An RNA Repair Operon Regulated by Damaged tRNAs

Highlights

- An RNA repair operon is activated by mutations that cause tRNA halves to accumulate

- Operon expression and accumulation of tRNA halves occur upon DNA damage

- The 5’ tRNA halves that accumulate end in 2’, 3’-cyclic phosphate

- The RtcR transcriptional activator oligomerizes upon binding these 5’ tRNA halves

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In Brief

Hughes et al. demonstrate that a bacterial RNA repair operon, containing the RtcB RNA ligase and the RtcA RNA cyclase, is regulated by binding of 5’ tRNA halves ending in 2’, 3’-cyclic phosphate to the RtcR transcriptional activator. These studies show how tRNA fragments can regulate bacterial gene expression.
An RNA Repair Operon Regulated by Damaged tRNAs

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SUMMARY

Many bacteria contain an RNA repair operon, encoding the RtcB RNA ligase and the RtcA RNA cyclase, that is regulated by the RtcR transcriptional activator. Although RtcR contains a divergent version of the CARF (CRISPR-associated Rossman fold) oligonucleotide-binding regulatory domain, both the specific signal that regulates operon expression and the substrates of the encoded enzymes are unknown. We report that tRNA fragments activate operon expression. Using a genetic screen in Salmonella enterica serovar Typhimurium, we find that the operon is expressed in the presence of mutations that cause tRNA fragments to accumulate. RtcA, which converts RNA phosphate ends to 2′, 3′-cyclic phosphate, is also required. Operon expression and tRNA fragment accumulation also occur upon DNA damage. The CARF domain binds 5′ tRNA fragments ending in cyclic phosphate, and RtcR oligomerizes upon binding these ligands, a prerequisite for operon activation. Our studies reveal a signaling pathway involving broken tRNAs and implicate the operon in tRNA repair.

INTRODUCTION

Most organisms contain repair systems that ligate RNA fragments generated by nuclease cleavage or removal of encoded intervening sequences. A major repair pathway involves RtcB, which joins pre-tRNA halves after intron excision in metazoans and archaea. In metazoans, RtcB also ligates mRNA encoding the XBP1 transcription factor as part of the unfolded protein response (Englert et al., 2011; Popow et al., 2011; Jurkin et al., 2014; Kosmaczewski et al., 2014). RtcB joins RNA 5′-OH ends to 2′, 3′-cyclic phosphate or 3′-phosphate RNA ends (Englert et al., 2011; Tanaka et al., 2011). RtcB is also present in bacteria, where its functions are less understood. The only reported substrate is 16S rRNA, since E. coli RtcB can re-ligate a 3′ fragment of 16S rRNA to the rRNA body after cleavage by a stress-induced endonuclease (Temmel et al., 2017). In vitro, E. coli RtcB can add a ppG cap to DNAs ending in 3′-phosphate (Das et al., 2013); however, the role of this activity in vivo is unclear.

In bacteria, RtcB is often expressed as part of a highly regulated “RNA repair” operon. In this operon, rtcB is adjacent to rtcA, which encodes an RNA cyclase that converts 3′-phosphate ends to 2′, 3′-cyclic phosphate (Genschik et al., 1998; Tanaka and Shuman, 2011). In some bacteria, including Salmonella enterica serovar Typhimurium (S. Typhimurium), the rtcBA operon also encodes orthologs of a human RNA-binding protein known as the Ro60 autoantigen and noncoding RNAs called Y RNAs (Chen et al., 2013; Das and Shuman, 2013; Burroughs and Aravind, 2016). The functions of bacterial Ro60 proteins (called Rsr for Ro sixty-related) and Y RNAs have only been studied in Deinococcus radiodurans, where Rsr is tethered by Y RNA to the 3′ to 5′ exoribonuclease polynucleotide phosphorylase (PNPase), specializing the nuclease for structured RNA decay (Chen et al., 2013). Transcription of the operon is often regulated by the enhancer binding protein RtcR, which is encoded adjacent to rtcBA and transcribed in the opposite direction (Genschik et al., 1998).

An impediment to studying the roles of the rtcBA operon has been a lack of information as to how the operon is regulated. RtcR is a member of the ρ4-dependent enhancer binding protein family, which typically features an N-terminal signal-sensing ligand-binding domain fused to a C-terminal AAA+ ATPase domain that multimerizes and interacts with ρ4 (Bush and Dixon, 2012). The N-terminal portion of RtcR contains a divergent form of the CARF (CRISPR-associated Rossman fold) domain (Makarova et al., 2014). Canonical CARF domains have been best characterized in type III CRISPR-associated Cas systems, where they are linked to effector domains such as ribonucleases. In these systems, binding of cyclic oligoadenylate (cOA) molecules to the CARF domain activates the adjacent effector (Kazlauskienė et al., 2017; Niewoehner et al., 2017). However, ligands that bind the RtcR domain have not been identified.

To determine how a rtcBA operon is activated, we performed a genetic screen to identify mutations that result in transcription of...
the S. Typhimurium rsr-yrlBA-rtcBA operon. We report that mutations that result in accumulation of tRNA fragments activate operon expression. Some mutations result in DNA damage, and we show that a feature of the DNA damage response in S. Typhimurium is the activation of one or more ribonucleases that cleave tRNAs in the anticodon loop, resulting in 5'0 fragments ending in 2',3'-cyclic phosphate. Consistent with the hypothesis that RNAs ending in 2',3'-cyclic phosphate are important for operon activation, overexpression of RtcA increases operon expression. We show that the RtcR CARF domain binds 5'0 tRNA fragments ending in 2',3'-cyclic phosphate and that RtcR forms oligomers upon ligand binding. Our studies identify a signaling pathway involving tRNA and implicate the operon in the repair of damaged tRNAs.

RESULTS

Identification of Mutations that Result in Operon Activation

To identify genes that, when mutated, result in activation of the rsr-yrlBA-rtcBA operon, we generated S. Typhimurium strains in which a lacZ reporter was inserted upstream of rsr under control of the rsr promoter (Figure 1A). We introduced the reporter into two different virulent strains, SL1344 and 14028s, because our early experiments revealed strain-specific differences in the extent to which the operon was repressed. Specifically, while β-galactosidase was fully repressed in the SL1344 Prsr-lacZ strain, we detected low levels of β-galactosidase in the 14028s Prsr-lacZ strain (Figures 1B and 1C). Expression of Prsr-lacZ was under control of the RtcR transcriptional activator, since β-galactosidase activity increased >2,500-fold in both strains when we expressed a constitutively active RtcR lacking part of the N-terminal ligand-binding domain (RtcRN; Genschik et al., 1998) (Figure 1C). Upon RtcRN expression, both Prsr-lacZ strains also appeared strongly blue when grown on X-gal-containing agar (Figure 1B).

We used a chloramphenicol-resistant derivative of pSAM, a mariner himar1C9 transposon delivery vector (Goodman et al., 2009), to create a library of transposon insertions in the Prsr-lacZ strains. Mutants that formed blue colonies on X-gal agar and exhibited at least a 50% increase in β-galactosidase activity compared to the parent strain were selected (Figure 1D). With these criteria, we obtained 45 mutants from 28,000 chloramphenicol-resistant 14028s Prsr-lacZ colonies and 26 mutants from 35,000 chloramphenicol-resistant SL1344 Prsr-lacZ colonies. Using ligation-mediated PCR, we mapped the transposon insertions to 28 distinct loci (Table S1). Six loci were identified in both strains, 17 were identified only in the 14028s strain and five only in the SL1344 strain. To confirm that the transposon insertion was responsible for the blue color, we used P22 phage transduction to transfer each transposon mutation into the parent Prsr-lacZ strain. Loci with at least two independent transposon insertions are shown in Figure S1, together with the insertion sites (red circles, 14028s; white circles, SL1344).
Mutations in Genes Involved in tRNA Metabolism and DNA Repair Activate the Operon

The genes we identified fell into three major functional categories. One group mapped within the rsr-yrIBA-rtcBA operon (Figure S1A). As expected, we recovered transposon insertions that, similar to the RtcRΔN mutation, truncate the RtcR N-terminal CARF domain to render the operon constitutively active (Genschik et al., 1998). As these mutations were only recovered in the 14028s strain, our screen may not have reached saturation. We also recovered transposon insertions from both strains within rtcB. One explanation is that the operon is normally expressed at low levels in both strains and that decreased RtcB ligase activity results, directly or indirectly, in increased levels of ligands that activate the operon.

A second category consisted of genes with roles in tRNA metabolism (Figure S1B). Insertions in truA, which encodes the pseudouridine synthase that modifies uridines at positions 38, 39, and 40 in the anticodon arm of some tRNAs (Cortese et al., 1974), activate the operon, as do insertions in pnp, which encodes the PNPase exoribonuclease. In E. coli, PNPase degrades aberrant pre-tRNAs (Li et al., 2002). We also obtained mutations in sraG, which encodes a small RNA that regulates pnp mRNA levels (Fontaine et al., 2016).

Surprisingly, the largest category consisted of genes involved in DNA replication, repair, and segregation (Figure S1C). These genes included polA, which encodes DNA polymerase I; mnhA, which encodes RNase H1, which cleaves the RNA strand of RNA-DNA hybrids that form during DNA replication and transcription (Hollis and Shaban, 2011); recC, which encodes a subunit of the RecBCD helicase-nuclease complex that functions in double-strand break repair (Dillingham and Kowalczykowski, 2009); ruvA and ruvC, whose products resolve Holliday junctions (Wyatt and West, 2014); anduvD, which encodes a helicase involved in nucleotide excision repair (Kisker et al., 2013). We also identified mutations in yebC, a transcriptional regulator that contributes to E. coli survival after ionizing radiation (Byrne et al., 2014). Other mutations disrupted tfsK, which encodes a DNA translocase with roles in chromosome segregation (Kaimer and Graumann, 2011); and parA and parB, which encode proteins required for plasmid segregation (Gerdes et al., 2010). We also obtained transposon insertions in several genes that function in other processes (Figure S1D).

To compare the extent to which the various mutations increased P

rsr-lacZ expression, we performed β-galactosidase assays. We focused on the three major categories (Figures S1A–S1C) and assayed cells between the mid-logarithmic and early stationary phases of growth. For all genes that were sites of transposon insertion in both strains (rtcB, pnp, sraG, ruvA, and yebC), β-galactosidase levels were >10-fold higher in the 14028s strain (Figures 1E and 1F). Due to the stronger induction, subsequent experiments were performed in the 14028s strain.

As the sraG RNA downregulates pnp mRNA (Fontaine et al., 2016), it was surprising that mutations in both pnp and sraG increased operon activation. Since sraG overlaps the pnp promoter, and our mutations are within or near this promoter, we examined PNPase levels by immunoblotting. PNPase was undetectable in sraG mutants (Figure 1G), indicating the transposon insertions abrogate PNPase synthesis.

To confirm that loss of function of the affected genes was responsible for operon activation, we generated strains lacking open reading frames (ORFs) that were sites of transposon insertion. We monitored operon activation using western blotting to detect expression of Rsr fused at the N terminus to three copies of the FLAG epitope. When strains containing deletions in truA, pnp, ruvA, or mnhA were grown to mid-logarithmic phase, FLAG3-Rsr increased compared to wild-type cells (Figure 2A). Although the low levels of FLAG3-Rsr in ΔrtcB strains were not significantly different from wild-type strains, strains lacking pnp and either truA or rtcB contained higher levels of FLAG3-Rsr than either deletion alone, revealing that deletions in PNPase, TruA, and RtcB act additively.

Since expression of the E. coli rtcBA operon increases in stationary phase (Temmel et al., 2017), we examined expression of the S. Typhimurium operon as a function of growth. Although P

rsr-lacZ expression did not change in the parental strain, expression increased more than 5-fold in the truA transposon mutant (truA::Mm) between mid-log and stationary phase (Figure 2B). Western blotting revealed that although the levels of FLAG3-Rsr in wild-type cells increased less than 1.5-fold, FLAG3-Rsr increased 2.8-fold in ΔuvA strains, 2-fold in Δpnp strains, and 5-fold in ΔtruA strains in stationary phase relative to mid-log phase (Figure 2C). Thus, mutations that disrupt certain genes involved in tRNA metabolism and DNA repair activate expression of the rsr-yrIBA-rtcBA operon and the effects are more severe in stationary phase.

Distinct tRNA Fragments Accumulate in Mutant Strains

Since one category of mutations that resulted in operon activation were predicted to affect tRNA metabolism, we examined tRNA levels in the mutant strains. We examined truA, since pseudouridines in tRNA anticodon arms contribute to structural stability (Arnez and Steitz, 1994); rtcB, since RtcB ligates eukaryotic and archaeal tRNAs after intron excision; and pnp, which degrades aberrant pre-tRNAs (Li et al., 2002). We grew strains lacking each gene to mid-log or stationary phase, isolated RNA, and performed northern blotting to detect two tRNAs that are TruA substrates, tRNAHis(GUG) and tRNALeu(UAG). Several 5′ fragments (Figures 2D and 2E) and 3′ fragments (Figures 2A and 2B) of these tRNAs were detected in wild-type and mutant strains during logarithmic growth and increased in cells in stationary phase.

Notably, some fragments in ΔtruA strains were altered in mobility and/or levels compared to wild-type strains. For both tRNAHis(GUG) and tRNALeu(UAG), some 5′ and 3′ fragments were reduced in ΔtruA strains, while others became prominent (Figures 2D, 2E, S2A, and S2B, lanes 2 and 10, red lines). Possibly, the decreased stability of these tRNAs in ΔtruA strains renders them susceptible to cleavage at distinct sites. Fragments of tRNAs that are not TruA substrates, such as tRNATrp(CCA), were unaffected in ΔtruA strains (Figure S2C).

To determine if accumulation of specific tRNA fragments was a common feature of our mutants, we examined strains carrying deletions in genes important for DNA replication and repair. Remarkably, we detected discrete tRNA fragments in some strains. This was most apparent in stationary phase, where we detected 5′ fragments of tRNATrp(CCA) and tRNAMet in strains that lacked ruvA or were deleted for the 3′ end of the essential
polA gene (polAΔC) (Figures 2F and 2G). These fragments, as well as 5’ and 3’ fragments of other tRNAs, such as tRNA_Cys(GCA), increased further in ΔruvA cells that also lacked pnp (Figures 2H and S2E–S2G), indicating that PNPase may degrade the fragments. Although we did not detect specific fragments in ΔyebC or ΔrecC strains, low levels of these fragments may be obscured by the background of nonspecific fragments that we show later to be irrelevant for operon activation.

Accumulation of tRNA Fragments and Operon Induction Occur through at Least Two Pathways, One of Which Requires RecA

Many of the identified genes, such as ruvA, polA, ruvC, yebC, uvrD and ftsK, were also isolated in a screen for E. coli mutations that result in expression of the SOS regulon, a gene network induced upon DNA damage (O’Reilly and Kreuzer, 2004). To determine if the SOS response was required for tRNA fragment accumulation or activation of the rsr-yrlBA-rtcBA operon, we examined strains lacking RecA, since expression of the SOS regulon initiates when activated RecA assists cleavage of the LexA repressor. Notably, the tRNA_{5’CCA} fragments that accumulated in ΔruvA cells were strongly reduced in ΔruvA ΔrecA cells (Figure 3A). Western blotting revealed that although the operon was activated in ΔruvA stationary phase cells, it was expressed similarly to wild-type cells in ΔruvA ΔrecA cells (Figure 3B).

In contrast, accumulation of tRNA fragments in ΔtruA cells was unaffected by recA deletion (Figures 3C and 3D), supporting the hypothesis that tRNA breakage occurs in these cells due to lack of pseudouridine in some anticodon stems. Activation of the rsr-yrlBA-rtcBA operon in ΔtruA strains was also unaffected by recA deletion (Figure 3E). Our results support a model in which tRNA fragments can be generated through at least two pathways, one involving tRNA fragility and a second requiring the RecA component of the SOS response. In this model, accumulation of tRNA fragments may result, directly or indirectly, in production of a ligand that binds the RtcR CARF domain to activate the operon. Consistent with this hypothesis, operon activation in both ΔtruA and ΔruvA cells requires RtcR (Figures 3F and 3G).

DNA Damaging Agents Cause tRNA Cleavage and Operon Activation

To obtain further evidence that tRNA fragment accumulation was linked to operon activation, we asked if treatment with DNA damaging agents results in tRNA cleavage. Upon treatment with the interstrand crosslinker mitomycin C (MMC), which leads to operon activation (Kurasz et al., 2018), FLAG3-Rsr expression was detected within one hour and peaked by 2 h (Figure 4A). Cleavage of the LexA repressor occurred with similar kinetics.
Operon induction required RecA and LexA cleavage, as FLAG3-Rsr was not detected in ΔrecA strains or strains carrying the uncleavable lexA3 allele (Little and Harper, 1979) (Figures 4A and S3A). (Deletions of lexA are lethal, because S. Typhimurium contains LexA-regulated prophages; Bunny et al., 2002; Lemire et al., 2011.) Similar to ΔruvA and ΔtruA strains, operon expression required RtcR (Figure S3B).

Upon MMC treatment, many tRNAs, including tRNA^{Trp(CCA)}, tRNA^{Tyr(GUA)}, and tRNA^{Cys(GCA)}, underwent cleavage, as both 5′ (Figures 4B–4H) and 3′ (Figures S3D and S3E) fragments accumulated. Fragments were detected within 1 h of adding MMC but were absent or reduced in ΔrecA and lexA3 strains (Figures 4B–4D and S3C–S3E). Levels of some full-length tRNAs, such as tRNA^{Trp(CCA)}, decreased concomitantly (Figures 4E and S3D), indicating the fragments derive from cleavage of mature tRNAs rather than reduced decay of preexisting fragments. Although the operon-encoded YrlA RNA contains a tRNA-like domain (Chen et al., 2014), fragments of this RNA were not detected (Figure S3F), indicating it is not a substrate for the RecA-dependent nuclease. Operon activation and tRNA fragment accumulation also occurred upon treatment with other DNA damaging agents, such as bleomycin, which cleaves single- and double-stranded DNA (Bolzán and Bianchi, 2018), and methyl methanesulfonate (MMS), which methylates DNA (Wyatt and Pittman, 2006) (Figures 4I, S3G, and S3H). Together, our data indicate that a tRNA anticodon nuclease is activated as part of the S. Typhimurium SOS response.

Inspection of the tRNA fragments that accumulated upon MMC treatment revealed some differences between the mutant strains. Most 5′ and 3′ fragments increased in cells lacking PNPase (Figures 4E–4G and S3E, lane 10). For some tRNAs that are truA substrates, the fragments that accumulated were altered in mobility in ΔtruA strains compared to wild-type cells and increased further in ΔtruA Δpnp strains (Figure 4H, lanes 9 and 14, red lines), implicating PNPase in their degradation. Fragments of tRNA^{Trp(CCA)} and tRNA^{Tyr(GUA)} were slightly increased in ΔrtcB Δpnp strains compared to Δpnp strains (Figures 4E, 4F, and S3D, lanes 10 and 15), supporting a possible role for RtcB in repairing broken tRNAs.

To further assess the link between tRNA fragments and operon activation, we examined the E. coli rtcBA operon, which is regulated by RtcR. Overexpression of two toxins that cleave tRNAs, but not MMC incubation, activates this operon (Engl et al., 2016; Kurasz et al., 2018). If tRNA fragments are important for activation, then failure of the E. coli operon to be expressed in the presence of MMC may be due to the absence of these fragments. Consistent with our model, tRNA fragments did not accumulate in E. coli during growth in MMC (Figures S3I and S3J). Western blotting to detect RtcB confirmed that the E. coli operon was not induced (Figure S3K).

Together, our results reveal that RecA-dependent tRNA cleavage and rsr-yrIBA-rtcBA operon activation occur during conditions that induce the SOS response in S. Typhimurium. Since tRNA fragments do not accumulate when the operon is activated by overexpressing constitutively active RtcRΔN (Figures 4E–4H, S3D, and S3E, lane 2), operon activation in itself does not cause fragment accumulation. Instead, this result, together with our data that the operon is activated in ΔtruA strains in the absence of DNA damage, supports a model in which tRNA cleavage results in a ligand that activates the operon.
We determined whether expression of the rsr-yrlBA-rtcBA operon is important for survival after MMC exposure. Wild-type strains expressing constitutively active RtcR (RtcRD\textsuperscript{N}) were more resistant to 1 μg/mL MMC than the same cells carrying an empty vector, while strains lacking RtcR (D\textsubscript{rtcR}) were less resistant than wild-type cells (Figure 4J). Quantitation revealed that wild-type strains overexpressing RtcR were 4.8-fold more resistant than cells carrying an empty vector, while D\textsubscript{rtcR} cells were 9.4-fold less resistant than wild-type cells (Figure 4K). The decreased resistance of D\textsubscript{rtcR} strains could be complemented by expressing either RtcRD\textsuperscript{N} or wild-type RtcR on a plasmid. Thus, operon activation confers a growth advantage in MMC.

### Most tRNA Cleavage Occurs in the Anticodon Loop, Leaving a Cyclic Phosphate End

To understand how tRNA cleavage could result in a ligand, we characterized the fragments that accumulate upon DNA damage. Since endonucleases can leave 2', 3'-cyclic phosphate, 3'-phosphate, or 3'-OH at the 5' end of the 5' fragment, we tested if pre-treatment with T4 polynucleotide kinase (PNK), which converts 2', 3'-cyclic phosphate and 3'-phosphate to 3'-OH, was needed for T4 RNA ligase (which requires 3'-OH) to ligate a 5'-phosphate-containing oligonucleotide to the 5' fragments (Figure S4A). Comparison of RNA from wild-type and D\textsubscript{pnp} strains, followed by northern blotting to detect specific tRNAs, revealed that ligation to the 5' fragments of all examined tRNAs increased in the presence of PNK (Figures S4B–S4E). To distinguish between 3'-phosphate and cyclic phosphate, we asked if treatment with calf intestinal phosphatase (CIP) (which removes 3'-phosphate, but not 2', 3'-cyclic phosphate) could substitute for PNK or if treatment with acid (which opens up cyclic phosphate) was also required. Maximal ligation of the oligonucleotide to the 5' tRNA fragments occurred in the presence of acid and CIP, indicating some fragments end in cyclic phosphate (Figures S4G–S4J). Thus, one or more metal-independent endoribonucleases (which leave 2', 3'-cyclic phosphate) (Yang, 2011) contribute to cleavage. As some ligation occurred with CIP alone, 5' fragments ending in 3'-phosphate were also present (Figures S4G–S4J). These species could derive from fragments ending in 2', 3'-cyclic phosphate or from cleavage by additional nucleases.

Using 3' rapid amplification of cDNA ends, we determined that cleavage occurred within the anticodon loops. For tRNA\textsuperscript{Trp(CCA)}, tRNA\textsuperscript{Trp(GUA)}, tRNA\textsuperscript{Cys(GCA)}, and tRNA\textsuperscript{Phe(GAA)}, most 5' fragments terminated at position 36, the last nucleotide of the anticodon,
with the apparent cleavage site between two adenines (Figure S4K, arrowheads; Table S2). For tRNA^{fMet}, most cleavage occurred after position 37, between two adenines (Figure S4K; Table S2). For tRNA^{Leu(UAG)}, cleavage was heterogeneous, with fragments ending at multiple sites (Figure S4K, arrows; Table S2). Slightly shorter 5′ fragments of most tRNAs were also recovered, which may represent exonucleolytic nibbling and/or other cleavages (arrows).

We tested if the abundant metal-independent endonuclease RNase I was important for cleavage. In strains lacking RNase I (ΔrnuA), we observed striking decreases in all nonspecific fragments (Figure 5A, lanes 10–17). However, the specific tRNA^{Trp(CCA)} and tRNA^{Leu(UAG)} fragments that accumulate in ΔrnuA strains were unchanged in ΔrnuA ΔrnuA strains (Figures 5A and 5B, lanes 6 and 14). Although we could not detect specific tRNA^{Glu(GCA)} or tRNA^{Trp(GLU)} fragments in Δrpu and ΔrnuA strains due to the background of nonspecific fragments, specific fragments were evident in Δrpu ΔrnuA and ΔrnuA ΔrnuA strains (Figures 5C and S5A, lanes 13 and 14). The RecA-dependent tRNA fragments detected in MMC were also unaffected (Figures 5D–5F and S5B, lanes 14–17). As expected if these fragments are important for formation of the ligand that binds RtcR, operon induction was unaffected in ΔrnuA strains (Figure 5G).

Despite much effort, we were unable to identify the endonuclease responsible for the RecA-dependent tRNA cleavage. We examined strains lacking 22 other toxins and potential nucleases, including three metal-independent nucleases encoded adjacent to consensus LexA sites. These are YafQ, the toxin component of an antitoxin:toxin cassette encoded downstream of the rsr-yrlBA-rtcBA operon (Figure S5C); HigB, which is encoded with its HigA antitoxin downstream of a LexA-regulated ORF; and HigB2, which is encoded on the opposite strand such that HigB and HigB2 may be controlled through the same palindromic LexA site (Figure S5D). All three toxins are part of the RelE family, which bind ribosomes and cleave translating mRNAs (Harms et al., 2018). Northern and western blotting of ΔrnuA strains carrying deletions of all three toxins revealed that they were not required for accumulation of tRNA^{Trp(CCA)} fragments or operon activation (Figures S5E and S5F). We tested strains deleted for 19 other toxins and potential nucleases, with similar negative results (Figures S5G–S5J). Since RNA decay pathways are often redundant (Houseley and Tollervey, 2009), multiple endonucleases may carry out tRNA cleavage.

**RtcA Is Important for Induction of the rsr-yrlBA-rtcBA Operon**

To test if 2′, 3′-cyclic phosphate RNA ends were important for operon activation, we examined the role of RtcA, which converts...
A

B

C

D

E

F

G

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3'-phosphate ends to 2', 3'-cyclic phosphate (Genschik et al., 1998). Operon activation decreased 4.8-fold in ΔtruA ΔrtcA cells compared to ΔtruA cells and was similar to wild-type cells (Figure 5H). Thus, the lower stability of some tRNA anticodon stems in ΔtruA strains may make them susceptible to nuclease that leave 3'-phosphate ends that are subsequently converted by RtcA to 2', 3'-cyclic phosphate. In contrast, operon activation was not significantly different between ΔruvA and ΔruvA ΔrtcA strains (Figure 5I), supporting the idea that tRNA halves that accumulate in ΔruvA strains derive from cleavage by a metal-independent endonuclease.

We also found that the low level of expression of the rsr-yrBA-rtcBA operon in wild-type 14028s strains in stationary phase was reduced in ΔrtcA strains (Figures 5H and 5I). This suggested that the RtcA levels in 14028s, but not SL1344 strains, might be sufficient to convert broken tRNA ends to 2', 3'-cyclic phosphates as part of a feedback loop resulting in low levels of operon expression. To test if increased RtcA levels were sufficient for operon activation, we expressed RtcA under control of the arabinose-inducible promoter in the SL1344 and 14028s strains. We detected RtcA in both strains in the absence of arabinose due to the leakiness of this promoter (Figure 5J). Importantly, operon expression increased, as Rsr levels were 4.5-fold higher in 14028s strains and 11-fold higher in SL1344 strains (Figure 5J). These fragments increased when PNPase was also deleted (Figure 6A), consistent with the enhanced operon activation in ΔruvA Δropn strains (Figure 2A). The fragments ended in cyclic phosphate, as ligation of an oligonucleotide to most tRNALeu(UAG) 5' fragments required treatment with acid and CIP (Figure S6A). Thus, tRNALeu(UAG) 5' fragments were good candidates for a ligand that activates the operon.

We tested whether 5' fragments of tRNALeu(UAG) or other tRNAs could bind the CARF domain. We expressed both the CARF domain and RtcR in E. coli (Figures S6B and S6C) and performed electrophoretic mobility shift assays (EMSAs) with the purified proteins. Initial studies in which we incubated 5' tRNA halves derived from tRNALeu(UAG), tRNAleu(GCU), tRNAleu(GAA), and tRNAleu(UAA) ending in 2', 3'-cyclic phosphate or 3'-OH revealed that both the isolated CARF domain and RtcR bound most efficiently to 5' halves of tRNALeu(UAG) (Figure 6B). We also tested tRNALeu(UAG) 5' halves ending at other nucleotides in the anticodon loop. Although all tested 5' halves ending in cyclic phosphate bound both the CARF domain and RtcR, fragments that ended after the G of the UAG were most efficiently bound (Figures 6B and 6C). Quantitative assays revealed that the amount of CARF domain required to shift 50% of these tRNALeu(UAG) halves ending in cyclic phosphate was ~200 nM, while more than 5 µM was required to shift 50% of the same RNAs ending in 3'-OH (Figure 6D). Specific binding to the CARF domain was not detected when the fragments ended in 3'-phosphate.

We also examined binding to full-length RtcR, in which the CARF domain is followed by AAA+ (ATPases associated with multiple cellular activities) and helix-turn-helix DNA-binding domains. Based on other bacterial enhancer proteins, RtcR is expected to be a dimer in its unliganded form. In the absence of ligand, the CARF domain negatively regulates transcription, most likely by preventing the AAA+ domain from oligomerizing (Genschik et al., 1998; Bush and Dixon, 2012). The amount of RtcR required to shift 50% of the tRNALeu(UAG) fragments ending in cyclic phosphate was slightly less than that of the isolated CARF domain (~125 nM; Figure 6E, arrowheads). Beginning at 125 nM RtcR, we also detected a larger complex. This complex, which could represent oligomerization, eventually became the predominant species (Figure 6E, asterisk). Formation of both

Figure 6. RtcR Oligomerizes on Binding tRNA Fragments Ending in Cyclic Phosphate

(A) RNA from the indicated strains was subjected to northern blotting to detect tRNA^{Leu(UAG)} 5' halves.

(B) T32P-labeled 5' tRNA halves (1 nM) ending after the anticodon in 2', 3'-cyclic phosphate (lanes 1–3, 7–9, and 13–15) or 3'-OH (lanes 4–6, 10–12, and 16–18) were incubated with no protein, 0.5 µM CARF domain, or 0.5 µM RtcR. Reactions contained 1 mM ATP. RNA-protein complexes (RNPs) were separated from naked RNA in native gels.

(C) Similar to (B), except that 5' tRNA^{Leu(UAG)} halves ended at the indicated positions.

(D and E) 5' tRNA^{Leu(UAG)} halves ending after the G of the anticodon were incubated with the indicated concentrations of CARF domain (D) or RtcR (E). RNAs ended in 2', 3'-cyclic phosphate (lanes 1–9), 3'-OH (lanes 10–18), or 3'-phosphate (lanes 19–27). In (E), arrowheads denote the first complexes formed, while asterisks denote complexes that could represent oligomers. Circles, complexes that form on all three RNAs at the highest RtcR concentrations. In (D) and (E), the samples were fractionated in two gels. Binding reactions in (E) contained 1 mM ATP.

(F) tRNA^{Leu(UAG)} halves ending in cyclic phosphate were mixed with the indicated concentrations of RtcR with or without 1 mM ATP. Complexes are designated with arrowheads, asterisks, and circles as in (E).

(G) Size exclusion chromatography was performed on RtcR (48 µM) alone or bound to 48 µM tRNA^{Leu(UAG)} halves ending in 3'-phosphate or 2', 3'-cyclic phosphate. Left, overlay of chromatograms. Right, proteins and RNA in the indicated peaks were fractionated in SDS-PAGE and denaturing polyacrylamide gels, respectively. The complex eluting at 20 µL is ATP from the binding reaction.
complexes, but not the complex formed with the isolated CARF domain, was enhanced when ATP was present, suggesting conformational changes mediated by the AAA+ domain contribute to their formation (Figures 6F and S6D). As both RtcR-containing complexes were reduced in levels when the fragments ended in 3'-OH and were barely detectable when the fragments ended in 3'-phosphate, tRNA 5' halves ending in cyclic phosphate are the preferred ligand for their formation. At the highest protein concentrations, a third complex that migrated slightly faster than the initial complex was detected (Figure 6E, circles). As formation of this complex was not dependent on the presence of either ATP or a cyclic phosphate RNA end, this complex may represent nonspecific interactions of the RNA fragment with RtcR.

To further characterize these complexes, we performed size exclusion chromatography and analyzed the composition of the peaks using gel electrophoresis. In these experiments, we mixed RtcR with equimolar amounts of 5' tRNALeu(UAG) halves to maximize complex formation. When the tRNA halves ended in cyclic phosphate, two RtcR/tRNA complexes formed that migrated at ~400 and 160 kDa, consistent with hexameric (358 kDa) and dimeric (119 kDa) forms of RtcR bound to three and one tRNA halves, respectively (12 kDa each). A third peak consisted of unbound tRNA halves (Figure 6G). Notably, when the tRNA halves terminated in 3'-phosphate, the 400-kDa complex was not detected, the 160-kDa complex was reduced, and the peak corresponding to unbound RNA increased (Figure 6G). We conclude that RtcR forms oligomers upon binding tRNA fragments ending in cyclic phosphate.

**DISCUSSION**

Although rtcBA and rsr-yrlBA-rtcBA RNA repair operons are widespread in bacteria, the signals that activate the operons have been obscure. We showed that tRNA 5' fragments ending in 2', 3'-cyclic phosphate bind the RtcR CARF domain. These tRNA fragments accumulate in the presence of mutations in genes that maintain tRNA homeostasis and upon activation of a LexA-regulated endoribonuclease. Since operon expression is important for S. Typhimurium survival after MMC exposure, our experiments uncover a signaling pathway involving tRNA and implicate rsr-yrlBA-rtcBA operon components in the repair of nucleic acid damage.

**A Signaling Pathway Involving tRNA**

Our data support a model in which 5' tRNA fragments ending in 2', 3'-cyclic phosphate bind the RtcR CARF domain, triggering oligomerization of the AAA+ domain. These fragments accumulate in ΔtruA strains, which contain hypomodified tRNAs with unstable anticodon arms (Figure 7A), in strains carrying mutations that result in DNA damage and in wild-type cells treated with DNA damaging agents (Figure 7B). These tRNA fragments also accumulate in strains lacking PNPase (Figure 7C). Binding of the fragments to RtcR triggers oligomerization, resulting in operon transcription (Figure 7D). Our proposal that 5' tRNA fragments ending in 2', 3'-cyclic phosphate are critical ligands is consistent with data that the E. coli operon is activated by ectopic expression of colicin D and S. Typhimurium LT2 VapC (Engl et al., 2016), toxins that cleave the anticodon loops of tRNAArg and tRNAMet, respectively (Masaki and Ogawa, 2002; Winther and Gerdes, 2011).

Although canonical CARF domains, such as those of Csm6 and Csx1, dimerize to form a symmetric binding pocket for cOA (Jia et al., 2019; Molina et al., 2019; García-Doval et al., 2020), structural analyses will be required to determine how the divergent RtcR CARF domain recognizes asymmetric tRNA fragments ending in cyclic phosphate. As the affinity of the RtcR CARF domain for the in-vitro-synthesized tRNA halves used in our assays is lower than the 0.5–5 nM affinity of canonical CARF domains for cOA ligands (Kazlauskienė et al., 2017; Niewoehner et al., 2017), other proteins and/or tRNA modifications, which are often present in the vicinity of the anticodon, may contribute to recognition. It is also possible that another tRNA 5' half may be a better ligand than the tRNALeu(UAG) fragment used in our studies or that tRNA 3' fragments contribute.

If binding of 5' tRNA fragments ending in 2', 3'-cyclic phosphate activates the operon, how is signaling regulated? For CARF domains activated by cOA, signaling is terminated by ring nuclease activity that is encoded within the CARF domain (Athukoralage et al., 2019; Jia et al., 2019) or supplied by stand-alone nuclease activities (Athukoralage et al., 2018). One possibility is that RtcA and RtcB function as part of a feedback loop to regulate cleaved tRNAs and terminate operon expression. PNPase may contribute to reducing operon expression by degrading tRNA fragments (Figure 7C). However, since RNAs ending in 3'-phosphate or 2', 3'-cyclic phosphate are not PNPase substrates (Singer, 1958), other enzymes must first convert these ends to 3'-OH.

It is curious that tRNA cleavage occurs upon DNA damage in S. Typhimurium. Since S. Typhimurium contains LexA-regulated prophages (Lemire et al., 2011), prophage-encoded endonucleases may carry out cleavage. In this case, the decreased levels of specific tRNAs may be peripheral to the DNA damage response, instead allowing the phage to reduce host protein synthesis or favor translation of specific proteins. It is also possible that tRNA cleavage, by decreasing protein synthesis and slowing division, allows more time for DNA repair. Given the growing evidence that RNA can template and otherwise enhance DNA repair (Michelini et al., 2018), another possibility is that the tRNA halves play a role in restoring genome integrity.

**Functions of the rsr-yrlBA-rtcBA Operon**

Our findings that (1) the operon is activated by tRNA cleavage, (2) RtcA is required for operon activation in ΔtruA cells, and (3) some tRNA fragments are present at slightly higher levels in ΔrtcB Δpnp cells than Δpnp cells support a role for RtcA and RtcB in tRNA repair (Figure 7E). Such a role would be consistent with the finding that RtcB seals anticodon loops after excision of intervening sequences in eukaryotes and archaea (Englert et al., 2011; Popow et al., 2011; Kosmaczewski et al., 2014). It would also be consistent with our observation that mutations in RtcB trigger operon expression, since tRNA halves might be expected to accumulate in ΔrtcB cells. Although we did not detect accumulation of tRNA halves in ΔrtcB cells, we did not examine the full complement of tRNAs. Our identification of
conditions that activate the operon should allow us to determine the diversity of RtcA and RtcB substrates.

Although our studies did not address the roles of Rsr and Y RNAs, several possibilities can be envisioned. Since *D. radiodurans* Rsr and Y RNA assist PNPase in degrading structured RNA (Chen et al., 2013), they could potentially function in tRNA decay. An alternative, but not exclusive, possibility is that the tRNA-like domain of YrIA RNA, which is not a substrate for the anticodon nuclease (Figure S3F), acts as a competitive inhibitor of this nuclease.

Finally, we note that RtcB, Rsr, and Y RNAs could potentially function in DNA repair. *E. coli* RtcB can ligate DNA 3'-PO₄ ends to DNA 5'-OH ends and can add a ppG cap to DNAs ending in 3'-phosphate, a modification that allows priming by DNA polymerase (Das et al., 2013, 2014). *D. radiodurans* Rsr contributes to survival after UV irradiation and Rsr and Y RNAs are among

Figure 7. Model for Operon Activation

(A) In ΔtruA strains, hypomodified tRNAs are susceptible to nuclease, leading to tRNA fragment accumulation. RtcA converts the ends of the 5' fragments to 2', 3'-cyclic phosphate (cP).

(B) In ΔruvA strains and after treatment with DNA damaging agents, a RecA-regulated endonuclease cleaves tRNAs, resulting in 5' halves ending in cyclic phosphate.

(C–E) We propose that 5' tRNA halves ending in cyclic phosphate can be degraded by PNPase (C); bind the CARF domain of a RtcR dimer, resulting in oligomerization and operon activation (D); and may also be repaired by RtcB (E).
the most upregulated genes during recovery of *D. radiodurans* from DNA damage (Chen et al., 2000; Tanaka et al., 2004). Since Rsr and Y RNAs are encoded adjacent to RtcB in diverse bacteria, it will be interesting to determine if activation in response to DNA damage is a conserved feature of rsr-yrIBA-rtcBA operons.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Monoclonal ANTI-FLAG M2 antibody produced in mouse | Sigma-Aldrich | Cat#F1804; RRID: AB_262044 |
| HRP anti- E. coli RNA polymerase Sigma 70 | BioLegend | Cat#663205; RRID: AB_2629596 |
| Rabbit anti- E. coli RecA | Narumi et al., 2001 | N/A |
| Rabbit anti- E. coli LexA | gifts of I. Narumi, Toyo University, Itakura, Gunma Japan | N/A |
| Rabbit anti- E. coli RtcB | Temmel et al., 2017 | N/A |
| HA Tag Monoclonal Antibody, HRP | Thermo Fisher Scientific | Cat#26183; RRID: AB_2533056 |
| Rabbit anti- E. coli RplE | gift of J. Zengel, University of Maryland, Baltimore County, MD | N/A |
| Rabbit anti- E. coli LexA | Abcam | Cat#ab174384; RRID: N/A |
| Rabbit IgG HRP Linked Whole Ab | Sigma-Aldrich | Cat#GENA934; RRID: AB_2722659 |
| Goat anti-Mouse IgG Fc Cross-Adsorbed Secondary Antibody, HRP | Thermo Fisher Scientific | Cat#31439; RRID: AB_228292 |

## Bacterial and Virus Strains

**List of Strains Used in This Study**

This paper

See Table S3

### Chemicals, Peptides, and Recombinant Proteins

| Chemical, Peptide, or Recombinant Protein | Source | Identifier |
|------------------------------------------|--------|------------|
| 2,6-Diaminopimelic acid                  | Sigma-Aldrich | Cat#D1377; CAS: 583-93-7 |
| Mitomycin C                              | Sigma-Aldrich | Cat#M4287; CAS: 50-07-7 |
| Methyl methanesulfonate                  | Sigma-Aldrich | Cat#129925; CAS: 66-27-3 |
| Bleomycin                                | Sigma-Aldrich | Cat#B8416; CAS: 9041-93-4 |
| Phenol solution (acid)                    | Sigma-Aldrich | Cat#P4682; CAS: 108-95-2 |
| X-gal                                    | MP Biomedicals | Cat#114063102; CAS: 7240-90-6 |
| DNazol                                   | Thermo Fisher Scientific | Cat#10503027 |
| RNAProtect Bacteria Reagent               | QIAGEN | Cat#76506 |
| Turbo DNase I                            | Thermo Fisher Scientific | Cat#AM2238 |
| Calf intestinal alkaline phosphatase     | Sigma-Aldrich | Cat#11097075001 |
| T4 polynucleotide kinase                 | New England Biolabs | Cat#M0201S |
| T4 RNA Ligase 2, truncated KQ             | New England Biolabs | Cat#M0373S |
| Polyethylene glycol 8000                  | Sigma-Aldrich | Cat#1546605; CAS: 25322-68-2 |
| RNaseOUT                                 | Thermo Fisher Scientific | Cat#10777019 |
| Superscript III Reverse Transcriptase    | Thermo Fisher Scientific | Cat#18080093 |
| Phusion DNA polymerase                   | New England Biolabs | Cat#M0530S |
| Factor Xa Protease                       | New England Biolabs | Cat#P8010S |
| ATP, [γ-32P]                             | PerkinElmer | Cat#BLU002Z250UC |
| Chloramphenicol                          | Sigma-Aldrich | Cat#C0378; CAS 56-75-7 |
| Carbendicillin                           | Sigma-Aldrich | Cat#C1389; CAS 4800-94-6 |
| Tetracycline                             | Sigma-Aldrich | Cat#T3383; CAS 64-75-5 |
| 2-Mercaptoethanol                        | Sigma-Aldrich | Cat#M6250; CAS 60-24-2 |
| 2-Nitrophenyl-β-D-galactopyranoside      | Sigma-Aldrich | Cat#N1127; CAS 369-07-3 |
| Pierce ECL Western Blotting Substrate    | Thermo Fisher Scientific | Cat#32106 |
| SYBR Gold Nucleic Acid Gel Stain         | Thermo Fisher Scientific | Cat#S11494 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Critical Commercial Assays |        |            |
| RNA Clean and Concentrator-5 | Zymo Research | Cat#R1013 |
| Zero Blunt PCR cloning kit | Thermo Fisher Scientific | Cat#K270020 |
| TruSeq RNA Library Prep Kit v2 | Illumina | Cat#RS-122-2001 |
| Bacteria Ribo-Zero Magnetic Kit | Illumina | Cat#MRZB12424 |
| Deposited Data |        |            |
| Raw and Analyzed Data | This paper | GEO: GSE153782 |
| Oligonucleotides |        |            |
| List of Oligonucleotides Used in This Study. | This paper | See Table S3 |
| Recombinant DNA |        |            |
| List of Plasmids used in this study. | This paper | See Table S3 |
| Software and Algorithms |        |            |
| ImageJ | NIH | https://imagej.net/Welcome |
| GraphPad Prism 8.0 | GraphPad | https://www.graphpad.com/scientific-software/prism/ |
| Pfam | El-Gebali et al., 2019 | N/A |
| CDD | Yang et al., 2020 | N/A |
| HMMSCAN (HMMER3 package) | Potter et al., 2018 | https://www.ebi.ac.uk/Tools/pfa/hmmer3_hmmscan/ |
| PSI-BLAST | Altschul and Koonin, 1998 | https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE=Proteins&PROGRAM=blastp&RUN_PSIBLAST=on |
| JACKHMMER | Johnson et al., 2010 | https://www.ebi.ac.uk/Tools/hmmer/search/jackhmmer |
| HHPRed | Zimmermann et al., 2018 | https://toolkit.tuebingen.mpg.de/tools/hhpred |
| Kalign program | Lassmann, 2019 | N/A |
| Pymol | Schrödinger | https://pymol.org/2/ |
| Illumina basecalling RTA 1.18.66.3 | Illumina | N/A |
| Trimmomatic version 0.30 software | Boiger et al., 2014 | http://www.usadellab.org/cms/?page=trimmomatic |
| Bowtie2 version 2.3.3. | Langmead and Salzberg, 2012 | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml |
| Cuffdiff | Trapnell et al., 2013 | http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/ |
| Other |        |            |
| Amersham Hybond-N | Cytiva | Cat#RPN303N |
| Ni-NTA Agarose | Thermo Fisher Scientific | Cat#R90101 |
| Superdex 200 Increase 10/300 Column | Cytiva | Cat#28990944 |
| Amicon Ultra-15 Centrifugal Filter Units | Millipore | Cat# UFC901024 |
| Whatman Optitran Nitrocellulose Blotting Membrane | Cytiva | Cat# 10439196 |

**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sandra Wolin (sandra.wolin@nih.gov).

**Materials Availability**
Bacterial strains and plasmids are available on request.
Stocker, 1981) (gift of Jorge Galan, Yale School of Medicine) and 14028s (Jarvik et al., 2010) (gift of Eduardo Groisman, Yale School of Medicine). Mutant strains were created using allelic exchange (Kaniga et al., 1994) using the donor strain All bacterial strains used in this study are listed in Table S3. Two virulent

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The accession number for the RNA sequencing data reported in this paper is Gene Expression Omnibus: GSE153782.

Data and Code Availability

The accession number for the RNA sequencing data reported in this paper is Gene Expression Omnibus: GSE153782.

METHOD DETAILS

Transposon mutagenesis

Plasmid pSRS_CM1 (Shames et al., 2017), also called pSB4807 (Fowler and Galan, 2018), a chloramphenicol-resistant derivative of pSAM_Bt (Goodman et al., 2009), was a gift of C. Fowler and J. Galan. This plasmid was transferred into donor strain E. coli β-2163Δnic35 (Demarre et al., 2005) and propagated at 37°C in LB containing DAP and carbenicillin. Conjugative-based transposon mutagenesis of recipient S. Typhimurium strains was performed by mixing donor and recipient strains in a 3:1 ratio. Bacterial conjugations were performed at room temperature for 24 h and transconjugants selected by plating on LB agar plates containing 15 mg/ml chloramphenicol and 40 mg/ml X-Gal (MP Biomedicals) to identify blue colonies. Approximately 63,000 colonies were screened (28,000 14028s and 35,000 SL1344) and blue colonies purified by streaking to single colonies on X-gal/chloramphenicol plates. Genomic DNA was isolated with DNAzol (Thermo Fisher), partially digested with Sau3AI and ligated to a DNA adaptor sequences. Transposon insertion sites of transposon insertion from genomic DNA.

β-Galactosidase assays

All strains were grown at 37°C in LB with the appropriate antibiotic. Expression of rtcR and rtcRΔN was induced from pBAD24 with 0% and 0.1% arabinose respectively. (Product is produced in 0% arabinose because the promoter is leaky). β-galactosidase activity was measured as described (Miller, 1972) from cells grown to OD600 between 0.320 and 1.160 (with most strains kept below 1.0) unless otherwise stated. Briefly, after cultures were incubated on ice for 20 minutes to stop growth, cells were pelleted and resuspended in Z buffer (60 mM Na2HPO4.7H2O, 40 mM NaH2PO4.H2O, 10 mM KCl, 1 mM MgSO4, 50 mM 2-mercaptoethanol, 1.0) unless otherwise stated. Briefly, after cultures were incubated on ice for 20 minutes to stop growth, cells were pelleted and resuspended in Z buffer (60 mM Na2HPO4.7H2O, 40 mM NaH2PO4.H2O, 10 mM KCl, 1 mM MgSO4, 50 mM 2-mercaptoethanol, adjusted to pH 7.0). Cells were diluted in Z buffer to 1 mL and permeabilized by adding 100 μl chloroform and 50 μl 0.1% sodium dodecyl sulfate and vortexing. After incubating at 28°C for 5 minutes, 200 μl 2-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich) was added. After yellow color developed, the reaction was stopped by adding 500 μl 1 M Na2CO3 and 1 mL transferred to a micro-centrifuge tube and the tube sedimented at 16,000 x g for 5 minutes. After measuring the OD420 and OD550 of the supernatant, units of activity were calculated according to the equation Miller units = 1000 x [(OD420 - 1.75 x OD550) / (reaction time (minutes) x volume of culture (ml)) / OD600 of original culture].

Construction of expression plasmids

The pRtcRN plasmid for overexpressing N-terminally truncated RtcR was described (Chen et al., 2013). To construct pRtcR, full-length RtcR was amplified from genomic DNA using 5′-GGAAATTCATGCGAAAAACCGTGCCCTTG-3′ and 5′-GCTCTAGATTATCCTGATACCAAGCTCCACCATGCGTG-3′, digested with EcoRI and Xbal and cloned into pBAD24. To construct pHA-RtcA (in which the HA epitope is fused to RtcA), full-length RtcA was amplified from genomic DNA with 5′-GGAAATTCATGTACCCATACGATGTTC and 5′-GGAATTCATGCGAAAAACCGTGCCCTTG-3′ and 5′-GCTCTAGATTATCCTGATACCAAGCTCCACCATGCGTG-3′, digested with EcoRI and Xbal and cloned into pBAD24.
Immunoblotting

Bacterial pellets were resuspended in 1X SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 12.5 mM EDTA, 0.02% bromophenol blue). Cells were lysed by boiling for 10 min. After centrifuging at 16,000 x g, cleared lysates were fractionated in SDS-polyacrylamide gels and transferred to nitrocellulose. Membranes were blocked overnight in 5% milk in TBS-T (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20) overnight at 4°C, washed twice with TBS-T at room temperature, and incubated with primary antibody diluted in 5% milk in TBS-T at room temperature for 1 hour. Membranes were washed in TBS-T before incubation with HRP-coupled secondary antibody diluted in 5% milk in TBS-T for 1 hour at room temperature followed by washing with TBS-T. HRP was detected by enhanced chemiluminescence (ECL) using the Pierce ECL Western Blotting Substrate (ThermoFisher). Antibodies used for immunoblotting are listed in the Key Resources Table. In Figure 4A, the anti-LexA antibody was a gift of Dr. I. Narumi (Toyo University).

RNA analyses and Northern blotting

Total RNA was extracted from bacterial pellets treated with RNAProtect Bacteria Reagent (QIAGEN) using hot acid phenol. Pellets (3 O.D. units) were resuspended in 400 μL of 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% SDS and 400 μL of acid phenol (pH 5) and incubated at 65°C for 30 minutes with occasional vortexing. The sample was then cooled briefly on ice, sedimented at 25°C for 10 minutes at 16,000 x g and the supernatant removed to a new microcentrifuge tube. After extraction with phenol:chloroform:isoamyl alcohol (50:49:1), RNA was precipitated with 2.5 volumes ethanol in 40 μL 3M NaOAc.

For 3' end analyses, 10 μg of RNA was incubated in 5 μL 10X Turbo DNase I buffer (Ambion) and 2 U Turbo DNase I (Ambion) at 37°C for 30 minutes followed by purification using RNA Clean and Concentrator-5 (Zymo Research Corporation). To remove 3' phosphates, RNA was first incubated with 10 mM HCl (Sigma) on ice for 4 hours, followed by incubation with 5 U of CIP (Roche), or 10 U T4 PNK (New England Biolabs) according to the manufacturers' instructions and purified using RNA Clean and Concentrator-5 kit (Zymo Research Corporation). Afterward, RNA (1.5 μg) was ligated overnight at 16°C to 750 ng of adenylated adapter (Table S3) using T4 RNA Ligase 2, truncated KQ (New England Biolabs) in the presence of 25% PEG 8000 and RNaseOUT (Thermo Fisher). Ligated RNAs were extracted with phenol:chloroform:isoamyl alcohol (50:49:1), precipitated with ethanol, and resuspended in 10 μL water.

While the sample was used for Northern analyses, the other half was subjected to reverse transcription using Superscript III (Thermo Fisher) and a primer complementary to the adenylated adapter (Table S3). After reverse transcription, cDNA was amplified using Phusion DNA polymerase (New England Biolabs), a tRNA-specific forward primer (Table S3), and the RT-adaptor reverse primer. Gel purified DNAs were cloned using Zero Blunt PCR cloning kit (Thermo Fisher) according to manufacturer instructions. At least 9 clones were sequenced for each sample. For Northern analyses, RNA was separated in 5% or 8% polyacrylamide/8.3 M urea gels and transferred to Hybond N (Cytiva) in 0.5X TBE at 150 mA for 16 h. Blots were hybridized with [32P]-labeled oligonucleotides as described (Tarn et al., 1995). Oligonucleotide probes are listed in Table S3. Radioactive signals were detected using a Typhoon FLA 7000 Phosphorimager (GE Healthcare).

RNA sequencing and analysis

Salmonella RNA was isolated from 14028s cells treated with or without MMC for two hours using hot acid phenol. The RNA library was prepared by the National Cancer Institute Sequencing Facility using the TruSeq RNA Library Prep Kit v2 (Illumina) following ribosomal RNA removal using the Ribovo-Zero Magnetic Kit for bacteria (Illumina). Sequencing was performed on a NextSeq 500 (Illumina) with 75 bp paired end reads. After trimming adapters and low quality sequence using Trimmomatic version 0.30 software, reads were aligned to the S. Typhimurium (14028s) genome using Bowtie2 software version 2.3.2. Mapped BAM files were used as input for Cuffdiff to determine differential gene expression between MMC-treated and control untreated samples.

Genome screening for candidate tRNA endoribonucleases

All proteins encoded by the genomes of S. Typhimurium SL1344 and 14028s strains were screened against a library of profiles constructed from Pfam (El-Gebali et al., 2019), CDD (Yang et al., 2020) and a custom database of profiles of RNase domains from diverse biological conflict systems using the RPSBLAST and HMMSearch program (HMMER3 package). Statistically significant hits (e < 10^-5) were checked for recovery of known and predicted endoribonuclease domains and these were set aside as potential candidates; for example, proteins containing domains that were members of the BECR fold (Zhang et al., 2014; Iyer et al., 2017), PIN domain superfamily (Matelska et al., 2017), SNase fold (Ponting, 1997), HEPN domain (Anantharaman et al., 2013), potential RNA endonucleases of the RNase H fold (Majorek et al., 2014) and metallo-beta-lactamase fold (Aravind, 1999), the RNase T2 domain (Watanabe et al., 1995), the RNaseE/G superfamily (Callaghan et al., 2005), and the SymE domain (Kawano et al., 2007) were considered valid hits. Proteins encoded by the two S. Typhimurium genomes were also subject to iterative sequence profile searches using PSI-BLAST (Altschul and Koonin, 1998) and JACKHMMER (Johnson et al., 2010) and profile-profile searches using the HHPred program (Zimmermann et al., 2018) in a further effort to unify them with known domains. Successful unification with any of the known RNase domains led to their inclusion in the candidate list. Genome contexts of all candidates were then isolated and screened for 1) links to known translation or ribosome assembly factors and 2) inclusion in biological conflict systems. Active site conservation patterns of each candidate were examined for potential loss of catalytic activity using multiple alignments [constructed with the Kalign program (Lassmann, 2019) and, if required, examination of homologous structures using PyMol (https://pymol.org/2/)]. Predicted inactive representatives of the above RNase domains were removed from further consideration. We arrived at a list of 40 potential RNases. To
prioritize, whole transcriptome sequencing of rRNA-depleted total RNA from wild-type cells treated with or without MMC was performed. Potential RNases that were expressed in the presence of MMC were prioritized for deletion.

**Survival assays**

Overnight cultures containing 100 μg/ml carbenicillin were diluted to OD₆₀₀ = 0.05 in LB containing 0.1% glucose, which reduces expression of genes controlled by the Pₐ₆₆₆ promoter (Guzman et al., 1995). After 2.5 h at 37°C, bacteria were diluted to OD₆₀₀ = 0.2 in LB containing 0.1% glucose and 1 μg/ml MMC. After 2 additional h at 37°C, bacteria were diluted and spotted or spread on LB agar plates. Colonies from both MMC- and mock-treated bacteria were counted to determine the fraction of surviving cells.

**MazEF purification and MazF activation**

To overexpress MazEF in E. coli, double-stranded DNA encoding the 41 C-terminal coding residues of the MazE antitoxin, a Factor Xa cleavage site and the coding sequence of the MazF toxin as a fusion protein (Park et al., 2012) was synthesized (gBlocks Gene Fragments, Integrated DNA Technologies), digested with Ndel and XhoI and inserted into pET28b (EMD Biosciences). After transforming the recombinant plasmid into E. coli BL21(DE3), cells were grown to OD₆₀₀ = 0.8, induced with 0.5 mM IPTG at 25°C for 4 h and harvested by centrifugation. After resuspending in 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 20 mM imidazole, cells were lysed by passing through a French Press at 8000 psi three times. After sedimenting the lysate at 40,000 rpm in a Beckman Type 50.2 Ti rotor, the supernatant was passed through a Ni-NTA agarose column (Thermo Fisher), washed with 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 30 mM imidazole until the OD₆₀₀ of the flowthrough was below 0.01. His-tagged MazEF was eluted with 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 250 mM imidazole. Further purification was by size exclusion chromatography using a Superdex 200 Increase 10/300 column (GE Healthcare Life Sciences) in 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% glycerol. Fractions containing MazEF were pooled, concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore), aliquoted and frozen in liquid nitrogen. MazF was activated by incubating 1 mg MazEF with 10 μg protease Factor Xa (NEB) in 10 mM Tris-HCl pH 8.0, 1 mM DTT, 2 mM CaCl₂ at 37°C for 3 h (Rouillon et al., 2019).

**Generation of 5′ tRNA halves for binding studies**

RNA oligonucleotides corresponding to 5′ tRNA halves containing 3′-OH or 3′-phosphate ends were purchased from Integrated DNA Technologies. To generate RNAs containing 2′, 3′-cyclic phosphate, activated MazF was incubated with RNA oligonucleotides containing a MazF cleavage site ACACUG at the 3′ end (Rouillon et al., 2019). For EMSAs, RNAs were were labeled at the 5′ end using [γ-³²P]-ATP and T4 PNK. After incubating at 70°C for 20 min to inactivate T4 PNK, activated MazF was added to 10 μg/μl and incubated for 2 h at 37°C to generate RNAs ending in 2′, 3′-cyclic phosphate. To remove the cyclic phosphate and generate a labeled RNA with 3′-OH, the reaction was incubated with T4 PNK (Amitsur et al., 1987). To generate labeled RNA ending with 3′-phosphate, the desired sequence was synthesized with an additional uridylate, labeled at the 5′ end with [γ-³²P]-ATP and T4 PNK, and incubated in 100 μl 1 M DL-lysine-Cl and 0.025 M Na₂O₃ (pH 8.3) at 45°C for 2.5 h (Neu and Heppel, 1964). Afterward, the RNA was extracted with phenol:chloroform:isomyl alcohol (50:49:1) and precipitated with 2.5 volumes ethanol. All labeled RNAs were purified from 15% polyacrylamide, 8.3M urea gels before use.

**Purification of full-length RtcR and the CARF domain**

Sequences encoding full-length S. Typhimurium RtcR and the CARF domain (amino acids 1-188) were amplified from genomic DNA using primers 5′-CGGGATCCGATGCGAAAAACGGTGGCCTTTG-3′ and 5′-CCGCTCGAGTTAGGTTGCAATGCCGGACTTCAG-3′ and primers 5′-CGGGATCCGATGCGAAAAACGGTGCCCCAGTCAAG-3′, and 5′-CCGCTCGAGTTAATTCTGTAAAACGTCCCACGTCAG-3′, respectively, digested with BamHI and XhoI and cloned into the same sites of pRSFDuet-1 (Novagen). The resulting plasmids were transformed into E. coli BL21(DE3). To express recombinant protein, cells were cultured in LB containing 20 mM Tris-HCl pH 7.5 and 50 μg/ml kanamycin to OD₆₀₀ = 0.7, 0.1mM IPTG was added and the culture incubated at 16°C for 20 h. His-tagged proteins were purified as described above, except that the lysis buffer was 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10% glycerol, 20 mM imidazole, the wash buffer was 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10% glycerol, 30 mM imidazole, the elution buffer was 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10% glycerol, 250 mM imidazole and the buffer used in gel filtration was 20 mM Tris-HCl pH 7.5, 250 mM NaCl, and 10% glycerol.

**RNA binding assays**

³²P-labeled RNAs in binding buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.05% Tween 20) were refolded by heating to 95°C for 2 min, frozen on dry ice and thawed on ice. Refolded RNAs were mixed with RtcR or the CARF domain in binding buffer in 5 μl total volume, incubated at 4°C for 30 min and at room temperature for 30 min. For RtcR, the reaction included 1 mM ATP except where stated. Reactions were fractionated in 6% polyacrylamide (80:1 acrylamide:bisacrylamide)/5% glycerol gels for CARF domain EMSAs and 4% polyacrylamide (80:1 acrylamide:bisacrylamide)/2.5% glycerol gels for RtcR EMSAs. Gels were run at 4°C, 5 V/cm for 20 min, then 10 V/cm in 0.5xTBE (50 mM Tris, 45 mM boric acid, 1.25 mM EDTA) until the bromophenol blue dye migrated 4 cm. The gels were dried and scanned using a Typhoon FLA 7000 Phosphorimagier (GE Healthcare Life Sciences). Fractions of bound RNA were quantitated using ImageJ (NIH). Results were analyzed with GraphPad Prism 8 and fitted by nonlinear regression using the equation for one site specific binding: $Y = B_{max}X/(K + X)$, where $Y$ is the fraction of bound RNA, $B_{max}$ is the maximum specific binding, $X$ is the protein concentration, and $K$ is the equilibrium binding constant.
Size exclusion chromatography

Size exclusion chromatography was performed on an ÄKTA Pure 25 using a Superdex 200 Increase 10/300 column, which was equilibrated and run in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 2% glycerol, 0.5% Tween 20, 1 mM ATP, with a flow rate at 0.25 ml/min. Next, 48 μM of RtcR and 48 μM of the refolded RNA were mixed in gel filtration buffer. After incubating 30 min on ice and at room temperature for 30 min to allow complex formation, 100 μl of the sample was injected onto the column. Elution volumes were monitored by measuring the absorbance at 280 nm. Proteins in peak fractions were analyzed using 4%–12% SDS-PAGE and staining with Coomassie blue, while RNAs was detected by fractionation in 8% polyacrylamide/8.3 M urea gels, followed by staining with SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

All results are presented as the mean (n = 3) of three biological replicates ± SEM. GraphPad Prism v8 was used for statistical analysis. P values were calculated using two-tailed unpaired t tests. *, p < 0.05; **, p < 0.01, ***, p < 0.001 indicate significant differences between samples; ns indicates not significant.
Supplemental Information

An RNA Repair Operon

Regulated by Damaged tRNAs

Kevin J. Hughes, Xinguo Chen, A. Maxwell Burroughs, L. Aravind, and Sandra L. Wolin
Figure S1, related to Figure 1. Location and orientation of transposon insertions resulting in P_{rsr}-lacZ expression.

(A-D) Transposon insertion sites in S. Typhimurium 14028s (red circles) and SL1344 (white circles) strains are indicated. Insertions in the Watson strand are shown above each locus and insertions in the Crick strand are shown below. Loci containing at least two independent insertions are shown, together with xerC and ndk, which are upstream from genes containing multiple insertions. Loci not described in the main text are pdxB, which encodes erythronate-4-phosphate dehydrogenase, which functions in the synthesis of pyridoxal 5'-phosphate, xerC, a site-specific DNA recombinase required for chromosome segregation (Blakely et al., 1993) (panel C), guaB, which encodes the inosine monophosphate dehydrogenase that functions in guanine biosynthesis, ndk, which encodes nucleotide diphosphate kinase, rodZ, a transmembrane protein required for the E. coli rod shape (Shiomi et al., 2008) and nhaA, which encodes the N+/H+ antiporter that maintains intracellular pH (Padan et al., 2005) (all panel D). The insertion in pdxB could have a polar effect on truA transcription.
Figure S2, related to Figure 2. Accumulation of tRNA fragments in mutant strains.

(A-D) Following growth to mid-log (lanes 1-8) or stationary phase (lanes 9-16), RNA was extracted from the indicated strains and subjected to Northern blotting. Blots were probed to detect 3' halves of tRNA^{His(GUG)} (A), tRNA^{Leu(UAG)} (B) and tRNA^{Trp(CCA)} (C). Fragments that differ in mobility or levels in ΔtruA strains grown in stationary phase are indicated with red lines. As a loading control, the blot was reprobed to detect 5S rRNA (D).

(E-G) RNA from the indicated strains grown to mid-log (lanes 1-5) or stationary phase (lanes 6-10) was subjected to Northern blotting to detect 5' halves of tRNA^{Cys(GCA)} (E) and 3' halves of tRNA^{Trp(CCA)} (F) and tRNA^{Cys(GCA)} (G). The asterisks denote tRNA precursors that accumulate in Δpnp strains. Fragments differing in mobility or levels in mutant strains grown in stationary phase are indicated with red lines.
Figure S3, related to Figure 4. DNA damage results in tRNA cleavage and operon induction

(A) After treating wild-type, ΔrecA and lexA3 strains carrying FLAG3-Rsr without (lanes 1-3) or with MMC (lanes 4-6) for 2 hours, lysates were immunoblotted to detect FLAG3-Rsr and RplE (loading control).

(B) Wild-type (lanes 1, 2, 7, 8) and ΔrtcR strains (lanes 3-6) carrying FLAG3-Rsr were grown without (lanes 1-6) or with MMC (lanes 7-12) and the resulting lysates subjected to Western blotting to detect FLAG3-Rsr and Rpl5. To confirm that lack of operon expression was due to loss of RtcR, ΔrtcR strains carrying plasmids expressing RtcR (lanes 5, 11) and His6-RtcR (lanes 6, 12) were examined. Strains carrying pRtcRΔN were also assayed (lanes 4, 10).
(C) After treating the strains without (A) (lanes 1-3) or with MMC (lanes 4-6) for 2 hours, RNA was extracted and subjected to Northern blotting to detect tRNA_{Trp(CCA)}^{5'} fragments.

(D-F) After two hours in MMC, RNA was extracted from the indicated strains and subjected to Northern analysis to detect 3’ fragments of tRNA_{Trp(CCA)}^{(D)}, tRNA_{Cys(GCA)}^{(E)} or 5’ fragments of YrlA RNA (F) (lanes 3-18). As a control, RNA from wild-type cells carrying the pRtcRΔN plasmid or an empty vector was assayed (lanes 1-2).

(G) After growing the indicated strains to mid-log, bleomycin was added and the strains incubated for 2 hours at 37°C. RNA was subjected to Northern analysis to detect 5’ fragments of tRNA_{Cys(GCA)}^{(D)} (lanes 3-18). Lanes 1-2, RNA from wild-type cells carrying either an empty vector or the pRtcRΔN plasmid.

(H) After treating wild-type and ΔrecA FLAG_{rsr} strains either without (lanes 1-2) or with MMS (lanes 3-4) for 2 hours, RNA was subjected to Northern blotting to detect 5’ halves of tRNA_{Trp(CCA)}^{ amounts.

(I and J) After growing E. coli MG1655 and S. Typhimurium 14028s strains with or without MMC, RNA was probed to detect 5’ fragments of tRNA_{Trp(CCA)}^{(I)} and tRNA_{fMet}^{(J)}.

(K) After growth with or without MMC for two hours, lysates of the indicated E. coli and S. Typhimurium strains were subjected to immunoblotting to detect RtcB, LexA and RplE.
**Figure S4, related to Figure 5. tRNA 5’ fragments end in cyclic phosphate**

(A) Schematic of strategy used to distinguish 5’ fragments ending in 2’, 3’-cyclic phosphate and 3’-phosphate from 5’ fragments ending in 3’-OH.

(B-E) Left panels: After growing wild-type (lanes 1-3) and Δpnp strains (lanes 4-6) with MMC for two hours, RNA was extracted and incubated without (lanes 1, 2, 4, 5) or with (lanes 3, 6) T4 PNK prior to adding T4 RNA ligase and a 5’-phosphate-containing oligonucleotide. After gel fractionation, Northern blotting was used to detect tRNA\textsubscript{Tyr} (GU) (B), tRNA\textsubscript{Cys} (GC) (C), tRNA\textsubscript{Trp} (CC) (D) and tRNA\textsubscript{Met} (E). Right panels: Quantitation. The asterisks in C and E denote shorter fragments of tRNA\textsubscript{Cys} (GC) and tRNA\textsubscript{Met} that end in 3’-OH.

(F) Schematic of strategy used to distinguish 5’ fragments ending in 2’, 3’-cyclic phosphate from 5’ fragments ending in 3’-phosphate.
To distinguish between 3'-phosphate and 2', 3'-cyclic phosphate, RNA from MMC-treated wild-type (lanes 2-7) and Δpnp strains (lanes 8-13) was incubated with acid (HCl) and calf-intestinal phosphatase (CIP, lanes 5 and 11) or CIP alone (lanes 6 and 12), prior to adding T4 RNA ligase and the 5'-phosphate containing oligonucleotide. Northern blotting was used to detect tRNA\textsubscript{Tyr(GUA)} (G), tRNA\textsubscript{Cys(GCA)} (H), tRNA\textsubscript{fMet} (I) and tRNA\textsubscript{Leu(UAG)} (J). As a positive control, T4 PNK was added in place of CIP (lanes 3 and 9). As negative controls, the indicated components were omitted from the reactions (lanes 2, 4, 7, 8, 10, 13). Lane 1, size markers. Right panels: Quantitation. The asterisks in H, I and J denote shorter fragments of tRNA\textsubscript{Cys(GCA)}, tRNA\textsubscript{fMet} and tRNA\textsubscript{Leu(UAG)} that end in 3'-OH.

(K) Cleavage sites of tRNA\textsubscript{Tyr(GUA)}, tRNA\textsubscript{Cys(GCA)}, tRNA\textsubscript{Trp(CCA)}, tRNA\textsubscript{Phe(GAA)}, tRNA\textsubscript{fMet} and tRNA\textsubscript{Leu(UAG)} determined by 3' RACE. For each tRNA, at least 9 cDNAs were sequenced (see Table S2). Arrowheads denote 3' ends present in more than half the sequences, while small arrows denote minor 3' ends.
Figure S5, related to Figure 5. Deletion of multiple candidate endonucleases does not affect tRNA cleavage or operon activation

(A) RNA from the experiment shown in Figures 5A-5C was subjected to Northern blotting to detect 5' fragments of tRNA_{Tyr}^{GUA}.

(B) RNA from the experiment shown in Figures 5D-G was subjected to Northern blotting to detect 5' fragments of tRNA_{Phe}^{GAA}.

(C and D) Genetic map of the dinJ-yafQ (C) and higB-higA and higB2-higA2 (D) neighborhoods. The sequences of the putative LexA sites are shown.

(E) RNA extracted from the indicated strains was subjected to Northern blotting to detect 5' fragments of tRNA_{Trp}^{CCA}.
(F) Lysates of the strains in (E) were subjected to immunoblotting to detect FLAG$_3$-Rsr and RplE (loading control).

(G and H) After growing for two hours in MMC, RNA was isolated from the indicated strains and subjected to Northern blotting to detect tRNA$_{Trp(CCA)}$ 5' fragments. In the last lanes of (G) and (H), RNA was extracted from strains lacking seven (G) or six (H) potential nucleases.

(I and J) After two hours of growth in MMC, lysates were prepared from the strains shown in (G and H) and immunoblotting was performed to detect FLAG$_3$-Rsr.
Figure S6, related to Figure 6. Binding of 5' tRNA halves to the RtcR CARF domain

(A) To determine whether the 5' tRNA\textsuperscript{Leu(UAG)} fragments that accumulate in ΔtruA Δpnp Δrna strains in stationary phase ended with 3'-OH, 3'-phosphate, or 2', 3'-cyclic phosphate, total RNA from these cells was incubated with acid (HCl) and or calf intestinal phosphatase (CIP) prior to adding T4 RNA ligase and the 5'-phosphate containing oligonucleotide. RNA in lane 1 received no acid or CIP. Northern blotting was used to detect tRNA\textsuperscript{Leu(UAG)}.

(B) To quantitate the purified protein, increasing amounts of CARF domain were subjected to SDS-PAGE together with 1, 2 and 4 µg of purified bovine serum albumin and visualized by Coomassie blue staining.

(C) Purified RtcR (1 µg) was subjected to SDS-PAGE and stained with Coomassie blue.

(D) To examine whether binding to the isolated CARF domain was dependent on ATP, 5' tRNA\textsuperscript{Leu(UAG)} halves ending in cyclic phosphate were mixed with the indicated concentrations of the isolated CARF domain with or without 1 mM ATP.
Table S2. 3’ ends of tRNA 5’ halves that accumulate in MMC (determined by 3’ RACE), related to Figures 5 and S4.

| tRNA          | 3' end sequences                                                                 | Number of clones |
|---------------|----------------------------------------------------------------------------------|------------------|
| tRNA_Cys(GCA) | CGGGATTTGCAAATCCGTTCTAGTCCGGTTCACTCCGGAACGCGCCTCCA                              | (8)              |
|               | GCGGATTGCA                                                                       | (1)              |
|               | GCGGATTGC                                                                       | (3)              |
| tRNA_Trp(GCA) | CCGGTCTCCAAACCCGGGTGGGAGTTCCAGATCTCCGCCCCTGCA                                   | (6)              |
|               | CCGGTCTCC                                                                         | (2)              |
|               | CCGGTCT                                                                            | (1)              |
| tRNA_Tyr(GUA) | GCAGACTGTAAATCTGCCCAGTCCGGGAAGTTCCATCTCTCCGACCAACCAA                              | (12)             |
| tRNA_Phe(GAA) | GGGGATTTGAAAATCCCGTGTCCTCGGAGTCCGAGTCCGGCAGCCA                                  | (10)             |
|               | GGGGATTTG                                                                           | (2)              |
| tRNA(fMet)    | TCGGGCTCATAACCCGAAAGTGTGGATCCGTTCAAATCCGGCCCCCGCAACCA                              | (8)              |
|               | TCGGGCTCA                                                                         | (4)              |
|               | TCGGGCT                                                                            | (2)              |
| tRNA_Leu(UAG)| CCAGATTTAGGTGTCTGCCCGCAAGTGCGAGTTCAAGTCGCCCTGCCACCA                               | (1)              |
|               | CCAGATTAG                                                                         | (4)              |
|               | CCAGATT                                                                         | (3)              |
|               | CCAGATT                                                                         | (5)              |
|               | CCAGAT                                                                         | (2)              |

Sequences of clones obtained from 3’ RACE. The number in parentheses denotes the number of independent clones for each sequence. A portion of the full length tRNA sequence is at the top in bold and the anticodon is underlined.