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Phage-Associated Mutator Phenotype in Group A Streptococcus[▽]

Julie Scott, Prestina Thompson-Mayberry, Stephanie Lahmamsi, Catherine J. King, and W. Michael McShan*

Department of Pharmaceutical Sciences, The University of Oklahoma College of Pharmacy, P.O. Box 26901, Oklahoma City, Oklahoma 73190

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Defects in DNA mismatch repair (MMR) occur frequently in natural populations of pathogenic and commensal bacteria, resulting in a mutator phenotype. We identified a unique genetic element in *Streptococcus pyogenes* strain SF370 that controls MMR via a dynamic process of prophage excision and reintegration in response to growth. In *S. pyogenes*, *mutS* and *mutL* are organized on a polycistronic mRNA under control of a common promoter. Prophage SF370.4 is integrated between the two genes, blocking expression of the downstream gene (*mutL*) and resulting in a mutator phenotype. However, in rapidly growing cells the prophage excises and replicates as an episome, allowing *mutL* to be expressed. Excision of prophage SF370.4 and expression of MutL mRNA occur simultaneously during early logarithmic growth when cell densities are low; this brief window of MutL gene expression ends as the cell density increases. However, detectable amounts of MutL protein remain in the cell until the onset of stationary phase. Thus, MMR in *S. pyogenes* SF370 is functional in exponentially growing cells but defective when resources are limiting. The presence of a prophage integrated into the 5′ end of *mutL* correlates with a mutator phenotype (10^{-7} to 10^{-8} mutation/generation, an approximately a 100-fold increase in the rate of spontaneous mutation compared with prophage-free strains [10^{-9} to 10^{-10} mutation/generation]). Such genetic elements may be common in *S. pyogenes* since 6 of 13 completed genomes have related prophages, and a survey of 100 strains found that about 20% of them are positive for phages occupying the SF370.4 *attP* site. The dynamic control of a major DNA repair system by a bacteriophage is a novel method for achieving the mutator phenotype and may allow the organism to respond rapidly to a changing environment while minimizing the risks associated with long-term hypermutability.

The ability to adapt to a changing environment is crucial to the success of any species. The mutation rate in bacteria has been estimated to be 0.003 mutation per genome ($\sim 5 \times 10^{-10}$ mutation per base) per replication (13), and therefore, a minimum population size is needed to ensure that that there are rare variants that are resistant to an antibiotic, for example. Accordingly, if the population density of a bacterial species is low, then at typical mutation rates rare mutants may not arise, leading to extinction.

A growing body of evidence indicates that bacteria from wild populations often avoid population extinction by altering their mutation rates. These strategies typically either reduce the fidelity of DNA replication or alter DNA repair mechanisms, resulting in a hypermutable state (49). As originally reported by LeClerc et al., the incidence of mutators among clinical isolates of pathogenic *Escherichia coli* and *Salmonella enterica* was found to be much higher than anticipated (>1%), with defects in DNA mismatch repair (MMR) being responsible for this (29). Subsequent studies found examples in many bacterial species; for example, 30% of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients and 57% of serogroup A epidemic isolates of *Neisseria meningitidis* were found to exhibit a mutator phenotype or be defective for MMR (18, 28, 43, 47). However, the appearance of mutator strains is not confined to pathogenic bacteria, since the frequency of the defects was essentially the same in commensal and pathogenic *E. coli* in the survey performed by Matic and colleagues (36). The evidence suggests that the frequency of mutators and thus the potential for evolution in wild populations of bacteria may be significantly different from the frequency of mutators and potential for evolution in laboratory strains.

Prokaryotic MMR has been most intensively studied in *E. coli*, where transient DNA hemimethylation patterns following replication are used to discriminate between the template strand and the newly synthesized strand containing the mismatch. The required proteins MutS, MutL, and MutH mediate MMR, recognizing the mismatch and cleaving the transiently unmethylated strand, allowing removal of the region containing the erroneous base and repair by resynthesis of the strand (35). Homologs of MutS and MutL appear to be universal; however, outside the gram-negative bacteria, homologs of MutH are not found. In gram-positive bacteria and eukaryotes, discrimination between the template strand and the strand needing repair does not appear to be based upon a transient hemimethylation state but may be based upon localization of MutS homologs by the DNA polymerase proliferating cell nuclear antigen (PCNA) clamp to base mismatches in newly replicated DNA. Following MutL incision of the strand, nuclease Exo1 is recruited to perform 5′→3′ excision through and beyond the site of the mismatch (25, 27, 51).

The genomes of temperate bacteriophages, upon integration into a host chromosome, can alter the genotype and phenotype of bacteria (6, 38). Sequencing and analysis of the genome of group A streptococcus (GAS) (*Streptococcus pyogenes*) serotype M1 strain SF370 revealed the presence of several endog-

* Corresponding author. Mailing address: Department of Pharmaceutical Sciences, The University of Oklahoma College of Pharmacy, P.O. Box 26901, Oklahoma City, OK 73190. Phone: (405) 271-6593. Fax: (405) 271-7505. E-mail: William-McShan@ouhsc.edu.

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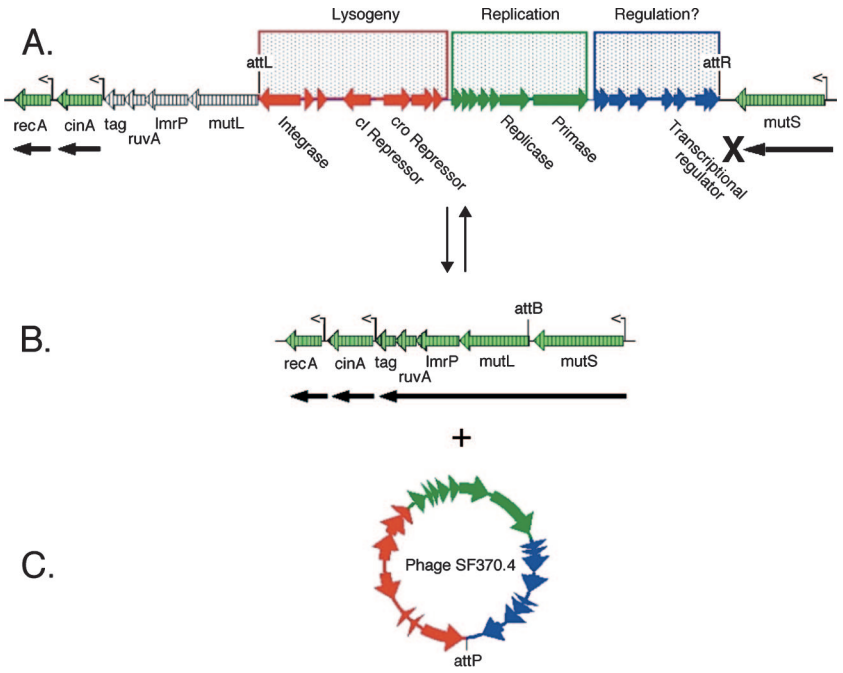

FIG. 1. *mutS-mutL* region of *S. pyogenes* SF370 and proposed mechanism of prophage SF370.4 excision. (A) Chromosomal region of the *S. pyogenes* SF370 chromosome that contains prophage SF370.4, which is integrated between the flanking host genes *mutS* and *mutL*. (B) In the absence of the prophage, a shared promoter is predicted to control *mutS* and *mutL*, as well as *lmrP*, *ruvA*, and *tag*, all of which are transcribed on a polycistronic mRNA. The presence of prophage SF370.4 truncates this mRNA after *mutS*, silencing the downstream genes until *cinA* and *recA*, each of which has its own promoters. As described in this report, activation of prophage SF370.4 leads to excision and release of the circular form of its genome (C) and restoration of the prophage-free MMR operon (B). Excision of the prophage leads to transcriptional activation of *mutL*, *lmrP*, *ruvA*, and *tag*, restoring MMR, Holliday junction resolution, and base excision repair. Transcriptionally active streptococcal genes are green, and the predicted mRNAs are indicated by arrows below the open reading frames. The small arrows above the open reading frames indicate the locations of the predicted promoters (46). Phage genes whose functions were predicted by homology to known genes are identified below the open reading frames in panel A.

enous bacteriophage genomes (prophages) (10, 12, 14). Prophage SF370.4, integrated between *mutS* and *mutL* (10), appears to be defective; the expected modules for integration, lysogeny control, replication, and regulation are present, but no identifiable genes for structural capsid proteins, host lysis, or DNA packaging are present (Fig. 1A). Thus, it is unlikely that this prophage could complete the lytic cycle and release new virions. However, the phage-bacterium DNA junctions (*attL* and *attR*) are intact, and direct sequence repeats define the ends of the prophage genome, a requirement for integrase-mediated integration and excision. For GAS genomes that lack this prophage, a genetic structure and promoter analysis predicted that *mutS* and *mutL* are transcribed together on a polycistronic message from a promoter located upstream of *mutS*. Since both genes are required for MMR (20), the presence of phage SF370.4 was expected to render the host defective for MMR so that it lacked *mutL* expression, resulting in a fixed mutator phenotype. However, here we show that in rapidly growing cells or following DNA damage, *S. pyogenes* strain SF370 expresses both *mutS* and *mutL*, while in stationary-phase cells only *mutS* is expressed. Further, the differential expression of *mutL* during growth results from the dynamic excision and reintegration of the SF370.4 prophage. This alteration in prophage integrative states results in a unique and sophisticated molecular mechanism to achieve a growth phase-dependent mutator phenotype in *S. pyogenes* strain SF370.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pyogenes* SF370, originally isolated from a wound infection, is a serotype M1 strain whose complete genome sequence has been determined (14) (Table 1). *S. pyogenes* NZ131 (= ATCC BAA-1633) is a serotype M49 strain that lacks any phage between *mutS* and *mutL* and was used as a source of phage-free DNA; its genome has also been completely sequenced (GenBank accession no. CP000829). Strain MGAS10394 is a serotype M6 strain whose genome has been determined and contains a prophage closely related to SF370.4 integrated into the same attachment site (2); it was obtained from the American Type Culture Collection (ATCC BAA-946). Strain JRS1 is a serotype M1 strain isolated from a case of streptococcal toxic shock syndrome in Oklahoma City, OK, that lacks an SF370.4-like prophage, as determined by DNA sequencing of the region (not shown). Bacteria were grown in Todd-Hewitt broth (Difco) supplemented with 2% yeast extract (THY medium) at 37°C; growth was monitored by determining the absorbance at 600 nm.

TABLE 1. Bacterial strains used in this study

S. pyogenes strain	Relevant genotype	Reference or source
SF370	*emm1* ϕSF370.4 *mutS*$^+$ *mutL* *ruvA*	14
NZ131	*emm49 mutS*$^+$ *mutL*$^+$ *ruvA*$^+$	52
MGAS10394	*emm6* ϕMGAS10394.8 *mutS*$^+$ *mutL*	2
JRS1	*emm1 mutS*$^+$ *mutL*$^+$	Clinical isolate, Oklahoma City

TABLE 2. Oligonucleotide primers used in this study

Gene target	Oligonucleotide primer	Size of amplified PCR product (bp)
mutS	5′AAGCGTGAGGTCGTTCAAGT 5′AGTGGCTGAGTTCTCGCATT	406
mutL	5′GAGGCTTTACCGTCTGTTGC 5′CCGGAAACTTCAAAATCCAA	413
mutS-mutL intergenic region (*attB*)	5′CAGGACTGCCAAAATCCCTA 5′TCGCTGGCCTTTCTACAACT	461
Phage SF370.4 *attP*	5′CTCAGGAAAATACGACAAGG 5′CACGCTTTTAGACACACTCA	892
Phage SF370.4 *int*	5′CTGCGGTACGTTCAGTCATC 5′TAAAGCGTTCAATCCCTGCT	563
attB[a]	5′ACACTCGCTGGCCTTTCTACA 5′CGAGGAAATTATGACAAACAT TATTG	90
attP[a]	5′ACTGCCAATCCAAGTCCAAT 5′TGAGAGGTGTAAACCTAATGA AACC	96
attL[a]	5′TGGTTGGCGAGAACTTCC 5′GGCAAAGAAAAAGAAGCTGTT	152
16S rRNA[b]	5′CACTCTCCCCTTCTGCACTC 5′AGCGTTGTCCGGATTTATTG	126
emm variable region[c]	5′TATTSGCTTAGAAAATTAA 5′GCAAGTTCTTCAGCTTGTTT	Variable

[a] Primers used for quantitative real-time PCR.
[b] Data from reference 3.

Nucleic acid preparation and PCR. DNA was isolated from streptococci as previously described (39, 44). The cells were harvested by centrifugation and resuspended in 100 μl Tris-EDTA buffer containing 50 U *Streptomyces globisporus* mutanolysin (Sigma-Aldrich, St. Louis, MO) and 5 mg lysozyme (Fisher Scientific, Pittsburgh, PA), and the suspensions were incubated at 37°C for 30 min. Cell lysis was carried out by addition of 0.5 ml GES reagent (5 M guanidium thiocyanate, 100 mM EDTA, 0.5% Sarkosyl), followed by vortexing and incubation on ice for 5 min. Lysis was quenched by addition of 0.25 ml of 7.5 M ammonium acetate, vortexing, and incubation on ice for 10 min. DNA was extracted by addition of 0.5 ml chloroform-isoamyl alcohol (24:1), which was mixed by vortexing until a uniform emulsion formed. Samples were centrifuged at full speed in a microcentrifuge for 10 min. The aqueous phase was retained, and DNA was precipitated by addition of 0.6 volume of isopropyl alcohol. Samples were centrifuged at 6,500 × *g* for 1 min and washed with 70% ethanol five times. Ethanol was removed by vacuum aspiration, and the pellets were dried and resuspended in 100 μl Tris-EDTA buffer containing 0.01 μg RNase A. For some experiments, cells in the growth media were harvested by centrifugation and then stored overnight in RNAlater (Ambion) at 4°C before they were processed as described above.

RNA was prepared using the RiboPure system for bacteria (Ambion) by following the manufacturer's recommended protocol. RNA samples were tested for a lack of DNA contamination by PCR using primers specific for the variable region of the *emm* gene (3). RNA samples were converted to cDNA using Superscript II (Invitrogen) and random hexamer priming by following the manufacturer's protocol.

Detection of prophage excision. PCR amplification of DNA (PCR) or cDNA (reverse transcription-PCR) sequences was performed using *Taq* DNA polymerase (Invitrogen), the buffers supplied by the manufacturer, and the recommended conditions. Primers were used to amplify specific internal regions of *mutS*, *mutL*, and the *mutS-mutL* intergenic region (Table 2). The *attP* site from the excised, circular prophage was amplified using specific primers (Table 2). When the phage was excised from the chromosome and its DNA was circularized, the *attP* primers generated a 486-bp PCR product. If the phage remained in the host strain's chromosome, no product was generated using the *attP* primers. Thermal cycling was performed by using initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at various temperatures (depending on the primer melting temperature) for 30 s, and synthesis at 72°C for 30 s, and a final extension at 72°C for 5 min.

Mitomycin C induction of MMR prophage. Mitomycin C induction of prophage was performed by using a modification of a previously described method (40). A single colony of strain SF370 from a tryptic soy agar plate supplemented with 5% sheep blood was used to inoculate THY broth, which was subsequently incubated overnight at 37°C. The overnight culture was diluted 1:20 into 100 ml fresh THY broth, and the culture was incubated at 37°C until early logarithmic growth began (A_{600}, 0.2). This culture was divided into two 50-ml cultures, and one of the latter cultures was treated with mitomycin C from *Streptomyces caespitosus* (Calbiotech, Spring Valley, CA) at a final concentration of 0.2 μg/ml. The cultures were incubated at 37°C for 1 h, and cells were harvested by centrifugation at 1,000 × *g* for 15 min at 4°C. The cell pellets were incubated at 65°C for 15 min to inactivate endogenous DNases, and chromosomal DNA was isolated as described above.

Kinetics of prophage excision during cell growth. Quantitative real-time PCR was used to observe prophage SF370.4 excision kinetics in the strain SF370 chromosome during growth. A single colony was used to inoculate 5 ml THY broth, which was then incubated at 37°C for 16 h. The overnight culture was diluted 1:20 in fresh, prewarmed THY broth. The culture was incubated at 37°C, and growth was monitored by determining the absorbance at 600 nm. Beginning 30 min postinoculation, samples were collected at 30-min intervals until 2 h postinoculation and then at 15-min intervals. Samples (30 ml) were collected when the culture density was low (A_{600}, <0.2), and 10-ml samples were collected later. Cells were harvested by centrifugation (3,500 × *g* for 10 min), suspended in 1 ml RNAlater (Ambion), and stored at 4°C for 24 h. Total DNA was then isolated as described above. Real-time PCR to detect phage SF370.4 *attP*, *attB*, and *attL* was carried out with a Bio-Rad iCycler equipped with the real-time optical fluorescent detection system using SYBR green PCR master mixture (Bio-Rad Laboratories, Hercules, CA) and the primer pairs shown in Table 2. The following program was employed for all PCRs: (i) an initial denaturation at 95°C for 3 min and (ii) 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. Following the final cycle, a melting curve analysis was performed for each sample to verify that a single product was produced. For each real-time PCR plate evaluated, primers for the 16S rRNA subunit housekeeping gene were also included for each sample for normalization of the data, and water blanks were used as negative controls. To determine the linear range of amplification for this primer set, initial PCRs were performed with serial dilutions of DNA containing from 150 to 0.015 ng as previously described (32). It was determined that 10 ng of DNA per reaction mixture was optimal under these conditions; accordingly, all DNA preparations were diluted so that they contained 10 ng/μl DNA. The products of three separate DNA isolations were analyzed by quantitative real-time PCR for all primer pairs, and the results were averaged.

MutS and MutL protein expression. An overnight broth culture of strain SF370 was diluted 1:20 into fresh, prewarmed THY broth and grown at 37°C. Growth was monitored by spectrophotometry, and an early-logarithmic culture was obtained (A_{600}, 0.2). Samples were removed when the A_{600} of the culture was 0.2, 0.3, 0.4, and 0.6, and the cells were quick-frozen in a dry ice-ethanol bath after they were harvested. All samples were stored at −80°C until they were processed. After thawing, the cells were collected by centrifugation and resuspended in 0.5 ml lysis buffer (20% Tween 20, 150 mM NaCl, 50 mM Tris; pH 8.0) supplemented with 0.05 ml of a protease inhibitor cocktail (Sigma). An equal volume of zirconium beads was added, and the cells were lysed by mechanical shearing using a bead beater (BioSpec Products, Bartlesville, OK). Shearing was performed using a 30-s pulse followed by 1 min of cooling of each sample, which was repeated five times. Cell debris and beads were removed by centrifugation, and the cell lysate was treated with a Bio-Rad 2D clean-up kit by following the manufacturer's protocol. Fifteen micrograms of protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nylon membrane by Western blotting, and probed with polyclonal rabbit antibodies to either *S. pyogenes* MutS or MutL using standard protocols (21). Rabbit antibodies were prepared by ProSci Incorporated, Poway, CA, using synthetic peptides corresponding to predicted antigenic sites from either *S. pyogenes* MutS (LEIGLTSRNKNAEN) or *S. pyogenes* MutL (IQENHTSLREL GKY) as haptens. Antibody binding was detected using an amplified alkaline phosphatase goat anti-rabbit immunoblot assay kit (Bio-Rad Laboratories, Hercules, CA) by following the manufacturer's recommended protocol. The relative

intensities of the MutS and MutL bands were quantified using the ImageJ software package (http://rsb.info.nih.gov/ij/).

Determination of the spontaneous mutation rates. A standard fluctuation test (30, 48) was used to estimate the mutation rates of *S. pyogenes* MMR prophage-containing strains SF370 and MGAS10394 and MMR prophage-free strains JRS1 and NZ131. A THY broth culture of the strain to be tested was started using an individual colony. After overnight incubation at 37°C, the cultures were diluted into fresh media to obtain final cell densities of <1,000 CFU/ml and dispensed to obtain 31 separate 1-ml aliquots. After incubation for 24 h at 37°C, one tube was used to determine the total number of CFU/ml by serial dilution, and the contents of the remaining tubes were mixed with 3 ml of melted soft agar (45°C) and poured onto THY medium plates containing 2 μg/ml ciprofloxacin, a DNA gyrase inhibitor. This concentration of antibiotic is 10 times the MIC (not shown). The cultures were incubated for 2 to 4 days to allow growth of ciprofloxacin-resistant colonies, and the number of resistant colonies per culture was determined. The mutation rate (with confidence limits) was calculated using the algorithm of Ma et al. (31), combined with the maximum likelihood estimation technique (54) and implemented by the *ft* software package (P. D. Sniegowski, University of Pennsylvania) (50). The results were plotted using the Prism4 software package. This experiment was repeated using the P_0 method of the Poisson distribution with 10-fold dilutions of the mutator SF370 cultures, as recommended by Rosche and Foster (48); this experiment gave similar estimates for the mutation rates (not shown). The MIC of ciprofloxacin was determined using the criteria recommended by the Clinical and Laboratory Standards Institute (Wayne, PA).

UV irradiation killing assay. Overnight cultures of strains SF370, MGAS10394, JRS1, and NZ131 were harvested by centrifugation and resuspended in sterile 0.1 M $MgSO_4$ at a final absorbance at 600 nm of 0.5. A calibrated 254-nm germicidal lamp (120 μW/cm^2) was prewarmed for 30 min prior to exposure of the strains. For each strain, 5 ml of a resuspended culture was placed in a sterile glass petri dish and exposed to the UV lamp. Since a homolog of photolyase is present in the GAS genome, the UV light treatment was carried out in a darkened room. At selected intervals (30, 60, 90, and 120 s), 1 ml was removed and serially diluted 10-fold using 0.1 M $MgSO_4$. Each dilution (2 μl) was spotted onto a THY agar plate and incubated in the dark at 37°C for 24 h.

Survey of GAS strains. One hundred *S. pyogenes* strains were randomly selected from the laboratory collection of J. J. Ferretti at the University of Oklahoma Health Sciences Center. This collection contains isolates obtained from worldwide locations during the last three decades. The *emm* type of each strain either had been previously determined serologically or was determined by performing PCR with primers that amplify the variable region. Approximately 1 μg of chromosomal DNA from each strain was added to enough deionized water to obtain a final volume of 0.15 ml. Sodium hydroxide (45 μl of a 1 M stock solution) was added to each sample, which was followed by heating at 65°C for 30 min. The solution was neutralized by addition of sodium acetate (pH 5) (65 μl of a 3 M stock solution). The samples were applied to a nylon membrane using a slot blot apparatus, washed twice with 2× SSC (34), and fixed to the membrane using UV light (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). A nonisotopic DNA probe for the SF370.4 integrase gene was prepared using a PCR DIG probe synthesis kit (Roche Diagnostics Corporation) by following the manufacturer's recommended protocol and reagents and using integrase-specific primers. The nylon membranes containing the DNA samples were hybridized to the probe and detected using a DIG DNA detection kit (Roche) by following the recommended protocol. DNA from strains SF370 and NZ131 were used as positive and negative controls, respectively. Strains giving positive results were confirmed by performing PCR for the *attL* junction between the phage integrase and *mutL*.

DNA sequencing and analysis. Automated DNA sequencing was performed at the University of Oklahoma Health Sciences Center Laboratory of Microbial Genomics. Prior to sequencing, PCR products were treated with shrimp alkaline phosphatase and exonuclease I by incubation at 37°C for 60 min, followed by inactivation of the enzymes by heating at 85°C for 15 min. Sequencing was performed using the same primers that were used for PCR. In some cases, the amplified PCR product was cloned for future study using the pGEM-T Easy vector (26). Computer predictions for promoter elements were performed using the Berkeley Drosophila Genome Project neural network promoter prediction server (46).

Nucleotide sequence accession number. The nucleotide sequence of the phage SF370.4 *attP* region has been deposited in the GenBank database under accession no. AY684192.

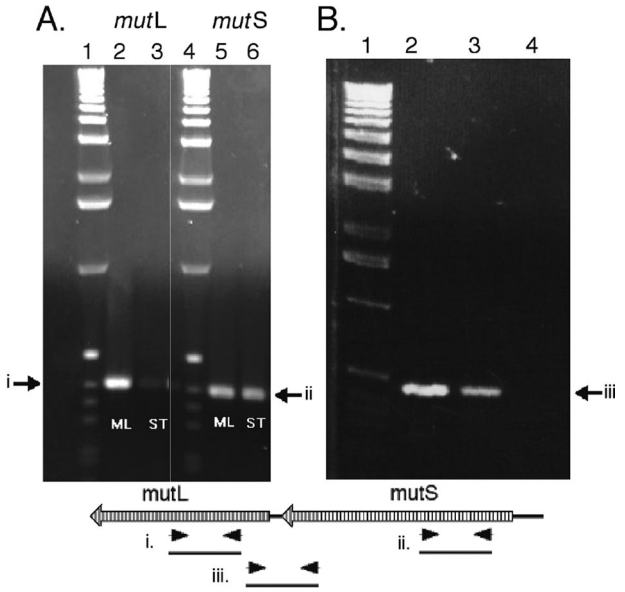

FIG. 2. Expression of *mutL* is growth dependent in GAS strain SF370. (A) cDNA from SF370 cells was synthesized from RNA isolated at mid-logarithmic (ML) or stationary (ST) phase, and PCR primers specific for *mutS* and *mutL* were used to amplify products specific for each gene (the targeted region of each gene in a phage-free chromosome is shown below the gels). The *mutS* message is detectable in both rapidly growing and stationary-phase cells (lanes 5 and 6), but *mutL* is expressed only in actively dividing cells (lane 2). Lanes 1 and 4, molecular weight standard (DNA kilobase ladder; Invitrogen); lane 2, *mutL*, mid-logarithmic cells; lane 3, *mutL*, stationary-phase cells; lane 5, *mutS*, mid-logarithmic cells; lane 6, *mutS*, stationary-phase cells. (B) Identification of the uninterrupted *mutS-mutL* mRNA in SF370. Primers specific for the phage-free junction between *mutS* and *mutL* amplify this region in SF370 cDNA obtained from actively dividing streptococci but not from genomic DNA. Lane 1, molecular weight standard; lane 2, NZ131 cDNA (phage-free strain; positive control); lane 3, SF370 mid-logarithmic cDNA; lane 4, SF370 chromosomal DNA.

RESULTS

Expression of MMR genes in GAS strain SF370 is related to the growth state of the cells. In contrast to the MutS and MutL genes in many eubacteria, the MutS and MutL genes in *S. pyogenes* are genetically linked together under control of a common promoter upstream of *mutS* and are thus predicted to be transcribed together on a polycistronic mRNA (Fig. 1). Additionally, three additional genes, *lmrP*, *tag*, and *ruvA*, are predicted to be components of this polycistronic mRNA. In the SF370 serotype M1 genome strain, as well as in several other *S. pyogenes* genome strains, a prophage is integrated between *mutS* and *mutL*; its attachment site overlaps the first five codons of the *mutL* coding region. Thus, the integration of phage SF370.4 at the 5′ end of *mutL* should interfere with or alter the transcription of this gene by separating it from the remainder of the normal mRNA, potentially inactivating MMR. Analysis of rapidly dividing *S. pyogenes* SF370 cells (mid-logarithmic phase) showed, however, that mRNAs for both *mutL* and *mutS* were transcribed (Fig. 2A, lanes 2 and 5, respectively), which should allow a functional MMR system. In contrast, when the cells reached stationary phase and stopped dividing, *mutS* was still transcribed (lane 6) but *mutL* was not

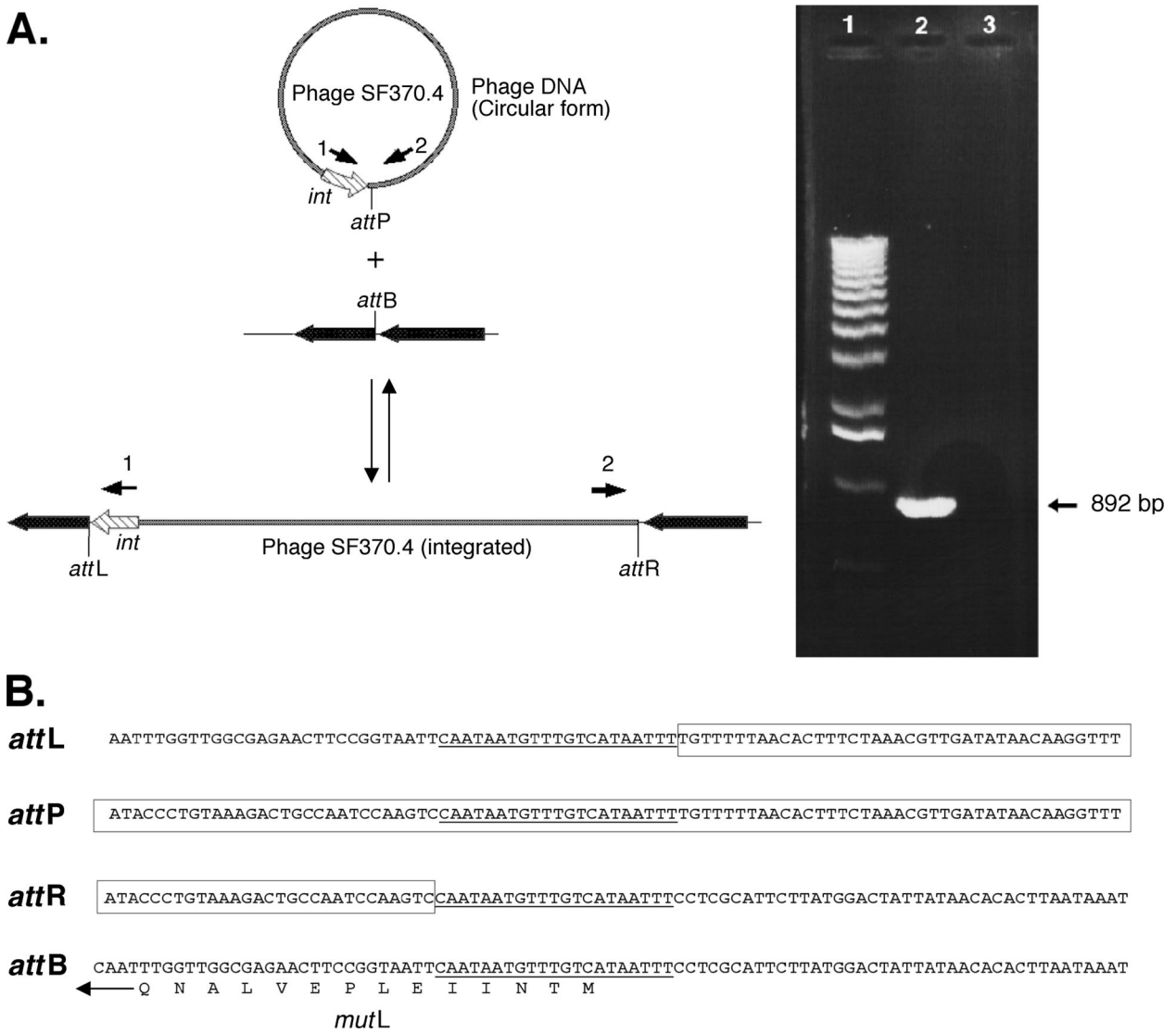

FIG. 3. The prophage SF370.4 chromosome excises from the host genome as a replicating circular molecule during exponential growth. (A) PCR primers (arrows 1 and 2) are located so that the phage *attP* region may be amplified by PCR only when the phage genome is excised from the bacterial chromosome and exists as free circular DNA. No product can be amplified from the integrated prophage in this reaction. Using DNA isolated from cells grown to mid-logarithmic stage, the specific PCR product was identified by gel electrophoresis (lane 2), and DNA sequencing confirmed the identity of the specific phage *attP* sequence. The orientation of the open reading frames matches the genome sequence. Lane 1, molecular weight standard; lane 2, *attP* region from the circular phage genome amplified from SF370 DNA isolated during mid-logarithmic growth; lane 3, chromosomal DNA isolated from a culture of strain SF370 after 18 h of growth at 37°C, showing no detectable *attP* PCR product. (B) Sequences flanking the integrated phage-host genome junctions (*attL* and *attR*) (14), the phage-free *mutS-mutL* junction (*attB*), and the circular phage genome *attP* site. The sequence shared by the phage and host genomes is underlined, and the initial amino acid residues of MutL are indicated below the *attB* sequence. Phage DNA sequences are enclosed in a box.

transcribed (lane 3), disabling MMR. The expression of *mutL* during logarithmic growth suggested that either the prophage was excised via integrase-mediated site-specific recombination, restoring the phage-free genetic constellation, or the phage genome harbored an alternative promoter for *mutL* that was activated during growth.

If prophage SF370.4 undergoes integrase-mediated excision during exponential growth, the event should restore a phage-free *mutS-mutL* sequence. Using cDNA as a template, the *mutS-mutL* intergenic region was readily detected by PCR amplification of a 461-bp product in the prophage-free genome of *S. pyogenes* strain NZ131 (Fig. 2B, lane 2), demonstrating that the two genes occupy a common transcript. The presence of the SF370.4 prophage in strain SF370 introduced over 13 kbp of intervening DNA that under typical PCR conditions prevents similar amplification from chromosomal DNA isolated from stationary-phase cells (Fig. 2B, lane 4). However, the phage-free *mutS-mutL* intergenic product was present in the SF370 cDNA from mRNA obtained from logarithmically growing streptococci (Fig. 2B, lane 3); its identity was confirmed by DNA sequencing (*attB*) (Fig. 3B). The restoration of the wild-type arrangement of the *mutS* and *mutL* genes and the

appearance of the associated mRNA transcript indicated that prophage excision from the *S. pyogenes* SF370 genome had occurred, allowing transcription from the *mutS* promoter to continue through to the downstream gene, *mutL*.

The phage SF370.4 genome is excised during exponential growth. The excision of phage SF370.4 should have released a circular free form of the prophage genome via Campbell-type homologous recombination (Fig. 3A). The attachment sites linking the phage genome to the bacterial genome in *S. pyogenes* SF370 could be identified by the 21-bp direct repeat (5′CAATAATGTTTGTCATAATTT3′) created by the integrative joining of the two genomes at the ends of the linear prophage (*attL* and *attR*) (Fig. 3B). The *attL* site encompassed the first five codons of *mutL*. Circularization of the phage genome upon excision brought the two ends together to create *attP* and simultaneously restored the prophage-free *attB* site on the bacterial genome (Fig. 3B). PCR performed with primers designed to amplify only the *attP* region in the circular genome (Fig. 3A) resulted in the predicted product when DNA from logarithmically growing cells was used as the reaction template, and DNA sequencing confirmed the specificity of the products (*attP*) (Fig. 3B). Using these primers, it was not possible to amplify a product from the integrated prophage. PCR performed with chromosomal DNA isolated from cells grown for 18 h at 37°C, which were in deep stationary phase, resulted in no product, indicating that most or all cells in the culture contained the prophage in the integrated state (Fig. 3A, lane 3).

Many prophages are induced by DNA-damaging agents, such as UV light or mitomycin C, and it was reasoned that such a challenge might promote population-wide induction of prophage SF370.4 if the phage repressor was sensitive to cleavage following an SOS response, as seen in phage lambda. Using PCR, the bacterial attachment site (*attB*) and the phage attachment site (*attP*) were amplified from total DNA isolated from mitomycin C-induced and uninduced mid-log-phase SF370 following incubation for 1 h after treatment (Fig. 4). Both the *attB* and *attP* PCR products (461 and 892 bp, respectively) (Fig. 4) were strongly amplified when DNA from the mitomycin C-induced cells was used; DNA sequencing confirmed the specificity of the products. By contrast, using an equimolar template, the DNA from uninduced but logarithmically growing SF370 produced decreased amounts of the *attB* product, and the *attP* PCR generated a secondary product that was >500 bp long (Fig. 4). This additional product was cloned and sequenced, which showed that it was the amplification product of a false priming site in an unrelated part of the genome (not shown). Therefore, this secondary *attP* PCR product appeared when the specific target (the circular phage genome) was absent, as in the case of SF370.4 prophage-free strain NZ131 (not shown), or when a mixed population of integrated and episomal prophage was present in logarithmically growing cells (Fig. 4). When complete or nearly complete prophage induction occurred after mitomycin C treatment, this secondary product was not detectable.

Excision of prophage SF370.4 and expression of MutL are growth dependent. The kinetics of prophage SF370.4 excision and reintegration were determined by examining the appearance and/or disappearance of the prophage-bacterium chromosome junctions that reflect the integrated or episomal state

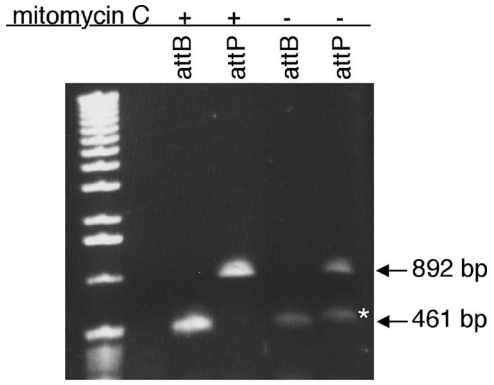

FIG. 4. Mitomycin C treatment enhances prophage excision. Equimolar amounts of chromosomal DNA from mitomycin C-induced (+) or uninduced (−) strain SF370 were used as templates to amplify *attB* and *attP* (the prophage-free chromosomal attachment site and the free, circular prophage attachment site, respectively). DNA was isolated 1 h postinduction. Both the *attB*- and *attP*-specific PCR products were strongly amplified when the mitomycin C-induced cells were used; DNA sequencing confirmed the specificity of the products. By contrast, using an equimolar template and the uninduced SF370 DNA resulted in amplification of decreased amounts of both products, and the *attP* PCR generated a secondary product (indicated by an asterisk) resulting from a false priming site in an unrelated part of the genome (not shown). Thus, when the specific target (*attP* on the circular phage genome) is absent (as it is in a prophage-free strain) (not shown) or when a mixed population of integrated and episomal prophage is present, this product can be amplified. Mitomycin C treatment of strain SF370 results in disappearance of this secondary PCR product.

of the prophage DNA. The integrated prophage DNA is defined by the left and right junctions with the bacterial chromosome (*attL* and *attR*, respectively); excision of the prophage eliminates these sequences, while simultaneously creating the prophage *attP* sequence and the bacterial *attB* sequence. As shown in Fig. 5, *attL* began to disappear during very early logarithmic growth, presumably about the time of DNA replication initiation. The disappearance of *attL* was coordinated with the appearance of *attB*, the prophage-free constellation of the bacterial chromosome. The episomal prophage-associated *attP* site was detected within minutes after the appearance of *attB* and the disappearance of *attL*, and it continued to be present in the cell after *attL* reappeared. The simultaneous presence of *attP* and *attL* during part of the growth cycle (between approximately 120 and 180 min [Fig. 5]) indicated that there was a mixed population of cells with both integrated and episomal forms of the prophage, in agreement with the results for the uninduced cells shown in Fig. 4. Further, the continued increase in *attP* levels following the reappearance of *attL* may have reflected an increased number of copies of the extrachromosomal prophage during this period.

Prophage excision during early exponential growth predicted that the levels of MutL protein should increase rapidly over the same interval. The amounts of MutS protein, by contrast, should have been constitutive and little affected by the prophage. Total cytoplasmic proteins recovered from strain SF370 cells over the course of exponential growth showed that there was a high level of MutL expression very early (A_{600}, 0.2), which decreased to nearly undetectable levels as the culture density increased as the culture reached stationary phase

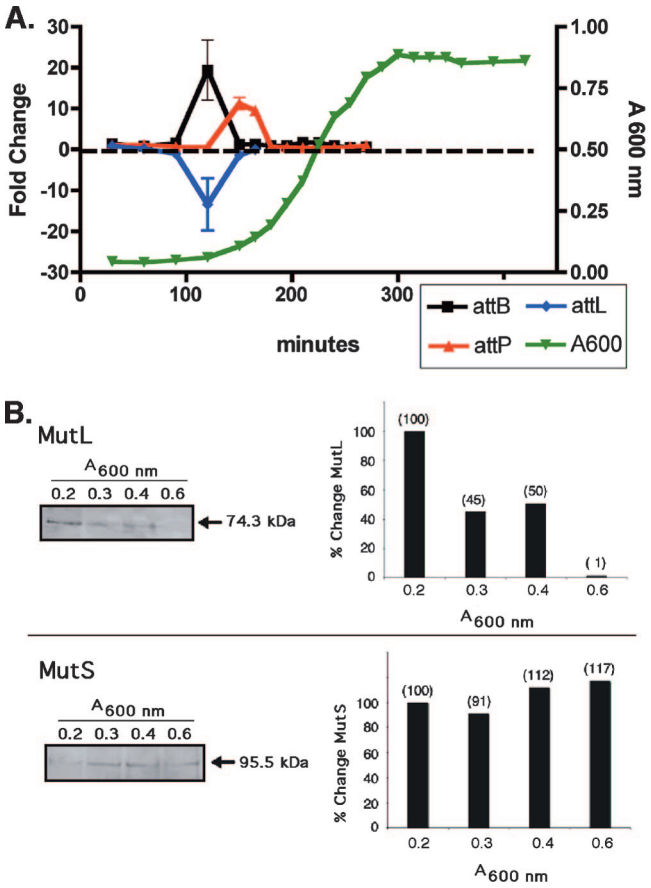

FIG. 5. Induction of prophage SF370.4 and expression of MutL in relation to growth. (A) The induction of prophage SF370.4 occurs near the beginning of exponential growth. Samples were removed from a culture of strain SF370 at timed intervals during growth. DNA was extracted and analyzed by quantitative real-time PCR to determine the presence and quantities of sequences specific for *attP*, *attB*, and *attL* relative to the 16S rRNA gene. The appearance of *attB* and the disappearance of *attL* at around 100 min were exactly coordinated, while *attP* was detectable after a short lag time. This delay in detection may have reflected the episomal prophage replication leading to a higher copy number. By 150 min, prophage reintegration had occurred, leading to the reappearance of *attL* and the disappearance of *attB* and *attP*. (B) Expression of protein MutL occurred early in logarithmic growth and diminished as stationary phase was approached, mirroring the kinetics of phage SF370.4 excision and reintegration. Growth of GAS strain SF370 was monitored by determining the absorbance at 600 nm, and samples for cytoplasmic protein analysis were taken when the culture density reached approximately 0.2, 0.3, 0.4, and 0.6. After extraction, proteins (3 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nylon membrane for hybridization to polyclonal anti-MutL or anti-MutS antibodies. The relative amounts of the 74.3-kDa MutL band were measured by densitometry and normalized to the sample harvested at an A_{600} of 0.2. The relative amount of MutL protein detected decreased to <5% of the maximum amount by the time that the cells reached stationary phase. A similar analysis of MutS expression showed that there was constant expression of the predicted 95.5-kDa protein during growth of GAS strain SF370. Thus, in contrast to MutL expression, MutS expression does not decrease as the cells move from the logarithmic phase to the stationary phase.

(Fig. 5). This loss is in contrast to the expression of MutL in the prophage-free strain NZ131, which was not decreased during logarithmic growth (not shown). The level of MutS, by contrast, was essentially constant over the same period. The results strongly suggest that prophage SF370.4 is excised from its attachment site in *mutL* early in exponential growth of a cell culture, allowing the expression of MutL to be restored. As the prophage returned to the integrated state, the levels of MutL in the cell decreased until they were very low. This pattern of MutL expression suggests that the cells alternate between a mutator phenotype and a wild-type phenotype with respect to MMR.

A mutator phenotype and sensitivity to killing by UV irradiation are associated with phage SF370.4. The loss of MutL expression following integration of prophage SF370.4 into the *S. pyogenes* SF370 genome would indicate that a mutator phenotype was conferred upon its host. Using a modified Luria-Delbrück fluctuation assay (30, 48), the mutation rate for spontaneous resistance to 10 times the MIC of ciprofloxacin, a DNA gyrase inhibitor, was determined for strains SF370, NZ131, MGAS10394, and JRS1. MGAS10394 is a serotype M6 genome strain (2) harboring a closely related prophage integrated into the same *attB* site as SF370.4, while JRS1 is a serotype M1 clinical isolate from a case of streptococcal toxic shock. Neither strain NZ131 nor strain JRS1 has a prophage integrated into the *mutL* gene, and both strains should be wild type for MMR. The mutation rates were estimated to be 3.3×10^{-7} and 3.2×10^{-9} mutation/generation for SF370 and NZ131, respectively; thus, the mutation rate for SF370 was almost 100-fold greater than the mutation rate for prophage-free strain NZ131 and was consistent with a mutator phenotype (Fig. 6A). An association between a prophage integrated into *mutL* and an increased mutation rate was also observed for strain MGAS10394 (6.8×10^{-8} mutation/generation). Strain JRS1, wild type for MMR, had a mutation rate of 5.3×10^{-10} mutation/generation (Fig. 6A).

The polycistronic mRNA containing *mutS* and *mutL* is also predicted to contain the downstream gene *ruvA*, and transcription of this gene would be interrupted by the presence of SF370.4, resulting in increased sensitivity to killing by UV irradiation (24). As shown in Fig. 6B, MMR prophage lysogen-containing strains SF370 and MGAS10394 were at least 100 times more sensitive to killing by 254-nm irradiation than SF370.4 prophage-free strains NZ131 and JRS1 with a 2-min exposure. Both strains were analyzed in stationary phase, when the differences should have been most pronounced due to the integrative state of prophage SF370.4. Ideally, an isogenic pair of strains with and without the prophage would be used for analysis, but attempts to cure the prophage have not been successful yet (unpublished results). However, comparison of the previously described genomes of SF370 and MGAS10394 (14) with the recently completed genome sequence of NZ131 (W. M. McShan, J. J. Ferretti, T. Karasawa, A. N. Suvorov, S. Lin, B. Qin, H. Jia, S. Kenton, F. Najar, H. Wu, J. Scott, B. A. Roe, and D. J. Savic, submitted for publication) allowed examination of other genes that might influence the mutation rate or UV sensitivity (*mutS2*, *ruvB*, etc.). No differences in the gene products between SF370 and the other genome strains were found, so it is probable that the difference in mutation rates is due to the presence of prophage SF370.4.

Frequency of MMR prophages in GAS. Related prophages integrating into the same *attB* site in *mutL* appear to be common genetic elements in *S. pyogenes*. Thirteen GAS genomes have been completed so far; 12 of these genomes have been published (2, 4, 5, 14, 19, 23, 41, 53, 55), and one has not been

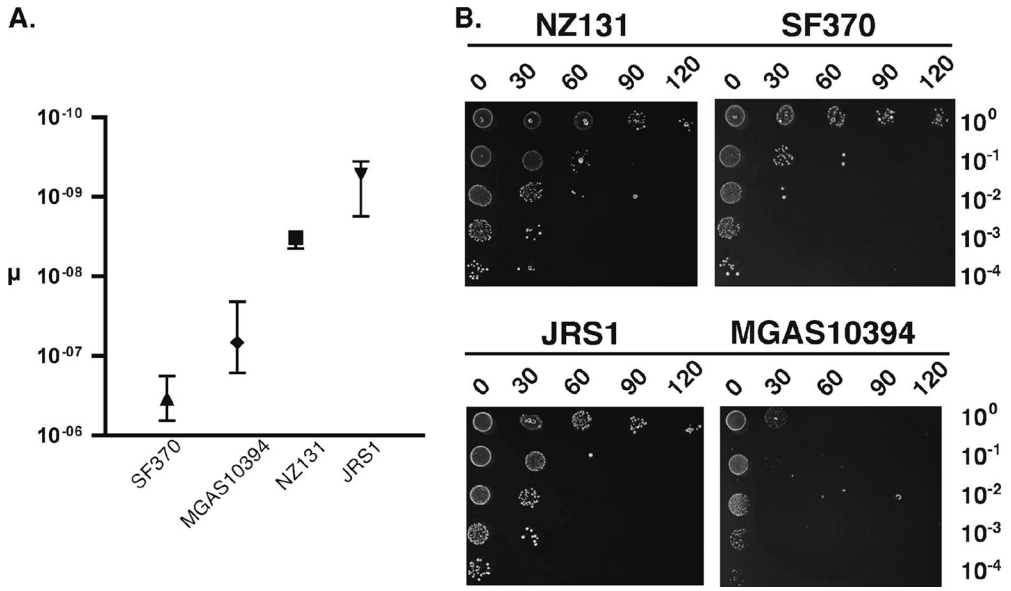

FIG. 6. Mutator phenotype associated with prophages integrated into *mutL*. (A) Calculated spontaneous rates of mutation (μ) (mutation/generation) to ciprofloxacin resistance of strains SF370 (serotype M1, MMR prophage positive), MGAS10394 (serotype M6, MMR prophage positive), NZ131 (serotype M49, MMR prophage negative), and JRS1 (serotype M1, MMR prophage negative). Here, prophage carriage refers to the presence or absence of phage SF370.4 or its close relative found in strain MGAS10394. For each strain, 30 parallel cultures were established with <1,000 CFU/culture, grown for 24 h at 37°C, and plated individually on selective media. After 48 to 96 h of incubation, colonies were enumerated, and mutation rates with 95% confidence limits were calculated using the maximum likelihood estimation technique (31, 54). Prophage-carrying strains SF370 and MGAS10394 both showed enhanced mutation rates compared to prophage-free strains NZ131 and JRS1. (B) Enhanced sensitivity of MMR prophage strains SF370 and MGAS10394 to killing by UV irradiation. Strains SF370, MGAS10394, JRS1, and NZ131 were exposed for 0 to 120 s to 258-nm light (120 μW/cm^2), and 10-fold dilutions were spotted onto an agar plate. Prophage-carrying strains SF370 and MGAS10394 showed ~100-fold-greater killing than prophage-free strains JRS1 and NZ131, consistent with the inhibition of *ruvA* expression. The protocol was performed in a darkened room to prevent photoreactivation.

published (M49 strain NZ131 [submitted]). Prophages closely related to SF370.4 have been found in six genomes (M2 strain MGAS10270, M4 strain MGAS10750, M5 strain Manfredo, M6 strain MGAS10394, and M28 strain MGAS6180). Although each prophage is unique, the prophages share extensive regions of identity or homology (Fig. 7). Particularly conserved are the lysogeny and DNA replication regions, and, as is the case for prophage SF370.4, none of the prophages contain genes for phage structural proteins or host lysis. Therefore, they all appear to have the potential to have a molecular lifestyle similar to that of SF370.4, causing their hosts to switch between a wild-type phenotype and a mutator phenotype. The possible exception is serotype M5 strain Manfredo, which has a deletion of 43 codons within *int* compared to the other strains and thus may be defective for integration and excision. The frequent appearance of SF370.4-like prophages in the genome strains, all of which were chosen for sequencing because of an association with severe human disease (2, 4, 5, 14, 19, 41, 53, 55), suggests that these prophages may be associated with hosts having increased pathogenicity.

One hundred randomly selected clinical isolates having diverse geographical origins and serotypes were screened for the presence of SF370.4-like phages by DNA hybridization to the phage SF370.4 *int* gene. Twenty of these isolates were positive for the presence of phage; these strains included serotype M1, M2, M18, M31, M37, M49, M58, M78, and M123 strains and strains with nontypeable serotypes (Table 3). The phage integrase hybridization probe had no homology to any bacterial DNA in the GenBank database except the nearly identical sequences from the related phages in the other genome strains (using BLASTN with a minimum e value of 10^{-4}). Bacteriophage integrases are very diverse at the protein level (1, 42), and given further variation at the DNA level due to the degeneracy of the code, the phage SF370.4 integrase probe can be expected to be a reasonable reporter for phages using the *mutL* attachment site. To confirm use of the *mutL attB* attachment site in the probe-positive strains, PCR was performed using primers to amplify the *mutL*-integrase junction (not shown). Therefore, both the results of genome sequencing and this brief survey indicate that SF370.4-related phages are frequent genetic elements in *S. pyogenes*. The mechanism of dissemination of these phages among the various serotypes remains unknown since none of the phages discovered by genome sequencing have any identifiable late genes for DNA packaging, capsids, or host lysis. Given the overall frequency of overall prophage carriage as shown by the multiple examples found in all of the sequenced genomes, this mechanism could well be generalized transduction. However, it is possible that there is some unknown packaging mechanism, perhaps employing a helper phage, that could generate infectious particles that could spread the phages in a manner similar to packaging and dissemination of the *Staphylococcus aureus* pathogenicity islands (57).

DISCUSSION

The evidence presented here shows that *mutL* expression in *S. pyogenes* SF370 is controlled by a dynamic process involving

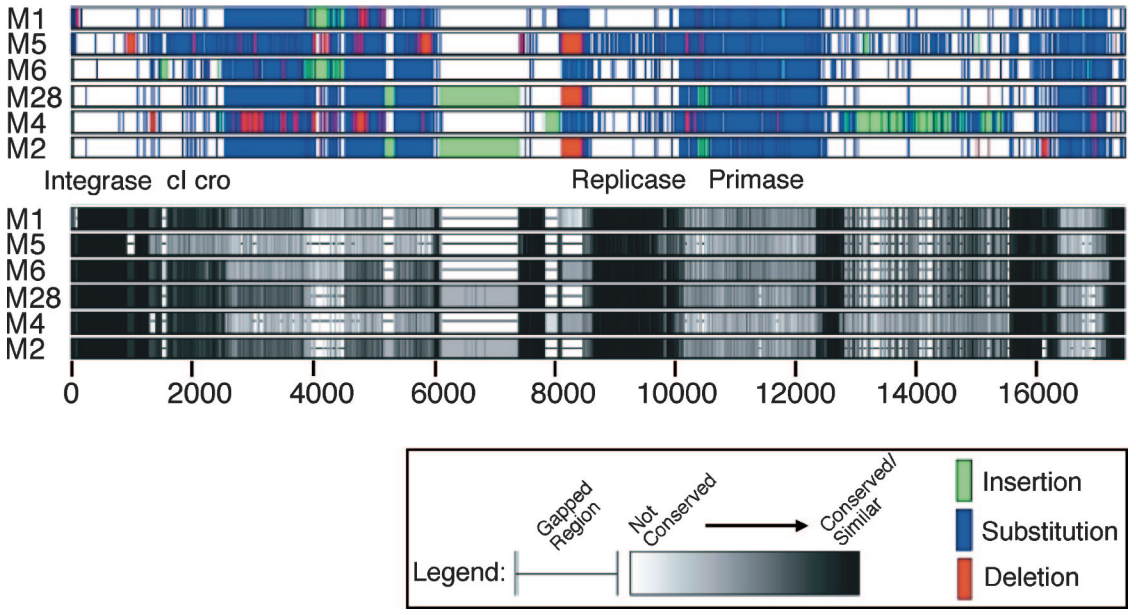

FIG. 7. Genomic MMR-converting phages. The prophages from *S. pyogenes* genome strains SF370, MGAS10394, Manfredo, MGAS10750, MGAS10270, and MGAS6180 that integrate into the same *attB* site at the 5′ end of *mutL* are compared. In the upper panel, the insertions, deletions, and base substitutions of the genomes are compared. The lower panel shows the levels of conservation of the genomes; black indicates the highest level of similarity. No identifiable capsid, DNA packaging, or lysis genes are present in any phage, but all six prophages contain either identical or highly conserved integration, control, and replication genes. The locations of several identifiable and conserved genes are indicated to provide a reference. Scale, 2,000 bp/tick.

prophage excision and reintegration in response to growth, activating MMR under conditions under which resources are not limiting. Further, induction of the prophage may occur at low population density since the highest levels of MutL expression were seen in early logarithmic growth phase, presumably during the initiation of DNA replication. The results presented here suggest that under the conditions used, there is a mixed population of bacteria, with individual cells having the prophage in either the integrated or episomal state. This situation indicates that the mutation rate of the population fluctuates between the wild-type and mutator rates, and the penetrance of the mutator phenotype could vary by availability of resources, environmental insult, or initiation of DNA replication. The presence of the prophage in strains SF370 and MGAS10394 correlates with a mutator phenotype compared to MMR prophage-free strains NZ131 and JRS1. While it is possible that other factors could be responsible for the mutator phenotype in these strains, the simultaneous sensitivity to UV irradiation in these strains, indicated by the inactivation of *ruvA* following prophage integration, supports the hypothesis that the presence of the prophage is the most direct explanation for the increase in the mutation rate. The creation of prophage-free isogenic mutants of these strains or the passage of the prophages to new hosts should allow a definitive answer to this question to be obtained. Further, the phenotypic changes resulting from inactivation of the other genes sharing the same mRNA with *mutL* and *ruvA* (*tag* and *lmrP*) have not been explored yet, and the integration of prophage SF370.4 into the MMR operon probably creates a complex mutator phenotype.

TABLE 3. GAS strains positive for SF370.4-related prophages

Strain	Serotype	Origin	Associated disease
338	NT[a]	Thailand	Pregnancy fever
342	M123	Thailand	Cellulitis
348	M49	Thailand	Ear discharge
386	M2	United States	Scarlet fever
402	M18	United States	Acute rheumatic fever
407	M78	USSR	Scarlet fever
410	M49	USSR	Scarlet fever
412	NT	USSR	Scarlet fever
429	NT	USSR	Unknown
431	M2	USSR	Unknown
443	NT	USSR	Unknown
485	M59	United States	Acute rheumatic fever
489	M1	United States	Septicemia
517	NT	United States	Unknown
532	M2	United States	Unknown
549	NT	United States	Unknown
687	M31	United States	Unknown
691	M1	United States	Severe invasive
761	NT	Unknown	Unknown
794	M37	United States	Unknown

[a] NT, nontypeable.

Excision during logarithmic cell division dictates that phage SF370.4 must be able to replicate its genome to prevent elimination from the population. Some temperate phages, such as coliphage P1, replicate as a plasmid in the temperate state, and phage SF370.4 may adopt a similar strategy when it is excised. The center of the integrated phage SF370.4 genome contains a region that is highly conserved in all of the related genome prophages (Fig. 7). This section of the genome contains putative replicase and primase genes that are homologous to DNA replication genes from plasmid pSt106 of *Streptococcus ther-*

mophilus (17). The lack of identifiable DNA packaging, structural, or lytic genes prevents the phage from entering a lytic cycle, and so replication of the circular phage genome as an autonomous element seems likely.

The excision of phage SF370.4 during exponential growth may occur by inactivation of its predicted repressor by proteolysis or by allosteric interaction with some protein or metabolite expressed by rapidly dividing streptococci. Phage SF370.4 thus appears to have evolved to function as a genetic switch to control a mutator phenotype, protecting rapidly growing cells from unwanted genetic changes while allowing the accumulation of random mutations, some of which might be adaptive when resources become limiting, or from the acquisition of new genetic material by horizontal transfer (37). Further, mitomycin C treatment of strain SF370 stimulated the excision of the prophage following the induction of an SOS-like response. Such a response in *S. pyogenes* presumably leads to an increased mutation rate through the induction of error-prone DNA replication, as seen in *E. coli* (45, 56), and thus the restoration of MMR following prophage induction by mitomycin C may counteract this increase in the mutation rate. It is unclear whether this balancing of error-prone DNA replication with restoration of normal MMR activity is the result of direct selection or is a circumstantial by-product of the evolution of prophage SF370.4. That is, the induction of prophage by the SOS response may be a genetic remnant from an ancestral phage that responded to cellular damage like a typical temperate prophage and entered the lytic cycle to escape from a damaged host. In *S. pyogenes*, there may be some common cellular signal during early logarithmic growth and induction of the SOS pathway. The induction of the SOS response in *E. coli* is controlled by induction of the RecA coprotease activity leading to the autocatalytic cleavage of the LexA repressor. In gram-positive bacteria, *lexA* equivalents have been found in some species, such as *Bacillus subtilis*, but a gene homolog has not been identified in the streptococci. A recent report identified a gene cassette that mediates the SOS response in *Streptococcus uberis* (58). One product of this cassette (HdiR) appears to function as a LexA equivalent in this species, and homologs of HdiR have been found in several other streptococcal species, including one of the genome strains of *S. pyogenes* (MGAS10394). Closer examination showed that this HdiR homolog is encoded on one of the temperate bacteriophages harbored by MGAS10394, and thus, while HdiR may play some role in the SOS response in *S. pyogenes*, it seems likely that a true LexA equivalent would be common to all GAS genomes rather than carried sporadically on a mobile element. A number of conserved hypothetical proteins containing predicted helix-turn-helix motifs are encoded in all of the sequenced *S. pyogenes* genomes, and it is possible that one of these proteins may function as the LexA equivalent. Clearly, this is a topic that needs more study in GAS.

A recent survey of the endogenous prophages found in the sequenced bacterial genomes showed that 41% of these phages are integrated into tRNA and transfer-messenger RNA genes, 31% are integrated into intergenic regions, and 28% are integrated into open reading frames for genes (16). Prophage site-specific integration occurs via a duplication between the phage and the host chromosome, and when integration occurs at gene targets, the duplication usually occurs at the 3′ end of the host target gene, leaving the target gene intact (via duplication); in at least one case, the duplication provides an alternative carboxy terminus for the specified protein (8, 9). By contrast, phage SF370.4 integrates into the 5′ end of *mutL*, blocking its transcription. Integration at the 5′ end of a host gene has also been found in another *S. pyogenes* SF370 prophage (phage SF370.1 integrates at the 5′ end of a dipeptidase gene) (38), and prophages acting as regulatory elements may be not uncommon in *S. pyogenes*. For example, in the strains with published genomes, prophages are integrated in the 5′ regions of the gamma-glutamyl kinase gene *proB*, *recX*, a HAD-like hydrolase gene, and a gene encoding a conserved hypothetical protein (2, 5, 41, 53).

Bioinformatic analysis suggests that several genes downstream from *mutL* can be predicted to be additional components of the polycistronic mRNA containing *mutS* and *mutL*; these genes are Spy2120 (encoding a predicted integral membrane protein related to the *Lactococcus lactis* multidrug exporter encoded by *lmrP*), *ruvA* (encoding a Holliday junction helicase subunit), and *tag* (encoding DNA-3-methyladenine glycosidase I). The *comX*-dependent competence damage protein gene *cinA* and rec*A* follow, completing a remarkable genetic group of recombination and repair genes. The same gene cluster is present in the genomes of group B streptococci and *Streptococcus mutans*, although the *lmrP* homolog is missing in *S. mutans*. This entire group of genes is responsible for a range of DNA repair functions, and therefore, in addition to MMR, the presence of phage SF370.4 may alter the expression of several DNA repair systems. For example, *ruvA* mutants have increased sensitivity to mutagens and an overall increased mutation rate (33), while in *tag* mutants the cell's sensitivity to alkylating mutagens is increased (60). Finally, although this operon is very similar in group B streptococci and *S. mutans*, the specific *attB* DNA sequence is unique to GAS, and so it is unlikely that prophage SF370.4 could integrate into these foreign species. This does not rule out the possible presence of equivalent prophages in the other streptococcal species, although none have been identified.

The endogenous bacteriophages of *S. pyogenes* are often vectors for toxin genes and other virulence factors, but the control of host gene expression (MMR) by a bacteriophage in response to the bacterial growth state and via cycles of repeated excision and integration is completely novel. A variety of bacterial stress responses that include mechanisms of inducing spontaneous mutations in slowly growing or nongrowing cells have been described as "adaptive mutations" (15). Some adaptive responses in *E. coli* have been shown to be influenced by environmental conditions (7), and the $MutS^+$ $MutL^-$ phenotype that results following integration of phage SF370.4 is strikingly similar to the limitation of MutL during stationary phase observed by Harris et al. in *E. coli* (22), suggesting that such a phenotype may be generally advantageous in situations where resources are limited. The nature of this advantage is unclear, however, since constitutive expression of MutS is energetically unfavorable. In the case of *S. pyogenes*, this may be due to lack of optimization of the phage integration site due to its relatively recent evolutionary appearance, or alternatively, the constitutive expression of MutS may contribute to maintaining some level of discrimination for RecA-mediated homeologous recombination between divergent DNA sequences

(59). It may well be that the observed system for control of MMR by prophage SF370.4 is indeed close to optimal, balancing the different selective pressures on the various repair systems coordinated by this element.

The frequent occurrence of MMR defects in natural populations of bacteria indicates that the benefit of increased mutability or the potential for horizontal genetic transfer (37) has a sufficiently high selective value to balance the risk of unfavorable mutations. Further, a bacterial species may undergo successive rounds of loss and regain of MMR function. The *mutS* and *mutL* genes from natural populations of *E. coli*, for example, exhibit high sequence mosaicism derived from diverse phylogenetic sources, while other housekeeping genes do not (11). This mosaicism was interpreted as having arisen from recurrent losses in MMR function, followed by reacquisition by horizontal transfer from wild-type strains. The phage-controlled system in *S. pyogenes* represents a sophisticated molecular alternative that does not require rare spontaneous mutations to inactivate MMR or the acquisition of exogenous DNA to reinstate the system. Indeed, the conditional expression of MMR in *S. pyogenes* fulfills the prediction of LeClerc et al. that "the ultimate pathogen would possess an elevated mutation rate that is transient (or conditional), providing genetic variation during the first few hours when the pathogen must survive, invade, and colonize its host" (29). A conditional mutator phenotype allows a bacterium to accumulate mutations that may provide an advantage during periods of stress, competition from other strains or species of bacteria, or limited resources. Conversely, a nonconditional mutator would eventually accumulate too many mutations that would prove to be deleterious to the cell. The ability to switch from mutator to nonmutator allows a cell to take advantage of both situations, ensuring its survival in the population. The specific selection advantage that MMR-converting prophages confer on their hosts and under what circumstances this occurs remain to be discovered, as do their mechanisms of dissemination through streptococcal populations. However, the widespread presence of prophages related to SF370.4 that are integrated into *mutL* in *S. pyogenes* strains suggests that these elements may confer a significant survival advantage to these strains.

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