Residues in the N-Terminal Domain of MutL Required for Mismatch Repair in *Bacillus subtilis*

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Mismatch repair is a highly conserved pathway responsible for correcting DNA polymerase errors incorporated during genome replication. MutL is a mismatch repair protein known to coordinate several steps in repair that ultimately results in strand removal following mismatch identification by MutS. MutL homologs from bacteria to humans contain well-conserved N-terminal and C-terminal domains. To understand the contribution of the MutL N-terminal domain to mismatch repair, we analyzed 14 different missense mutations in *Bacillus subtilis* MutL that were conserved with missense mutations identified in the human MutL homolog MLH1 from patients with hereditary nonpolyposis colorectal cancer (HNPCC). We characterized missense mutations in or near motifs important for ATP binding, ATPase activity, and DNA binding. We found that 13 of the 14 missense mutations conferred a substantial defect to mismatch repair *in vivo*, while three mutant alleles showed a dominant negative increase in mutation frequency to wild-type *mutL*. We performed immunoblot analysis to determine the relative stability of each mutant protein *in vivo* and found that, although most accumulated, several mutant proteins failed to maintain wild-type levels, suggesting defects in protein stability. The remaining missense mutations located in areas of the protein important for DNA binding, ATP binding, and ATPase activities of MutL compromised repair *in vivo*. Our results define functional residues in the N-terminal domain of *B. subtilis* MutL that are critical for mismatch repair *in vivo*.

Accurate DNA replication is critical for the maintenance of chromosomal DNA in all living cells (18). To ensure the faithful duplication of genetic material, replicative polymerases have proofreading activity (3' to 5' exonuclease) capable of correcting base-pairing errors formed during DNA synthesis (for a review, see references 18 and 31). Despite the high fidelity of replicative DNA polymerases, base-pairing errors still occur, and these errors can lead to deleterious mutations (for a review, see reference 30). The mismatch repair (MMR) pathway enlists two highly conserved proteins, MutS and MutL, to identify and correct base-pairing errors and insertion and deletion loops formed during DNA synthesis (32, 44). In doing so, MMR is capable of increasing the fidelity of the DNA replication pathway several-hundred-fold in a variety of organisms (8, 16, 20). In addition to its role in correcting replicative errors, MMR proteins also recognize DNA lesions produced by the oxidative by-products of intracellular metabolism (6, 43), as well as lesions formed by exogenous sources of DNA damage (52). Moreover, in Gram-negative bacteria, MMR has been shown to function in antirecombination, which prevents recombination between DNA from divergent sources, providing a genetic barrier between bacterial species (41).

MMR is critically important for human health. MMR deficiencies allow for some bacterial pathogens to persist within their host (e.g., see reference 36), while other pathogens require MMR for colonization of the intestine or ascent to the bladder during urinary tract infections (7, 10). Furthermore, MMR defects in bacteria can accelerate the acquisition of resistance to antibiotic treatments (5, 11, 36). Defects in the MMR pathway in humans cause an increased risk for the development of sporadic cancers, including hereditary nonpolyposis colorectal cancer (HNPCC) and Turcot's syndrome (16, 24, 35, 37). These are a few examples that highlight the practical importance of MMR to health care.

In the Gram-positive bacterium *Bacillus subtilis*, MutS recognizes a mismatch and recruits MutL to the site of mismatch recognition (13, 46, 48). Like most bacteria and all eukaryotic organ-

isms, *B. subtilis* lacks the canonical methylation-directed pathway characterized in *Escherichia coli* (9, 14, 15). In *E. coli*, Dam methylase and MutH represent the two proteins required for generating and sensing the methylation signal (33). In *B. subtilis*, MutH and Dam are not present and d(GATC) sequences are not methylated *in vivo* (12). The hallmark of the methylation-independent mismatch repair pathway is the presence of an endonuclease-containing MutL homolog (27, 28, 38). *B. subtilis* MutL contains two domains located at the N and C termini of the protein. Based on sequence homology with *E. coli* and other systems, the *B. subtilis* MutL N-terminal domain is a GHKL (DNA gyrase, Hsp90, bacterial histidine kinases, and MutL) family ATPase with a DNA binding cleft (2, 3, 21, 26). A structure for the N-terminal domain of *B. subtilis* *mutL* has not been solved. The C-terminal domain is comprised of the endonuclease active site, a binding site for the replication processivity clamp DnaN and a zinc binding loop (38). The N- and C-terminal domains are connected by a flexible linker (2, 3, 21, 26).

The C-terminal endonuclease active site is highly conserved from *B. subtilis* to humans and possesses the consensus active site motif DQHA(X)₂E(X)₄E (27, 28, 38). Strikingly, the endonuclease active site in *B. subtilis* MutL is identical to the site in *Saccharomyces cerevisiae* and the human MutL homologs PMS1 and PMS2 (27). Recently, the crystal structure was solved for the C-terminal domain of *B. subtilis* MutL (38). Mutation of residues found within the endonuclease active site, the zinc binding loop, or a site shown to bind DnaN within the C-terminal domain of MutL de-

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creases or abolishes MMR activity *in vivo* (38, 39). It is important to note that *E. coli* MutL lacks the endonuclease active site, as well as endonuclease activity and the zinc binding loop (22, 27). Furthermore, mutation of the DnaN interaction site within the C-terminal domain of *E. coli* MutL causes only a mild effect on mutagenesis, while mutation of the same site in *B. subtilis* MutL increases mutagenesis substantially (38, 39). Therefore, the effect of altering critical residues in the C-terminal domain of *B. subtilis* MutL has been well characterized, while the N-terminal domain has remained virtually unexplored in Gram-positive bacteria.

In this work, we characterized the N-terminal domain of *B. subtilis* using a mutational approach. Since *B. subtilis* MutL shares amino acid sequence conservation with the human MutL homolog MLH1, we characterized 14 *B. subtilis* *mutL* alleles with missense mutations corresponding to potentially pathogenic human *MLH1* polymorphisms found in patients diagnosed with HNPCC. We analyzed these variants for their protein stability, mutation rate, and ability to exert a negative effect on MMR in the presence of the wild-type *mutL* gene *in vivo*. We found that mutant MutL proteins with residue changes in or near motifs important for ATP binding, ATPase activity, or DNA binding showed a wide range of defects in MMR. Mutations located near the ATP binding site consistently conferred the most striking increases in mutagenesis. We found that three mutant proteins (MutL S40A, G63R, and G94S) failed to accumulate *in vivo*, which is consistent with the complete defect in MMR conferred by each corresponding allele. We also found that three missense mutations [*mutL*(E30A), *mutL*(N34H), and *mutL*(R176G)] conferred a dominant negative phenotype, supporting the notion that the corresponding mutation in human MLH1 would have a pathogenic role leading to genome instability in HNPCC patients. Together, our results identify residues in the N-terminal domain of *B. subtilis* MutL that are critical for MMR, and we test corresponding *MLH1* missense mutations for MMR defects in a tractable bacterial system.

MATERIALS AND METHODS

Bacteriology. Each *B. subtilis* *mutL* allele was constructed through site-directed mutagenesis (47) of the wild-type *mutL* gene and confirmed by DNA sequence analysis. All primers used in this study are available upon request. Integration at the ectopic *amyE* locus of the wild-type strain (PY79) was achieved by double crossover using standard procedures (25). To construct strains deficient for the native *mutL* allele, chromosomal DNA from LAS393 (*mutL::spc*) was used to transform each strain containing the *amyE::P_{spac}-mutL* allele. All strains used in this study are listed in Table 1. Unless otherwise stated, antibiotics and isopropylthio- β -galactoside (IPTG) were used at the following concentrations: 100 μ g/ml spectinomycin (Spc), 5 μ g/ml chloramphenicol (Cat), and 1 mM IPTG.

Mutation rates. Each strain to be tested was grown overnight on LB agar plates with the appropriate antibiotics, and 1 mM IPTG was prepared just prior to use. IPTG was used to induce expression of each mutant protein regulated by a *P_{spac}* promoter from the ectopic *amyE* locus. Following overnight growth on solid medium, 3-ml liquid LB cultures containing 1 mM IPTG were inoculated with a single colony and grown for approximately 3 h to reach late exponential growth (optical density at 600 nm [OD₆₀₀] between 1.0 and 1.2). A 1-ml sample of culture was removed and concentrated by centrifugation at 10,000 rpm. The supernatant was then aspirated carefully, as to not disturb the cell pellet. Subsequently, the cells were resuspended in 100 μ l of a 0.85% saline solution. The cell suspension was then serially 10⁶-fold diluted into 0.85% saline. To obtain a count of the total number of viable cells in each culture, 100 μ l of the final dilution was plated on LB while 100 μ l of the original cell resuspension

TABLE 1 List of strains

Strain	Characteristics	Source or reference
NJB1	PY79 (SP β ⁰)	46, 48
NJB21	<i>amyE::P_{spac}-mutL⁺</i> (cat)	46, 48
NJB22	<i>amyE::P_{spac}-mutL</i> (G18A) (cat)	This study
NJB23	<i>amyE::P_{spac}-mutL</i> (P24L) (cat)	This study
NJB24	<i>amyE::P_{spac}-mutL</i> (G63R) (cat)	This study
NJB25	<i>amyE::P_{spac}-mutL</i> (S102R) (cat)	This study
NJB26	<i>amyE::P_{spac}-mutL</i> (E30A) (cat)	This study
NJB27	<i>amyE::P_{spac}-mutL</i> (N34H) (cat)	This study
NJB28	<i>amyE::P_{spac}-mutL</i> (K80E) (cat)	This study
NJB29	<i>amyE::P_{spac}-mutL</i> (G94S) (cat)	This study
NJB30	<i>amyE::P_{spac}-mutL</i> (R176G) (cat)	This study
NJB31	<i>amyE::P_{spac}-mutL</i> (G234V) (cat)	This study
NJB32	<i>amyE::P_{spac}-mutL</i> (R256C) (cat)	This study
NJB33	<i>amyE::P_{spac}-mutL</i> (S40A) (cat)	This study
NJB34	<i>amyE::P_{spac}-mutL</i> (N60S) (cat)	This study
NJB35	<i>amyE::P_{spac}-mutL</i> (A17V) (cat)	This study
NJB37	<i>amyE::P_{spac}-mutL⁺</i> (cat) <i>mutL::spc</i>	This study
NJB38	<i>amyE::P_{spac}-mutL</i> (G18A) (cat) <i>mutL::spc</i>	This study
NJB39	<i>amyE::P_{spac}-mutL</i> (P24L0) (cat) <i>mutL::spc</i>	This study
NJB40	<i>amyE::P_{spac}-mutL</i> (G63R) (cat) <i>mutL::spc</i>	This study
NJB41	<i>amyE::P_{spac}-mutL</i> (S102R) (cat) <i>mutL::spc</i>	This study
NJB42	<i>amyE::P_{spac}-mutL</i> (E30A) (cat) <i>mutL::spc</i>	This study
NJB43	<i>amyE::P_{spac}-mutL</i> (N34H) (cat) <i>mutL::spc</i>	This study
NJB44	<i>amyE::P_{spac}-mutL</i> (K80E) (cat) <i>mutL::spc</i>	This study
NJB45	<i>amyE::P_{spac}-mutL</i> (G94S) (cat) <i>mutL::spc</i>	This study
NJB46	<i>amyE::P_{spac}-mutL</i> (R176G) (cat) <i>mutL::spc</i>	This study
NJB47	<i>amyE::P_{spac}-mutL</i> (G234V) (cat) <i>mutL::spc</i>	This study
NJB48	<i>amyE::P_{spac}-mutL</i> (R256C) (cat) <i>mutL::spc</i>	This study
NJB49	<i>amyE::P_{spac}-mutL</i> (S40A) (cat) <i>mutL::spc</i>	This study
NJB50	<i>amyE::P_{spac}-mutL</i> (N60S) (cat) <i>mutL::spc</i>	This study
NJB51	<i>amyE::P_{spac}-mutL</i> (A17V) (cat) <i>mutL::spc</i>	This study
LAS393	<i>mutL::spc</i>	46, 48

was plated on LB agar with 100 μ g/ml rifampin (Rif) to determine the number of Rif^r CFU. Following overnight growth, both the LB and LB-Rif plates were scored for colony-forming units. Mutation rate analysis (17) was conducted using the Ma-Sandri-Sarkar maximum likelihood estimator (MSS-MLE) method as described previously (23).

Immunoblotting. A single colony, obtained from overnight growth on LB agar plates with the appropriate antibiotics, was used to inoculate a 20-ml liquid LB culture. Cultures were incubated at 37°C to reach mid-exponential growth (OD₆₀₀ of 0.6 to 0.9). Cells were concentrated by centrifugation for 10 min at 10,000 rpm, followed by aspiration of the supernatant immediately following centrifugation. Cell pellets were resuspended in 1 ml of lysis buffer [10 mM Tris-HCl (pH 7.0), 0.5 mM EDTA, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and 1 $\times$ protease inhibitor cocktail from the Thermo Scientific protease inhibitor cocktail kit]. After resuspension, the samples were subjected to sonication for lysis. Sonication was conducted at 20 Hz for three 40-s treatments per sample. After sonication, cell extracts were centrifuged for 20 min at 6,000 rpm to remove insoluble debris. Concentrations of soluble protein extracts were determined using the Pierce BCA protein assay kit by monitoring the absorbance of 562-nm wavelengths in a Tecan plate reader.

Soluble extracts were resolved by SDS-PAGE (47) after loading 15 μ g of total protein per extract. Following transfer to a nitrocellulose membrane, each membrane was blocked in phosphate-buffered saline-Tween (PBS-T) with 5% milk for 1 h, followed by incubation overnight with a 1:500 primary antibody dilution against MutL or DnaN in blocking buffer as described previously (29). Washing in blocking buffer removed excess

A

BsMutL	1	MAKVIQLSDE-LSNKIA	AGEVVERPASVVKELVENAIDADSTVIEIDIEEAGLASIRVLD	59
		+A VI+ DE + N+IA	AGEV++RPA+ +KE++EN +DA ST I++ ++E GL I++ D	
hMLH1	3	VAGVIRRLDETVPNRI	AGEVIQRANAIKEMIENCLDAKSTSIQVIVKEGGLKLIQIQD	62
BsMutL	60	NGEGMENEDCKRAFR	RHATSKIKDENDLFRVRTLGRGEALPSIASVSHLEITTSTGEG-	118
		NG G+ ED R TS	K++ DL + T GFRGEAL SI+ V+H+ ITT T+G	
hMLH1	63	NGTGIRKEDLDIVCER	FTSKLQSFEDLASISTYGRGEALASISHVAHVTTITTKTADGK	122
BsMutL	172	DVVNR	I...234 GYIALPEITRASNYMSSVVNGRYIKNFPLVKAV	167
		+VV R	GYI+ + + +N R +++ L KA+	
hMLH1	176	EVVGR	Y...243 GYISNANYS-VKKCIFLLFINHRLVESTSLRKAI	176

B

FIG 1 Homology model of *B. subtilis* MutL N-terminal domain with the locations of amino acid substitutions indicated. (A) A sequence alignment of the N-terminal domains of *B. subtilis* MutL (BsMutL) and human MLH1 (hMLH1) is shown. We show the primary structure alignment relevant to the location of the missense mutations that were tested in this study. Colored residues correspond to the colors used in panel B. (B) A model of the MutL N-terminal domain dimer is shown. The amino acid residue changes are modeled on the left monomer. Residues predicted to be important for ATP binding, ATP hydrolysis, and DNA binding are colored in orange, red, and purple, respectively. Mutants with functions that are unclear based on homology are colored in green.

antibody before incubation of the membrane for 2 h in secondary antibody (goat anti-rabbit immunoglobulin-horseradish peroxidase [HRP] conjugate). For secondary antibodies, we used a working dilution of 1:2,000 in PBS-T with 5% milk. Following incubation, the membrane was washed once in PBS-T with 5% milk, followed by two washes in PBS-T to remove excess antibody. HRP was detected using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific), followed by exposure to Blue Lite autorad film (GeneMate).

RESULTS AND DISCUSSION

Identification of N-terminal *mutL* missense mutations defective for MMR. To characterize the N-terminal domain of *B. subtilis* MutL in MMR, we chose a mutational approach. In order to identify potential residues important for MutL function, we used the International Society for Gastrointestinal Hereditary Tumors (InSiGHT database) to identify missense mutations in the human MutL homolog *MHL1* from patients with hereditary nonpolyposis colorectal cancer (HNPCC; www.insight-group.org/) that altered residues conserved in *B. subtilis* MutL. We chose to study the corresponding *MLH1* missense mutations from HNPCC patients in *B. subtilis* for two reasons. First, very little is known about the N-terminal domain of *B. subtilis* MutL and *B. subtilis* MutL is well conserved with human MLH1 (Fig. 1A). Second, using *B. subtilis* as a model system, this approach allowed us to determine and quantify the severity of the defect to MMR caused by missense mutations corresponding to *MLH1* polymorphisms identified in

HNPCC patients. This is important because it allowed us to functionally assess the corresponding missense mutations in a genetically tractable bacterial system *in vivo*. Some studies have characterized human *MLH1* and *MSH6* polymorphisms in the corresponding *S. cerevisiae* genes, yielding important insights into the effects on MMR in a genetically tractable eukaryotic organism (e.g., see references 19, 50, and 51). To our knowledge, a bacterial system has not been used for this purpose. Therefore, we were interested in using *B. subtilis* as a rapid experimental system for analysis of human *MLH1* missense mutations and to functionally characterize the N-terminal domain of *B. subtilis* MutL.

To this end, we cloned 14 *B. subtilis* *mutL* missense mutations that correspond to human *MLH1* missense mutations (Table 2). We also show the location of each amino acid substitution on a model of the *B. subtilis* MutL N-terminal domain (Fig. 1B). Each *B. subtilis* mutant *mutL* allele was expressed ectopically from an IPTG-regulated promoter from the *amyE* locus in a strain background disrupted for the native *mutL* gene. We performed a titration of IPTG to determine the level required to express MutL to wild-type levels *in vivo* while also determining the mutation rate (Fig. 2). Expression of *mutL* with 25 μ M IPTG yielded a low protein level and a high spontaneous mutation rate. Induced expression with 100 μ M or 1 mM IPTG yielded protein levels similar to those of *mutL* expressed from its native promoter, and both concentrations yielded a mutation rate identical to that of the wild-

TABLE 2 *B. subtilis* *mutL* missense mutation conserved in human *MLH1*^a

<i>B. subtilis</i> <i>mutL</i> missense mutation	Human <i>MLH1</i> missense mutation	No. of reported occurrences in humans ^b	Potential function ^c
A17V	A21V	6	ATP binding
G18A	G22A	11	ATP binding
P24L	P28L	32	ATP hydrolysis
E30A	E34A	4	ATP hydrolysis
N34H	N38H	8	ATP hydrolysis
S40A	S44A	5	ATP hydrolysis
N60S	N64S	20	Unclear
G63R	G67R	74	ATP binding
K80E	K84E	20	ATP hydrolysis
G94S	G98S	8	ATP binding
S102R	S106R	7	ATP binding
R176G	R182G	14	DNA binding
G234V	G244V	6	DNA binding
R256C	R265C	53	ATP binding

^a A list of *MLH1* mutations, the corresponding *B. subtilis* missense mutations, the reported incidence in HNPCC patients, and the possible MutL functions impaired by each mutation is shown.

^b The occurrence of each human *MLH1* missense mutation was obtained from the International Society for Gastrointestinal Tumors (www.insight-group.org).

^c Potential function refers to the predicted biochemical function affected by each mutation based on the corresponding missense mutation location in *S. cerevisiae* Mlh1 or *E. coli* MutL where the biochemical defect has been determined (2, 26).

type control strain PY79 (Fig. 2). Since induced expression with 1 mM IPTG resulted in a protein level more comparable to that of native MutL, we chose to express each mutant protein with 1 mM IPTG for the analysis that follows.

FIG 2 Ectopic MutL expression. (A) The levels of MutL protein (left to right) from the wild-type control strain PY79, a strain with the *mutL::spc* allele, and samples with *mutL* expressed from *amyE* with the native *mutL* gene disrupted (*mutL::spc*) with the indicated concentrations of IPTG are shown. All samples were derived from cell extracts prepared as described in Materials and Methods. The sample load was normalized to total protein in each extract. (B) The mutation rate corresponding to each concentration of IPTG used in panel A is shown. Error bars represent the 95% confidence interval.

TABLE 3 Missense mutations in *B. subtilis* *mutL* cause defects in MMR^a

Genotype	Mutation rate × 10 ^{-9b}	Relative mutation rate	Mutations per culture
Wild type (PY79)	3.9 (2.0–5.8)	1.0	2.5
<i>amyE::P_{spac}-mutL</i> ⁺	3.8 (2.0–5.6)	1.0	2.3
<i>mutL::spc</i>	192.1 (184.1–200.1)	50.1	117.2
<i>mutL</i> (A17V)	180.1 (173.1–187.2)	47.0	126.6
<i>mutL</i> (G18A)	35.7 (29.9–41.4)	9.3	19.8
<i>mutL</i> (P24L)	81.0 (74.8–87.2)	21.1	54.1
<i>mutL</i> (E30A)	139.6 (132.0–147.2)	36.4	83.4
<i>mutL</i> (N34H)	177.0 (169.3–184.8)	46.2	110.3
<i>mutL</i> (S40A)	149.0 (142.5–155.4)	38.9	112.3
<i>mutL</i> (N60S)	2.5 (0.9–3.9)	0.7	1.4
<i>mutL</i> (G63R)	145.9 (137.3–154.5)	38.1	76.1
<i>mutL</i> (K80E)	150.7 (142.8–158.5)	39.3	89.2
<i>mutL</i> (G94S)	160.7 (153.5–168.0)	41.9	107.0
<i>mutL</i> (S102R)	24.9 (20.1–29.7)	6.5	15.3
<i>mutL</i> (R176G)	35.0 (30.3–39.6)	9.1	26.2
<i>mutL</i> (G234V)	98.1 (91.5–104.6)	25.6	65.0
<i>mutL</i> (R256C)	141.5 (134.0–148.9)	36.9	87.4

^a The mutation rate, the relative mutation rate, and the mutations conferred per culture for each *mutL* allele examined are shown. Mutation rates and mutations per culture (17) were calculated by FALCOR (Fluctuation Analysis Calculator) using the MSS-MLE method (23). Calculations are based on data collected through rifampin resistance by fluctuation analysis for 14 to 50 independent cultures per strain. The relative mutation rate reflects normalization of mutation rates to that obtained for *P_{spac}-mutL*⁺.

^b The error shown in the parentheses reflects the 95% confidence interval.

In the mutation rate analysis described above and in the analysis described below, we determined the mutation rate by performing a fluctuation analysis using rifampin resistance as an indicator for spontaneous mutagenesis. Rifampin resistance in *B. subtilis* is conferred by one of several nucleotide substitutions in the *rpoB* gene (13, 34), encoding the beta subunit of RNA polymerase (4). To assess the proficiency of each *mutL* allele in MMR, we used the Ma-Sandri-Sarkar maximum likelihood estimator (MSS-MLE) method for calculation mutation rates (see Materials and Methods) (23). Prior to calculation of mutation rates for each mutant *mutL* allele, we compared the mutation rates calculated for the wild-type strain (PY79) and the wild-type control (*mutL*⁺), expressing the only cellular copy of *mutL* from the ectopic *amyE* locus with the native *mutL* gene disrupted (*mutL::spc*). We found that the wild-type and ectopically expressed *mutL*⁺ strains yielded mutation rates that were nearly identical, with rates of 3.9×10^{-9} and 3.8×10^{-9} Rif mutations/generation, respectively (Fig. 2 and Table 3). Thus, the mutation rates conferred by *mutL*⁺ (PY79) and *amyE::P_{spac}-mutL*⁺ in a strain background disrupted for the native *mutL* locus (*mutL::spc*) were remarkably close, confirming that ectopic expression of functional *mutL* complements the *mutL::spc* allele (Fig. 2B and Table 3). We then tested a *mutL*-deficient strain (*mutL::spc*) and found a mutation rate of 192.1×10^{-9} mutations/generation (Table 3). Because *mutL* is required for MMR function, this mutation rate is representative of a complete loss of mismatch correction in *B. subtilis* and consistent with previously published data (13, 46).

With this assay established, we examined 14 different *mutL* alleles expressed from the *amyE* locus with the native *mutL* gene disrupted (see genotype in Table 1). Each *mutL* allele was inserted at the *amyE* locus by double-crossover integration (see Materials and Methods), and each novel *B. subtilis* *mutL* allele was se-

FIG 3 Expression level of mutant MutL proteins *in vivo*. An immunoblot with the relative level of each mutant MutL protein *in vivo* is shown. (A and B) Whole-cell extracts probed for MutL or as a loading control DnaN on a duplicate blot prepared from the same extract are shown. The *mutL*⁺ represents the wild-type *mutL* allele expressed from *amyE* with 1 mM IPTG, the same expression condition used for each mutant protein tested.

quenced prior to use. We performed immunoblot analysis to determine the relative accumulation of each mutant protein *in vivo*. This experiment was performed because an HNPCC-associated missense mutation in human *MLH1* could cause an MMR defect by simply destabilizing the protein *in vivo*, resulting in lower steady-state protein levels, compromising MMR *in vivo*. The absence of the immunoreactive band from each blot prepared from the *mutL::spc* strain (Fig. 2A and 3A and B) indicates that the antiserum we used was specific for MutL. The majority of the mutant MutL proteins accumulated to wild-type levels *in vivo*, including MutL with the A17V, G18A, P24L, E30A, N60S, S102R, or R176G change (Fig. 3). MutL proteins corresponding to N34H, K80E, G234V, or R256C were reduced *in vivo* with levels clearly lower than the wild-type control (Fig. 3). We also found that several mutant proteins failed to be detected by immunoblot analysis. These mutant proteins include MutL with the S40A, G94S, or G63R substitutions. Based on this result, we suggest that the MutL S40A, G96S, and G63R proteins are unstable and degraded *in vivo* (Fig. 3).

We then determined mutation rates for each of the 14 *mutL* alleles that were cloned (Table 3). The three mutants that failed to accumulate *in vivo* conferred a mutation rate comparable to the control strain defective for *mutL* (*mutL::spc*). Of the 11 *mutL* alleles that accumulated, we found that 10 conferred mutation rates significantly higher than the control expressing *mutL*⁺ under identical growth conditions (Table 3). We tested missense mutations in or near motifs important for ATP binding, ATPase activity, and DNA binding in the MutL N-terminal domain (Fig. 1B and Table 3).

mutL alleles with mutations hypothesized to impair ATP hydrolysis [*mutL*(E30A), *mutL*(N34H), *mutL*(S40A), *mutL*(K80E), and *mutL*(G94S)] showed, on average, the highest increase in the mutation rate relative to missense mutations in or adjacent to motifs important for ATP binding or DNA binding (Table 3). Mutations that impaired ATP hydrolysis were likely to affect protein stability as judged by protein accumulation *in vivo* (Fig. 3). Of the ATPase mutants, only the *mutL*(E30A) mutant accumulated to levels equivalent to *mutL*⁺. In both *E. coli* and *B. subtilis*, MutL is a weak ATPase, with the *B. subtilis* protein showing the lower

rate of hydrolysis ($k_{\text{cat}} = 0.3 \text{ min}^{-1}$) (38). In *B. subtilis*, the mechanistic role of ATPase activity in MMR is unclear; however, ATP binding by MutL is required for activation of *B. subtilis* endonuclease activity (38). MutL endonuclease activity requires that MutL is bound to ATP, as MutL ADP fails to nick DNA (38). In *E. coli*, ATP hydrolysis by MutL is critical for MMR *in vivo*, although the precise role is unclear, as an ATPase-deficient MutL mutant shows wild-type levels of MutH incision and UvrD loading *in vitro* (26, 42, 49). Even though the mechanistic role of MutL ATPase activity during MMR is not fully understood, it is clear that the integrity of this site is important for functional MMR in *E. coli* (26, 42, 49), *S. cerevisiae* (e.g., see reference 51), *B. subtilis* (Table 3), and humans (40). One possibility is that, following ATP hydrolysis, MutL changes conformation, altering protein contacts important for a step in repair following endonuclease cleavage of the DNA.

Increased mutation rates were also conferred by five mutant alleles with missense mutations near the site of ATP binding. These alleles include *mutL*(A17V), *mutL*(G18A), *mutL*(P24L), *mutL*(G63R), and *mutL*(S102R). However, the relative mutation rates caused by these missense mutations varied more than the mutant proteins with substitutions near the motif important for ATP hydrolysis. The relative mutation rates ranged from 6.5 to 47 (Table 3). The G63R mutant showed a complete defect in MMR, consistent with the failure of the mutant protein to accumulate *in vivo* (Fig. 3). Missense mutations in the DNA binding cleft of the N-terminal domain conferred an increase in the mutation rate compared to the *mutL*⁺ strain. As with alleles hypothesized to be altered for ATP binding, the two missense mutations constructed in conserved DNA binding cleft residues varied in the mutation rate. The *mutL*(G234V) mutation conferred a relative mutation rate of 25.6-fold, and the *mutL*(R176G) missense mutation caused a relative mutation rate of only 9.1-fold compared with ectopically expressed *mutL*⁺. Based on these results we speculate that DNA binding in the N-terminal cleft may not be as important for MMR *in vivo* as DNA binding in the C-terminal domain of *B. subtilis* MutL.

***mutL*(E30A), *mutL*(N34H), and *mutL*(R176G) are dominant negative to native *mutL*.** A dominant negative effect caused by a *mutL* mutant would provide further insight into its functional ability. This would distinguish between a mutant protein that is unfolded and/or inactive relative to a protein that can cause a defect in the pathway when the wild-type protein is present. Additionally, it could provide insight into a pathogenic mutation in the corresponding *MLH1* gene, which would cause an increase in mutation frequency in the presence of a wild-type allele in a diploid organism (24). Thus, we quantified the dominant negative effect of each mutant allele by determining the mutation rates of *B. subtilis* strains expressing both the mutant and wild-type *mutL* alleles (Table 4). In each strain tested, the native *mutL* locus remained intact and the missense mutants were expressed at the ectopic *amyE* locus from the *P_{spac}* promoter using the same level of IPTG as above. This will ensure that the mutant MutL proteins are expressed at the same level as the natively expressed wild-type MutL.

We found that three missense mutants conferred a dominant-negative increase in mutation rate to native *mutL*. Two of these mutants, the E30A and N34H mutants, are hypothesized to affect ATP hydrolysis. The E30A mutant protein conferred a slight increase in mutation rate, with a relative rate of 3.1-fold compared

TABLE 4 *mutL*(E30A), *mutL*(N34H), and *mutL*(R176G) show a dominant negative increase in mutation frequency to native *mutL*^a

Genotype	Mutation rate $\times 10^{-9b}$	Relative mutation rate	Mutations per culture
wild type (PY79)	3.7 (1.8–5.4)	1.0	2.2
<i>amyE::P_{spac}mutL</i> ⁺	3.5 (1.5–5.3)	1.0	2.0
<i>mutL::spc</i>	159.0 (151.1–167.0)	45.4	93.5
<i>mutL</i> (A17V)	4.1 (1.8–6.3)	1.2	2.9
<i>mutL</i> (G18A)	2.3 (0.8–3.6)	0.7	1.3
<i>mutL</i> (P24L)	3.7 (1.5–5.7)	1.1	2.3
<i>mutL</i> (E30A)	10.8 (7.5–14.1)	3.1	7.9
<i>mutL</i> (N34H)	102.3 (95.7–108.8)	29.2	68.2
<i>mutL</i> (S40A)	2.6 (0.8–4.1)	0.7	1.5
<i>mutL</i> (N60S)	2.7 (0.4–4.4)	0.8	1.2
<i>mutL</i> (G63R)	2.3 (0.7–3.6)	0.7	1.4
<i>mutL</i> (K80E)	6.2 (3.5–8.9)	1.8	4.0
<i>mutL</i> (G94S)	1.6 (0.4–2.4)	0.5	0.9
<i>mutL</i> (S102R)	5.0 (2.5–7.5)	1.4	3.1
<i>mutL</i> (R176G)	48.5 (41.1–55.8)	13.8	22.3
<i>mutL</i> (G234V)	5.2 (2.3–7.8)	1.5	2.6
<i>mutL</i> (R256C)	2.9 (0.8–4.6)	0.8	1.7

^a The mutation rate, relative mutation rate, and mutation per culture determined for each mutant allele when expressed in the presence of the wild-type *mutL* gene are shown. Mutation rates and mutations per culture were calculated by FALCOR (Fluctuation AnaLysis CalculatOR) using the MSS-MLE method (17, 23). Calculations were based on data collected through rifampin resistance by fluctuation analysis for 14 to 50 independent cultures per strain. Relative mutation rate reflects normalization of mutation rates to that obtained for *P_{spac}mutL*⁺.

^b The error shown in parentheses reflects the 95% confidence interval.

to that for the wild-type control. However, the N34H mutant yielded a relative rate of 29.2-fold. These data suggest that both the *mutL*(E30A) and *mutL*(N34H) mutants are dominant to the wild-type *mutL* allele, causing a partial defect in the MMR pathway. We find these data interesting since a dominant negative phenotype has been shown in HNPCC alleles and *E. coli mutL* bearing missense mutations affecting ATP hydrolysis (1, 45). The third mutant, the *mutL*(R176G) mutant, is a conserved DNA binding residue. It has a relative dominant negative mutation rate of 13.8-fold (Table 4). The missense mutation corresponding to R176G in *S. cerevisiae mlh1* (R182G) has been tested functionally and has also been found to be dominant negative, placing our data in good agreement with prior work (50). We speculate that the dominant negative results correspond to mutant MutL proteins forming mixed dimers with MutL⁺, decreasing mismatch repair activity *in vivo*, or that the mutant MutL is able to compete with wild-type MutL for sites of repair, decreasing MMR efficiency. We suggest the latter, since each dominant negative allele caused a partial decrease in MMR and not a complete loss in the pathway.

Conservation of MutL function between *B. subtilis* and eukaryotic systems. Several of the missense mutations that we tested in *B. subtilis* MutL have been examined in *S. cerevisiae mlh1* or in human MLH1 (50, 51). A previous study found that the *S. cerevisiae mlh1* missense mutations corresponding to *B. subtilis mutL*(P24L) and *mutL*(N60S) result in an intermediate increase in the mutation rate, while the *mutL*(R256C) mutant causes an increase in the mutation rate lower than those for the *mutL*(P24L) and *mutL*(N60S) mutant alleles (51). The *mutL*(K80E) allele results in a mutation rate close to that of a strain lacking *mlh1* (51). In *B. subtilis*, we found that the *mutL*(P24L) allele causes an inter-

mediate increase in the mutation rate, the *mutL*(N60S) allele is wild type for mutagenesis, and the *mutL*(K80E) and *mutL*(R256C) alleles confer an increase in the mutation rate comparable to that for a strain lacking *mutL*. In addition, our analysis found that *mutL*(K80E) and *mutL*(R256C) result in low protein levels *in vivo*, explaining our finding that the mutation rate conferred by these alleles approaches that of a *mutL*-deficient strain. We also found that the MutL S40A, G63R, and G94S proteins failed to accumulate *in vivo*. Studies of the corresponding human mutant protein revealed effects on expression for MLH1 G67R and R265C, corresponding to *B. subtilis mutL*(G63R) and *mutL*(R256C), respectively (50). Thus, two of the mutants that we found to have low or no accumulation in *B. subtilis* were shown to decrease in accumulation following expression in human cell culture.

The *mutL*(N60S) missense mutation has a different effect on MMR in *B. subtilis* than the corresponding missense mutation in *S. cerevisiae mlh1* (N64S), which caused a substantial defect in the MMR pathway (51). In addition, we found that the S40A mutant did not accumulate *in vivo* and that the corresponding change in human MLH1 showed greater than 75% expression, suggesting a difference in stability between the *B. subtilis* and human mutant proteins (50). Although we did observe some differences in the behavior of *B. subtilis* MutL relative to *S. cerevisiae* or human MLH1, most of the mutant proteins that we examined showed similar results on mutagenesis and protein accumulation. With these results, we suggest that *B. subtilis* MutL serves as an effective model for functional analysis of conserved *mutL* missense mutations identified in less experimentally tractable eukaryotic organisms.

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