGGCCCTCCCCGAGCCCAAGCCCCTC-3’ and 5’-ATATAGATCTTTAAGGTTCTCGGGGTAGAGGTG-3’, respectively; the underlines in the forward and reverse primers represent NdeI and BglII sites, respectively. The DNA fragments generated fragments were digested by NdeI and BamHI and cloned into the corresponding sites of pET-11a vector (Novagen). The amplified fragments were cloned into pET-15b/smMutL and pET-15b/ttMutL CTD as templates. The primer sets used for the first and second mutagenesis reactions were 5’-ATCATAGCTCAACATGCT-GCTCAAGAA-3’ and 5’-ATGGTGAGCTATGATATAGAGTTGAGCTATGATTAGAAT-3’, respectively. Plasmid expressing D351A/E357Q double mutants of aqMutL and aqMutL CTD were also constructed through twice PrimeSTAR mutagenesis reactions using pET-11a/aqmutL and pET-11a/aqmutL CTD as templates. The primer sets for the first and second processes were 5’-TTCGGCAGACCTCCTTTGGAAGAAG-3’ and 5’-GTGCTGGGCCACGAGTACGTAATC-3’, respectively. Expression plasmid for R372A aqMutL was also generated by mutagenizing pET-11a/aqmutL plasmid using the same procedure. The following primer set was used: 5’-GCCTTGAGGATTTCG-TAAAGCCGGA-3’ and 5’-GGAATAATCTGACAA-AAA-3’ and 5’-TATCCTGTTCAAAGGAGGTGCTGGGC-3’, respectively. Expression plasmid for D364A mutant of ttMutL was also mutagenized using pET-11a/ttmutL plasmid using the same procedure. The following primer set was used: 5’-GGATCCTCAGTATTTCCCTA ATTCCCTCAAACTAG-3’ and 5’-ATATCATATGCGCGTTAAGGTGGACAGGGAGGA-3’, respectively. Nucleotide sequencing analyses revealed that all constructions were error-free.

**Expression and Purification of Proteins**—aqMutL, aqMutL CTD, ttMutL, *A. aeolicus* β-clamp, *T. thermophilus* β-clamp, and their mutants were prepared as tag-free forms, whereas ttMutL CTD, smMutL CTD, and *S. mutans* β-clamp, and their mutants were prepared in His-tagged forms. aqMutL, aqMutL CTD, ttMutL, His-tagged ttMutL CTD, and *T. thermophilus* β-clamp were prepared as previously described (19, 43, 50). Mutants of aqMutL, aqMutL CTD, ttMutL, and His-tagged ttMutL CTD were prepared by the same procedure as for wild type proteins.

Rosetta2(DE3) pLysS was transformed with pET-11a/*A. aeolicus* β-clamp. One liter of LB medium (Difco) containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol was inoculated with 5 ml of the overnight preculture. After 4 h of cultivation at 37 °C, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.2 mM. The cells were further cultivated at 37 °C for 3 h and then harvested by centrifugation. The cells were lysed by sonication in buffer I (50 mM Tris-HCl (pH 8.0) and 0.1 mM DTT) and heated at 80 °C for 20 min. The lysate was immediately chilled on ice for 10 min. After centrifugation at 15,000 × g for 10 min, the supernatant was loaded onto a Toyopearl SuperQ-650 column (20 ml) (TOSOH). The column was washed with 100 ml of buffer I and further washed with 100 ml of buffer I containing 50 mM NaCl. Proteins were eluted with 100 ml of buffer I containing 300 mM NaCl. The fractions containing *A. aeolicus* β-clamp were detected by SDS-PAGE, collected, and dialyzed against one liter of buffer I three times at 4 °C. The protein solution was concentrated with a Vivaspin concentrator (Vivasience). Protein concentrations were determined on the basis of values of absorbance at 278 nm.
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theoretical molar extinction coefficients for the proteins were calculated using previously described method (51).

Rosetta2(DE3) pLysS was transformed with pET-16b/S. mutans mutL CTD. The 5-ml overnight preculture was inoculated into 1 liter of LB medium containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol. After a 3-h cultivation at 37 °C, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.2 mM. The cells were further cultivated at 37 °C for 3 h and then harvested by centrifugation. The cells were lysed by sonication in buffer I. After centrifugation at 15,000 g for 10 min, the supernatant was loaded onto a Talon resin (5 ml) (Clontech). The column was washed with 100 ml of buffer I and 100 ml of buffer I containing 5 mM imidazole. Proteins were eluted with a 100-ml gradient of 5–200 mM imidazole in buffer I. Fractions containing His-tagged smMutL CTD were collected and dialyzed against 1 liter of buffer I 3 times at 4 °C. His-tagged smMutL, His-tagged D474A/E480Q smMutL, and His-tagged S. mutans β-clamp were also prepared by the same procedure as that for His-tagged smMutL CTD.

Electrophoretic Mobility Shift Assay—The 5′-terminal Cy5-labeled 30-mer single-stranded DNA, 5′-CGGTATCTTGAC-TATGACCCTCTACGAGC-3′ was synthesized (BEX Co., Tokyo, Japan) and annealed to complementary single-stranded DNA (BEX Co.) to obtain 30 bp of dsDNA. The 100 nm 30-bp dsDNA was incubated with various concentrations of T. thermophilus β-clamp or A. aeolicus β-clamp in 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin for 30 min at 25 °C. The concentrations of proteins used are indicated at the top of the figure panels. The reaction mixture (10 µl) was loaded onto a 9% polyacrylamide gel and then electrophoresed in TBE buffer (89 mM Tris borate and 2 mM EDTA). DNA in the gel was detected using LAS3000 image analyzer (Fuji Film, Tokyo, Japan).

Crystallization and Structure Determination—aqMutL CTD was crystallized by the hanging drop vapor diffusion method. Then 1 µl of 29.4 mg/ml protein solution was mixed with an equal volume of 200 mM sodium acetate (pH 4.6) containing 100 mM cadmium chloride and 30% (v/v) polyethylene glycol 400. The drop was equilibrated against 50 µl of the reservoir solution at 20 °C.

The crystal of aqMutL CTD was cryo-cooled without an additive cryoprotectant at −173 °C. The data collection was performed as described previously with some modifications. In summary, 20 ng/ml concentrations of substrate pUC19 plasmid DNA was incubated with various concentrations of MutL CTDs in the buffer containing 50 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM MnCl2, 5 mM MgSO4, 0.5 mM ZnCl2, 0.1 mM DTT, and 5% glycerol. The concentrations of proteins, reaction temperatures, and incubation periods are indicated in the legends for figures. The reactions were terminated by the addition of one-fifth volume of the loading buffer (5 mM EDTA, 1% SDS, 50% glycerol, and 0.05% bromophenol blue). The reaction solutions were loaded onto a 1.0% agarose H14 (Takara) gel containing 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA and electrophoresed in the same buffer. The gels were stained with SYBR Green I (Thermo Fisher Scientific), and bands of DNA fragments were detected using LAS3000 imaging system (Fuji Film). The amounts of digested and undigested DNA were quantified using Multi Gauge version 3.0 software (Fuji Film).

Endonuclease Assay Using Linear dsDNA—A 573-bp DNA fragment was amplified from the ttMutL CTD coding region with primers 5′-ATGAGCCCCACCGG-3′ and 5′-TCACGGCGGCTCCG-3′ using pET-16b/ttnuL CTD plasmid as the template. The amplified DNA fragment was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and suspended in 5 mM Tris-HCl (pH 8.0). The 0.14, 0.57, or 2.2 µM 573-bp linear dsDNA was incubated with various concentrations of MutL proteins in a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM MnCl2, 5 mM MgSO4, 0.5 mM ZnCl2, 0.1 mM DTT, and 5% glycerol. The protein concentrations, reaction temperatures, and reaction periods are described in the leads to figures. The reactions were terminated by the addition of an equal volume of sample buffer (5 mM EDTA, 80% deionized formamide, 10 mM NaOH, 50% glycerol, 0.1% xylene cyanol, and 0.1% bromophenol blue). The reaction solutions were loaded onto a 12% polyacrylamide gel containing 8 M urea and electrophoresed in a buffer containing 20 mM Tris, 10 mM acetic acid, and 0.5 mM EDTA. When the reaction was performed using 2.2 µM substrate dsDNA, 1/4 of the reaction solution was loaded onto the gel to make it possible to precisely measure the high signal of unreacted substrate. Gels were stained with SYBR Gold (Thermo Fisher Scientific), and DNA fragments were detected using LAS3000 imaging system. The amounts of DNA fragments were quantified using Multi Gauge version 3.0 software. The rate of reaction was calculated by quantifying the proportion of products to unreacted substrate.

Author Contributions—K. F. and T. Y. conceived and designed the work. K. F. constructed the expression plasmids, prepared the proteins, crystallized the protein, and performed the endonuclease assays of MutL proteins. K. F., S. B., and T. K. performed the crystallography. K. F. and T. Y. wrote the paper with help from S. B. and T. K.

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