Activation of the mismatch-specific endonuclease EndoMS/NucS by the replication clamp is required for high fidelity DNA replication

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ABSTRACT

The mismatch repair (MMR) system, exemplified by the MutS/MutL proteins, is widespread in Bacteria and Eukarya. However, molecular mechanisms how numerous archaea and bacteria lacking the mutS/mutL genes maintain high replication fidelity and genome stability have remained elusive. EndoMS is a recently discovered hyperthermophilic mismatch-specific endonuclease encoded by nucS in Thermococcales. We deleted the nucS from the actinobacterium Corynebacterium glutamicum and demonstrated a drastic increase of spontaneous transition mutations in the nucS deletion strain. The observed spectra of these mutations were consistent with the enzymatic properties of EndoMS in vitro. The robust mismatch-specific endonuclease activity was detected with the purified C. glutamicum EndoMS protein but only in the presence of the β-clamp (DnaN). Our biochemical and genetic data suggest that the frequently occurring G/T mismatch is efficiently repaired by the bacterial EndoMS-β-clamp complex formed via a carboxy-terminal sequence motif of EndoMS proteins. Our study thus has great implications for understanding how the activity of the novel MMR system is coordinated with the replisome and provides new mechanistic insight into genetic diversity and mutational patterns in industrially and clinically (e.g. Mycobacteria) important archaeal and bacterial phyla previously thought to be devoid of the MMR system.

INTRODUCTION

In all three domains of life, the fidelity of DNA replication is crucial for faithful transfer of genetic information between generations, as uncorrected errors may lead to mutations potentially causing cell death, cancer and neurogenerative diseases (1). The fidelity of DNA replication is ensured by several distinct mechanisms including the capacity of DNA polymerases to select the correct nucleotide to be incorporated by the replisome (2). During the replication process, errors occasionally occur on the nascent (daughter) strand, and are detected and corrected by either the proofreading activity of DNA polymerases or the post-replicative mismatch repair (MMR) system (3). The functional role of the core MMR proteins MutL and MutS in the maintenance of the replication fidelity is exemplified by the fact that the genetic inactivation of the MMR system typically lowers the replication fidelity by two or three orders of magnitude and results in the accumulation of transition mutations. MMR also acts on mismatches and strand misalignments during recombination and controls recombination between similar, but non-identical sequences (4). Consequently, MMR inhibits interspecies recombination and gene exchange, and thus has wide implications for understanding the evolution of species (5).

In Escherichia coli and some other γ-proteobacteria, the ATP-dependent assembly of the MutS and MutL heteroduplex at the sites of mismatched bases leads to the activation of the MutH endonuclease activity, which selectively cleaves transiently non-methylated dam methylation sites (GATC), thus facilitating the selective unwinding and degradation of the mismatch by different exonucleases (6). The resulting daughter strand ‘nick’ created by MutH thus directs MMR to the neosynthesized strand (methyl-directed MMR). In E. coli, MutS and MutL strongly interact with the replication clamp (β-subunit) and the catalytic α-subunit of the
DNA polymerase III (7), indicating that MMR and DNA replication are intimately coupled. In most other species, including humans, for which the mismatch repair systems have been characterized, hemimethylation does not play a role in strand discrimination (8). In these cases, the strand specificity signal have been proposed to correspond to DNA strand discontinuities created by Okazaki fragment processing and/or processing of frequently misincorporated ribonucleotides (8). These daughter strand ‘nicks’ may facilitate the asymmetric loading of the MutSo-MutLo complex mediated by the replication clamp PCNA (9), resulting in strand-specific MMR due to the selective incision made by MutL in the vicinity of the mismatch (nick-directed MMR). Therefore, at least two independent solutions coordinated by replication clamps have emerged during evolution to differentiate the newly synthesized DNA strand with the replication errors (mismatches) from the template strand with the correct genetic information.

Previous bioinformatics studies failed to identify the genes encoding the canonical MMR proteins in the majority of archaeal species and many actinobacteria (10), thus raising the question of how high fidelity DNA replication in these species is achieved. Recent biochemical (11) and structural (12) studies revealed a mismatch-specific endonuclease activity for the archaeal EndoMS/NucS family proteins, which use a dual base flipping mechanism for mismatch recognition (13). These proteins were first identified as PCNA-interacting endonuclease acting on branched DNA substrates (14–16). Quite recently, a bacterial homolog of PCNA-interacting endonuclease acting on branched DNA substrates (17). This endonuclease activity with substrates bearing a single mismatch has been implicated in mutation avoidance through genetic studies, but in contrast to the archaeal proteins, the purified and correctly folded M. smegmatis EndoMS/NucS lacked nuclease activity with substrates bearing a single mismatch within a duplex DNA (17).

Here we report that the purified Corynebacterium glutamicum EndoMS/NucS (Cg/EndoMS) homodimers showed a mismatch-specific endonuclease activity that was markedly stimulated by the β-clamp encoded by dnaN in vitro. The C-terminal five residues of the Cg/EndoMS were found to be essential for the β-clamp interaction and were required for mismatch avoidance activity. The replication clamp may thus direct the EndoMS to mismatches that are left unrepaird by the replisome. Our study provides new mechanistic insights into coordination of the novel mismatch repair activity and causally links the biochemical and cellular DNA repair activities of EndoMS proteins. These data provide the biochemical basis for understanding the rate and the spectrum of spontaneous mutations in archaea, actinobacteria and other bacterial phyla, which were previously thought to lack the MMR system.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains XL1-Blue and XL10-Gold were used as hosts for cloning experiments. E. coli strains JM109 and BL21 CodonPlus (DE3)-RIL were used for the overproduction of His-tagged EndoMS/NucS and β-clamp proteins, respectively. E. coli strains were grown at 37°C on solid or liquid Luria-Bertani media. C. glutamicum strains were grown at 30°C on solid or liquid brain heart infusion (BHI) media (Oxoid). Antibiotics for the selection of recombinant E. coli strains were used at the following final concentrations: ampicillin (Amp), 100 μg/ml; kanamycin (Kan), 20 μg/ml; chloramphenicol (Cam), 30 μg/ml and tetracycline (Tet), 10 μg/ml. For the selection of C. glutamicum transformants, kanamycin and chloramphenicol were added at concentrations of 20 and 10 μg/ml, respectively.

**Construction of plasmids and C. glutamicum mutants**

The nucS gene of the C. glutamicum ATCC 13032 strain was deleted by allelic exchange using the non-polar kanamycin resistance cassette aphA-3 (867 pb), subcloned from pUC18K (Table 1). First, this cassette was inserted between the EcoRI and BamHI sites of pMCS5, which is unable to replicate in C. glutamicum. Then, the 500 bp fragment located upstream from nucS and the 510 bp fragment located downstream from nucS were PCR-amplified with the primer pairs SK94/SK95 and SK96/SK97 (Supplementary Table S1), respectively. The two PCR products were subsequently digested by NdeI/EcoRI and BamHI/PstI and inserted into the flanking regions of the aphA-3 cassette previously cloned into pMCS5, yielding pHM455 (Table 1) (Figure 1A). This plasmid was used as a suicide vector and introduced into C. glutamicum ATCC 13032 competent cells by electroporation as described previously (18). Transformants were selected on BHI plates containing kanamycin after an incubation at 30°C for 3 days. The allelic exchange was then checked by PCR using the following primer pairs: SK83/SK84, SK153/H50 (absence of nucS) and H17/SK154 (integration of aphA-3 at the correct locus) (see Supplementary Table S1 and Supplementary Figure S1). A deletion of 15 nucleotides from the 3′ terminus of nucS was performed by PCR-amplification using the primer pair SK177/SK169 (Supplementary Table S1). The PCR product was digested by NdeI and EcoRI and subsequently used to replace the upstream region of nucS previously cloned into pHM455, yielding pHM476. This plasmid was then introduced into ATCC 13032 competent cells by electroporation and transformants were selected as previously described. The allelic exchange was monitored by PCR-amplification and sequencing using the following primer pairs: SK177/SK84, SK153/H50 and H17/SK154 (see Supplementary Table S1 and Supplementary Figure S1).

**Complementation of the C. glutamicum ΔnucS mutant**

To produce the trans complementation plasmid pHM473, the nucS gene was PCR-amplified with the primer pair SK163/SK164 and inserted between the HindIII/EcoRI sites of the non-integrative E. coli - C. glutamicum shuttle vector pXMJ19 carrying the cat gene (Table 1). The pHM473 plasmid was then transferred into the C. glutamicum ΔnucS::aphA-3 (KanR) strain by electroporation. Transformants were selected on BHI plates containing chloramphenicol (10 μg/ml) and kanamycin (20 μg/ml) after an incubation at 30°C for 2 days.
Table 1. Bacterial strains and plasmids used in this study

| Strains or plasmids | Genotype/characteristics | Reference or origin |
|---------------------|--------------------------|---------------------|
| **E. coli strains**  |                          |                     |
| XL1-Blue            | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacP3 ΔM15 Tn10 (Tet')] | Agilent |
| XL10-Gold           | Tc1 Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F proAB lacIqZΔM15 Tn10 (Tet') amyC3'] | Agilent |
| JM109               | endA1, recA1, gyrA96, thi, hsdRI7 (rk', mk'), relA1, supE44, Δ(lac-proAB), [F' traD36, proAB, lacP2 ΔM15], | Takara Bio |
| BL21 CodonPlus (DE3)-RIL | E. coli B F- ompT hsdS (BmB) dcm+ Tetr gal λ (DE3) endA Hte [argU leuW Cgl Cam'] | Agilent |
| **C. glutamicum strains** |                          |                     |
| Cg ATCC 13032       | Wild-type parental strain | (20) This work |
| Cg (∆nucS)          | Cg 13032 (∆nucS::aphA-3) (replacement of nucS by a non-polar kanamycin resistance cassette (aphA-3)) | This work |
| Cg (∆nucSΔter)      | Cg 13032 (nucSΔter::aphA-3) (replacement of nucS by a copy of nucS with its 15 last nucleotides deleted, followed by a non-polar kanamycin resistance cassette) | This work |
| Cg (∆nucS) + pXMJ19 | Cg 13032 (∆nucS::aphA-3) transformed with the empty pXMJ19 vector | This work |
| Cg (∆nucS) + pHM473  | Cg 13032 (∆nucS::aphA-3) transformed with the pHM473 plasmid carrying a wild-type copy of the nucS gene | This work |
| **Plasmids**        |                          |                     |
| pMCS5               | ori pMB1 (colE1), plac, lacZa, Amp′, MCS of pUC18 / Unable to replicate in C. glutamicum (suicide vector) | MoBiTec |
| pXMJ19              | ori pMB1 (colE1), plac, lacPl, Cam′, MCS of pUC18 / E. coli-C. glutamicum (shuttle vector) | (37) This work |
| pQE80L              | ori colE1, pT3, lacP, Amp′, 6xHis tag (N-ter) | Qiagen |
| pG-KJE8             | ori pI54, paraB, dnaK, dnaJ, gprE, Cam′ | Takara Bio |
| pUC18K              | ori pMB1 (colE1), plac, lacZa, Amp′, Kan′ / Promotor less non-polar kanamycin cassette (aphA-3) cloned into pUC18 | (38) This work |
| pHM449              | nucS of Cg ATCC 13032 cloned into pQE80L (BamHI / PstI) | This work |
| pHM455              | nucS + erm cassette flanked with the upstream and downstream regions of nucS and cloned into pHM55 (Amp′, Kan′) | This work |
| pHM473              | nucS of Cg ATCC 13032 cloned into pXMJ19 (HindIII / EcoRI) | This work |
| pHM476              | nucS with its 15 last nucleotides deleted followed by the aphA-3 cassette and cloned into pMC55 (Amp′, Kan′) | This work |
| pHM477              | A stop codon was introduced into nucS in pHM449 to delete five amino acids from the C-terminus of CglEndoMS | This work |
| pET-Cgβ-clamp       | dnaN of Cg ATCC 13032 cloned into a modified version of pET28a (NdeI / NotI), ori pMB1 (colE1), pT7, Amp′, 6xHis tag (N-ter) | This work |

Estimation of mutation frequencies

*C. glutamicum* and its derivatives were grown overnight using 2 ml 96-deep-well plates at 30°C in BHI liquid medium, including antibiotics when required. Appropriate dilutions were plated on BHI medium with or without rifampicin (Rif) at 100 µg/ml. Rifampicin resistant (RifR) colonies originating from the independent cultures obtained were isolated and sequenced for the wild-type strain 13032 and the ∆nucS mutant, respectively. Spontaneous mutations conferring rifampicin resistance were then identified from these mutants, by PCR-amplification and sequencing a well characterized RifR-determining region in the rpoB gene (NP_599733; NCgI0471), using the primer pair SK157/SK158 [Supplementary Table S1 (19)]. Where indicated, streptomycin was used in analogous experiments at the final concentration of 100 µg/ml.

Cloning of the *C. glutamicum* genes encoding EndoMS/NucS and β-clamp (DnaN) for protein purification

The nucS (CAF19919; NCgI1168) and dnaN (NP_599254; NCgI0002) genes, encoding the CgEndoMS/NucS and the DNA polymerase III β-subunit (β-clamp) respectively, were amplified by PCR from *C. glutamicum* ATCC 13032 genomic DNA, using *Pfu* DNA polymerase (Agilent) and the following primer pairs: SK83/SK84 for nucS and Cgβ-clamp-F/Cgβ-clamp-R for dnaN [Supplementary Table S1 (20)]. The amplified nucS gene was inserted between the BamHI and PstI sites of pQE80L (Qiagen), to generate pHM449 (Table 1). To delete the last five amino acids of the EndoMS/NucS protein, a stop codon was introduced into the nucS gene on the pHM449 plasmid by PCR-mediated mutagenesis (QuickChange site-directed mutagenesis kit; Agilent), using the primer pair ΔCter-F/ΔCter-R (Supplementary Table S1). The DNA product corresponding to dnaN was digested by NdeI and NotI (New England Biolabs), and inserted into the respective sites of a modified pET28a vector (Novagen). In this modified vector, the recognition sequence for thrombin was changed to that for Tobacco Etch Virus (TEV) protease, and the Kan-resistance gene was changed to an Amp-resistance gene. The resulting plasmid was designated as pET-Cgβ-clamp (Table 1). Both inserted sequences were confirmed by sequencing.

Protein expression and purification

His-tagged CgEndoMS and CgEndoMS-ΔCter were prepared from *E. coli* JM109 cells transformed with pHM449 and pHM477, respectively. The chaperone plasmid pG-KJE8 was co-transformed with both plasmids. *E. coli* cells
Figure 1. The functional role of Cg/EndoMS/NucS in mutation avoidance. (A) The physical and genetic organization of the C. glutamicum nucS locus are shown. Approximate positions of the oligonucleotides used for construction and confirmation of the ΔnucS::aphA-3 (KanR) are also indicated. (B) The plates showing the formation of spontaneous RifR colonies for wildtype (left panel) and ΔnucS::aphA-3 (right panel) strains. The plates demonstrate the significant increase in the spontaneous mutation rate for the deletion strain. (C) Mutation frequencies observed for the C. glutamicum wild type, ΔnucS, ΔnucS/pXMJ19 (empty plasmid control), ΔnucS/pHM473 (carrying nucS in trans) and nucSΔCter strains. The last strain lacks the five last amino acid residues of Cg/EndoMS/NucS. The line indicates the geometric mean of the four different biological replicates. Numbers refer to relative fold differences compared to Cg/EndoMS-WT. (D) Relative nucS expression levels for the different strains are shown. The values shown are averages of two biological replicates determined using RT-PCR as described in the materials and methods section.

were cultured in LB medium, containing 50 μg/ml ampicillin, 34 μg/ml chloramphenicol, 10 ng/ml tetracycline and 0.5 mg/ml L-arabinose, at 37°C until the culture attained an OD600 of 0.5. IPTG was then added to a final concentration of 0.2 mM, and the cells were further grown for 18 h at 25°C. The cells were collected, and were resuspended in buffer A (25 mM HEPES, pH 7.5, 1 M NaCl, and 5 mM imidazole), and were disrupted by sonication. The soluble cell extracts were applied to TALON metal affinity resin (Clontech), and the resin was washed with buffer A. The bound fraction was eluted with 25 mM HEPES, pH 7.5, 0.5 M NaCl and 100 mM imidazole, and the eluate was applied to Superdex 200 5/150 column (GE Healthcare), which was eluted with 25 mM HEPES, pH 7.5, 0.3 M NaCl, 0.5 mM EDTA, and 0.1 mM DTT. The fraction containing Cg/EndoMS was stored at −80°C. His-tagged Cg/β-clamp was prepared from E. coli BL21 CodonPlus (DE3)-RIL (Agilent) cells transformed with pET-Cg/β-clamp. E. coli cells were cultured in LB medium, containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol, at 37°C until the culture attained an OD600 of 0.5. IPTG was then added to a final concentration of 1 mM, and the cells were further grown for 18 h at 25°C. The cells were collected, and were resuspended in buffer B (25 mM HEPES, pH 7.5, 0.5 M NaCl and 5 mM imidazole), and were disrupted by sonication. The soluble cell extracts were applied to TALON metal affinity resin, and the resin was washed with buffer B. The bound fraction was eluted with 25 mM HEPES, pH 7.5, 0.3 M NaCl and 100 mM imidazole, and the eluate was applied to Superdex 200 5/150 column, which was eluted with 25 mM HEPES, pH 7.5, 0.3 M NaCl, 0.5 mM DTT and 0.1 mM EDTA. The protein concentrations were calculated by measuring the absorbance at 280 nm. The theoretical molar extinction coefficients of Cg/EndoMS, Cg/EndoMS-ΔCter, and Cg/β-clamp are 19 940, 19 940 and 21 430 M⁻¹ cm⁻¹.
respectively. The purity of the proteins was confirmed by SDS-PAGE.

Gel filtration chromatography analyses

Purified recombinant Cg/EndoMS (1.2 nmol), Cg/EndoMS-Dct (1.2 nmol), Cg/β-clamp (2 nmol) were applied to a Superdex 200 3.2/30 column (GE Healthcare), and were eluted at a flow rate of 40 µl/min in buffer containing 25 mM HEPES, pH 7.5, 0.5 mM DTT, 0.1 mM EDTA and 0.15 M NaCl using SMART system (Amersham Pharmacia). The standard marker proteins, including thyroglobulin (MW, 670 000), γ-globulin (MW, 158 000), ovalbumin (MW, 44 000) and myoglobin (MW, 17 000), were subjected to gel filtration as a control.

Cleavage assay of mismatch-containing DNA

Cleavage reactions of the Cy5-labeled substrate dsDNAs, formed with the oligonucleotides listed in Supplementary Tables S2 and S3, by the proteins were generally performed at 30°C in an optimized reaction mixture containing 25 mM Bis-Tris, pH 6.4, 2.5 mM MnCl₂, 5 mM DTT and 0.1% Triton X-100. The modified reaction conditions are described in each section. The reaction was quenched by the addition of 25 mM EDTA and 0.3%/SDS, and the products were analyzed by either 10% native polyacrylamide gel electrophoresis (PAGE) or 8 M urea-15% PAGE in TBE buffer (89 mM Tris, 89 mM boric acid and 2.5 mM EDTA, pH 8.3). The products were visualized with a Typhoon Trio+ image analyzer (GE Healthcare).

Electrophoretic mobility shift assay

Cg/EndoMS (0.8 µM as a monomer) and Cg/β-clamp (8 µM as a monomer) were incubated with 5 nM 5′-Cy5-labeled ssDNA (45 nt) or dsDNA (45 bp) in a reaction solution (25 mM Bis-Tris, pH 6.4, 2.5 mM MnCl₂, 5 mM DTT and 0.1% Triton X-100) at 30°C for 30 min. An aliquot was fractionated on an 8% PAGE in TBE buffer and was visualized and quantified with a Typhoon Trio+ image analyzer.

Homology modeling of the bacterial EndoMS/NucS

We constructed a model of the C. glutamicum EndoMS/NucS dimer with the modelling program Modeller (21) version 9.18 by using the EndoMS-dsDNA structure carrying a mismatch (PDB code 5GKE) as the template. Five initial models were constructed and the best model was chosen using molpdf and the DOPE score and by evaluating the stereochemical quality with PROCHECK (22). Molecular graphics and structures superposition were performed with the UCSF Chimera package (23). Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

RNA extraction, reverse transcription and real-time PCR quantification

Total RNA was extracted and purified from the C. glutamicum wild type strain and the mutants carrying a deletion of 15 nucleotides from the 3′ terminus of nucS using a Qiagen miRNeasy Mini kit (Qiagen, Courtaboeuf, France) with a final elution volume of 50 µl. The concentration of the RNA fraction was quantified using a Nanodrop 2000c system (Thermo Fisher Scientific). The RT-PCR was performed with a Maxima First Strand cDNA Synthesis Kit for RT-PCR (Thermo Scientific). For each strain, 200 ng of total RNA was used for the synthesis of the first-strand complementary DNA (cDNA). We used 50 pg of cDNA for the real-time PCR assay with SensiFast™ SYBR No-ROX reagents (Bioline Reagent) and the MiniOpticon System (BioRad). The real-time PCR was performed using a concentration range of nucS and 16S rRNA (house-10 genes to calculate the quantitative expression ratio of nucS to 16S rRNA in each sample from two separate experiments. The following primer pairs were used: qPCR-NucS-F/qPCR-NucS-R (nucS) and qPCR-16S-F/qPCR-16S-R (16S rRNA; NCg1r101) (Supplementary Table S1). Where indicated, the percentage of the expression ratio of nucS to 16S for the wild type sample was set to 100%.

RESULTS

Identification and preparation of a C. glutamicum EndoMS

Previous structural studies have demonstrated that the EndoMS proteins possess a two-domain structure (12,16). The amino-terminal dimerization domain has been implicated in the mismatch (MM) binding, whereas the carboxy-terminal region bears an endonuclease active site, which is structurally related to many families of restriction endonucleases (11,12). As we previously reported, the EndoMS homologs were identified not only in archaeal, but also in some of bacterial phyla, particularly actinobacteria (11,16). EndoMS also reportedly interacts with proliferating cell nuclear antigen (PCNA) via a PCNA-interacting peptide (PIP) motif at its C-terminal region, although the PIP motif is not conserved in all homologs (11,12). The β-clamp, identified as the β subunit of DNA polymerase III, in Bacteria has the same functions as the eukaryotic and archaeal PCNAs. The sequence, [EK]-[LY]-[TR]-L-F, which is similar to the consensus sequences of the β-clamp binding motif (24) was found in the C-terminal region of the putative sequences of the actinobacterial EndoMS homologs (Supplementary Figure S1A). Among the bacterial sequences, we selected the C. glutamicum EndoMS (encoded by nucS) and the β-clamp (encoded by dnaN) homologs for further studies. Cg/EndoMS shares only 29.2%, 26.5% and 20.7% amino acid identities, with the EndoMS proteins from Thermococcus kodakarenensis, Pyrococcus abyssi and Methanopyrus kandleri, respectively, but the critical residues for the mismatch binding and nuclease activity are highly conserved among them.

The functional role of Cg/EndoMS in mutation avoidance

The nucS gene is surrounded by the genes NCg1167 (upstream) and NCg1169 (downstream) genes, which have been annotated as ‘universal stress protein UspA’ and ‘nucleoside-diphosphate-sugar epimerase’ in the C. glutamicum genome, respectively (Figure 1A). These proteins have
predicted functions in multiple stress responses (including oxidative stress) and the metabolism of nucleotide sugars that act as activated donors in glycosylation reactions. Our strategy to inactivate the nucS gene, by replacing it with the aphA-3 gene conferring kanamycin resistance (KanR), is also illustrated in Figure 1A. The obtained plasmid-borne deletion-insertion construct (ΔnucS::aphA-3) was inserted into the suicide plasmid to yield the plasmid pHM455 (Table 1), which was transferred to C. glutamicum by electroporation. We screened twenty KanR colonies by PCR for the absence of nucS resulting from an expected double recombination event. This led to the identification of one C. glutamicum ΔnucS::aphA-3 candidate strain where nucS had been replaced with aphA-3. The correct chromosomal localization of aphA-3 was confirmed through additional PCR analyses (see Supplementary Figure S1B and C). The isolation of this deletion strain allowed the subsequent testing of whether nucS is required for mutation avoidance by estimating the mutation frequencies of RifR in the wild type and ΔnucS::aphA-3 C. glutamicum strains (Figure 1B). Using four biological replicates and by comparing the geometric means of the measured mutation rates, we observed that the lack of nucS increased the spontaneous mutation rate by a factor of 205 in comparison to the wild type strain (Figure 1C). This hypermutable phenotype was suppressed by expressing nucS in trans from the plasmid pHM473 (Figure 1C and D), thus establishing the role of nucS in mutation avoidance. To support this result, we measured mutation frequencies using a different marker gene. The mutation frequencies observed with streptomycin were 1.37 $\times$ 10$^{-8}$ and 1.47 $\times$ 10$^{-6}$ for the wild type and ΔnucS strains, respectively. To investigate the possible functional importance of the putative β-clamp interaction motif in Cgl/EndoMS (see Figure 2A), we deleted 15 nucleotides corresponding to five carboxy-terminal residues from the chromosomal nucS gene and measured the level of RifR colony production (Table 1). Our results revealed that although this construct was still transcribed in cells, the five carboxyterminal residues, ELTLF, are absolutely required for the mutation avoidance function of Cgl/EndoMS in our assays (Figure 1C and D).

**Purification of Cgl/EndoMS and Cglβ-clamp**

The recombinant His-tagged proteins, Cgl/EndoMS, Cglβ-clamp, and Cgl/EndoMS-ΔCter, among which the latter lacks five carboxyterminal residues of the wild type Cgl/EndoMS, were successfully overproduced by cultivating the E. coli cells bearing the expression plasmids, pHM449, pET-Cglβ-clamp and pHM477 with IPTG induction (Table 1). As shown in Supplementary Figure S2, the three proteins were purified to near homogeneity. The migrations of the protein bands for His-tagged Cgl/EndoMS and Cgl/EndoMS-ΔCter were obviously slower than those estimated from the calculated molecular weights, 27 014.5 and 26 410.8, respectively, probably because of the open conformation of the EndoMS protein without a DNA substrate. The migration positions of the protein band for the His-tagged Cglβ-clamp protein corresponded well to the calculated molecular weight of 44 872.9. The gel filtration of Cgl/EndoMS and Cgl/EndoMS-ΔCter showed similar chromatograms. The estimated molecular weights of the peak tops indicated the relative molecular masses estimated by the standard curve from the marker proteins. Aliquots of each fraction were subjected to an SDS-12% PAGE followed by Coomassie Brilliant Blue staining (lower part). Protein size markers were run in lanes m, and their sizes are indicated on the left sides of the gels. The loading samples (20%) were run in lanes i.

Figure 2. Properties of Cgl/EndoMS-WT and Cglβ-clamp. (A) The two-domain structure of the EndoMS/NucS proteins. 'MM binding' and 'endonuclease' refer to mismatch binding and endonuclease active site domains, respectively. The positions of the experimentally determined and putative replication clamp binding motif for the Thermococcales and actinobacterial EndoMS/NucS proteins are shown. (B–D) Gel filtration chromatography analyses of Cgl/EndoMS-WT (B), Cgl/EndoMS-ΔCter (C), and Cglβ-clamp (D). Elution profiles, monitored by the absorbance at 280 nm, are shown in the upper part. The arrowheads indicate the elution positions of the standard marker proteins, and the numbers in parentheses on the peak tops indicate the relative molecular masses estimated by the standard curve from the marker proteins. Aliquots of each fraction were subjected to an SDS-12% PAGE followed by Coomassie Brilliant Blue staining (lower part). Protein size markers were run in lanes m, and their sizes are indicated on the left sides of the gels. The loading samples (20%) were run in lanes i.
the peaks were 61 000 and 29 000 or 28 000, which appeared to be a dimer and a monomer, respectively (Figure 2B and C). This result suggests that, similar to its archaeal orthologues, Cg/EndoMS is mainly a dimer in solution. The main peak was isolated and applied to the same column again. However, this rechromatography resulted in the same profiles with two peaks, suggesting that the dimer form of Cg/EndoMS is unstable in both cases of WT and ΔCter. This might also reflect the presence of the monomer–dimer equilibrium for the two Cg/EndoMS variants. The elution profile of the Cgβ-clamp showed a clear single peak, with an estimated molecular weight of ~77 900, suggesting that it exists as a stable homodimer in solution (Figure 2D). A similar result was previously described for the E. coli β-clamp (25).

The mismatch-specific cleavage activity of Cg/EndoMS is activated by the β-clamp

To investigate whether Cg/EndoMS has mismatch DNA-specific endonuclease activity, the purified protein was mixed with oligodeoxynucleotides containing a mismatched base-pair, and the cleavage reactions were monitored by native PAGE. We found that in comparison to T. kodakarenensis EndoMS (20 nM), Cg/EndoMS had less activity with the mismatched DNA, even when more than twice the protein concentration (50 nM) was used (Figure 3A, lanes 1 and 3). However, the cleavage activity was markedly stimulated by the addition of the Cgβ-clamp, and maximal stimulation was reached at equimolar amounts of the Cgβ-clamp and Cg/EndoMS (Figure 3A, lanes 3–7, and 3B). Furthermore, no stimulation was observed when the Cg/EndoMS-ΔCter mutant was used (lanes 8–12). SPR analysis with immobilized Cgβ-clamp were performed to quantify the physical interactions of Cg/EndoMS with Cgβ-clamp (Figure 3C). A KD value of 88 nM was estimated through global fitting analysis, accounting for both association and dissociation phases using a 1:1 interaction model. The apparent binding affinity of EndoMS to β-clamp is comparable to that of P. abyssi EndoMS to PCNA (KD = 15 nM) (16). On the other hand, Cg/EndoMS-ΔCter did not show any response with Cgβ-clamp even at a high concentration up to 2 μM (Figure 3C). These results indicated that the Cgβ-clamp has a critical function in the activation of the mismatch-specific endonuclease activity of Cg/EndoMS, and that the last five residues of Cg/EndoMS are crucial for the interaction with the Cgβ-clamp. To determine the exact cleavage site of Cg/EndoMS in the mismatch-containing DNA, synthetic DNA substrates, labeled with Cy5 at the 5’-terminus of the upper or lower strand were used as substrates. The products generated by Cg/EndoMS with and without the Cgβ-clamp were analyzed by denaturing PAGE. These experiments revealed that Cg/EndoMS cleaved the third phosphodiester bond on the 5’ side of the mismatched base in both strands, to produce a cohesive end with a five-nucleotide long 5’-protrusion (Figure 4). The cleavage pattern by Cg/EndoMS was not different with or without Cgβ-clamp (Figure 4B and C), and was exactly the same as that observed in the presence of TkoEndoMS (11). This observation is also fully compatible with our structural models of the Cg/EndoMS dimer using the TkoEndoMS-dsDNA structure (PDB code}

![Figure 3. Cleavage of mismatch-containing DNA by Cg/EndoMS-WT and Cg/EndoMS-ΔCter with Cgβ-clamp. (A) The 5’-Cy5-labeled DNA substrates (5 nM) containing the GT base pair were incubated with the proteins. The products were analyzed by native 10% PAGE followed by laser scanning (cropped gel image). Lanes 1, positive control (20 nM TkoEndoMS, as a monomer); 2, negative control (no proteins); 3–7, 50 nM Cg/EndoMS-WT (as a monomer); 8–12, 50 nM Cg/EndoMS-ΔCter (as a monomer); 13, no EndoMS. The indicated concentrations (as a monomer) of Cgβ-clamp were added to the reactions. Representative results are shown. The band assignments are indicated on the side of the panels: p, cleaved products. (B) Quantification of the cleaved products. Independent data points from three measurements are plotted. (C) The physical interactions of Cg/EndoMS with Cgβ-clamp were characterized by SPR using a BIACORE 3 system (Biacore Inc.). Purified Cgβ-Clamp was immobilized on the sensor Chip CM5, and various concentrations (indicated on the right side of the sensorgrams) of Cg/EndoMS-WT and Cg/EndoMS-ΔCter were loaded onto the chip for 2 min at a flow rate of 30 μl/min in running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P20). Regeneration was achieved using a 1-min injection of 0.025% SDS. Each background response was subtracted. Data were analyzed using BIAevaluation v.3 software (Biacore Inc.)]
Figure 4. Cleavage pattern of mismatch-containing DNA by Cgl/EndoMS. (A) The dsDNA substrate (45 bp) containing the mismatched base pair and the cleaved products are shown schematically. The 5'-Cy5-labeled upper strand (B) and the 5'-Cy5-labeled lower strand (C) were used to make the dsDNA. The numbers indicate the length of the each strand cleaved by Cgl/EndoMS. (B and C) The substrates (5 nM) were incubated with various proteins. The products were separated by 8 M urea–12% PAGE in TBE buffer followed by laser scanning. The size markers were loaded in lanes 1 in panels b and c, and the sizes are indicated on the left side of each band. Lanes 2, no proteins; 3, 0.4 μM Cglβ-clamp (as monomer); 4, 0.4 μM Cgl/EndoMS (as monomer); 5, 0.4 μM Cgl/EndoMS (as monomer) and 0.4 μM Cglβ-clamp (as monomer); 6, 0.04 μM Tko/EndoMS (as monomer). (D) Catalytic site structure superposition of EndoMS/NucS model (green) and template 5GKE (gray). DNA and protein are depicted in stick and ball-and-stick representations respectively. Residue numbering is relative to the template sequence. R191 is not a conserved amino acid, but in the case of Cgl/EndoMS/NucS R173 is predicted to present a similar interaction with DNA phosphate. (E) Mismatch base recognition site structure superposition of EndoMS/NucS model (green) and template 5GKE (gray). The DNA and protein are depicted in stick-and-ribbon and ball-and-stick representations, respectively. Residue numbering is relative to the template sequence.

5GKE), indicating the excellent superimposition of the catalytic and substrate binding sites (Figure 4D and E).

DNA binding ability of Cgl/EndoMS

A recent report revealed that the EndoMS/NucS protein from M. smegmatis has some ssDNA binding ability, although no cleavage activity for mismatched DNA was shown in vitro (17). For comparison with the M. smegmatis EndoMS/NucS, the DNA binding ability of Cgl/EndoMS was examined by an electrophoresis mobility shift assay (EMSA). The EMSA revealed that Cgl/EndoMS cleaved the G/T-containing DNA and no shifted band was observed. In the case of normal dsDNA and ssDNA, some shifted bands were observed as reported for M. smegmatis EndoMS/NucS (Supplementary Figure S3). These shifts may reflect nonspecific binding.

Optimized reaction conditions for actinobacterial EndoMS with mismatch-containing DNA

To further characterize Cgl/EndoMS and Cglβ-clamp, the cleavage reactions were performed under various conditions. The effects of divalent cations, temperature, and pH on the activity are shown in Supplementary Figure S4. Whereas the mismatch endonuclease activity of
TkoEndoMS can use both Mg\(^{2+}\) and Mn\(^{2+}\) for catalysis and functions in a broad pH range ranging from 6.0 to 11.0, the activity of the bacterial protein is strictly Mn\(^{2+}\)-dependent and has a very narrow pH optimum around 6.4. In agreement with the mesophilic lifestyle of \textit{C. glutamicum}, the activity of Cg/EndoMS rapidly diminishes above 30°C. In the future, comparisons of bacterial and archaeal EndoMS orthologues will be interesting to further investigate the structural basis of their peculiar catalytic differences, although they do not seem to play major roles in the determination of the substrate specificity as described below.

**In vitro mismatch specificity of the Cg/EndoMS protein**

To investigate the mismatch specificity of the Cg/EndoMS nuclease, we used substrates containing all possible combinations of mismatched bases in cleavage reactions in the presence of the Cgβ-clamp (Figure 5). We observed very clear endonuclease activities not only with the G/T mismatch-containing dsDNA substrate used in the above experiments and preferred by the TkoEndoMS, but also with DNA duplexes containing T/T and, to a lesser extent, G/G mismatches. However, T/C, A/G, C/C and A/C mismatches were either cut very poorly or not at all. We conclude that the preferred \textit{in vitro} substrates of Cg/EndoMS are G/T (mismatch resulting in ‘transition’ mutations if left unrepaired) and T/T (‘transversion’ mismatch), which were cleaved with high efficiencies under these experimental conditions. Notably, the other possible ‘transition mismatch’, A/C, was not cleaved at all under these optimized conditions. We also stress that a previous study indicated that the EndoMS proteins lack ‘nicking’ activity that would cleave only one of the two strands of the DNAs with the mismatch-containing DNA substrates (11). Nevertheless, there is a possibility that Cg/EndoMS, in particular complexed with the DNA-bound Cgβ-clamp, might introduce a nick into a dsDNA. To address this point, a normal DNA or DNAs containing C/C or A/C mismatches were reacted with Cg/EndoMS with or without β-clamp. The reaction mixtures were fractionated by electrophoresis on a denaturing gel in parallel with a native gel. A cleaved band was detected from the G/T mismatch, but not from either the C/C or A/C mismatch, indicating that Cg/EndoMS did not clearly cleave even one of the two strands of the DNAs in the vicinity of these mismatches (Supplementary Figure S5).

**Accumulation of transition mutations in the absence of the Cg/EndoMS**

To investigate the mutational patterns of the \textit{C. glutamicum} wild type and ΔnucS strains, we isolated and sequenced independent Rif\(^{R}\) mutants for the two strains (Figure 6). Many different types of mutations within the rpoB gene can cause resistance to rifampicin (19,26). We found that up to 98% of base substitutions in the isolated Rif\(^{R}\) mutants were transition mutations (Supplementary Table S4, Figure 6A). The fact that transitions are strongly favored over transversions among the sequenced base substitutions is in agreement with earlier studies on spectra of spontaneous mutations in Bacteria [see for instance (27)]. In particular, our results suggested that the relative level of the appearance of A:T to G:C transitions were favored approximately three to four-fold in the absence of EndoMS (Figure 6A). \textit{A priori}, these transitions could result from either non-repaired C:A or G:T mismatches, as indicated schematically in Figure 6B.

**DISCUSSION**

Our previous study, searching for proteins involved in mismatch repair (MMR) in archaea that lack the homologs for MutS/MutL proteins, revealed an endonuclease family dubbed EndoMS that is encoded by \textit{nucS}. This protein family cleaves both strands of double-stranded DNA carrying mismatches \textit{in vitro}, thus generating double-strand breaks (11). In addition, a structural analysis of this archaeal homodimeric EndoMS, complexed with a double-stranded DNA carrying G/T mismatches, revealed that these mismatched bases were flipped out into binding sites located in the N-terminal domain of EndoMS. The active site for cleavage, which resembles that of the restriction endonuclease, is located in the C-terminal domain (12), and the archaeal EndoMS structures in the absence of DNA, together with earlier SAXS analyses (14), indicated the movement of the C-terminal catalytic domain brought about by the DNA binding that is required for activity. The \textit{nucS} genes are widely distributed in the domain Archaea, but not in most Bacteria and not at all in Eukarya. It is remarkable that a \textit{nucS} homolog was also found in Actinobacteria, which lack \textit{mutS/mutL} homologs. A recent report showed that the \textit{nucS} gene in \textit{M. smegmatis} cells is required for mutation avoidance and antirecombination in the cells (17). This genetic study strongly supported the MMR function of EndoMS/NucS in this mycobacterial species. However, the purified EndoMS from \textit{M. smegmatis} did not show the mismatch-specific endonuclease activity \textit{in vitro}. Therefore, in the absence of combined biochemical and genetic studies in a single archaeal or actinobacterial species lacking canonical MutS/MutL-directed MMR proteins, the existence and specificity of an alternative MMR system was not fully understood or demonstrated.

Here, we have linked biochemical and genetic evidence for the mutation avoidance activities of the EndoMS family of proteins in \textit{C. glutamicum}. We provide convincing evidence indicating that the physical interaction between the \textit{C. glutamicum} replication β-clamp (DnaN) and the EndoMS homodimer is required for the recognition and cleavage of the mismatches \textit{in vitro} and \textit{in vivo} to protect corynebacterial cells from accumulation of mutations. Our biochemical data revealed that the physical interaction of the β-clamp substantially activates the mismatch-specific cleavage activity of the EndoMS/NucS proteins, and the carboxyl-terminal β-clamp-binding motif of Cg/EndoMS is necessary for this activation and mutation avoidance in the cells. \textit{A priori}, the replication clamp could act directly on the catalytic step of EndoMS proteins or modulate its DNA binding properties.

In the first scenario, the clamp could increase the catalytic rate constant up to four orders of magnitude, as has been demonstrated with other DNA repair nucleases (28). This is not fully compatible with our observations indicating ‘only’ four to five fold activation. In the future experiments, we will thus address whether the replication clamp helps EndoMS...
to scan DNA molecules that carry mismatches in order to recognize and process them.

Our observations already suggest a functional coupling of EndoMS/NucS-directed mismatch repair with the replisome. The role of the replication clamp, the β-clamp in Bacteria and PCNA in Archaea, is important for coordinating this non-canonical MMR, and thus diminishing the formation of unwanted double-strand chromosomal breaks by free EndoMS/NucS. After a double-strand break occurs at the mismatch site by EndoMS/NucS, this break would be processed by end resection to produce 3′-protruding ends, followed by homologous recombination. A recent articles showing that Corynebacteria are polyploidic organisms, resembling archaea (29), strongly supports the involvement of the homologous recombination in the repair of double

Figure 5. Preference for base-pair mismatches. (A) Five nanomolar Cy5-labeled dsDNA (45 bp), containing single base-pair mismatches (G/T, G/G, T/T, T/C, A/G, C/C, A/C, A/A), were incubated with various proteins as indicated on the panels. The products were separated by 10% PAGE. The representative results are shown. The base pairs are indicated on the top of each panel. The band assignments are indicated on the side of the panels, s, substrates; p, cleaved products. (B) Quantification of the cleaved products by 0.4 μM CglEndoMS and 0.2 μM Cglβ-clamp, and by 0.1 μM TkoEndoMS, as shown in lanes 4 and 7 in (A). The bars show the averages and the error bars are standard error of the mean (s.e.m.) from three independent experiments.
orthologues may share a common DNA repair function.

fying that the actinobacterial and archaeal EndoMS
out strand discrimination.

strand breaks created around the repaired mismatches with-
out strand discrimination.

CgoEndoMS shows the order of preferences for G/T, T/T
and G/G mismatches in vitro and these substrate preferences
are quite similar to those of TkoEndoMS (11), signi-
fying that the actinobacterial and archaeal EndoMS/NucS
orthologues may share a common DNA repair function.

The large majority of the RifR base substitutions occurring
in the absence of EndoMS are transitions (Figure 6A) indi-
cating that they must result from unrepaired purine mispair-
ing with a wrong pyrimidine (G/T or C/A transition mis-
matches). Strikingly, whereas G/T mismatches act as pre-
ferred substrates for EndoMS/NucS proteins in vitro (Fig-
ure 5), C/A mismatches are processed very poorly (or not
all). This is different from E. coli MutS/MutL system that
corrects both transition mismatches [see for instance the
early reference (30)]. The fact that both MMR systems work
efficiently on the G/T mismatch is likely to have a biological
meaning, as G/T mismatches are very common replication
errors (8). This may reflect the relatively high capacity of at
least some DNA polymerases to efficiently create G/T mis-
matches (31) that are stabilized by rare tautomeric forms
of guanosine and thymidine (32). Transitions may thus oc-
cur more frequently in the cell than transversions because
molecular mechanisms generating and stabilizing them are
different. It thus appears feasible that G/T mismatches
frequently escaping proofreading activity of the replisome are
efficiently repaired by the EndoMS/β-clamp complex. We
stress that the EndoMS/NucS system is quite different from
the very short patch repair system (Vsr) found for instance in
E. coli (30). Although EndoMS and VSR endonucle-
ases both efficiently cleave G/T mismatches, the Vsr system
corrects G/T mismatches resulting from 5-methylcytosine
deamination in a very specific sequence context. Moreover,
the recognition of the G/T mismatch is achieved differ-
ently in the two systems. Cytosine methylation does not ap-
ppear to occur in Corynebacteria and Mycobacteria, as ex-
perimentally suggested by PaccBio sequencing (33,34). Our
combined biochemical and genetic study thus suggests that
EndoMS/NucS proteins have been fine-tuned to correct the
frequently occurring transition mismatches in cells. We do
not exclude the possibility that other types of mismatches
(for instance T/T or G/G mismatches) resulting in transver-
sions will also be processed by this novel DNA repair path-
way, when they occur in cells.

In conclusion, we demonstrated the existence of a non-
canonical MMR system in C. glutamicum, one of the most
important industrial organisms used for the production
of L-glutamate and L-lysine and other various products
(35,36). The measured frequencies and the spectra of tran-
sition mutations for the wild type and ΔnucS C. glutami-
icum strains are consistent with the enzymatic properties of
EndoMS/NucS-β–clamp complex in vitro. Our study may
also be applicable for the industrial engineering of the use-
ful strains and could provide a rational format for under-
standing the extent of genetic variation and the develop-
ment of antibiotic resistant phenotypes in important human
pathogens including Mycobacterium tuberculosis.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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