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Depletion of the Cellular Amounts of the MutS and MutH Methyl-Directed Mismatch Repair Proteins in Stationary-Phase *Escherichia coli* K-12 Cells

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The MutL, MutS, and MutH proteins mediate methyl-directed mismatch (MDM) repair and help to maintain chromosome stability in *Escherichia coli*. We determined the amounts of the MDM repair proteins in exponentially growing, stationary-phase, and nutrient-starved bacteria by quantitative Western immunoblotting. Extracts of null mutants containing various amounts of purified MDM repair proteins were used as quantitation standards. In bacteria growing exponentially in enriched minimal salts-glucose medium, about 113 MutL dimers, 186 MutS dimers, and 135 MutH monomers were present per cell. Calculations with the in vitro dissociation constants of MutS binding to different mismatches suggested that MutS is not present in excess, and may be nearly limiting in some cases, for MDM repair in exponentially growing cells. Remarkably, when bacteria entered late stationary phase or were deprived of a utilizable carbon source for several days, the cellular amount of MutS dropped at least 10-fold and became barely detectable by the methods used. In contrast, the amount of MutH dropped only about threefold and the amount of MutL remained essentially constant in late-stationary-phase and carbon-starved cells compared with those in exponentially growing bacteria. RNase T2 protection assays showed that the amounts of *mutS*, *mutH*, and *mutL*, but not *miaA*, transcripts decreased to undetectable levels in late-stationary-phase cells. These results suggested that depletion of MutS in nutritionally stressed cells was possibly caused by the relative instability of MutS compared with MutL and MutH. Our findings suggest that the MDM repair capacity is repressed in nutritionally stressed bacteria and correlate with conclusions from recent studies of adaptive mutagenesis. On the other hand, we did not detect induction of MutS or MutL in cells containing stable mismatches in multicopy single-stranded DNA encoded by bacterial retrons.

The MutS, MutL, and MutH proteins play crucial roles in methyl-directed mismatch (MDM) repair in *Escherichia coli* (Fig. 1) (35–37, 45). MDM repair corrects mismatched base pairs and small bulge loops that arise as replication errors and thereby helps to set the spontaneous mutation rate (39, 57, 58). The MutL and MutS proteins also prevent homeologous recombination between related bacterial species (32, 46, 64, 65), suppress chromosomal rearrangements (41), and play an ancillary role in very-short-patch repair, which corrects mismatches that arise by deamination of 5-methyl-cytosine residues in certain contexts (29, 66). Together, these diverse functions indicate that the MutS, MutL, and MutH MDM repair proteins are part of a major system that maintains the genetic integrity and stability of bacterial chromosomes (36, 37, 45). The strand-break-directed mismatch repair exemplified by the *E. coli* MDM repair system is ancient and ubiquitous, and homologs of *E. coli* MutS and MutL have been found in yeasts, humans, and other organisms (7, 14, 23, 26a, 38, 42, 47). The recent discovery that human colon and sporadic cancers are caused by mutations in genes encoding homologs of *E. coli* MutL and MutS dramatically underscores the importance of this kind of repair system in chromosome protection and maintenance (7, 14, 26a, 38, 45).

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A biochemical outline of the steps involved in *E. coli* MDM repair has been worked out mainly by Modrich and his associates (1a, 9, 28, 35). MutS is thought to initiate MDM repair by binding to mismatched base pairs and small bulge loops (Fig. 1, top) (25, 39, 57, 58). MutS shows a binding preference for certain mismatches in vitro that is correlated with repair efficiency of mismatched phage DNA in vivo (57, 58). The biochemical function of MutL is unknown, although it may act as a molecular matchmaker that allows formation of a MDM repair complex containing MutS, MutL, and MutH at mismatched base pairs and unmethylated d(GATC) sites (21, 51). Formation of this complex is accompanied by ATP hydrolysis and activation of latent MutH d(GATC)-specific endonuclease activity, which cleaves newly replicated hemimethylated DNA strands 5′ or 3′ to mismatches (Fig. 1, top) (1a, 22, 63). The second stage of MDM repair also requires the MutL and MutS proteins and involves the excision of DNA from the nick introduced by activated MutH to just past the mismatch (Fig. 1, middle) (35–37). In the last stage of MDM repair, the long patch of single-stranded DNA is filled in and closed by ligation (Fig. 1, bottom) (35–37).

Little is known about the structure, expression, regulation, or coordination of the *mutS*, *mutL*, and *mutH* genes that encode the MutS, MutL, and MutH MDM repair proteins, respectively. *mutL* is a member of a complex superoperon with genes that encode the AmiB cell wall degradative amidase and the MiaA tRNA modification enzyme (62, 62a). The transcriptional organizations of *mutS* and *mutH* are largely unknown (20, 54). The cellular amounts of the MDM repair proteins

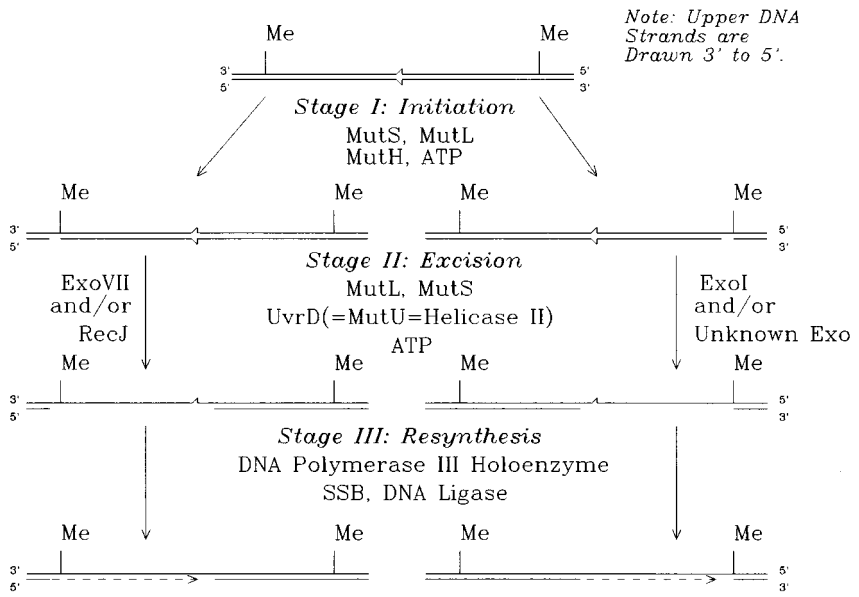

FIG. 1. Stages of MDM repair in *E. coli*. The figure is redrawn from information in references 35 to 37. MutS recognizes and binds to mismatches, MutL presumably acts as a molecular matchmaker in the MDM repair complex, and MutH is a d(GATC)-specific endonuclease (see the text). MutH is required for only the initiation stage, whereas MutS and MutL are required for both the initiation and excision stages (see the text). Me, methyl; SSB, single-stranded DNA binding protein.

have never been determined directly. Amounts of 10 to 30 molecules of MutL, MutS, and MutH per cell were estimated from β-galactosidase activities of gene fusions between the individual *mutHLS* genes and *lacZ* (10). The amount of MutH was also estimated at about 20 copies per cell from comparisons of the in vitro repair activity of purified MutH added to extracts of *mutH* mutants with that of extracts from wild-type bacteria (63).

Several pieces of indirect evidence imply that MDM repair capacity decreases in stressed bacterial cells. Schaaper and Radman showed that competent bacterial cells grown to late log phase in very rich medium had about fivefold-less MDM repair activity than cells from early log phase (53). Foster and Trimarchi (17) and Rosenberg and coworkers (48) independently found that frameshift mutations that accumulated in F′ *lac* genes during long-term starvation for a carbon source were similar to those expected for mutants with decreased MDM repair (24, 56). Recently, Longerich et. al provided additional support for the hypothesis that mismatch repair is disabled in starved cells (29a). They demonstrated that essentially the same spectrum of frameshift mutations was obtained in non-growing, starved *mutHLS*$^+$ cells as that in exponentially growing *mutS* or *mutL* mutants, which lacked MDM repair (29a).

In this paper, we present quantitation of the cellular amounts of the MDM repair proteins and transcripts in exponentially growing and stationary-phase bacterial cells. Our results support the idea that MDM repair capacity drops as cells enter stationary phase because of depletion of the MutS, and to a lesser extent the MutH, proteins. On the other hand, an increased load of mismatched base pairs, which saturated MDM repair, did not induce greater amounts of the MDM repair proteins.

MATERIALS AND METHODS

Materials. Bradford (5) and D_c (Lowry) protein assay kits and a bovine serum albumin (BSA) standard were purchased from Bio-Rad Laboratories (Hercules, Calif.). Polyvinylidene difluoride membranes (PVDF-plus, 0.45-μm pore size) were purchased from Micron Separations, Inc. (Westboro, Mass.). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (whole molecules) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Promega Corp. (Madison, Wis.).

Bacterial strains and culture conditions. The following *E. coli* K-12 strains were used: MG1655 (prototroph) (C. Gross collection [2]); NU426 [W3110 *sup*(Am) prototroph] (C. Yanofsky collection [2]); TX2652 (CC106 *mutL*::Ω), TX2928 (CC106 *mutH*::Tn*5*), and TX2929 (CC106 *mutS*::Tn*5*) (laboratory collection [13, 62a]); FC29 {*ara* Δ(*lac proB*)$_{XIII}$ *thiA* [F′Δ(*lacI lacZ*) *lacY*$^+$ *lacA*$^+$ *pro*$^+$]} (P. Foster collection [8, 15]); and CC107(pT110) and CC107(pT161) (W. Maas collection [31]).

Luria-Bertani (LB) broth and agar containing 10 g of NaCl per liter (50) were prepared from capsules marketed by Bio-101 (La Jolla, Calif.). Vogel-Bonner (1× E) minimal salts were prepared as described elsewhere (11). Enriched minimal salts-glucose medium (EMMG) contained 1× E salts, 0.01 mM $FeSO_4$, 0.4% (wt/vol) glucose, and 0.5% (wt/vol) vitamin assay Casamino Acids (Difco Laboratories, Detroit, Mich.). M9 minimal medium was prepared as described previously (34).

Sample preparation for quantitative Western blotting (immunoblotting). Cells from a single colony of each bacterial strain were added to 5 ml of EMMG, and the cultures were grown overnight with shaking (300 rpm) at 37°C. The overnight cultures (0.4 ml) were added to 80 ml of fresh EMMG in a 500-ml side-arm flask, which was shaken at 37°C (Fig. 2). Samples for Western immunoblotting were taken at culture densities of 50 Klett (660 nm) units (5.0×10^8 cells per ml) and 200 Klett units (2.2×10^9 cells per ml) and at 4 h (5.9×10^9 cells per ml), 19 h (5.7×10^9 cells per ml), and 43 h (6.5×10^9 cells per ml) after a density of 200 Klett units was reached (Fig. 2). The proportion of living cells was checked by using the Live/Dead BacLight bacterium viability kit (Molecular Probes Inc., Eugene, Oreg.). Fewer than 1% dead cells were detected at all sampling points, even 43 h after cultures reached a turbidity of 200 Klett (660 nm) units.

For Western immunoblotting, 5 to 10 ml of the cultures was removed at each sampling point, and cells were collected by centrifugation at 5,000 × *g* for 7 min at room temperature. Pellets were washed once with 1× E salts and suspended in 1× E salts at 0.01, 0.05, and 0.1 of the volume centrifuged for cultures at 50 Klett (660 nm) units, at 200 Klett units, and in stationary phase, respectively (see Results). This resuspension scheme gave final extracts containing 6 to 8 mg of protein per ml. Portions of the cell suspensions (20 μl) were diluted 20-fold in 0.5% (wt/vol) sodium dodecyl sulfate (SDS) and boiled for 10 min. Total protein concentrations were determined by using the Bio-Rad D_c (Lowry) protein assay kit with BSA in 0.5% SDS as the standard. The protein concentrations of all samples were in the linear range of the protein assay standard curve (data not shown). The remaining cell suspensions were lysed by adding an equal volume of 2× Laemmli dissociation solution (27) and boiling for 10 min. The resulting extracts were used for quantitative Western immunoblotting. Protein concentrations were not determined directly in the final extracts, because the Laemmli dissociation solution contained 2-mercaptoethanol, which interfered with the protein assay.

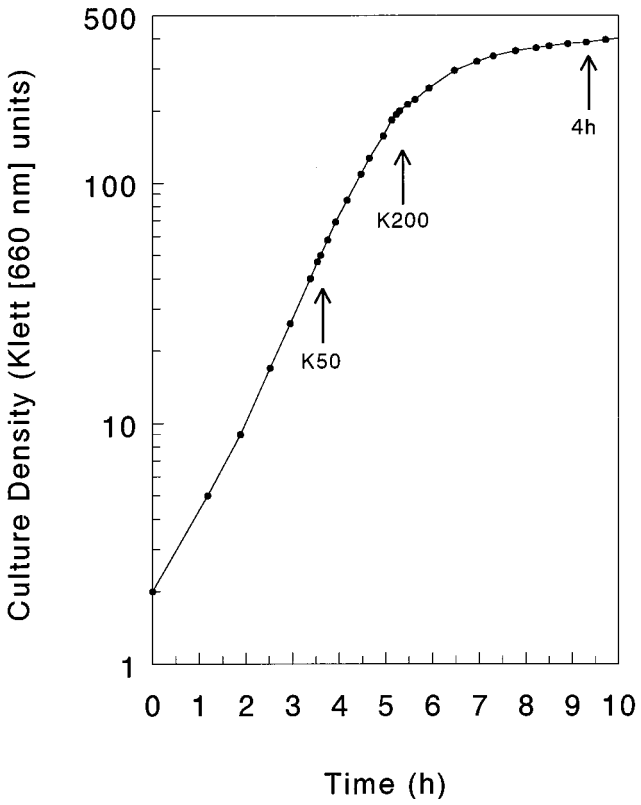

FIG. 2. Typical growth curve of strain MG1655 in EMMG medium at 37°C showing the points (arrows) at which samples were taken for quantitative Western immunoblotting and RNase T2 protection assays (see Materials and Methods). K50, 50 Klett units; K200, 200 Klett units; 4h, 4 h after the culture reached a density of 200 Klett units.

For determinations of the number of cells per milliliter, 0.1 ml of the original culture was added to 0.9 ml of cold 1× E salts, and the mixture was agitated vigorously on a vortex mixer. The number of CFU per milliliter of culture was determined by serial dilution of the culture into 1× E salts and spreading the dilutions onto LB agar plates, which were incubated at 37°C overnight. Microscopic examination of the first and subsequent dilutions for the Live/Dead BacLight test (described above) showed that the vast majority of the cells spread onto agar plates were individual cells. No aggregates containing multiple cells were observed, and only a small minority of the cells were present as divisional pairs that had not separated. Consequently, the number of CFU per milliliter accurately reflected the number of cells per milliliter in our determinations. From the protein concentrations and the number of CFU per milliliter at each sampling point, the amounts of total protein present in 10^9 cells were calculated to be 339, 330, 242, 274, and 230 μg at cell densities of 50 and 200 Klett units and at 4, 19, and 43 h after a density of 200 Klett units was reached, respectively. These values were used to calculate the numbers of MutH, MutL, and MutS molecules per cell.

Preparation of protein standards for quantitative Western blotting. His-6-tagged *E. coli* MutH, MutL, and MutS proteins (referred to hereafter as His-6-MutH, His-6-MutL, and His-6-MutS, respectively) were purified as described previously (13). Protein concentrations of the purified His-6-MutH, His-6-MutL, and His-6-MutS were determined by the Bradford protein assay (5) with BSA as the standard. The His-6-tagged fusion proteins were digested with thrombin (Novagen, Madison, Wis.) to remove 17 of 20 amino acids from the amino-terminal leader peptides, including the His-6-affinity tag. Proteins were then separated by SDS–11% (wt/vol) polyacrylamide gel electrophoresis (PAGE) (5.6% stacking) and visualized by staining with Coomassie brilliant blue R-250. Digestion of His-6-MutH and His-6-MutS with thrombin seemed to remove the His-6-affinity tag without obvious cleavage at internal sites. In contrast, thrombin cleaved His-6-MutL at one or two sites in a nonconserved segment of MutL, which may form a hinge region (60), resulting in 40- and 27-kDa polypeptide fragments. Therefore, thrombin-treated His-6-MutS and His-6-MutH and untreated His-6-MutL were used as the standards for quantitative Western immunoblotting.

Antibody production and preabsorption of antisera with insertion mutant lysates. In early experiments, polyclonal anti-MutL and anti-MutH, -MutL, and -MutS antisera, which were generous gifts from Paul Modrich (Duke University) and Martin Marinus (University of Massachusetts Medical Center), respectively, were used in blotting experiments. In final experiments, we prepared supplies of polyclonal antibodies in rabbits against purified His-6-MutH, His-6-MutL, and His-6-MutS as described previously (68). To reduce the background in the blots, antisera were preabsorbed to lysates prepared from *mutHLS* insertion mutants. Strains TX2652(CC106 *mutL*::Ω), TX2928(CC106 *mutH*::Tn*5*), and TX2929 (CC106 *mutS*::Tn*5*) were grown overnight in LB medium containing 50 μg of kanamycin per ml at 37°C with shaking to saturation. Cells were collected by centrifugation at 5,000 × *g* for 7 min at 4°C. Pellets were resuspended in 0.1 volume of 1× Laemmli dissociation solution (27), and cells were lysed by boiling for 10 min. Each mutant lysate (400 μl) and 40 μl of the corresponding undiluted antiserum were added to 40 ml of 20 mM Tris-HCl (pH 8.2)–0.9% (wt/vol) NaCl–1% (wt/vol) nonfat dry milk. The mixture was incubated at 4°C for about 48 h in a 50-ml plastic centrifuge tube that was gently inverted. The resulting preabsorbed, diluted antisera were used directly for quantitative Western blotting.

Quantitative Western blotting. Quantitative Western blotting was carried out by a procedure described previously (68) with the following changes. Denatured total protein (100 μg for MutL and MutS or 150 μg for MutH) from cells harvested at various densities and times (see Results) was loaded per lane and fractionated by SDS–11% (for MutL and MutS) or –15% (wt/vol) (for MutH) PAGE (5.6% stacking) (27). Quantitation standards run on the same gels were mixtures of various amounts of purified denatured proteins mixed with 100 or 150 μg of denatured total protein prepared from individual *mutHLS* mutants at a culture density of 50 Klett units. Proteins were electrotransferred from SDS-PAGE gels to PVDF-plus membranes in a Transphor TE50 unit (Hoefer Scientific Instruments, San Francisco, Calif.) containing 25 mM Tris–192 mM glycine (pH 8.5)–20% (vol/vol) methanol at 1.4°C for 6 to 7 h at 100 V. The membranes were incubated in blocking solution (40 ml of 20 mM Tris-HCl [pH 8.2], 0.9% [wt/vol] NaCl, 5% [wt/vol] nonfat dry milk) at 4°C with gentle shaking overnight. The membranes were incubated with 40 ml of preabsorbed antisera (described above) for 3 h at room temperature with gentle shaking. Subsequent steps were the same as those described previously (68). Air-dried membranes were scanned on a UC 630 Maxcolor scanner (UMAX Data System Inc., Taiwan), and images were saved into the Adobe Photoshop 2.5 program and analyzed by the Image 1.42f program. The amounts of MutL, MutS, and MutH in cellular extracts were determined from the linear detection range of standard curves generated for the standards described above. The number of MutH monomers (molecular mass of 25 kDa [63]), MutL dimers (molecular mass of 139 kDa [21]), and MutS dimers (molecular mass of 194 kDa [58]) per cell was calculated from the amount of MDM repair protein (in nanograms) determined in Western blots, the molecular masses of the MDM repair proteins, Avogadro's number (6.023×10^{23} molecules per mol), the total amount of protein (in micrograms) analyzed by Western blotting, and the amount of total protein (in micrograms) per 10^9 cells harvested at the indicated culture densities or times (see Results).

Long-term starvation of FC29 cells for carbon source. Strain FC29 was grown overnight in 1× M9 minimal salts (34) containing 0.4% (wt/vol) glucose and 20 μg of thiamine per ml at 37°C with rotary shaking (300 rpm) to early stationary phase. Cells were collected by centrifugation at 5,000 × *g* for 7 min at room temperature. Cell pellets were washed twice with 1× M9 salts and resuspended in 0.1 volume of 1× M9 salts. The suspension (0.1 ml; $\approx 3 \times 10^9$ cells) was spread onto 1× M9 minimal salts plates containing 0.2% (wt/vol) lactose, 20 μg of thiamine per ml, and 1.5% (wt/vol) Bacto-agar (Difco Laboratories). The plates were incubated at 37°C for 8 days (8, 15). Each day, the cells were washed off 20 plates with 1× M9 salts (final volume of 20 ml), and protein samples were prepared for quantitative Western immunoblotting as described above.

RNase T2 protection assays of chromosomal transcripts. Total RNA was purified from the same cultures from which protein samples were prepared for Western blotting. RNA was prepared by adding portions of the bacterial cultures directly to lysis solutions without intervening steps as described previously (61). RNase T2 protection assays of transcripts from the bacterial chromosomes were completed as described previously (61). The RNA for detection of cotranscripts from the *mutL-miaA* region was synthesized with phage T7 RNA polymerase and plasmid pTX315 linearized with *Bsa*HI as described previously (62). An RNA probe, which covered the last 890 nucleotides (nt) of the *mutS* coding region and 85 nt downstream of *mutS*, was synthesized with phage SP6 RNA polymerase and plasmid pTX413 linearized with *Eco*RI. pTX413 contained a 975-bp *Hin*cII-to-*Hin*dIII insert from Kohara phage 449 (26, 49) cloned into the pGEM3Z vector (Promega, Inc.). An RNA probe, which covered 668 nt of the region upstream from *mutH* and the first 296 nt of the *mutH* coding region, was synthesized with phage RNA polymerase SP6 and plasmid pTX411 linearized with *Eco*RI. pTX411 contained a 964-bp *Hin*dIII-to-*Hin*cII insert from Kohara phage 462 (26, 49) cloned into pGEM3Z. A series of labeled RNA molecules of known lengths were used as size standards to determine the lengths of the protected probes (61). Each hybridization reaction mixture contained 15 to 30 μg of total bacterial RNA.

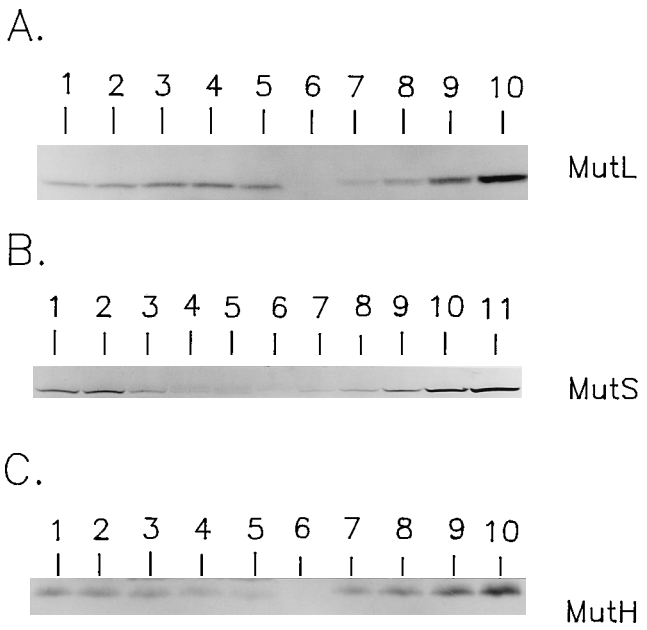

FIG. 3. Typical quantitative Western immunoblots used to determine the cellular amounts of the MutL (A), MutS (B), and MutH (C) proteins in *E. coli* prototroph MG1655 grown to various stages (see Fig. 2) in EMMG at 37°C. Cellular amounts were determined from standard curves generated with the quantitation standards listed (see Materials and Methods). (A) Lanes: 1 to 5, MutL (amounts) in 100 μg of total protein from MG1655 grown to densities of 50 (7.9 ng) and 200 (8.8 ng) Klett units and to 4 h (9.9 ng), 19 h (10.7 ng), and 43 h (8.6 ng) after a density of 200 Klett units was reached, respectively; 6 to 10, quantitation standards containing 100 μg of total protein from TX2652(CC106 *mutL*::Ω) grown in EMMG at 37°C to a density of 50 Klett units to which the following amounts of purified His-6-MutL were added (see Materials and Methods): none (*mutL*::Ω extract only), 3, 6, 12, and 24 ng, respectively. (B) Lanes: 1 to 5, MutS in 100 μg of total protein from MG1655 grown to densities of 50 (17.4 ng) and 200 (18.9 ng) Klett units and to 4 h (9.3 ng), 19 h (3.3 ng), and 43 h (2.8 ng) after a density of 200 Klett units was reached, respectively; 6 to 11, quantitation standards containing 100 μg of total protein from TX2929(CC106 *mutS*::Tn*5*) grown in EMMG at 37°C to a density of 50 Klett units to which the following amounts of purified MutS were added (see Materials and Methods): none (*mutS*::Tn*5* extract only), 3.8, 7.5, 15, 30, and 60 ng, respectively. (C) Lanes: 1 to 5, MutH in 150 μg of total protein from MG1655 grown to densities of 50 (2.5 ng) and 200 (2.0 ng) Klett units and to 4 h (1.7 ng), 19 h (1.2 ng), and 43 h (1.1 ng) after a density of 200 Klett units was reached, respectively; 6 to 10, quantitation standards containing 150 μg of total protein from TX2928(CC106 *mutH*::Tn*5*) grown in EMMG at 37°C to a density of 50 Klett units to which the following amounts of purified MutH were added (see Materials and Methods): none (*mutH*::Tn*5* extract only), 1.5, 3.0, 6.0, and 12 ng, respectively.

RESULTS

Quantitation of MutL, MutS, and MutH amounts in exponentially growing bacteria. We performed quantitative Western immunoblot analyses to determine the cellular amounts of the MutL, MutS, and MutH proteins in *E. coli* cells growing exponentially in EMMG medium at 37°C (see Materials and Methods) (Fig. 2). Cells of prototrophic W3110 (NU426) and MG1655 strains were harvested for extraction at a density of 50 Klett (660 nm) units (≈5×10^8 cells per ml) (Fig. 2, K50). Standards for immunoblots were extracts from *mutL*, *mutS*, and *mutH* null mutants to which various amounts of purified MutL, MutS, and MutH proteins were added (Fig. 3). To this end, His-6-MutL, His-6-MutS, and His-6-MutH proteins were purified as described previously (13), and the His-6-tag affinity label was removed from His-6-MutS and His-6-MutH by treatment with thrombin protease (see Materials and Methods). His-6-MutL was cleaved by thrombin at a likely hinge region in the middle of the MutL polypeptide (see Materials and Methods) (60); consequently, His-6-MutL was used as a standard. We did not observe changes in transfer efficiency during Western analysis when His-6-MutS, His-6-MutH, and His-6-PdxB (39a) were compared with their thrombin-cleaved derivatives (data not shown). This result suggested that the His-6 affinity tag probably did not greatly influence protein transfer of the His-6-MutL standard.

Extracts of *mutL*, *mutS*, and *mutH* null mutants lacked the corresponding intact MutL, MutS, and MutH MDM repair proteins (Fig. 3, lanes 6). Densitometer tracings of the sample bands were within the linear detection range of the His-6-MutL (Fig. 3A, lanes 7 to 10), MutS (Fig. 3B, lanes 7 to 11), and MutH (Fig. 3C, lanes 7 to 10) standards (data not shown; see Materials and Methods). On the basis of separate blots of the same set of samples of MG1655 (Fig. 4), the following numbers of molecules per cell (expressed as means ± standard errors) were calculated (see Materials and Methods): for MutL, 113 ± 11 dimers; for MutS, 186 ± 4 dimers; and for MutH, 135 ± 2 monomers.

Depletion of MutS and MutH, but not MutL, in stationary-phase cells. We extended our quantitative Western analyses of the MDM repair proteins to cultures in transition phase (density of 200 Klett units) (Fig. 2, K200), in early stationary phase (4 h after a density of 200 Klett units was reached) (Fig. 2, 4 h), and in late stationary phase (19 and 43 h after a density of 200 Klett units was reached). The amount of MutL protein persisted throughout all phases of growth (Fig. 3A and 4), even to 6 days in stationary phase (data not shown). In contrast, the amount of MutS protein was maintained in transition-phase cells (K200) but dropped threefold in early-stationary-phase cells (4 h) and became difficult to detect above background in

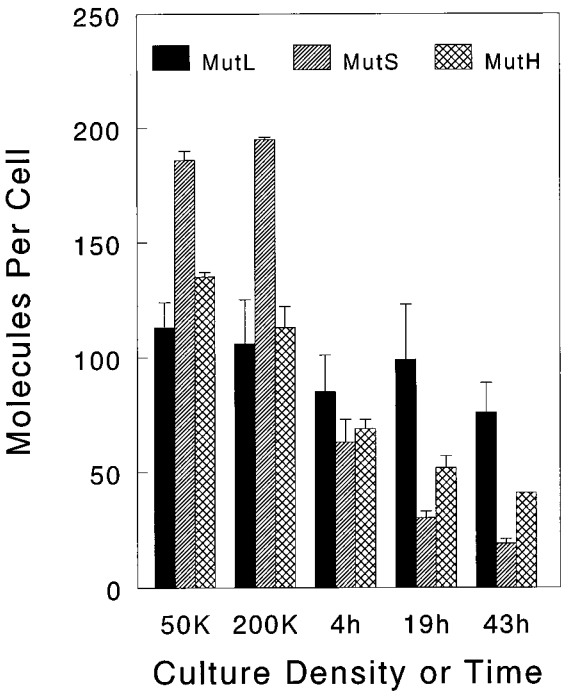

FIG. 4. Numbers of MutL dimers, MutS dimers, and MutH monomers per MG1655 cell at various phases of growth (Fig. 2) in EMMG medium at 37°C. The number of molecules per cell was calculated by using the cellular amounts of the MutHLS proteins (e.g., Fig. 3), the amount of protein per viable cell at different phases of growth, and other parameters listed in Materials and Methods. The numbers of molecules per cell shown are the averages of two (for MutS and MutH) or three (for MutL) quantitative Western immunoblots from one set of protein samples. Repeated experiments with different sets of samples of MG1655 and NU426 showed less than 20% variation from the values shown.

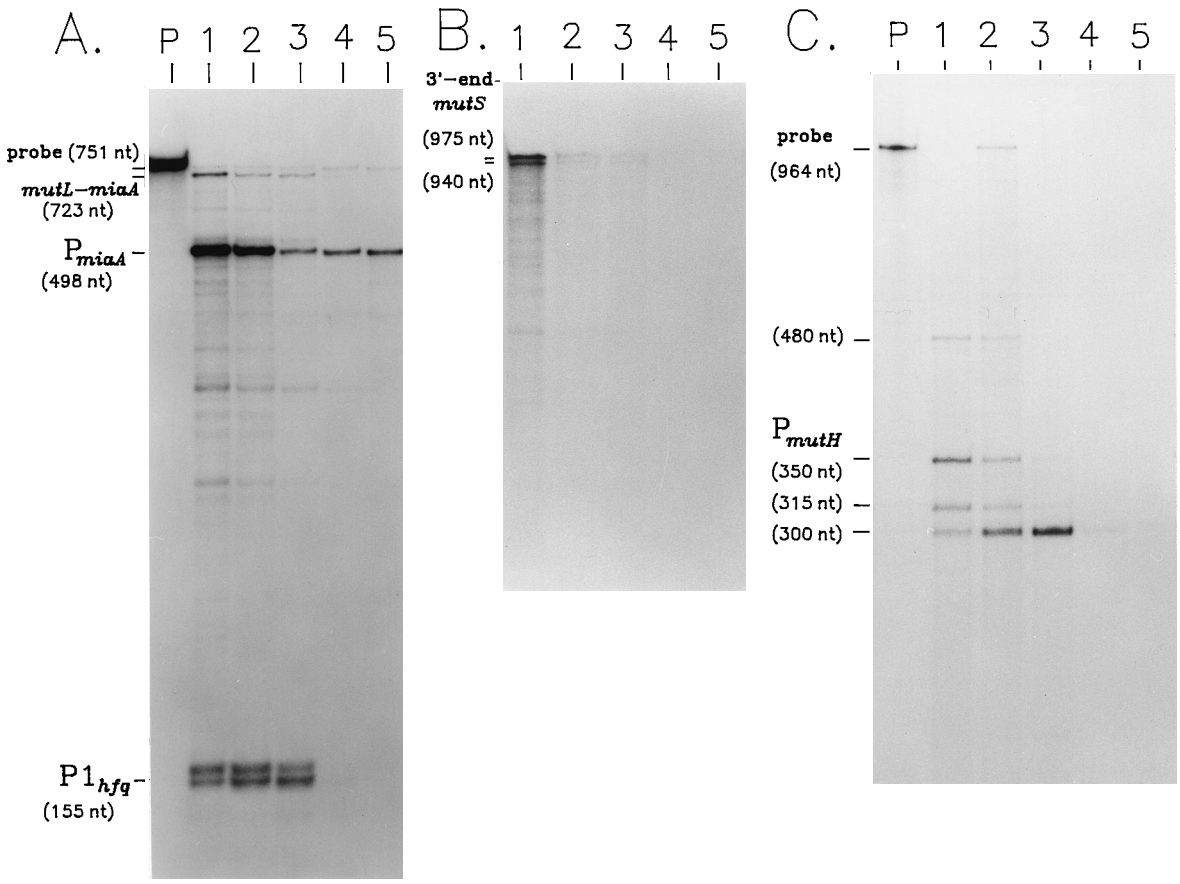

FIG. 5. Disappearance of *mutL*, *mutS*, and *mutH* transcripts in stationary-phase bacteria. Total RNA preparations from MG1655 grown to various stages were hybridized to RNA probes for the *mutL-miaA* cotranscript (A), the 3′ region of *mutS* (B), and the 5′ region of *mutH* (C), and the resulting hybrids were analyzed by RNase T2 protection assays (see Materials and Methods). (A) Lanes: P, intact RNA probe for transcripts from the *mutL-miaA* region; 1 to 5, protected species from hybrids formed between the *mutL-miaA* probe and RNA from cells grown to densities of 120 and 200 Klett units and to 4, 19, and 67 h after a density of 200 Klett units was reached, respectively. Bands corresponding to the intact *mutL-miaA* probe, the *mutL-miaA* cotranscript, and transcripts initiated from the σ^{70}-dependent P_{miaA} and σ^{32}-dependent $P1_{hfq}$ internal promoters are indicated on the left (59, 62). The faint, uppermost bands in lanes 1 to 5 represent small amounts of undigested probe. (B) Lanes: 1 to 5, protected species from hybrids formed between the probe for the 3′ region of the *mutS* transcript and RNA from cells grown as described for panel A, except that the reaction mixture in lane 5 contained RNA isolated 43 h after the bacteria reached a density of 200 Klett units. The intact probe is not shown. The 975- and 940-nt bands corresponded to a full-length-protected and a slightly shorter protected species from the 3′ end of the *mutS* transcript, respectively. (C) Lanes: P, intact RNA probe for transcripts from the 5′ region of *mutH*; 1 to 5, protected species from hybrids formed between the *mutH* probe and RNA from cells grown as described for panel B. The same four protected bands (480, 350, 315, and 300 nt) were detected with another RNA probe that ended at the same site in *mutH* (data not shown). This result indicated that the four bands were specific to the *mutH* coding region and not the other end of the RNA probe. The 350-nt band, marked P_{mutH}, corresponded to transcripts initiated from a promoter detected previously (20). The other three protected bands probably corresponded to additional promoters or processed transcripts (see the text). The uppermost band in lane 2 represents undigested probe.

19- and 43-h cultures (Fig. 3B and 4). We estimated that the amount of cellular MutS declined at least 10-fold in stationary-phase cells compared with that in exponentially growing and transition-phase cells. Likewise, the amount of cellular MutH dropped noticeably, about two- to threefold, in early- and late-stationary-phase cells compared with that in exponentially growing and transition-phase cells (Fig. 3C and 4). Thus, the amounts of the MutS and MutH MDM repair proteins were considerably reduced in stationary-phase cells compared with those in exponentially growing cells. Similar results were obtained when different sets of samples of MG1655 and NU426 were analyzed by quantitative Western blotting.

To understand the mechanism by which the amounts of MutS and MutH protein decreased in stationary-phase cells, we quantitated segments of the mRNA transcripts from the *mutL*, *mutS*, and *mutH* genes (Fig. 5). RNase T2 protection assays with RNA isolated from the same MG1655 cultures from which protein samples were prepared showed that the amounts of *mutL*, *mutS*, and *mutH* transcripts decreased significantly in stationary-phase cells. The *mutL-miaA* cotranscript and transcripts initiated at the σ^{70}-dependent P_{miaA} (59, 62) and σ^{32}-dependent $P1_{hfq}$ promoters (59, 62) were detectable in exponentially growing cells (at 120 Klett units), in cells at 200 Klett units, and in cells 4 h after a density of 200 Klett units was reached (Fig. 5A, lanes 1 to 3, respectively). After 19 and 67 h, the *mutL-miaA* cotranscript and the $P1_{hfq}$-initiated transcript were undetectable (Fig. 5A, lanes 4 and 5), whereas the P_{miaA}-initiated transcripts were present at about 20% of the amount detected in exponentially growing cells (Fig. 5A, lanes 1, 4, and 5).

Transcripts (940 and 975 nt) from the promoter-distal 3′ region of *mutS* were readily detectable in late-exponential-phase cells (120 Klett units) (Fig. 5B, lane 1). However, the amounts of the *mutS* transcript from this region and from the *mutS* promoter-proximal region (data not shown) decreased significantly as cells entered transition and early stationary phase (Fig. 5B, lanes 2 and 3) and became undetectable in cells after 19 h (Fig. 5B, lanes 4 and 5). Likewise, the amount of

promoter-proximal *mutH* transcript decreased in early-stationary-phase cultures (Fig. 5C, lane 3) and became barely detectable in 19- and 43-h cultures (Fig. 5C, lanes 4 and 5). The band marked as P_{mutH} (350 nt) probably corresponded to a transcript initiated from a promoter detected previously (20). We have not yet determined whether the three remaining *mutH* transcript bands (480, 315, and 300 nt) correspond to transcripts initiated from separate promoters or to processed transcripts. Similar results of diminishing amounts of *mutL*, *mutS*, and *mutH* transcripts as cells entered stationary phase were obtained with RNA obtained from the W3110 prototrophic strain NU426 (data not shown).

MutL and MutS amounts in cells subjected to long-term starvation. Studies of adaptive mutagenesis suggested that MDM repair may be decreased in bacteria subjected to long-term starvation for carbon source (3, 16, 17, 29a, 48, 55). Many adaptive mutagenesis protocols have determined the reversion frequency of a *lac* allele contained on an F′ element in a mutant with a deletion of the *lac* operon in its chromosome (8, 15, 48). In these studies, bacteria were grown overnight in minimal salts-glucose medium and then washed, concentrated, and spread onto minimal salts-lactose plates (8, 15, 48).

On the basis of results presented above, we expected that the cellular amount of MutS protein would be depleted in the nutrient-stressed cells used for adaptive mutagenesis experiments. Therefore, we determined the cellular amounts of MutS and MutL in strain FC29 subjected to an adaptive mutagenesis plating protocol. FC29 contains Δ*lacZ* mutations in its chromosome and on an F′ element, so postplating revertants cannot accumulate and begin to grow on minimal salts-lactose plates. Each day for 8 days, we washed nondividing cells from the plates, prepared extracts, and performed quantitative Western analyses (see Materials and Methods). In agreement with the results described above for strains MG1655 and NU426 (Fig. 2 to 4, 4 h), we detected intact MutS protein in FC29 cells grown in M9 minimal salts-glucose medium to early stationary phase (data not shown). After 1 day on the M9 minimal salts-lactose plates, we could no longer detect MutS protein above background in strain FC29 (data not shown). In contrast, the amount of MutL protein decreased slightly after 1 day of starvation and then remained essentially constant for the remaining 7 days of the experiment (data not shown). Thus, the amount of cellular MutS again declined in nutrient-stressed cells, whereas the amount of MutL persisted.

Cellular MutL and MutS amounts remained unchanged by overexpression of T × C mismatched msDNA. Maas and coworkers recently showed that overexpression of retron-encoded multicopy single-stranded DNA (msDNA) containing a nonrepairable T × C mismatch saturated the MDM repair system (30, 31). The increased mutation frequency caused by overexpression of the mismatched msDNA was suppressed by a multicopy plasmid containing the $mutS^+$ gene (30). In addition, mutation frequency was not increased when msDNA lacking a mismatched base pair was overexpressed from a multicopy plasmid (30, 31).

We wanted to determine whether cells responded to an increased load of mismatched base pairs by increasing the amounts of the MDM repair proteins. Consequently, we measured the amounts of the MutS and MutL proteins in strain CC107 overexpressing mismatched msDNA from plasmid pT161 or nonmismatched msDNA from plasmid pT110. In exponential- and transition-phase cells grown in EMMG containing 50 μg of ampicillin per ml at 37°C, we detected both MutS and MutL, but we did not observe any difference in the amounts in the two strains. In late-stationary-phase cells, the amount of MutS decreased to below the level of detection and the amount of MutL remained essentially constant in both strains. Control experiments confirmed the previous finding (31) that CC107(pT161) grown in EMMG containing 50 μg of ampicillin per ml showed a mutator phenotype compared with CC107(pT110) for the accumulation of rifampin-resistant mutants (data not shown). Thus, the amounts of the MutL and MutS proteins were not induced by an increased number of T×C mismatched base pairs that saturated the MDM repair system.

DISCUSSION

This report addresses two important physiological issues about MDM repair in *E. coli*. First, we determined the cellular amounts of the three MDM repair proteins in exponentially growing cells. We found that MutS (≈186 dimers per cell) was present in slight excess over MutL (≈113 dimers per cell) and MutH (≈135 monomers per cell) (Fig. 3 and 4). Our results showed that the MDM repair proteins were present in about 10-fold-greater amounts in exponentially growing cells than estimated previously by indirect methods (see Introduction). Second, we showed that the levels of the MDM repair proteins were regulated in cells at different phases of growth. In particular, we showed that the amount of MutS protein decreased at least 10-fold in late-stationary-phase cells compared with that in exponential-phase cells (Fig. 2 to 4). In the same cells, the amount of MutH protein decreased somewhat (approximately threefold), whereas the amount of MutL protein remained constant even after several days in stationary phase (Fig. 3 and 4) (see Results). This study is the first to demonstrate regulation of the amounts of MDM repair protein in *E. coli.*

The cellular amounts of the MutHLS repair proteins are comparable to that of the Dam d(GATC)-specific methyltransferase (≈130 molecules per cell) (4) but are small compared with those of many biosynthetic enzymes (≈500 to 60,000 molecules per cell [67, 68]) or proteins of the transcriptional and translational machinery (≈5,000 to 25,000 molecules per cell [6]). To surmise whether the MDM repair proteins were present in an excess amount in exponentially growing cells, we performed the following calculation. The bacteria used in this study grew in EMMG at 37°C with a doubling time of about 40 min (Fig. 2). Bacteria growing at this rate contain about 2.3 genome equivalents of DNA per cell (6), which corresponds to about 10.8×10^6 bp of DNA per cell (26, 49). The mutation rate in a *mutL* mutant, which still ensures DNA fidelity by base selection and proofreading, is about 4.5×10^{-8} per base pair replicated (52). Therefore, the average *E. coli* cell will not accumulate even one mismatched base pair per round of replication, and one mismatch per cell may be taken as an upper limit. *E. coli* cells growing under these conditions have an average volume of about 1.2×10^{-12} ml (12), which means that the cellular concentration of one mismatched base pair or 186 dimers of MutS would be about 1.4×10^{-9} or 2.6×10^{-7} M, respectively. The apparent dissociation constants (K_d) for binding of MutS (corrected for dimers) to different mismatched base pairs contained in phage genomes could be divided into three classes (57): strong (G × T and A × C; K_d [average] of $\approx 23 \times 10^{-9}$ M); medium (A × A, T × T, and G × G; K_d [average] of $\approx 67 \times 10^{-9}$ M), and weak (A × G, C × T, and C × C; K_d [average] of $\approx 187 \times 10^{-9}$ M). If only one MutS dimer is assumed to bind to one mismatched base pair per cell, then $[\text{MutS}]_{\text{total}} \approx [\text{MutS}]_{\text{free}}$, and the binding equation can be simplified to $[\text{mismatched DNA}]_{\text{free}}/[\text{mismatched DNA}]_{\text{total}} \approx K_d/(K_d + [\text{MutS}]_{\text{total}})$ (43). Thus, MutS will be bound about 92, 80, and 58% to the tight-, medium-, and weak-binding classes

of mismatched base pairs in any instant of time. These average occupancy times must be overestimates, because nonspecific binding of MutS to nonmismatched DNA, which is known to occur in vitro, was not taken into consideration in our calculation (13, 22, 25). Although mismatch binding by MutS is only one factor that determines DNA correction efficiency (57), these approximations strongly imply that the MutHLS repair proteins are not in excess and may, in fact, be nearly limiting in some cases for mismatch recognition in exponentially growing cells. Consistent with this conclusion, expression of MutS and MutL from multicopy plasmids seemed to increase the efficiency of error correction in cells grown to early stationary phase (16).

The large decrease in the amount of MutS in late-stationary-phase (Fig. 3 and 4) and carbon-limited cells (see Results) supports the hypothesis that MDM repair is deficient in nutrient-deprived bacteria (3, 16, 17, 29a, 48, 55). These previous studies examined aspects of adaptive mutagenesis by using strains and starvation protocols similar to those used here (Results). On the basis of the considerations discussed above, a 10-fold drop in the amount of cellular MutS should significantly reduce the MDM repair capacity, especially since MutS recognizes mismatches in the first step of MDM repair (Fig. 1). This limitation in the amount of MutS might be exaggerated by multiple chromosome copies, which seem to be present in stationary-phase *E. coli* cells (1). As noted previously (17, 29a, 48), defective MDM repair would account for the type of −1 deletion mutations that arose in iterated repeats on an F′ element in starving cells. Moreover, recent analyses of adaptive mutagenesis showed that replication of F′ episomes, probably from the *oriT* transfer origin, takes place in nondividing, nutritionally stressed cells (18, 19, 40, 44). Our results suggest that this episome replication occurs in cells deficient in MDM repair. Finally, the observation that expression of MutS and MutL from multicopy plasmids suppressed adaptive mutagenesis in *polB* mutants (16) is consistent with the conclusion that the amounts of MutS and possibly functional MutL declined in carbon-starved bacteria.

The depletion of MutS in nutritionally stressed cells was probably caused by an abrupt decrease in the amount of the *mutS* transcript (Fig. 5) and the relative instability of MutS compared with MutL and MutH (Fig. 3 and 4). The amounts of the *mutL* and *mutH* transcripts also dropped to undetectable levels as cells entered late stationary phase (Fig. 5), but the amount of MutL, in particular, persisted at a nearly constant level in stationary-phase and starved cells for several days (Fig. 4) (Results). In contrast to *mutHLS*, transcripts from the σ^{70}-dependent P_{miaA} internal promoter (59, 62), which is located in the *mutL* coding region, were detectable in late-stationary-phase bacteria for at least 67 h (Fig. 5). Our present experiments cannot distinguish whether the abrupt drop in *mutHLS* transcript levels was caused by decreased transcription initiations, increased transcript degradation, or both. We also cannot tell from our current results whether MutL and MutH that persisted in late-stationary-phase cells were functional and whether MutS is relatively unstable compared with MutL and MutH under all growth conditions. Ongoing studies will address these issues and the mechanisms that ensure similar cellular amounts of MutS, MutL, and MutH (Fig. 4) from the separate *mutS* (61.5 min), *mutL* (94.8 min), and *mutH* (63.9 min) genes in exponentially growing bacteria.

Recently, Radman and colleagues discussed the implications of repair in nonstressed and stressed cells for evolution and the development of disease (33, 45). In their arguments, they assumed that bacterial MDM repair was essentially "constitutive" and emphasized the importance of SOS-inducible error-prone repair for evolutionary adaptation (45). The results presented here show that the amounts of the MDM repair proteins are not constitutive and that the amounts of MutS and MutH are regulated downward in stationary-phase and starved bacteria. Nonetheless, our findings fit with their general argument that bacterial cells maintain chromosome fidelity during the "easy-life" conditions but still need mechanisms that allow potentially beneficial mutations to accumulate in "hard-life" situations (45). Besides induction of SOS, the MDM repair and other fidelity functions of MutS (see Introduction) are likely repressed in nutrient-limited bacteria. Our results also imply that the MDM repair level is regulated by controlling the amount of the MutS component that binds to mismatches and initiates repair. The amounts of MutL or MutH, which interact with MutS after it binds to mismatches (Fig. 1), remained the same or only moderately decreased, respectively, as bacteria entered late stationary phase (Fig. 3 and 4).

On the other hand, we did not detect induction of MutS or MutL in cells containing stable T × C mismatches in retron-encoded msDNA (Results). Overexpression of T × C mismatch-containing msDNA from the plasmids used here caused a mutator phenotype (Results) (31) that was suppressed by expression of MutS from a multicopy plasmid (30). These results imply that the amount of MutS was titrated below a critical level by the presence of excess stable T × C mismatches (30). Thus, it appears that the amount of cellular MutS can be repressed but not increased in response to the stress conditions tested so far. It remains to be determined whether other situations that titrate MDM repair and lead to a strong mutator phenotype, such as the growth of *mutD* mutants in rich medium (10, 53), change the cellular amounts or levels of activity of the MDM repair proteins. In *mutD* mutants, limitation for MutL and MutH, rather than MutS, may diminish MDM repair capacity (53).

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