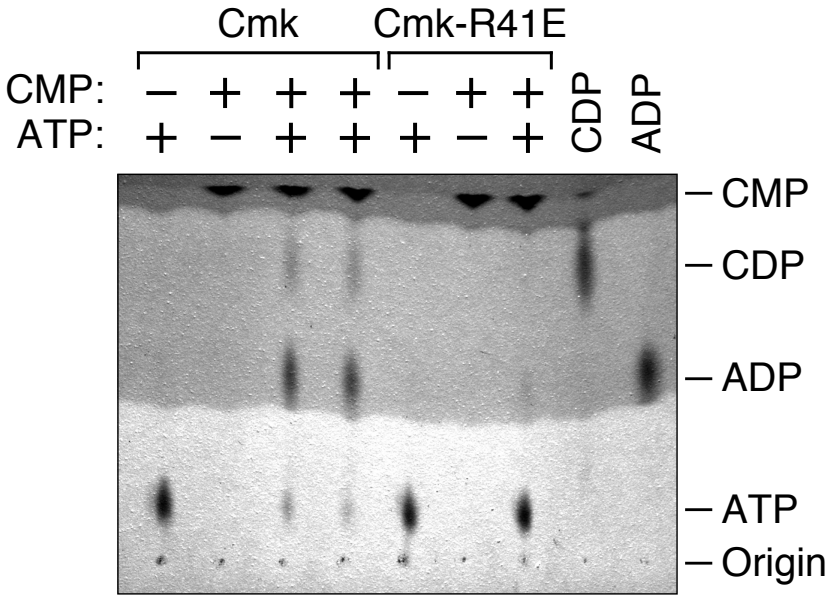


**Figure S1. Inactivation of cytidylate kinase by an R41E amino acid substitution.** The catalytic activity of wild-type and mutated cytidylate kinase (Cmk) was compared *in vitro* by using thin layer chromatography on PEI-cellulose to examine their ability to convert CMP + ATP into CDP + ADP. Nucleotides were detected by UV shadowing on a fluorescent TLC plate. CDP and ADP not treated with either form of the enzyme were included as markers. Lanes 3 and 4 are identical except that the phosphorylation of CMP by wild-type cytidylate kinase was allowed to proceed three times longer in lane 4.

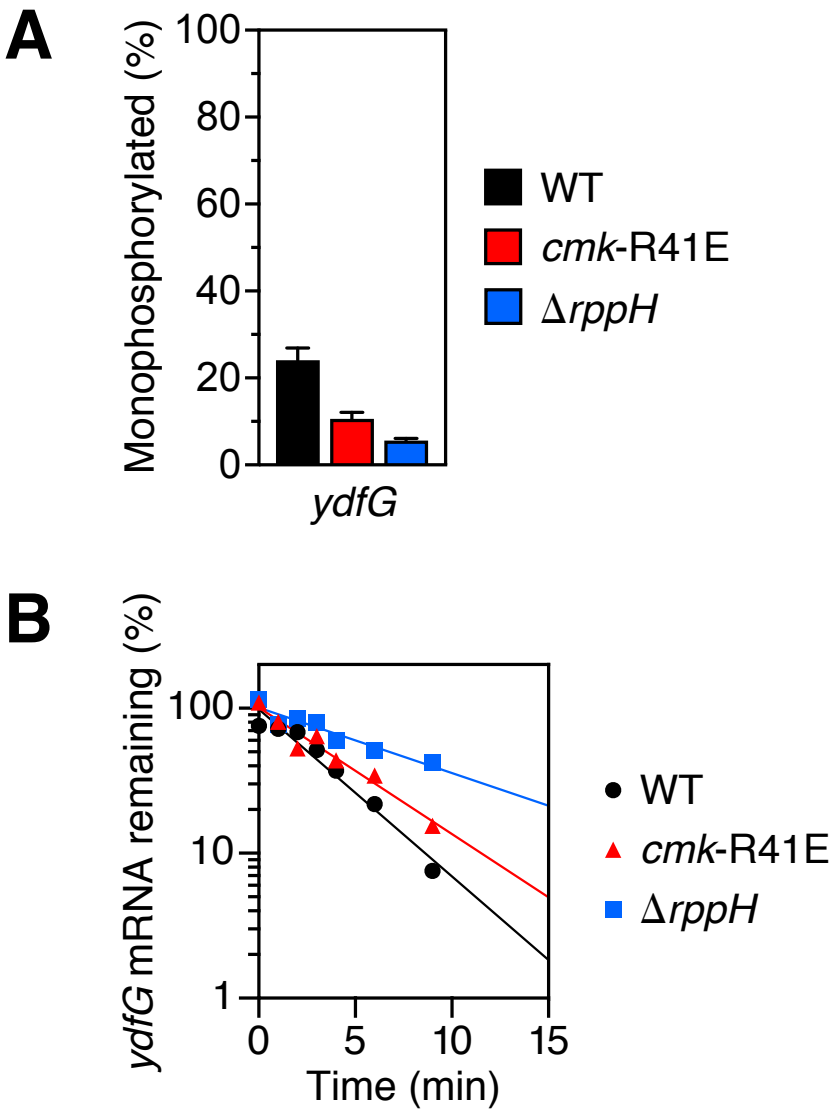


**Figure S2. Influence of cytidylate kinase on the phosphorylation state and decay rate of *ydfG* mRNA in *E. coli*.**

A. Effect of cytidylate kinase on the percentage of *ydfG* 5' ends that were monophosphorylated, as determined by PABLO. Each value is the average of three biological replicates. Error bars correspond to standard deviations.

B. Effect of cytidylate kinase on the half-life of *ydfG* mRNA. The decay of *ydfG* mRNA was monitored as a function of time after inhibiting transcription with rifampicin, plotted semilogarithmically, and analyzed by linear regression. Representative experiments are shown.

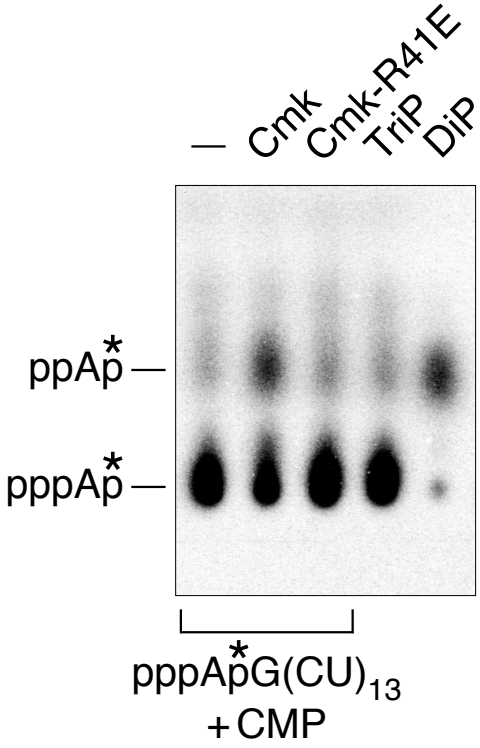


**Figure S3. Ability of cytidylate kinase to convert the 5' triphosphate of an A-initiated RNA to a diphosphate *in vitro*.**

Triphosphorylated $AG(CU)_{13}$ bearing a single radiolabeled phosphate (*) between the first two nucleotides was synthesized by *in vitro* transcription, and the ability of cytidylate kinase (Cmk) to transfer the 5'-terminal $\gamma$-phosphate to CMP was analyzed *in vitro* by subsequent alkaline hydrolysis and thin layer chromatography on PEI-cellulose to examine the RNA reaction products. Radiolabeled nucleotides were detected by autoradiography. The alkaline hydrolysis products of triphosphorylated $AG(CU)_{13}$ (TriP) and diphosphorylated $AG(CU)_{13}$ (DiP) not treated with the enzyme were included as markers. p, phosphate. –, no enzyme added.

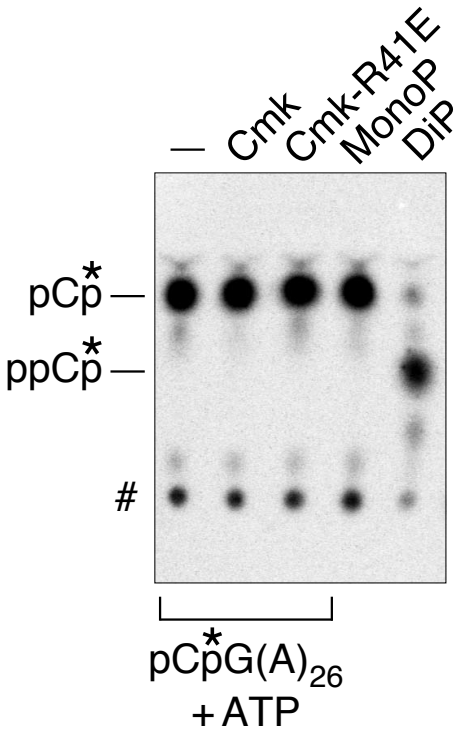


**Figure S4. Inability of cytidylate kinase to phosphorylate the 5' end of a monophosphorylated C-initiated RNA *in vitro*.**

Monophosphorylated $CG(A)_{26}$ bearing a single radiolabeled phosphate (*) between the first two nucleotides was synthesized by *in vitro* transcription, and the ability of cytidylate kinase (Cmk) to phosphorylate its 5' end in the presence of ATP was analyzed *in vitro* by subsequent alkaline hydrolysis and thin layer chromatography on PEI-cellulose to examine the RNA reaction products. Radiolabeled nucleotides were detected by autoradiography. The alkaline hydrolysis products of monophosphorylated $CG(A)_{26}$ (MonoP) and diphosphorylated $CG(A)_{26}$ (DiP) not treated with the enzyme were included as markers. p, phosphate. –, no enzyme added. #, radiolabeled pppGp released from a triphosphorylated $G(A)_{26}$ contaminant by alkaline hydrolysis.

**Table S1A. Effect of Δ*cmk* on the 5′ phosphorylation state of A-initiated transcripts.**

| | Monophosphorylated (%) | | | |
|---|---|---|---|---|
| | **Wild-type** | **Δ*rppH*** | **Δ*cmk*** | **Δ*dapF*** |
| ***yeiP*** | 37.3 ± 8.6 | 4.5 ± 1.0 | 11.4 ± 2.0 | 13.6 ± 2.7 |
| ***rpsT* P1** | 57.1 ± 2.9 | | 37.3 ± 6.5 | |
| ***trxB*** | 40.6 ± 9.4 | | 27.3 ± 9.0 | |
| ***yajQ*** | 67.5 ± 3.4 | | 51.7 ± 3.9 | |
| ***ydfG*** | 20.9 ± 2.1 | | 12.5 ± 2.2 | |
| ***yfcZ*** | 55.0 ± 8.0 | | 36.0 ± 3.5 | |

**Table S1B. Similar effect of *cmk*-R41E, *cmk*Δ95-142, and Δ*cmk* on the 5′ phosphorylation state of *yeiP* mRNA.**

| Monophosphorylated (%) | | | | | |
|---|---|---|---|---|---|
| **Wild-type** | **Δ*cmk*** | ***cmk*-R41E** | ***cmk*-R41E + WT *cmk*** | ***cmk*Δ95-142** | ***cmk*Δ95-142 + WT *cmk*** |
| 35.6 ± 3.1 | 13.0 ± 3.7 | 15.5 ± 4.2 | 37.3 ± 8.0 | 16.0 ± 4.3 | 37.8 ± 7.4 |

**Table S1C. Epistatic effect of *cmk* and *dapF* mutations on the 5′ phosphorylation state of A-initiated transcripts.**

| Monophosphorylated (%) | | | | |
|---|---|---|---|---|
| | **Wild-type** | ***cmk*-R41E** | **Δ*dapF*** | ***cmk*-R41E Δ*dapF*** |
| ***yeiP*** | 46.1 ± 7.0 | 20.2 ± 2.5 | 22.7 ± 0.9 | 20.7 ± 2.9 |
| ***ydfG*** | 33.0 ± 2.7 | 14.8 ± 1.5 | 16.5 ± 3.6 | 14.2 ± 2.5 |

**Table S1D. Effect of *cmk*-R41E on the 5′ phosphorylation state of C-initiated transcripts.**

| | Monophosphorylated (%) | | |
|---|---|---|---|
| | **Wild-type** | **Δ*rppH*** | ***cmk*-R41E** |
| ***rpsT* P2** | 14.6 ± 2.1 | 3.3 ± 0.5 | 45.5 ± 6.5 |
| ***efp*** | 16.5 ± 1.3 | 4.5 ± 0.4 | 27.9 ± 2.7 |
| ***efp*-C1A** | 33.7 ± 9.3 | 4.4 ± 1.8 | 14.8 ± 1.6 |

**Table S1E. Cmk-independent phosphorylation state of processed R1.3-*yeiP* mRNA.**

| Monophosphorylated (%) | | | |
|---|---|---|---|
| **Wild-type** | **Δ*rppH*** | ***cmk*-R41E** | ***cmk*-R41E Δ*rppH*** |
| 99.6 ± 6.0 | 100.0 ± 5.4 | 100.6 ± 6.9 | 102.8 ± 8.0 |

**Table S1F. Effect of *cmk*-R41E on the 5′ phosphorylation state of *efp* and *efp*-C1A mRNA in cells lacking RppH.**

| | Monophosphorylated (%) | | | |
|---|---|---|---|---|
| | **Wild-type** | **Δ*rppH*** | ***cmk*-R41E** | ***cmk*-R41E Δ*rppH*** |
| ***efp*** | 19.8 ± 4.2 | 4.8 ± 1.4 | 32.1 ± 4.5 | 11.9 ± 0.9 |
| ***efp*-C1A** | 35.3 ± 1.0 | 5.5 ± 0.4 | 18.7 ± 3.2 | 5.4 ± 0.1 |

**Table S2. mRNA half-lives.**

| | Half-life (min) | | | | | |
|---|---|---|---|---|---|---|
| | **Wild-type** | **Δ*rppH*** | **Δ*cmk*** | ***cmk*-R41E** | ***cmk*Δ95-142** | ***cmk*-R41E Δ*rppH*** |
| ***yeiP*** | 1.4 ± 0.1 | 14.8 ± 2.5 | 2.9 ± 0.2 | 3.3 ± 0.2 | 2.9 ± 0.1 | |
| ***ydfG*** | 2.4 ± 0.2 | 12.1 ± 2.6 | | 3.6 ± 0.5 | | |
| ***rpsT* P2** | 1.8 ± 0.2 | 9.0 ± 0.6 | | 2.8 ± 0.2 | | |
| ***efp*** | 1.4 ± 0.1 | 5.1 ± 0.6 | | 2.0 ± 0.1 | | |
| ***efp* monoP** | | | | | | 1.1 ± 0.1 |
| ***efp* triP/diP** | | | | | | 12.2 ± 2.3 |

**Table S3A. Measurements used to calculate the capping efficiencies of *yeiP* standards synthesized by *in vitro* transcription.**

| Measured values | | | | 5′ homogeneity | |
|---|---|---|---|---|---|
| $Y_{TS}$ | $Y_{DS}$ | $Y_{MS}$ | $E_{LS}$ | $D_S$ | $M_S$ |
| | | | | | |
| 0.106 ± 0.041 | 0.625 ± 0.120 | 0.000 ± 0.000 | 0.751 ± 0.057 | 0.930 | 0.978 |
| | | | | | |

$Y_{TS}$, $Y_{DS}$, and $Y_{MS}$ are the fractional PACO ligation yields obtained for the triphosphorylated, diphosphorylated, and monophosphorylated standards, respectively. $E_{LS}$ is the PABLO ligation efficiency of fully monophosphorylated RNA. The Y and E values correspond to the mean and standard deviation of 2-3 independent measurements. $D_S$ and $M_S$ are the calculated fractions of those standards that are diphosphorylated and monophosphorylated, respectively.

**Table S3B. Calculated capping efficiencies of *yeiP* standards synthesized by *in vitro* transcription.**

| | Capping efficiencies | | |
|---|---|---|---|
| | $E_T$ | $E_D$ | $E_M$ |
| | | | |
| Monte Carlo | $0.140^{+0.056}_{-0.057}$ | $0.870^{+0.192}_{-0.189}$ | $-0.003^{+0.001}_{-0.001}$ |
| Direct calculation | 0.142 | 0.890 | – 0.003 |
| | | | |

$E_T$, $E_D$, and $E_M$ are the fractional capping efficiencies calculated for triphosphorylated, diphosphorylated, and monophosphorylated RNA, respectively, with 0 being no capping and 1 being complete capping. Each efficiency determined by Monte Carlo simulation corresponds to the peak of an asymmetric distribution, and the error range (±) corresponds to a confidence level of 68.3%, equivalent to one standard deviation. For comparison, the capping efficiencies were also calculated directly; these values agree well with the peak values obtained by Monte Carlo simulation, but only the simulation allowed error ranges to be determined. A minor discrepancy versus the theoretical minimum (0.000) resulted from the small mathematical correction factors that were used.

**Table S3C. Measurements used to calculate the phosphorylation state of *yeiP* mRNA in *E. coli*.**

| | PACO | | PABLO | |
|---|---|---|---|---|
| | $Y_D$ | $E_{LD}$ | $Y_M$ | $E_{LM}$ |
| | | | | |
| Wild-type | 0.349 ± 0.028 | 0.623 ± 0.022 | 0.240 ± 0.025 | 0.673 ± 0.013 |
| | | | | |
| Δ*rppH* | 0.653 ± 0.009 | 0.706 ± 0.009 | 0.029 ± 0.009 | 0.652 ± 0.050 |
| | | | | |
| *cmk*-R41E | 0.551 ± 0.042 | 0.617 ± 0.010 | 0.105 ± 0.030 | 0.673 ± 0.013 |
| | | | | |

The phosphorylation state of *yeiP* mRNA was compared in wild-type, Δ*rppH*, and *cmk*-R41E *E. coli* cells by PACO and PABLO. $Y_D$ and $Y_M$ are the fractional PACO and PABLO ligation yields. $E_{LD}$ and $E_{LM}$ are the PABLO ligation efficiencies of fully monophosphorylated RNA. The values listed correspond to the mean and standard deviation of measurements on three biological replicates.

**Table S3D. Phosphorylation state of *yeiP* mRNA in *E. coli*.**

| | | DiP | MonoP |
|---|---|---|---|
| | | | |
| Wild-type | Monte Carlo | $0.582^{+0.187}_{-0.149}$ | $0.311^{+0.041}_{-0.039}$ |
| | Direct calculation | 0.661 | 0.312 |
| | | | |
| Δ*rppH* | Monte Carlo | $0.969^{+0.301}_{-0.219}$ | $-0.024^{+0.021}_{-0.026}$ |
| | Direct calculation | 1.110 | – 0.032 |
| | | | |
| *cmk*-R41E | Monte Carlo | $0.943^{+0.321}_{-0.219}$ | $0.085^{+0.049}_{-0.049}$ |
| | Direct calculation | 1.090 | 0.083 |
| | | | |

The phosphorylation state of *yeiP* mRNA was compared in wild-type, Δ*rppH*, and *cmk*-R41E *E. coli* cells. Each fractional value determined by Monte Carlo simulation corresponds to the peak of an asymmetric distribution, and the error range (±) corresponds to a confidence level of 68.3%, equivalent to one standard deviation. For comparison, the fractional amounts of diphosphorylated and monophosphorylated *yeiP* mRNA were also calculated directly; these values agree well with the peak values obtained by Monte Carlo simulation, but only the simulation allowed error ranges to be determined. Minor discrepancies versus the theoretical maximum (1.000) or minimum (0.000) resulted from the small mathematical correction factors that were used and are either within the margin of error or close to it.

**Table S4. Strains used in this study**

| Strain | Genotype | Source |
|---|---|---|
| BW25113 | *rrnB3* Δ*lacZ4787 hsdR514* Δ*(araBAD)567* Δ(*rhaBAD)568 rph-1* | Datsenko and Wanner, 2000 |
| MH5 | BW25113 Δ*rppH* | Deana et al., 2008 |
| MH410 | BL21(DE3) Δ*rna rne*-131 | Foley et al., 2015 |
| MH314 | BW25113 Δ*dapF* | This study |
| MH379 | BW25113 Δ*cmk* | This study |
| MH453 | BW25113 *cmk*Δ95-142 | This study |
| MH454 | BW25113 *cmk*-R41E | This study |
| MH515 | BW25113 *dapF*-FLAG | This study |
| MH507 | BW25113 Δ*rppH dapF*-FLAG | This study |
| MH508 | BW25113 *cmkR41E dapF*-FLAG | This study |
| MH161 | BW25113 *rppH*-FH | Luciano et al., 2012 |
| MH511 | BW25113 Δ*dapF rppH*-FH | This study |
| MH512 | BW25113 *cmkR41E rppH*-FH | This study |
| MH514 | BW25113 Δ*dapF cmk*-R41E | This study |
| MH476 | BW25113 *rnc-105* | This study |
| MH487 | BW25113 Δ*rppH cmk*-R41E | This study |
| MH471 | BW25113 Δ*efp* | This study |
| MH489 | BW25113 Δ*efp* Δ*rppH* | This study |
| MH488 | BW25113 Δ*efp cmk*-R41E | This study |
| MH506 | BW25113 Δ*efp* Δ*rppH cmk*-R41E | This study |

**Table S5. Plasmids used in this study**

| Plasmid | Description | Source |
|---|---|---|
| pYeiP-GFP | pCM128 derivative expressing the 5′ UTR and first 20 amino acids of *yeiP* fused in-frame to superfolder GFP | This study |
| pPM30 cmk | *cmk* cloned between the *EcoR*I and *BamH*I sites of pPM30 | This study |
| pYeiP1 | *yeiP* cloned between the *EcoR*I and *Pst*I sites of pBR322fd | Richards et al., 2012 |
| pYdfG1 | *ydfG* cloned between the *EcoR*I and *Pst*I sites of pBR322fd | Luciano et al., 2012 |
| pTrxB1 | *trxB* cloned between the *EcoR*I and *Pst*I sites of pBR322fd | Richards et al., 2012 |
| pYajQ1 | *yajQ* cloned between the *EcoR*I and *Pst*I sites of pBR322fd | This study |
| pYfcZ1 | *yfcZ* cloned between the *EcoR*I and *Pst*I sites of pBR322fd | Luciano et al., 2012 |
| pEfp1 | *efp* cloned between the *Cla*I and *Pst*I sites of pBR322fd | This study |
| pEfp1-C1A | pEfp1 with A substituted for the original C at the +1 transcription start site | This study |
| pR1.3-yeiP | T7 Φ gene R1.3 stem-loop fused to the *yeiP* 5′ UTR of pYeiP1 | This study |
| pRE112 rppH-FH | Allelic exchange vector for adding a C-terminal FLAG-$His_6$ tag to chromosomally encoded RppH | Luciano et al., 2012 |
| pRE112 dapFFLAG | Allelic exchange vector for adding a C-terminal FLAG tag to chromosomally encoded DapF | This study |
| pRE112 cmkΔ95-142 | Allelic exchange vector for making the chromosomal mutant *cmkΔ95-142* | This study |
| pRE112 cmkR41E | Allelic exchange vector for making the chromosomal mutant *cmk-R41E* | This study |
| pPlacHis6-cmk | pACYC177 derivative expressing N-terminally $His_6$-tagged Cmk under $P_{lac}$ control | This study |
| pPlacHis6-cmkR41E | pACYC177 derivative expressing N-terminally $His_6$-tagged Cmk-R41E under $P_{lac}$ control | This study |

**Table S6 - Oligonucleotides used in this study**

| Name | Sequence | Purpose |
|---|---|---|
| DZyeiP69 | GTA ATT CAG TAG GCT AGC TAC AAC GAC ATA CCT TTT | 10-23 DNAzyme for cutting 69 nucleotides from the 5′ end of *yeiP* and *yeiP*-U2G, and 137 nucleotides from the 5′ end of R1.3-*yeiP* |
| DZtrxB95 | GCC GCG TAG AGG CTA GCT ACA ACG AAG CAG CGG TG | 10-23 DNAzyme for cutting 95 nucleotides from the 5′ end of *trxB* |
| DZyfcZ106 | GGA GTT GTC CAG GCT AGC TAC AAC GAA ATG GTG CCA | 10-23 DNAzyme for cutting 106 nucleotides from the 5′ end of *yfcZ* |
| DZydfG87 | CCC TTG TTG AAG GCT AGC TAC AAC GAA AAA CGA CGA | 10-23 DNAzyme for cutting 87 nucleotides from the 5′ end of *ydfG* |
| DZyajQ96 | ACT CCA CTT CGG GCT AGC TAC AAC GAG GCT CGC GTT | 10-23 DNAzyme for cutting 96 nucleotides from the 5′ end of *yajQ* |
| DZefp87 | TCT AAC ATG AGG CTA GCT ACA ACG ATT TAA GAC CA | 10-23 DNAzyme for cutting 87 nucleotides from the 5′ end of *efp* and *efp*-C1A |
| DZrpsT152 | TCA GAC TGA AGG CTA GCT ACA ACG AGG CGC GCT TC | 10-23 DNAzyme for cutting 152 nucleotides from the 5′ end of *rpsT* P1 and 63 nt from the 5′ end of *rpsT* P2 |
| X22 | GAA CAA TAT GAA TGA TAA CTT G | X oligo for PACO/PABLO analysis |
| X91 | CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC ACC CCC CCC CCC CCC CCC CCC CCC CCC CCC CAC CCC CCC GAA CAA TAT GAA TGA TAA CTT G | X oligo for PACO/PABLO analysis of R1.3-*yeiP* |
| Y-yeiP | AGT CGA AAA TGT CAA AAA TAT CAA GTT ATC ATT CAT ATT GTT C | Y oligo for PABLO analysis of *yeiP* |
| Y-yeiP-U2G | AGT CGA AAA TGT CAA AAA TCT CAA GTT ATC ATT CAT ATT GTT C | Y oligo for PABLO analysis of *yeiP*-U2G |
| Y-yeiP-R1.3L | TGT AGA TCT ATT GGT TAA ATG CAA GTT ATC ATT CAT ATT GTT C | Y oligo for PABLO analysis of R1.3-*yeiP* |
| Y-trxB | AGT TAT CAT TCA TAT TGT TC | Y oligo for PABLO analysis of *trxB* |
| yfcZ Y1 | GGT GCC AGA TAA ACG TTC AAG TTA TCA TTC ATA TTG TTC | Y oligo for PABLO analysis of *yfcZ* |
| ydfG Y1 | CCT CAA CGC TTT TGT GTC AAG TTA TCA TTC ATA TTG TTC | Y oligo for PABLO analysis of *ydfG* |
| yajQ Y1 | CTC TCC CTT CAT TTT TGA TCA AGT TAT CAT TCA TAT TGT TC | Y oligo for PABLO analysis of *yajQ* |

| | | |
|---|---|---|
| Y-efp | TGG TAG CTA AGC CAC AAA ATG CAA GTT ATC ATT CAT ATT GTT C | Y oligo for PABLO analysis of *efp* |
| Y-efp-C1A | TGG TAG CTA AGC CAC AAA ATT CAA GTT ATC ATT CAT ATT GTT C | Y oligo for PABLO analysis of *efp*-C1A |
| Y-P1 | ACT CGT TAC GTA GTG ATC AAG TTA TCA TTC ATA TTG TTC | Y oligo for PABLO analysis of *rpsT* P1 |
| Y-P2 | TCT ATA TGG ACA ATT CAA AGC AAG TTA TCA TTC ATA TTG TTC | Y oligo for PABLO analysis of *rpsT* P2 |
| yeiP probe | TTC GTT CGC TCT TGG CAT CG | Northern blot probe for *yeiP*, *yeiP*-U2G, and R1.3-*yeiP* |
| ydfG probe | CAC CAA AAC CTG CCG TTG CTC CAG T | Northern blot probe for *ydfG* |
| trxB probe | GTG TTT GGT CGT GCC CAT GAG | Northern blot probe for *trxB* |
| yfcZ probe | CAC CAA AAC CTG CCG TTG CTC CAG T | Northern blot probe for *yfcZ* |
| yajQ probe | CAG AGA CAA TAT CGA AAG ATG GC | Northern blot probe for *yajQ* |
| efp probe | ACG TTG CCA TAA GGC CCT CT | Northern blot probe for *efp* and *efp*-C1A |
| rpsT probe | GTC CAA CTC CCA AAT GTG TTC | Northern blot probe for *rpsT* P1 and *rpsT* P2 |
| RACE-1 | CGA CTG GAG CAC GAG GAC ACT GAC ATG GAC TGA AGG AGT AGrA rArA | RNA oligonucleotide for 5′ RACE |
| RACE+ | CGA CTG GAG CAC GAG GAC ACT GA | Forward primer for 5′ RACE |
| RACEnest | GGA CAC TGA CAT GGA CTG AAG GAG TA | Forward nested primer for 5′ RACE |
| efp rev | GAC CAT TCC ACA GAG TTA CG | *efp*-specific primer for 5′ RACE |
| rpsTP2 rev | ATC TGT GCA GTC AGG TTA GC | *rpsT* P2-specific primer for 5′ RACE |
| CGAA sense | AAA AAA GAT CTC GCG GAA TTC AAA TTA ATA CGA CTC ACT ATT CGA | Sense template for $CG(A)_{26}$ synthesis by in vitro transcription |
| CGAA antisense | TTT TTT TTT TTT TTT TTT TTT TTT TTC GAA TAG TGA GTC GTA TTA ATT TGA ATT CCG CGA GAT CTT TTT T | Antisense template for $CG(A)_{26}$ synthesis by in vitro transcription |
| AGCU sense | AAA AAA GAT CTC GCG GAA TTC AAA TTA ATA CGA CTC ACT ATT AGC | Sense template for $AG(CU)_{13}$ synthesis by in vitro transcription |

| | | |
|---|---|---|
| AGCU antisense | AGA GAG AGA GAG AGA GAG AGA GAG AGC TAA TAG TGA GTC GTA TTA ATT TGA ATT CCG CGA GAT CTT TTT T | Antisense template for $AG(CU)_{13}$ synthesis by in vitro transcription |