Supplemental Text

A bacterial DNA repair pathway specific to a natural antibiotic

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Running Title: MrfAB are a novel excision repair pathway

Supplemental text

Supplemental Methods

Strain construction

Strains were constructed using CRISPR/Cas9 genome editing (P. E. Burby & Simmons, 2017a, 2017b) or double crossover recombination(P. E. Burby, Simmons, Schroeder, & Simmons, 2018).

PEB320 (Δ*mrfAB*): PY79 was transformed with pPB88 to delete *mrfAB* using CRISPR/Cas9 genome editing. Deletion of *mrfAB* was verified by PCR genotyping using oPEB452/462.

PEB337 (Δ*mrfA*, Δ*uvrAB*): PEB316 was transformed with pPB84 to delete *uvrAB* using CRISPR/Cas9 genome editing. Deletion of *uvrAB* was verified by PCR genotyping using oPEB424/432.

PEB339 (Δ*mrfB*, Δ*uvrAB*): PEB316 was transformed with pPB84 to delete *uvrAB* using CRISPR/Cas9 genome editing. Deletion of *uvrAB* was verified by PCR genotyping using oPEB424/432.

PEB369 (Δ*mrfA*, *amyE::*P_{xyl}*-mrfA*): PEB316 was transformed with pPB109. Replacement of *amyE* with P_{xyl}*-mrfA* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct. Genomic DNA from the resulting strain, PEB347, was used to transform PEB316, and replacement of *amyE* was verified by an inability to utilize starch.

PEB371 (Δ*mrfB*, *amyE::*P_{xyl}*-mrfB*): PY79 was transformed with pPB110. Replacement of *amyE* with P_{xyl}*-mrfB* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct. Genomic DNA from the resulting strain, PEB345, was used to transform PEB318, and replacement of *amyE* was verified by an inability to utilize starch. Retention of the *mrfB* deletion allele was verified by PCR genotyping using oPEB461/462.

PEB505 (Δ*mrfA*, *amyE::*P_{xyl}*-mrfA-K82A*): PEB316 was transformed with pPB159 digested with the restriction enzymes ScaI and BsaI. Replacement of *amyE* with P_{xyl}*-mrfA-K82A* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB507 (Δ*mrfA*, *amyE::Pxyl-mrfA-DE185-186AA*): PEB316 was transformed with pPB160 digested with the restriction enzymes ScaI and BsaI. Replacement of *amyE* with P_{xyl}*-mrfA-DE185-186AA* by double crossover recombination was verified by testing for an inability to

utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB509 (Δ*mrfA*, *amyE::Pxyl-mrfA-T134V*): PEB316 was transformed with pPB161 digested with the restriction enzymes ScaI and BsaI. Replacement of *amyE* with P_{xyl}*-mrfA-T134V* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB511 (Δ*mrfA*, *amyE::Pxyl-mrfA-S222A*): PEB316 was transformed with pPB162 digested with the restriction enzymes ScaI and BsaI. Replacement of *amyE* with P_{xyl}*-mrfA-S222A* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB513 (Δ*mrfA*, *amyE::Pxyl-mrfA-ΔC*): PEB316 was transformed with pPB163 digested with the restriction enzymes ScaI and BsaI. Replacement of *amyE* with P_{xyl}*-mrfAΔC* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB515 (Δ*mrfA*, *amyE::Pxyl-mrfA-C718A & C720A*): PEB316 was transformed with pPB164 digested with the restriction enzymes ScaI and BsaI. Replacement of *amyE* with P_{xyl}*-mrfA-C718A & C720A* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB517 (Δ*mrfA*, *amyE::Pxyl-mrfA-C718A, C720A, C724C, & C727A*):): PEB316 was transformed with pPB165 digested with the restriction enzymes ScaI and BsaI. Replacement of *amyE* with P_{xyl}- *mrfA-C718A, C720A, C724C, & C727A* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB519 (Δ*mrfB*, *amyE::Pxyl-mrfB-D107A*): PEB318 was transformed with pPB166 digested with the restriction enzymes ScaI and KpnI. Replacement of *amyE* with P_{xyl}*-mrfB-D107A* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB521 (Δ*mrfB*, *amyE::Pxyl-mrfB-E109A*): PEB318 was transformed with pPB167 digested with the restriction enzymes ScaI and KpnI. Replacement of *amyE* with P_{xyl}*-mrfB-E109A* by double crossover recombination was verified by testing for an inability to utilize starch and for

the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB523 (Δ*mrfB*, *amyE::Pxyl-mrfB-D172A*): PEB318 was transformed with pPB168 digested with the restriction enzymes ScaI and KpnI. Replacement of *amyE* with P_{xyl}-*mrfB-D172A* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB525 (Δ*mrfB*, *amyE::Pxyl-mrfB-D262A*): PEB318 was transformed with pPB169 digested with the restriction enzymes ScaI and KpnI. Replacement of *amyE* with P_{xyl}-*mrfB-D262A* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB527 (Δ*mrfB*, *amyE::Pxyl-mrfB-H258A*): PEB318 was transformed with pPB170 digested with the restriction enzymes ScaI and KpnI. Replacement of *amyE* with P_{xyl}-*mrfB-H258A* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB529 (Δ*mrfB*, *amyE::Pxyl-mrfB*-Δ*C*): PEB318 was transformed with pPB171 digested with the restriction enzymes ScaI and KpnI. Replacement of *amyE* with P_{xyl}- *mrfB*-Δ*C* by double crossover recombination was verified by testing for an inability to utilize starch and for the absence of a spectinomycin resistance marker found on the plasmid, but not on the integrated construct.

PEB812 (Δ*mrfAB*, Δ*uvrAB*): PEB320 was transformed with pPB84 to delete *uvrAB* using CRISPR/Cas9 genome editing. Deletion of *uvrAB* was verified by PCR genotyping using oPEB424/432.

PEB822 (Δ*uvrABC*): PEB309 was transformed with pPB85 to delete *uvrC* using CRISPR/Cas9 genome editing. Deletion of *uvrC* was confirmed by PCR genotyping using oPEB443/444.

PEB824 (Δ*mrfAB*, Δ*uvrC*): PEB320 was transformed with pPB85 to delete *uvrC* using CRISPR/Cas9 genome editing. Deletion of *uvrC* was confirmed by PCR genotyping using oPEB443/444.

PEB826 (Δ*mrfAB*, Δ*uvrABC*): PEB812 was transformed with pPB85 to delete *uvrC* using CRISPR/Cas9 genome editing. Deletion of *uvrC* was confirmed by PCR genotyping using oPEB443/444.

PEB828 (*recA::recA-gfp*): PY79 was transformed with chromosomal DNA from LAS40.

PEB830 (Δ*mrfAB*, *recA::recA-gfp*): PEB320 was transformed with chromosomal DNA from LAS40. Retention of the Δ*mrfAB* allele was verified by PCR genotyping using oPEB452/462.

PEB832 (Δ*uvrABC*, *recA::recA-gfp*): PEB822 was transformed with chromosomal DNA from LAS40. Retention of the Δ*uvrAB* allele was verified by PCR genotyping using oPEB424/432, and retention of the Δ*uvrC* allele was verified by PCR genotyping using oPEB443/444.

PEB834 (Δ*mrfAB*, Δ*uvrABC*, *recA::recA-gfp*): PEB826 was transformed with chromosomal DNA from LAS40. Retention of the Δ*mrfAB* allele was verified by PCR genotyping using oPEB452/462. Retention of the Δ*uvrAB* allele was verified by PCR genotyping using oPEB424/432, and retention of the Δ*uvrC* allele was verified by PCR genotyping using oPEB443/444.

PEB866 (Δ*mrfA*, *amyE::*P_{xyl}*-Bc-mrfA*): The *mrfA* homolog from *Bacillus cereus* (CUB17870.1) was codon optimized and used to generate a gBlock (IDT). The gBlock oPEB1044 was used in an overlap PCR reaction with two other PCR amplicons (*amyE* upstream and P_{xyl} generated using oPEB370/383 and a chloramphenicol resistance cassette and *amyE* downstream generated with oPEB557/377) using oPEB370/377. The resulting PCR product containing *amyE-up-*P_{xyl}*-Bc-mrfA-camR-amyE-down* was gel extracted and used to transform PEB316. Replacement of *amyE* with P_{xyl}*-Bc-mrfA-camR* was verified by testing for an inability to utilize starch.

PEB870 (Δ*mrfB*, *amyE::*P_{xyl}*-Bc-mrfB*): The *mrfB* homolog from *Bacillus cereus* (CUB17873.1) was codon optimized and used to generate a gBlock (IDT). The gBlock oPEB1046 was used in an overlap PCR reaction with two other PCR amplicons (*amyE* upstream and P_{xyl} generated using oPEB370/383 and a chloramphenicol resistance cassette and *amyE* downstream generated with oPEB557/377) using oPEB370/377. The resulting PCR product containing *amyE-up-*P_{xyl}*-Bc-mrfB-camR-amyE-down* was gel extracted and used to transform PEB318. Replacement of *amyE* with P_{xyl}*-Bc-mrfB-camR* was verified by testing for an inability to utilize starch.

PEB872 (Δ*mrfB*, *amyE::*P_{xyl}*-Pa-mrfB*): The *mrfB* homolog from *Pseudomonas aeruginosa* (CRP88025.1) was codon optimized and used to generate a gBlock (IDT). The gBlock oPEB1045 was used in an overlap PCR reaction with two other PCR amplicons (*amyE* upstream and P_{xyl} generated using oPEB370/383 and a chloramphenicol resistance cassette and *amyE* downstream generated with oPEB557/377) using oPEB370/377. The resulting PCR product containing *amyE-up-*P_{xyl}*-Pa-mrfB-camR-amyE-down* was gel extracted and used to transform PEB318. Replacement of *amyE* with P_{xyl}*-Pa-mrfB-camR* was verified by testing for an inability to utilize starch.

PEB898 (Δ*mrfA*, *amyE::*P_{xyl}*-Sp-mrfA*): The *mrfA* homolog from *Streptococcus pneumoniae* (COD01438.1) was codon optimized and used to generate a gBlock (IDT). The gBlock oPEB1047 was used in an overlap PCR reaction with two other PCR amplicons (*amyE* upstream and P_{xyl} generated using oPEB370/383 and a chloramphenicol resistance cassette and *amyE* downstream generated with oPEB557/377) using oPEB370/377. The resulting PCR product

containing *amyE-up-*P_{xyl}*-Sp-mrfA-camR-amyE-down* was gel extracted and used to transform PEB318. Replacement of *amyE* with P_{xyl}*-Sp-mrfA-camR* was verified by testing for an inability to utilize starch.

PEB900 (Δ*mrfB*, *amyE::*P_{xyl}*-Sp-mrfB*): The *mrfB* homolog from *Streptococcus pneumoniae* (COD01468.1) was codon optimized and used to generate a gBlock (IDT). The gBlock oPEB1048 was used in an overlap PCR reaction with two other PCR amplicons (*amyE* upstream and P_{xyl} generated using oPEB370/383 and a chloramphenicol resistance cassette and *amyE* downstream generated with oPEB557/377) using oPEB370/377. The resulting PCR product containing *amyE-up-*P_{xyl}*-Sp-mrfB-camR-amyE-down* was gel extracted and used to transform PEB318. Replacement of *amyE* with P_{xyl}*-Sp-mrfB-camR* was verified by testing for an inability to utilize starch.

PEB902 (Δ*mrfA*, *amyE::*P_{xyl}*-Pa-mrfA*): The *mrfA* homolog from *Pseudomonas aeruginosa* (CRP88044.1) was codon optimized and used to generate a gBlock (IDT). The gBlock oPEB1043 was used in an overlap PCR reaction with two other PCR amplicons (*amyE* upstream and P_{xyl} generated using oPEB370/383 and a chloramphenicol resistance cassette and *amyE* downstream generated with oPEB557/377) using oPEB370/377. The resulting PCR product containing *amyE-up-*P_{xyl}*-Pa-mrfA-camR-amyE-down* was gel extracted and used to transform PEB318. Replacement of *amyE* with P_{xyl}*-Pa-mrfA-camR* was verified by testing for an inability to utilize starch.

Plasmid construction

Plasmids were constructed via Gibson assembly as described (P. E. Burby et al., 2018; Gibson, 2011). Plasmids for the bacterial two-hybrid assays were constructed using 0.2% glucose in the media for selection of clones and cultures grown for plasmid isolation.

pPB88: Plasmid pPB88 was constructed using four PCR products: 1) the vector pPB41 was amplified using oPEB217/218; 2) Cas9/CRISPR::*mrfA* was amplified using pPB75 as a template with oPEB232/234; 3) the sequence upstream of *mrfA* for the editing template was amplified using oPEB448/464; and 4) the sequence downstream of *mrfB* for the editing template was amplified using oPEB465/460. Clones were verified via Sanger sequencing using oPEB227, oPEB253, and oPEB454.

pPB97: Plasmid pPB97 was constructed using two PCR products: 1) the vector pET28b-10xHis-Smt3 was amplified using oPEB56/57; and 2) the MrfB ORF was amplified using oPEB545/546. Clones were verified via Sanger sequencing using oPEB58, oPEB527, and oPEB547.

pPB109: Plasmid oPEB109 was constructed using four PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were

amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; and 4) the *mrfA* ORF was amplified using oPEB562/563. Clones were verified via Sanger sequencing using oPEB345, oPEB348, oPEB543, and oPEB544.

pPB110: Plasmid oPEB110 was constructed using four PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; and 4) the *mrfB* ORF was amplified using oPEB564/565. Clones were verified via Sanger sequencing using oPEB345, oPEB348, and oPEB547.

pPB159: Plasmid oPEB159 was constructed using five PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; 4) the 5′portion of the *mrfA-K82A* ORF was amplified using oPEB562/721; and 5) the 3′portion of the *mrfA-K82A* ORF was amplified using oPEB720/563. Clones were verified via Sanger sequencing using oPEB345, oPEB348, oPEB543, and oPEB544.

pPB160: Plasmid oPEB160 was constructed using five PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; 4) the 5′portion of the *mrfA-DE185-186AA* ORF was amplified using oPEB562/723; and 5) the 3′portion of the *mrfAK-DE185-186AA* ORF was amplified using oPEB722/563. Clones were verified via Sanger sequencing using oPEB345, oPEB348, oPEB543, and oPEB544.

pPB161: Plasmid oPEB161 was constructed using five PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; 4) the 5′portion of the *mrfA-T134V* ORF was amplified using oPEB562/725; and 5) the 3′portion of the *mrfA-T134V* ORF was amplified using oPEB724/563. Clones were verified via Sanger sequencing using oPEB345, oPEB348, oPEB543, and oPEB544.

pPB162: Plasmid oPEB162 was constructed using five PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; 4) the 5′portion of the *mrfA-S222A* ORF was amplified using oPEB562/727; and 5) the 3′portion of the *mrfA-S222A* ORF was amplified

using oPEB726/563. Clones were verified via Sanger sequencing using oPEB345, oPEB348, oPEB543, and oPEB544.

pPB163: Plasmid oPEB163 was constructed using four PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; and 4) the *mrfA*ΔC ORF was amplified using oPEB562/728. Clones were verified via Sanger sequencing using oPEB345, oPEB348, oPEB543, and oPEB544.

pPB164: Plasmid oPEB164 was constructed using four PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; and 4) the *mrfA-C718A & C720A* ORF was amplified using oPEB562/729. Clones were verified via Sanger sequencing using oPEB345, oPEB348, oPEB543, and oPEB544.

pPB165: Plasmid oPEB165 was constructed using four PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; and 4) the *mrfA-C718A, C720A, C724A, & C727A* ORF was amplified using oPEB562/730. Clones were verified via Sanger sequencing using oPEB345, oPEB348, oPEB543, and oPEB544.

pPB166: Plasmid oPEB166 was constructed using five PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; 4) the 5′portion of the *mrfB-D107A* ORF was amplified using oPEB564/732; and 5) the 3′ portion of the *mrfB-D107A* ORF was amplified using oPEB731/565. Clones were verified via Sanger sequencing using oPEB345, oPEB348, and oPEB547.

pPB167: Plasmid oPEB167 was constructed using five PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; 4) the 5′portion of the *mrfB-E109A* ORF was amplified using oPEB564/734; and 5) the 3′ portion of the *mrfB-E109A* ORF was amplified using oPEB733/565. Clones were verified via Sanger sequencing using oPEB345, oPEB348, and oPEB547.

pPB168: Plasmid oPEB168 was constructed using five PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream

portion of *amyE* was amplified using oPEB557/377; 4) the 5′ portion of the *mrfB-D172A* ORF was amplified using oPEB564/736; and 5) the 3′ portion of the *mrfB-D172A* ORF was amplified using oPEB735/565. Clones were verified via Sanger sequencing using oPEB345, oPEB348, and oPEB547.

pPB169: Plasmid oPEB169 was constructed using five PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; 4) the 5′ portion of the *mrfB-D262A* ORF was amplified using oPEB564/766; and 5) the 3′ portion of the *mrfB-D262A* ORF was amplified using oPEB765/565. Clones were verified via Sanger sequencing using oPEB345, oPEB348, and oPEB547.

pPB170: Plasmid oPEB170 was constructed using five PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; 4) the 5′ portion of the *mrfB-H258A* ORF was amplified using oPEB564/768; and 5) the 3′ portion of the *mrfB-H258A* ORF was amplified using oPEB767/565. Clones were verified via Sanger sequencing using oPEB345, oPEB348, and oPEB547.

pPB171: Plasmid oPEB171 was constructed using four PCR products: 1) the vector pPB47 was amplified using oPEB116/117; 2) the upstream portion of *amyE* and the P_{xyl} promoter were amplified using oPEB370/383; 3) the chloramphenicol resistance cassette and the downstream portion of *amyE* was amplified using oPEB557/377; and 4) the *mrfB*ΔC ORF was amplified using oPEB564/737. Clones were verified via Sanger sequencing using oPEB345, oPEB348, and oPEB547.

pPB263: Plasmid pPB263 was constructed using two PCR products: 1) the vector pUT18C was amplified using oPEB1017/1018; and 2) the *mrfA* ORF was amplified using oPEB1026/1027. Clones were verified via Sanger sequencing using oPEB543, oPEB544, oPEB1024, and oPEB1025.

pPB264: Plasmid pPB264 was constructed using two PCR products: 1) the vector pUT18 was amplified using oPEB1016/1012; and 2) the *mrfA* ORF was amplified using oPEB1028/1029. Clones were verified by Sanger sequencing using oPEB543, oPEB544, oPEB1019, and oPEB1023.

pPB265: Plasmid pPB265 was constructed using two PCR products: 1) the vector pKT25 was amplified using oPEB1014/1015; and 2) the *mrfB* ORF was amplified using oPEB1030/1031. Clones were verified by Sanger sequencing using oPEB547, oPEB1021, and oPEB1022.

pPB266: Plasmid pPB266 was constructed using two PCR products: 1) the vector pKNT25 was amplified using oPEB1012/1013; and 2) the *mrfB* ORF was amplified using oPEB1032/1033. Clones were verified by Sanger sequencing using oPEB547, oPEB1019, and oPEB1020.

pPB273: Plasmid pPB273 was constructed using two PCR products: 1) the vector pKT25 was amplified using oPEB1014/1015; and 2) the *mrfB*ΔC ORF was amplified using oPEB1030/1056. Clones were verified by Sanger sequencing using oPEB1021 and oPEB1022.

pPB274: Plasmid pPB274 was constructed using two PCR products: 1) the vector pKT25 was amplified using oPEB1014/1015; and 2) the *mrfB*ΔN ORF was amplified using oPEB1057/1031. Clones were verified by Sanger sequencing using oPEB1021 and oPEB1022.

pPB283: Plasmid pPB283 was constructed using two PCR products: 1) the vector pUT18 was amplified using oPEB1016/1012; and 2) the *mrfA*ΔN ORF was amplified using oPEB1053/1029. Clones were verified by Sanger sequencing using oPEB543, oPEB544, oPEB1019, and oPEB1023.

pPB284: Plasmid pPB284 was constructed using two PCR products: 1) the vector pUT18 was amplified using oPEB1016/1012; and 2) the *mrfA*ΔC ORF was amplified using oPEB1028/1054. Clones were verified by Sanger sequencing using oPEB543, oPEB544, oPEB1019, and oPEB1023.

pPB285: Plasmid pPB285 was constructed using two PCR products: 1) the vector pUT18 was amplified using oPEB1016/1012; and 2) the *mrfA-N* ORF was amplified using oPEB1028/1055. Clones were verified by Sanger sequencing using oPEB1019 and oPEB1023.

gBlocks used in this study

oPEB1043:

caaaggggaaatgggatcctaaggaggtatacatATGGCGTACGAACTGGCGAAACGGACTGCGGACGCTGAACAG
AAGCTCGCTACTCGCGACGGACTTCCTGCCCGGGACGGGGCCCTGTTATCTGCTCGCCTTCAGAGAAGATATCAAGA
CCGTATTACGGGAAGCTTTGCGATCCCTGGACGTGAGGGCCGTTACGCTCCAATACCTGACTCTGTTCCACCTGCCC
TGGCAGCAGCCTTAAAGGCGCGTGGTATTGAACAGCTTTACAGCCATCAAGCTGAGGCCTGGGAGGCCTCTCAACGC
GGAGAGCACGTCGCGATCGTAACGCCGACAGCATCCGGCAAGAGCCTGTGCTATACCTTGCCTGTTGTTTCTGCAGC
TATGCAGGATAAGGCGAAGGCGCTCTACCTCTTCCCTACTAAGGCACTGGCTCAAGACCAGGTCGCCGAGCTCCTGG
AGTTGAACAGAGCAGGAGATCTGGGTGTCAAAGCATTCACTTTCGATGGCGATACGCCGGGGGATGCACGTCAAGCT
ATTCGCTTACATGGCGATATTGTCGTGAGTAACCCAGATATGTTACATCAAGCGATACTCCCACATCATACCAAATG
GGCACAGTTTTTTGAGAATTTGCGTTATATAGTGATCGATGAAGTTCATACGTACCGCGGAGTATTCGGGTCCCATG
TGACTAACGTATTGAGACGGCTCAAAAGAATCTGCGCGTTTTACGGCGTACAACCTCAGTTCATTCTCTGTTCTGCA
ACCATTGGCAATCCTCAGGCGCATGCAGAAGCACTCATCGAGGCTCCTGTAACTGCTGTTACTGAATCTGGCGCACC
TACAGGGCCGAAGCAAGTACTTTTGTGGAACCCACCGGTGATAAACCCGGATTTAGGGCTCCGTGCTAGCGCGAGAA
GTCAAAGCAATCGCATAGCCAGAATAGCTATCAAGTCTGGCCTTAAAACTTTAGTATTCGCCCAAACTCGCCTCATG
GTAGAAGTTTTGACGAAGTACTTAAAAGACATTTTCGATCACGACCCGCGTAAACCGCCGCGTATCCGCGCGTACAG
AGGAGGTTATTTACCGACTGAACGGCGGGAAACTGAAAGAGCCATGCGGGCCGGTAATATCGACGGGATAGTATCTA
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oPEB1044:

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oPEB1048:

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Supplemental alignments

MrfA alignment

```
Pa-MrfA      MAYELAKRTADAEQKLATRDGLPARDGALLSARLQRRYQDRITGSFAIPGREGRYAPIPD   60
Sp-MrfA      -----------------------MKKKSLSELIQELKNHENIVHWHEEEPREAKTMPMPE   37
Bs-MrfA      -----------------------MKKKSLTELISDLKGNENVVNWHEIEPREAKTRPMPE   37
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Bc-MrfA      -----------------------MKKKSLTELISELKGNENIVNWHEIEPREARTRPMPE   37
                                     :. :* .   : : ::.:.  .    **.:  *:*:

Pa-MrfA      SVPPALAAALKARGIEQLYSHQAEAWEASQRGEHVAIVTPTASGKSLCYTLPVVSAAMQD   120
Sp-MrfA      QVDPNIRAALEKRGIERLFTHQYSAFQTVQNGESIVAVTPTASGKTLCYNLPVLQSIAED   97
Bs-MrfA      SIDERIKAALSKRGIDELYTHQYSAFQYVQKGESIVTVTPTASGKTLCYNLPVLQSIAQD   97
Bc-MrfA      SIDERIKAALSKRGIDELYTHQFSAFQYVQKGESIVTVTPTASGKTLSYNLPVLQSIAQD   97
             .:    : ***. ***:.*::** .*::  *.** :. ********:*.*.***:.:  :*

Pa-MrfA      -KAKALYLFPTKALAQDQVAELLELNRAGDLGVKAFTFDGDTPGDARQAIRLHGDIVVSN   179
Sp-MrfA      ASSRALYLFPTKALAQDQKSELNEIIDETGMDIKSFTYDGDTSPAIRQKVRKAGHIVITN   157
Bs-MrfA      ETNRALYLFPTKALAQDQKSELNEIIDEMGIDIKSFTYDGDTSPAIRQKVRKAGHIVITN   157
Bc-MrfA      ETNRALYLFPTKALAQDQKSELNEIIDEMGIDIKSFTYDGDTSPAIRQKVRKAGHIVITN   157
              . :************** :** *:     .:.:*:**:****     ** :*  *.**::*

Pa-MrfA      PDMLHQAILPHHTKWAQFFENLRYIVIDEVHTYRGVFGSHVTNVLRRLKRICAFYGVQPQ   239
Sp-MrfA      PDMLHSAILPHHTKWVSLFENLKYIVIDELHTYRGVFGSHVANVIRRLMRICAFYGSKPS   217
Bs-MrfA      PDMLHSAILPHHTKWVSLFENLKYIVIDELHTYRGVFGSHVANVIRRLKRICRFYGSDPV   217
Bc-MrfA      PDMLHSAILPHHTKWVSLFENLKYIVIDELHTYRGVFGSHVANVIRRLKRICRFYGSDPV   217
             *****.*********..:****:******:**********:**:*** *** *** .*

Pa-MrfA      FILCSATIGNPQAHAEALIEAPVTAVTESGAPTGPKQVLLWNPPVINPDLGLRASARSQS   299
Sp-MrfA      FICTSATIANPRELAEQLTGKSVRLIDDNGAPAGRKHFAFYNPPIVNKPLHIRKSATVEV   277
Bs-MrfA      FICTSATIANPKELGEQLTGKPMRLVDDNGAPSGRKHFVFYNPPIVNKPLNIRRSATAEV   277
Bc-MrfA      FICTSATIANPKELGEQLTGKPMRLVDDNGAPSGRKHFVFYNPPIVNKPLNIRKSATAEV   277
             **  ****.**:   .* *     :  : :.***:* *:. ::***::*  * :* **  :

Pa-MrfA      NRIARIAIKSGLKTLVFAQTRLMVEVLTKYLKDIFDHDPRKPPRIRAYRGGYLPTERRET   359
Sp-MrfA      NELAKTFLKNKIQTIVFARSRVRVEIILSHIQEIVKKE-IGAKSVRGYRGGYLSKERREI   336
Bs-MrfA      NELAKEFLKNKVQTIVFARSRVRVEIILSHIQELVKKE-IGTKSIRGYRGGYLPKERREI   336
Bc-MrfA      NELAKEFLKNKVQTIVFARSRVRVEIILSHIQELVKKE-IGTKSIRGYRGGYLPKERREI   336
             *.:*:  :*. ::*:***::*: **:: .:::::..::      :*.****** .****

Pa-MrfA      ERAMRAGNIDGIVSTSALELGVDIGSLDVVILNGYPGSVAATWQRFGRAGRRQQPALGVM   419
Sp-MrfA      ERGLRDGSILGVVSTNALELGVDIGQLQVCVMTGYPGSVASAWQQAGRAGRRQGEALIVM   396
Bs-MrfA      ERGLREGDILGVVSTNALELGVDIGQLQVCVMTGYPGSVASAWQQAGRAGRRHGESLIIM   396
Bc-MrfA      ERGLREGEILGVVSTNALELGVDIGQLQVCVMTGYPGSVASAWQQAGRAGRRHGESLIIM   396
             **.:* *.* *:***.*********.*:* ::.*******::**: ******:  :* :*

Pa-MrfA      VASSQPLDQYVVRHPDFFAEASPEHARIAPDQPLILFDHIRCAAFELPFRVGDGFGPIDP   479
Sp-MrfA      VANSDPIDQYIVRHPDYFFKRSPESARINPDNLIILVDHLKCAAYELPFRADETFGENDA   456
Bs-MrfA      VANSTPIDQYIVRHPEYFFNRSPESARINPENLIILVDHLKCAAYELPFRADEEFGAMEV   456
Bc-MrfA      VANSTPIDQYIVRHPEYFFNRSPESARINPENLIILVDHLKCAAYELPFRADEEFGPMDV   456
             **.* *:***:****::* : *** *** *:: :**.**::***:*****..: **  :

Pa-MrfA      EVFLEALAETEVIHREGERWEWIADSYPANAVSLRAVADGNFVVVDRSDGRQ-QIIAEVD   538
Sp-MrfA      RDILEYLEEEGVLHENRERYHWASESFPASNISLRSASQENVVIVDRSETADVKIIGEMD   516
Bs-MrfA      SDILEYLQEEAVLHRNGERYHWASESFPASNISLRSASQENVVIVDQSDIANVRIIGEMD   516
Bc-MrfA      SDILEYLQEEAVLHRNGERYHWASESFPASNISLRSASQENVVIVDQSDIANVRIIGEMD   516
               :** * *  *:*.: **:.* ::*:**. :***:.:: *.*:**:*:  : :**.*:*

Pa-MrfA      YSAAALTLYEGAIHMVQSTPYQVETLDWEGRKAYVTRTHVDYYTDSIDFTKLKVLDRFDG   598
Sp-MrfA      RFSAMTLLHDEAIYLHEGVQYQVEKLDWDHKKAYVRKVDVEYYTDANLAVQLKVLDIDRT   576
Bs-MrfA      RFSAMTLLHDEAIYLHEGVQYQVEKLDWDHKKAYVRKVDVEYYTDANLAVQLKVLEIDKT   576
Bc-MrfA      RFSAMTLLHDEAIYLHEGVQYQVEKLDWDHKKAYVRKVDVEYYTDANLAVQLKVLEIDKT   576
               :*   *:: **:: :.. ****.***: :**** :..*:****:   .:****:

Pa-MrfA      GVAGRGDSHHGEVHVVRRVAGYKKIRYYTHENIGYGPVNLPDQELHTTAVWWQLPQALLL   658
Sp-MrfA      DSRKKTALHFGDVTVNALPTIFKKIKMTTFENIGSGPIHLPEEELHTSAAWLELKETDSE   636
Bs-MrfA      KEKSRTSLHYGDVTVNALPTIFKKIKMTTFENIGSGPIHLPEEELHTSAAWLEIKTADED   636
Bc-MrfA      KEKSRTSLHYGDVTVNALPTIFKKIKMTTFENIGSGPIHLPEEELHTSAAWLEIKTADED   636
                 :   *.*:* *    : :***:  *.**** **::**::****:*.* ::  :
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Pa-MrfA         RAFASRQDPLDGFLGAAYALHIVATVAVMADARDLQKSVGNGDGSWFAIADQSGRGQLRG   718
Sp-MrfA         IGEKT---LEQLLLGIAHVLQHIVPVYVMCDRNDVHVV----------------------   671
Bs-MrfA         IGEKT---LEQLLLGISNVLQHIVPVYIMCDRNDVHVV----------------------   671
Bc-MrfA         IGEKT---LEQLLLGISNVLQHIVPVYIMCDRNDVHVI----------------------   671
                 .  :     : :** : .*: :. * :*.* .*::

Pa-MrfA         SEGDPGGVELLQEFVPTVYLYDNFPGGVGLSEPLWQRQAELVQRARELVQRCDCKAGCPA   778
Sp-MrfA         -----PQIKAAHTGLPTIFLYDHYPGGIGLADEVYKRFDEINEGAERLIRQCPCQDGCPS   726
Bs-MrfA         -----SQIKAAHTGLPTIFLYDHYPGGIGLAEEVFKRFSDINEAAKQLITHCPCHDGCPS   726
Bc-MrfA         -----SQIKAAHTGLPTIFLYDHYPGGIGLAEEVFKRFSDINEAAKQLIKQCPCHDGCPS   726
                       ::  :  :**::***::***:**:: :::*  :: : *..*: :* *: ***:

Pa-MrfA         CVGPVLAAQEEDETSPRALALRVLDLFDAEACRHVPDVVVTTRDPMELLAP   829
Sp-MrfA         CIGSEIEGIDAKKAI-----LRLLNYV-----------------------   748
Bs-MrfA         CIGTEIEGIKAKERI-----LQLLDQMS----------------------   749
Bc-MrfA         CIGTEIEGIKAKERI-----LQLLVQMA----------------------   749
                *:*  : . . .:       *::*  .
```

MrfB alignment

```
Pa-MrfB         MSLSLDKLRLLRRQAGDPKASTPAVPDVPPAPPAPVAANDARQPPAERSVFAWVEQEIRH   60
Sp-MrfB         MSLKNKLKR-MKNHLN--------VP------------------------------EAEK   21
Bs-MrfB         MSLKGKLQR-MKKHMA--------LD------------------------------EGEQ   21
Bc-MrfB         MSLKGKLQR-MKKHMV--------LD------------------------------EGEH   21
                ***. .  * ::.:          :                               * .:

Pa-MrfB         KPTGAAAPTP-ASAPLRRPEVGSLHRLLGLRTRSGATPARASA-----QDRQLPGEEIAP   114
Sp-MrfB         KPAASQLSVPDIQVPFREE-----WEALGV------KPFFFEDEYCLIRETVYPL-SHRH   69
Bs-MrfB         KIEAGKQENHFDDIPFLEE-----WEAFGM------KPFIFEDEYCLIREVEYPL-SHRH   69
Bc-MrfB         KIEAGQRENNFAEIPFLEE-----WEAFGM------KPFFFEDEYCLIREVEYPL-SHRH   69
                *  ..       . *: .       . :*:      .*   .      ::   *  .

Pa-MrfB         GLFLIESLQPQAIPAQPLSLDFARRDGEHVAARDLLFFDTETTGLAGGTGTRAFMIGAAD   174
Sp-MrfB         GRYSFSELDDVMALWNKGGLT-HTLSAKGYEKSQLFFFDTETTGLGGGAGNMIFLLGHAR   128
Bs-MrfB         GLYSFSELEEVITLWNQSGLS-HTLSAKGYNKNNLFFFDTETTGLGGGAGNTIFLLGHAR   128
Bc-MrfB         GLYRFSELDEVITLWNQSSLS-HTLSAKGYNKNSLFFFDTETTGLGGGAGNTIFLLGHAR   128
                * : :..*:      :  .*     ..:     .*:*********.**:*.  *::* *

Pa-MrfB         WHVCPQRGEGLRIRQLLMATMAAEDAMLATFAGWLQPSTVFCSYNGRSYDAPLLKARYRL   234
Sp-MrfB         VYE-----DRVAVKQHLLPKPGNETALYKSFLSEV-DITSLVTYNGKAFDWPQVKTRHTL   182
Bs-MrfB         VYE-----DRVTVKQHLLPKPGNEVALYQSFLSEV-DITSLVTYNGKAFDWPQVKTRHTL   182
Bc-MrfB         VYE-----DRVTVKQHLLPKPGNEVALYQSFLSEV-DITSLVTYNGKAFDWPQVKTRHTL   182
                 :      : : ::* *: . . * *:  :* . :   * : :***:::* * :*:*: *

Pa-MrfB         ARQRDP-ISALDHVDLLYPTRRRYRGTWENCKLSTIERQLLRVVREDDLPGSEAPGAWLR   293
Sp-MrfB         LRDRLPKLPDFGHFDLLHGARRLWKHKLERVSLSAVENEELAFKRDEDTPGYLAPMLYFQ   242
Bs-MrfB         IRDRLPKLPEFGHFDLLHGARRLWKHKMDRVSLGTVEKEELGIRRLEDTPGYLAPMLYFH   242
Bc-MrfB         IRDRLPKLPEFGHFDLLHGARRLWKHKMDRVSLGTVEKEELGIHRQEDTPGYLAPMLYFH   242
                 *:* * :  :.*.***: :** :: . :. .*.::*.: * . * :* **  **  :::

Pa-MrfB         FLRGGDAVNLRRVADHNHQDVVTLALLLQRLVREEQR---------E-RETLALVGQ---   340
Sp-MrfB         FLKAEDPALLKGVLSHNEQDVLSLIALYIHMSKKIFASSDQT---SERQEAYAMAKWFIA   299
Bs-MrfB         FIKAQEPDLLKGVLHHNEMDVLSLISLYIHMSKKILSESHAPK---EHSEAYAMAKWFMA   299
Bc-MrfB         FIKAQEPDLLKGVLHHNEMDVLSLISLYIHMSKKILSASYASKEHIEHSEAYAMAKWFMA   302
                *::. :   *: *  **. **::*  *  :: ::            *  *: *:.

Pa-MrfB         ------------------------------------------------------------   340
Sp-MrfB         HKETDRAVSQLEALQGKDFEDSDRALFDLAMLYKKQNRRQDAVPLWEKLTDSDLHTCRHH   359
Bs-MrfB         HKETDQAIKQLERLIEKSFEDQDSARLDLSLLYKKQNRLEEAVPLWEKLSRSQNQKCRYA   359
Bc-MrfB         HKETDQAVKQLERLKEKSFEDQDRARLDLSLLYKKQNRLEEAVPLWEKLSRSQNQKCRYT   362
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Pa-MrfB      ---------------------------------------------------------- 340
Sp-MrfB      SAVELAIYFEHHAKDYKKALQAAQQAAED-GEISEKEAEKLHVRIARLKRKYSS 412
Bs-MrfB      AVIELAKYFEHKKKEFGKALQVAEQSLSDAACLSEKETEKLHVRIARLKRKYSS 413
Bc-MrfB      AVIELAKYFEHKKKEFGKALYIAEQLLSDAAFLSEKESEKLQVRIARLKRKYSS 416
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Supplemental Figures

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MrfA      53 DELYTHQYSAFQYVQKGESIVTVTPTASGKTLCYNLPVLQSIAQDETNRALYLFPTKALA
Hrq1p    289 ENFYSHQADAINSLHQGENVIITTSTSSGKSLIYQLAAIDLLLKDPESTFMYIFPTKALA
                * **  *      **     * * *** * * *        *      * *******

MrfA     113 QDQKSELNEIIDEMGIDIKSF--TYDGDTSPAIRQKVRKAGHIVITNPDMLHSAILPHHT
Hrq1p    349 QDQKRAFKVILSKIPELKNAVVDTYDGDTEPEERAYIRKNARVIFTNPDMIHTSILPNHA
             ****     *             ****** *  *   **      ***** *  *** * 

MrfA     171 KWVSLFENLKYIVIDELHTYRGVFGSHVANVIRRLKRICR-FYGSDPV-FICTSATIANP
Hrq1p    409 NWRHFLYHLKLVVVDELHIYKGLFGSHVALVMRRLLRLCHCFYENSGLQFISCSATLKSP
              *      **  * **** * * ****** * *** * *  **      **  ***   *

MrfA     229 KELGEQLTG-KPMRLVDDNGAPSGRKHFVFYNPPIVNKPLNIRRSATAEVNELAKEFLKN
Hrq1p    469 VQHMKDMFGINEVTLIHEDGSPTGAKHLVVWNPPILPQHERKRENFIRESAKILVQLILN
                     *     *    * * * ** *  ****       *     *          *

MrfA     288 KVQTIVFARSRVRVEIILSHIQEL---VKKEIGTKSIRGYRGGYLPKERREIERGLREGD
Hrq1p    529 NVRTIAFCYVRRVCELLMKEVRNIFIETGREDLVTEVMSYRGGYSASDRRKIEREMFHGN
              * ** *   *   *               *        *****    ** ***    * 

MrfA     345 ILGVVSTNALELGVDIGQLQVCVMTGYPGSVASAWQQAGRAGRRHGESLIIMVANSTPID
Hrq1p    589 LKAVISTNALELGIDIGGLDAVLMCGFPLSMANFHQQSGRAGRRNNDSLTLVVASDSPVD
                * ******** *** *    * * * * *   ** ******   **   **   * *

MrfA     405 QYIVRHPEYFFN----RSPESARINPENLIILVDHLKCAAYELPFRADEEFGAMEVSDIL
Hrq1p    649 QHYVAHPESLLEVNNFESYQDLVLDFNNILILEGHIQCAAFELPINFERDKQYFTESHLR
             *  * ***         *         *  **  *  *** ***            *   

MrfA     461 EYLQEEAVLHRNGERYHWASE--SFPASNISLRSASQENVVIVDQSDIANVRIIGEMDRF
Hrq1p    709 KICVER--LHHNQDGYHASNRFLPWPSKCVSLRGGEEDQFAVVDITNGRNI-IIEEIEAS
                 *   ** *   **        *    ***         **     *  ** *    

MrfA     519 SAMTLLHDEAIYLHEGVQYQVEKLDWDHKKAYVRKVDVEYYTDANLAVQLKVLEIDKTKE
Hrq1p    766 RTSFTLYDGGIFIHQGYPYLVKEFNPDERYAKVQRVDVDWVTNQRDFTDVDPQEIELIRS
                  * *  *  * *  * *     *   * *  ***   *           **     

MrfA     579 KSRTSLHYGDVTVNALPTIFKKIKMTTFENIGSG-PIHLPEEELHTSAAWLEIKTADEDI
Hrq1p    826 LRNSDVPVYFGKIKTTIIVFGFFKVDKYKRIIDAIETHNPPVIINSKGLWIDMPKYALEI
                                *   *      *      * *         *         *

MrfA     638 GEK---TLEQLLLGISNVLQHIVPVYIMCDRNDVHV--------VSQIKAAHTGLPTIFL
Hrq1p    886 CQKKQLNVAGAIHGAQHAIMGMLPRFIVAGVDEIQTECKAPEKEFAERQTKRKRPARLIF
               *          *         *  *

MrfA     687 YDHYPG--GIGLAEEVFKRFSDINEAAKQLITHCPCHDGCPSCI
Hrq1p    946 YDSKGGKYGSGLCVKAFEHIDDIIESSLRRIEECPCSDGCPDCV
             **   *  * **    *    ** *     *  *** **** *
```

Motif I, Motif Ia, Motif Ib, Motif II, Motif III, Motif IV, Motif V,
Motif VI, conserved cysteines

Figure S1. Helicase motifs of MrfA. An alignment of MrfA to Hrq1 from *S. cerevisiae*. Helicase motifs are highlighted as shown. The alignment was constructed using SIM (Huang & Miller, 1991).

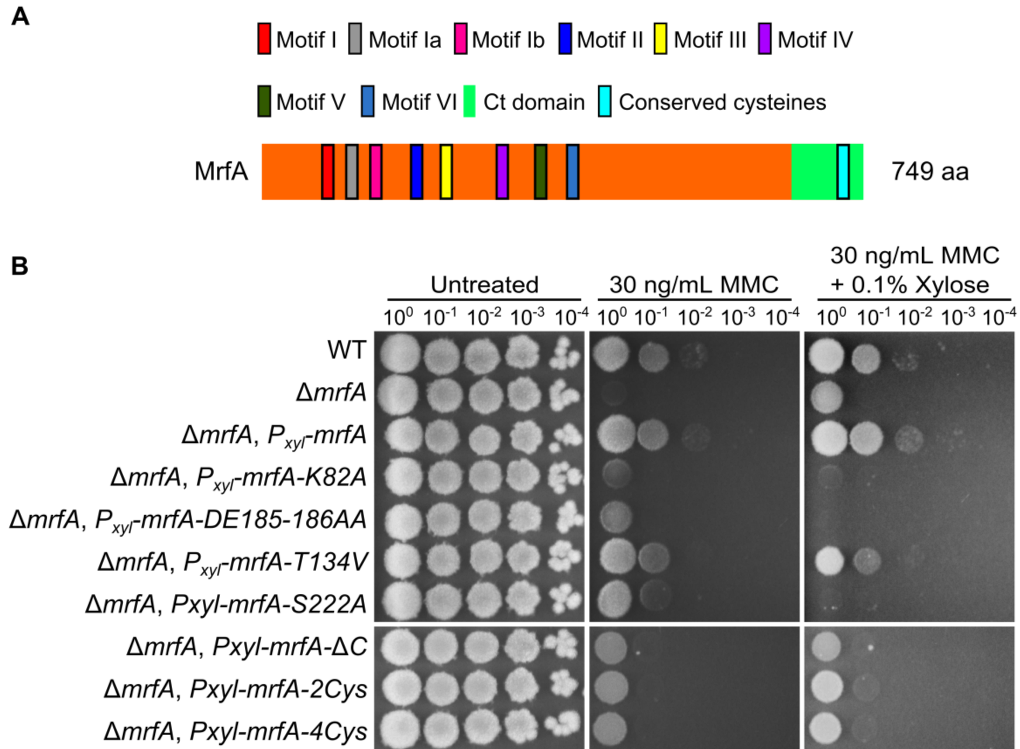


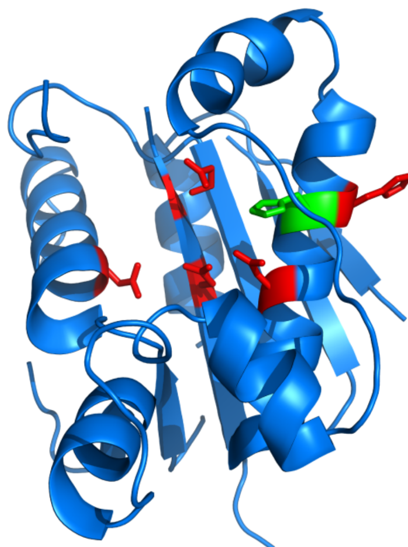
Figure S2. MrfA helicase motifs and conserved cysteines are required for function. (A) A schematic of MrfA depicting putative helicase motifs, C-terminal (Ct) domain, and conserved cysteines. **(B)** Spot titer assay using strains with the indicated genotypes spotted on the indicated media.

A

```
MrfB      VEYPLSHRHGLYSFSELEEVITLWNQSGLSHTLSAKGYNKNNLFFFDTETTGLGGGA---     117
ExoI      --------------------------------MMNDGKQQSTFLFHDYETFGTHPA----     24
DnaQ      ---------------------------------MST--AITRQIVLDTETTGMNQIGAHY     25
ExoX      -----------------------------------------MLRIIDTETCGLQ------     13
                                                      . * ** *

MrfB      -GNTIFLLGHAR-VYEDRVT--------------------------VKQHLLPKPGNEVA     149
ExoI      -LDRPAQFAAIRTDSEFNVIGEPEVFYCKPADDYLPQPGAVLITGITPQEARAKGENEAA     83
DnaQ      EGHKIIEIGAVE-VVNRRLTGNNFHVYLKPDRLV--DPEAFGVHGIADEFLLDKP----T     78
ExoX      --GGIVEIASVD-VIDGKIV-NPMSHLVRPDRPI--SPQAMAIHRITEAMVADKP----W     63
                 :.      : .:                           .      *

MrfB      LYQSFLS--EVDITSLVTYNGKAFD-------------------WPQVKTRHTLIRDRLP     188
ExoI      FAARIHSLFTVPKTCILGYNNVRFDDEVTRNIFYRNFYDPYAWSWQHDNSRW-----DLL     138
DnaQ      FAEVADEFMDYIRGAELVIHNAAFDIG----------FMDYEFSLLKR---------DIP     119
ExoX      IEDVIPHY---YGSEWYVAHNASFDRR----------VLPE-----------------MP     93
          :                  :.   **                                 :

MrfB      KLPEFG-HFDLLHGARRLWKHKMDRVSLGTVEKEELGIRRLEDTPGYLAPMLYFHFIKAQ     247
ExoI      DVMRACY---ALRPEGINWPENDDGLPSFRL----------------------EHLTKAN     173
DnaQ      KTNTFCKVTDSLAVARKMFPGKRNSLDALCA----------------------RYEIDNS     157
ExoX      G-EWIC----TMKLARRLWPGIKYSNMALYK----------------------TRKLNVQ     126
                     :      :                                      . .

MrfB      EPDLLKGVLHHNEMDVLSLISLYIHMSKKILSESHA-------------------P----     284
ExoI      GIE--HSNAHDAMADVYATIAMAK------------------------------------     195
DnaQ      -----KRTLHGALLDAQILAEVYLAM--------------TGGQTSMAFAMEGETQQQQG     198
ExoX      TPP--GLHHHRALYDCYITAALLIDIMNTSGWTAEQMADITGRPSLMTTFTFGK---YRG     181
                   *    *      :

MrfB      ----KEHSEAYAMAKWFMAH----------------------------------------     300
ExoI      ----LVKTRQPRLFDYLFTHRNKHKLMALIDVPQMKPLVHVSGMFGAWRGNTSWVAPLAW     251
DnaQ      EATIQRIVRQASKLRVVFATDEEIAA-----HEARLDLVQKKGGSCLWRA----------     243
ExoX      KAVSDVAERDPGYLRWLFNNLDSMS------PELRLTLKHYLENT---------------     220
                  .       .:
```

Putative catalytic residues, Putative catalytic residue from structural model

B



C

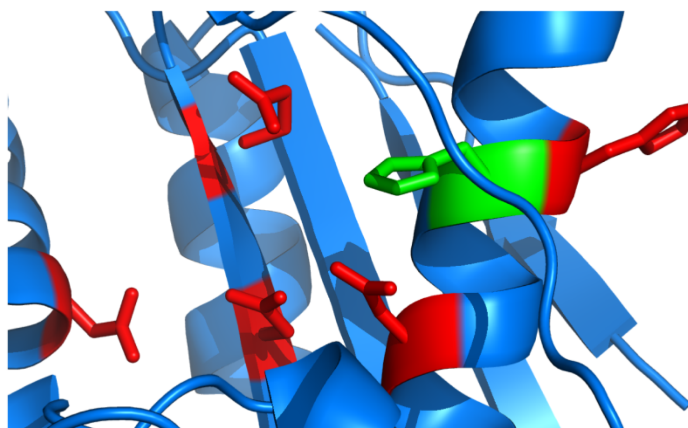


Figure S3. Putative catalytic residues of MrfB. (A) Alignment of the exonuclease domain of MrfB to ExoI (SbcD), DnaQ, and ExoX from *E. coli* using Clustal Omega (Sievers & Higgins, 2014). Putative catalytic residues are highlighted in red, and a putative non-conserved catalytic residue is highlighted in green. **(B)** A structural model of MrfB, modelled on DNA polymerase epsilon catalytic subunit A (pdb structure c5okiA (Grabarczyk, Silkenat, & Kisker, 2018)), was generated using Phyre2 (Kelley, Mezulis, Yates, Wass, & Sternberg, 2015). The model depicting amino acids 100-270 of MrfB is shown as a cartoon in blue, and the putative catalytic residues are colored as in A. **(C)** A close up view of the putative catalytic residues from the model shown in B.

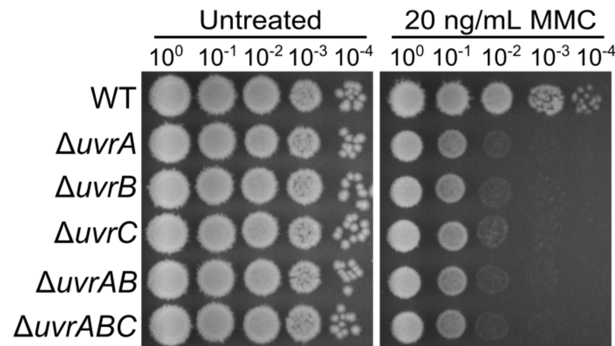


Figure S4. UvrABC function in the same pathway. Spot titer assay using the indicated *uvr* deletion strains grown on the indicated media.

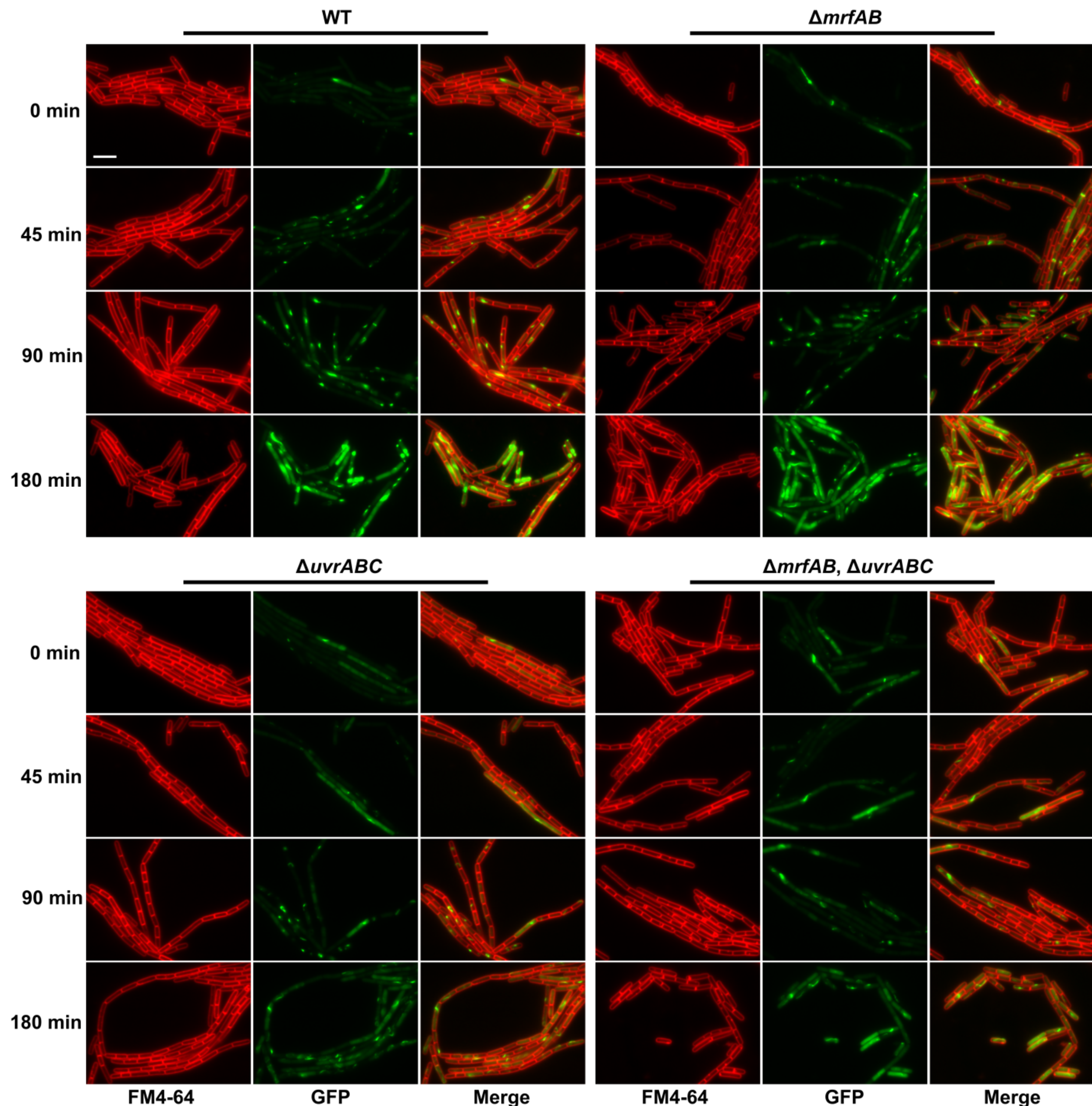


Figure S5. MrfAB are not required for unhooking interstrand DNA cross-links. Representative micrographs of cells from the indicated genotypes that also contain RecA-GFP expressed from the native locus. Time of imaging post MMC treatment (5 ng/mL) is indicated (rows). Membranes, stained with FM4-64 are shown in red, RecA-GFP is shown in green, and both are shown in the merged images. The white scale bar indicates 5 μm. The images for WT and Δ*mrfAB*, Δ*uvrABC* are also in figure 5 and are shown here for comparison.

Supplemental Tables

Table S1. Strains used in this study.

Strain	Genotype	Reference
PY79	PY79	(Youngman, Perkins, & Losick, 1984)
LAS40	*recA∷recA-gfp*	(Simmons, Grossman, & Walker, 2007)
PEB125	Δ*recU∷erm*	(P. E. Burby & Simmons, 2017b)
PEB307	Δ*uvrA*	(P. E. Burby et al., 2018)
PEB308	Δ*uvrB*	(P. E. Burby et al., 2018)
PEB309	Δ*uvrAB*	(Peter E Burby, Simmons, & Simmons, 2018)
PEB310	Δ*uvrC*	(P. E. Burby et al., 2018)
PEB316	Δ*mrfA* (*yprA*)	(P. E. Burby et al., 2018)
PEB318	Δ*mrfB* (*yprB*)	(P. E. Burby et al., 2018)
PEB320	Δ*mrfAB*	This study
PEB337	Δ*mrfA*, Δ*uvrAB*	This study
PEB339	Δ*mrfB*, Δ*uvrAB*	This study
PEB369	Δ*mrfA*, *amyE::*P_{xyl}*-mrfA*	This study
PEB371	Δ*mrfB*, *amyE::*P_{xyl}*-mrfB*	This study
PEB505	Δ*mrfA*, *amyE::*P_{xyl}*-mrfA-K82A*	This study
PEB507	Δ*mrfA*, *amyE::Pxyl-mrfA-DE185-186AA*	This study
PEB509	Δ*mrfA*, *amyE::Pxyl-mrfA-T134V*	This study
PEB511	Δ*mrfA*, *amyE::Pxyl-mrfA-S222A*	This study
PEB513	Δ*mrfA*, *amyE::Pxyl-mrfA-*Δ*C*	This study
PEB515	Δ*mrfA*, *amyE::Pxyl-mrfA-C718A & C720A*	This study
PEB517	Δ*mrfA*, *amyE::Pxyl-mrfA-C718A, C720A, C724C, & C727A*	This study
PEB519	Δ*mrfB*, *amyE::Pxyl-mrfB-D107A*	This study
PEB521	Δ*mrfB*, *amyE::Pxyl-mrfB-E109A*	This study
PEB523	Δ*mrfB*, *amyE::Pxyl-mrfB-D172A*	This study
PEB525	Δ*mrfB*, *amyE::Pxyl-mrfB-D262A*	This study
PEB527	Δ*mrfB*, *amyE::Pxyl-mrfB-H258A*	This study
PEB529	Δ*mrfB*, *amyE::Pxyl-mrfB-*Δ*C*	This study
PEB812	Δ*mrfAB*, Δ*uvrAB*	This study
PEB822	Δ*uvrABC*	This study
PEB824	Δ*mrfAB*, Δ*uvrC*	This study
PEB826	Δ*mrfAB*, Δ*uvrABC*	This study
PEB828	*recA∷recA-gfp*	This study
PEB830	Δ*mrfAB*, *recA∷recA-gfp*	This study
PEB832	Δ*uvrABC*, *recA∷recA-gfp*	This study
PEB834	Δ*mrfAB*, Δ*uvrABC*, *recA∷recA-gfp*	This study
PEB866	Δ*mrfA*, *amyE::*P_{xyl}*-Bc-mrfA*	This study

PEB870	Δ*mrfB*, *amyE::*P_{xyl}*-Bc-mrfB*	This study
PEB872	Δ*mrfB*, *amyE::*P_{xyl}*-Pa-mrfB*	This study
PEB898	Δ*mrfA*, *amyE::*P_{xyl}*-Sp-mrfA*	This study
PEB900	Δ*mrfB*, *amyE::*P_{xyl}*-Sp-mrfB*	This study
PEB902	Δ*mrfA*, *amyE::*P_{xyl}*-Pa-mrfA*	This study

Table S2. Plasmids used in this study.

Plasmid number	Plasmid name	Reference/Source
pUC19	pUC19	NEB (3041S)
pKT25	pKT25	Euromedex (EUP-25C)
pKNT25	pKNT25	Euromedex (EUP-25N)
pUT18	pUT18	Euromedex (EUP-18N)
pUT18C	pUT18C	Euromedex (EUP-18C
pPB41	pPB41	(P. E. Burby & Simmons, 2017a, 2017b)
pPB47	pPB47	(P. E. Burby et al., 2018)
pPB73	pPB41-CRISPR::*uvrB*	(P. E. Burby et al., 2018)
pPB74	pPB41-CRISPR::*uvrC*	(P. E. Burby et al., 2018)
pPB75	pPB41-CRISPR::*mrfA*	(P. E. Burby et al., 2018)
pPB84	pPB73-Δ*uvrAB* editing template	(Peter E Burby et al., 2018)
pPB85	pPB74-Δ*uvrC* editing template	(P. E. Burby et al., 2018)
pPB88	pPB75-Δ*mrfAB* editing template	This study
pPB97	pET-28b-10xHis-Smt3-MrfB	This study
pPB109	pPB47-*amyE::Pxyl-mrfA-camR*	This study
pPB110	pPB47-*amyE::Pxyl-mrfB-camR*	This study
pPB159	pPB47-*amyE::Pxyl-mrfA-K82A-camR*	This study
pPB160	pPB47-*amyE::Pxyl-mrfA-DE185-186AA-camR*	This study
pPB161	pPB47-*amyE::Pxyl-mrfA-T134V-camR*	This study
pPB162	pPB47-*amyE::Pxyl-mrfA-S222A-camR*	This study
pPB163	pPB47-*amyE::Pxyl-mrfA-ΔC-camR*	This study
pPB164	pPB47-*amyE::Pxyl-mrfA-C718A & C720A-camR*	This study
pPB165	pPB47-*amyE::Pxyl-mrfA-C718A, C720A, C724A, & C727A-camR*	This study
pPB166	pPB47-*amyE::Pxyl-mrfB-D107A-camR*	This study
pPB167	pPB47-*amyE::Pxyl-mrfB-E109A-camR*	This study
pPB168	pPB47-*amyE::Pxyl-mrfB-D172A-camR*	This study
pPB169	pPB47-*amyE::Pxyl-mrfB-D262A-camR*	This study
pPB170	pPB47-*amyE::Pxyl-mrfB-H258A-camR*	This study
pPB171	pPB47-*amyE::Pxyl-mrfB-ΔC-camR*	This study
pPB263	pU-T18-MrfA	This study
pPB264	pU-MrfA-T18	This study
pPB265	pK-T25-MrfB	This study
pPB266	pK-MrfB-T25	This study
pPB273	pK-T25-MrfBΔC	This study
pPB274	pK-T25-MrfBΔN	This study
pPB283	pU-MrfAΔN-T18	This study
pPB284	pU-MrfAΔC-T18	This study
pPB285	pU-MrfA-N-T18	This study

Table S3. Oligonucleotides used in this study.

Primer name	Sequence
oPEB56	ACCTCCAATCTGTTCGCGGTG
oPEB57	taaTCGAGCACCACCACCACCAC
oPEB58	GCTAGTTATTGCTCAGCGG
oPEB116	ctctcgtttcatcggtatcattac
oPEB117	cgcttcgttaatacagatgtaggt
oPEB217	GAACCTCATTACGAATTCAGCATGC
oPEB218	GAATGGCGATTTTCGTTCGTGAATAC
oPEB227	CCGTCAATTGTCTGATTCGTTA
oPEB232	GCTGTAGGCATAGGCTTGGTTATG
oPEB234	GTATTCACGAACGAAAATCGCCATTCCTAGCAGCACGCCATAGTGACTG
oPEB253	GAAGGGTAGTCCAGAAGATAACGA
oPEB345	actcctttgtttatccaccgaac
oPEB348	TTATTTTTGACACCAGACCAACTG
oPEB370	cacctacatctgtattaacgaagcgTCAATGGGGAAGAGAACCGCTTAAG
oPEB377	ggtaatgataccgatgaaacgagagAACAAAATTCTCCAGTCTTCACATCG
oPEB383	atgtatacctccttaggatcccatttcc
oPEB424	agaatgaatcgtgaaatgatcacc
oPEB432	acggatcgatatgattctctaagc
oPEB443	aaaccggaatccttcagacaatac
oPEB444	cttctaacggcacttggtaatttt
oPEB448	GCATGCTGAATTCGTAATGAGGTTCcgagttgattaggttctgaaatcc
oPEB452	tcttgtcatgcttgtaaaggtagc
oPEB454	agaaaatgatgggagaaggaatag
oPEB460	GCATAACCAAGCCTATGCCTACAGCatggtgtgatgacagctacctttta
oPEB461	AGCCATGGAAGTCAGTGATATTCT
oPEB462	tctttattcggttctttccagttc
oPEB464	gggaatattctttacacctctttgtcaagtac
oPEB465	tgtacttgacaaagaggtgtaaagaatattccccgggaaagcgcaaaagacgacttgtttcgccatga atttt
oPEB527	TAAAAGACAGGGTAAGGAAATGGA
oPEB543	CAATCAGACAAAAGGTGAGAAAAG
oPEB544	AGAAATCGAAAGAGGACTGAGAGA
oPEB545	GCTCACCGCGAACAGATTGGAGGTATGTCATTAAAAGGGAAACTCCAAC
oPEB546	CTCAGTGGTGGTGGTGGTGGTGCTCGAttaTTAAGAGGAATATTTCCTCTTTAGCCGGGCAATTCTCA CATGCAGTT
oPEB547	TAAGCGAGGTTGACATTACATCAC
oPEB557	taaCGGTTTCCATATGGGGATTGGTG
oPEB562	aatgggatcctaaggaggtatacatATGAAAAAGAAATCACTGACTGAACT
oPEB563	ACCAATCCCCATATGGAAACCGttaTTACGACATTTGATCCAACAGCTG

oPEB564	aatgggatcctaaggaggtatacatATGTCATTAAAAGGGAAACTCCAAC
oPEB565	ACCAATCCCCATATGGAAACCGttaTTAAGAGGAATATTTCCTCTTTAGCCGGGCAATTCTCACATGCAGTT
oPEB720	GTAACGCCAACAGCATCAGGAGCTACGTTATGCTACAACCTCCCAGTC
oPEB721	TGGGAGGTTGTAGCATAACGTAGCTCCTGATGCTGTTGGCGTTACGGT
oPEB722	ACCTTAAGTATATCGTCATCGCTGCACTTCATACGTATCGAGGTGTGTTC
oPEB723	ACACCTCGATACGTATGAAGTGCAGCGATGACGATATACTTAAGGTTTTCAAAC
oPEB724	GGGCATTGATATTAAAAGCTTTGTATATGACGGGGATACGTCTCCGGCA
oPEB725	CCGGAGACGTATCCCCGTCATATACAAAGCTTTTAATATCAATGCCCATTTCATC
oPEB726	GTGATCCAGTTTTTATTTGTACTGCTGCAACGATTGCCAACCCAAAGGAA
oPEB727	CCTTTGGGTTGGCAATCGTTGCAGCAGTACAAATAAAAACTGGATCACTTCCA
oPEB728	CAATCCCCATATGGAAACCGttaTTAAGGGACAATATGCTGCAGCACA
oPEB729	TCGCCACCAATCCCCATATGGAAACCGttaTTACGACATTTGATCCAACAGCTGCAAAATTCTTTCCTTTGCTTTTATCCCTTCTATTTCCGTACCTATACAAGACGGACAGCCGTCATGAGCAGGAGCATGTGTAATCAGTTGTTTCGCCGCTT
oPEB730	TCGCCACCAATCCCCATATGGAAACCGttaTTACGACATTTGATCCAACAGCTGCAAAATTCTTTCCTTTGCTTTTATCCCTTCTATTTCCGTACCTATAGCAGACGGAGCGCCGTCATGAGCAGGAGCATGTGTAATCAGTTGTTTCGCCGCTT
oPEB731	ACAAAAACAACCTCTTTTTCTTTGCTACAGAAACAACCGGTCTTGGGGGT
oPEB732	CCCCAAGACCGGTTGTTTCTGTAGCAAAGAAAAAGAGGTTGTTTTTGTTATACCCT
oPEB733	CAACCTCTTTTTCTTTGATACAGCTACAACCGGTCTTGGGGGTGGA
oPEB734	CTCCACCCCCAAGACCGGTTGTAGCTGTATCAAAGAAAAAGAGGTTGTTTTTGTT
oPEB735	GACCTACAACGGCAAAGCCTTTGCTTGGCCGCAGGTGAAAACAAGGCA
oPEB736	GCCTTGTTTTCACCTGCGGCCAAGCAAAGGCTTTGCCGTTGTAGGTCAC
oPEB737	CAATCCCCATATGGAAACCGttaTTATGGCGCATGTGATTCTGAAAGGAT
oPEB765	TGTCCTGCATCATAATGAAATGGCTGTGTTATCACTCATTTCATTGTACATC
oPEB766	ACAATGAAATGAGTGATAACACAGCCATTTCATTATGATGCAGGACACCT
oPEB767	TCTTTTAAAAGGTGTCCTGCATGCTAATGAAATGGATGTGTTATCACTCATTTC
oPEB768	GTGATAACACATCCATTTCATTAGCATGCAGGACACCTTTTAAAAGATCC
oPEB1012	CATagctgtttcctgtgtgaaattg
oPEB1013	GGTGAAGGTCAAGGACAAGGCCAAGCCTGCAGGTCGACTCTAGAGGA
oPEB1014	TTGGCCTTGTCCTTGACCTTCACCGGGATCCTCTAGAGTCGACCCTG
oPEB1015	TAActaagaattcggccgtcgttt
oPEB1016	GGTGAAGGTCAAGGACAAGGCCAACCGAGCTCGAATTCAGCCGCCA
oPEB1017	TTGGCCTTGTCCTTGACCTTCACCCTCTAGAGTCGACCTGCAGTGG
oPEB1018	TAActaagtaatatggtgcactctcagt
oPEB1019	caggctttacactttatgcttcc
oPEB1020	GTAACCAGCCTGATGCGATT
oPEB1021	ATTATGCCGCATCTGTCCAACT
oPEB1022	gcaaggcgattaagttgggtaa
oPEB1023	GATTTTCCACAACAAGTCGATG
oPEB1024	TTCTCGCCGGATGTACTGGAAAC
oPEB1025	tggcttaactatgcggcatcaga

oPEB1026	GGTGAAGGTCAAGGACAAGGCCAAATGAAAAAGAAATCACTGACTGAACT
oPEB1027	actgagagtgcaccatattacttagTTATTACGACATTTGATCCAACAGCTG
oPEB1028	acaatttcacacaggaaacagctATGAAAAAGAAATCACTGACTGAACT
oPEB1029	CTCGGTTGGCCTTGTCCTTGACCTTCACCCGACATTTGATCCAACAGCTGCA
oPEB1030	CCCGGTGAAGGTCAAGGACAAGGCCAAATGTCATTAAAAGGGAAACTCCAAC
oPEB1031	aaaacgacggccgaattcttagTTATTAAGAGGAATATTTCCTCTTTAGCCGGGCAATTC
oPEB1032	gataacaatttcacacaggaaacagctATGTCATTAAAAGGGAAACTCCAAC
oPEB1033	AGGCTTGGCCTTGTCCTTGACCTTCACCAGAGGAATATTTCCTCTTTAGCCGGGCAATTC
oPEB1053	cggataacaatttcacacaggaaacagctATGAAAGGAGAGAGCATCGTTACCGTAA
oPEB1054	GAGCTCGGTTGGCCTTGTCCTTGACCTTCACCAGGGACAATATGCTGCAGCACATTC
oPEB1055	CGAGCTCGGTTGGCCTTGTCCTTGACCTTCACCTTTTTGCACATATTGAAAAGCGGAA
oPEB1056	ttgtaaaacgacggccgaattcttagTTATGGCGCATGTGATTCTGAAAGGAT
oPEB1057	GGATCCCGGTGAAGGTCAAGGACAAGGCCAAATGAAAGAACACAGTGAAGCCTATG

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