REPORT

Bacterial noncoding Y RNAs are widespread and mimic tRNAs

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ABSTRACT

Many bacteria encode an ortholog of the Ro60 autoantigen, a ring-shaped protein that is bound in animal cells to noncoding RNAs (ncRNAs) called Y RNAs. Studies in *Deinococcus radiodurans* revealed that Y RNA tethers Ro60 to polynucleotide phosphorylase, specializing this exoribonuclease for structured RNA degradation. Although Ro60 orthologs are present in a wide range of bacteria, Y RNAs have been detected in only two species, making it unclear whether these ncRNAs are common Ro60 partners in bacteria. In this study, we report that likely Y RNAs are encoded near Ro60 in >250 bacterial and phage species. By comparing conserved features, we discovered that at least one Y RNA in each species contains a domain resembling tRNA. We show that these RNAs contain nucleotide modifications characteristic of tRNA and are substrates for several enzymes that recognize tRNAs. Our studies confirm the importance of Y RNAs in bacterial physiology and identify a new class of ncRNAs that mimic tRNA.

Keywords: noncoding RNAs; tRNA-like domain; Ro60 autoantigen

INTRODUCTION

A class of noncoding RNAs (ncRNAs) whose function is only now being elucidated is the Y class of ncRNAs. These ~100 nt ncRNAs were first identified because they are bound by the Ro 60 kDa protein (Ro60), a common autoantigen in patients with the rheumatic diseases systemic lupus erythematosus and Sjogren's syndrome (Lerner et al. 1981). Y RNAs were identified subsequently in a variety of vertebrate species, the nematode *Caenorhabditis elegans* and the radiation-resistant bacterium *Deinococcus radiodurans* (Sim and Wolin 2011). All characterized Y RNAs are between 80 and 130 nt and fold into structures containing a large internal loop and a long stem that contains the Ro60 binding site (Kohn et al. 2013; Wolin et al. 2013). Most vertebrate species contain between two and four distinct Y RNAs (Wolin et al. 2013).

A least one role of Y RNAs is to influence the subcellular location and function of Ro60, a ring-shaped protein that is proposed to function in ncRNA quality control. Studies of *Xenopus laevis* Ro60 revealed that it binds the single-stranded ends of misfolded ncRNA precursors in its central cavity (Stein et al. 2005; Fuchs et al. 2006). Y RNAs bind on the outer surface of the Ro60 ring and mask a nuclear accumulation signal, thus retaining Ro60 in the cytoplasm (Stein et al. 2005;

Sim et al. 2009). Also, as the misfolded ncRNA and Y RNA binding surfaces overlap, Y RNAs could regulate binding of Ro60 to misfolded ncRNAs (Stein et al. 2005; Fuchs et al. 2006).

Because Ro60 and Y RNA orthologs have not been detected in yeast, *D. radiodurans* has been used as a model single-celled organism for elucidating function. Consistent with a role in ncRNA surveillance, studies on this bacterium revealed that both Ro60 and Y RNA assist degradation of structured RNAs by exoribonucleases. Specifically, Y RNA tethers the Ro60 ortholog Rsr (Ro-sixty related) to the exoribonuclease polynucleotide phosphorylase (PNPase) to form RYPER (Ro60/Y RNA/PNPase Exoribonuclease RNP), an RNA degradation machine (Chen et al. 2013; Wolin et al. 2013). As in vertebrates, Rsr is a ring (Ramesh et al. 2007) and RYPER is configured such that single-stranded RNA could pass through the Rsr ring into the PNPase cavity for degradation (Chen et al. 2013). The presence of Rsr and Y RNA increases the effectiveness of PNPase in degrading structured RNAs (Chen et al. 2013). Rsr also functions with the exoribonucleases RNase II and RNase PH to mature 23S rRNA during heat stress (Chen et al. 2007). In this case, the Y-RNA-free Rsr carries out rRNA maturation, and bound

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Y RNA inhibits this process (Chen et al. 2007). These data have resulted in a model in which Y RNA functions to both tether Ro60 orthologs to other proteins and to regulate access of substrates to the Ro60 cavity (Chen et al. 2013).

To determine the extent to which functions identified in *D. radiodurans* are conserved, it is necessary to study Ro60 and Y RNA in other bacteria. However, while ~5% of sequenced bacterial genomes and some mycobacteriophages contain recognizable Ro60 proteins (Pedulla et al. 2003; Sim and Wolin 2011), Y RNAs have not been identified by homology searching using either metazoan or *D. radiodurans* Y RNAs. Notably, during a recent characterization of the Ro60 ortholog in the enteric bacterium *Salmonella enterica* serovar Typhimurium (Chen et al. 2013), we discovered that *S. Typhimurium* Rsr was associated with two ncRNAs that we named YrIA (Y RNA-like) and YrIB. As in *D. radiodurans*, the two *S. Typhimurium* ncRNAs were encoded within 2 kb of Rsr (Chen et al. 2000, 2013). Consistent with a function similar to the *D. radiodurans* RNA, *S. Typhimurium* YrIA was part of a complex that also contained Rsr and PNPase (Chen et al. 2013).

Given the intimate relationship between Ro60 and Y RNAs in metazoans and the broad distribution of bacterial Ro60 proteins, it seemed likely that Y RNAs were equally widespread. To identify additional Y RNAs, we performed homology searches using the newly identified *Salmonella* RNAs. We report that RNAs resembling *S. Typhimurium* YrIA are encoded adjacent to Ro60 orthologs in >250 bacterial and phage species. Surprisingly, comparison of conserved features revealed that at least one RNA in each species contains a domain that strikingly resembles tRNA. These domains resemble tRNAs in vivo as the RNAs contain modified nucleotides characteristic of tRNAs and are substrates for enzymes that recognize tRNAs. Although the *D. radiodurans* Y RNA is evolutionarily distant from the newly identified RNAs, this RNA also contains some tRNA-like features. Our studies vastly expand the number of bacterial Y RNAs and reveal that members of this family constitute a new class of tRNA mimics.

RESULTS AND DISCUSSION

Potential YrIA RNAs are widely present in Ro60-containing bacteria

To identify additional bacterial Y RNAs, we first searched GenBank manually using the *D. radiodurans* and *S. Typhimurium* ncRNAs. RNAs used in these initial BLASTN searches were the major ncRNA bound by *D. radiodurans* Rsr, called Yrn1 here (Y RNA-1) (Chen et al. 2000), and two ncRNAs bound by *S. Typhimurium* Rsr, YrIA and YrIB (Fig. 1A,B; Chen et al. 2013). Although searches with Yrn1 did not reveal possible orthologs, and searches with YrIB only identified orthologs in other *S. enterica* serovars, a search with YrIA revealed possible ncRNAs in the α -proteo-

bacterium *Rhizobium etli* and the γ -proteobacterium *Pseudomonas fulva* (Fig. 1C). By performing iterative searches with each newly identified putative RNA, we identified YrIA-related sequences in other bacterial phyla and in several mycobacteriophages that were known to encode a Ro60 ortholog (Fig. 1D).

To increase detection of YrIA orthologs, we used Infernal (Nawrocki and Eddy 2013) to build consensus RNA secondary structure models and search bacterial genomes systematically (see Materials and Methods and Supplemental files). Genome sequences were collected for all bacteria and bacteriophages that were annotated in GenBank as possessing a protein containing a TROVE (Telomerase, Ro, Vault) domain, the name given to the HEAT repeat domain that forms the Ro60 ring (Bateman and Kickhoefer 2003). Importantly, even when entire genomes were searched, nearly all sequences that were the best matches to the Infernal model were within 4 kb of the likely Ro60 ortholog and encoded on the same DNA strand (Supplemental Tables S1 and S2). Using these criteria, we identified YrIA-related sequences in 254 bacterial species and 22 bacteriophages.

Bacterial Y RNAs can be modeled to resemble tRNAs

By comparing the putative YrIA RNAs, we identified conserved features. One short motif near the 5' end of each RNA, a GNCGAAN₀₋₁G (N is any nucleotide), is also present in *S. Typhimurium* YrIB and overlaps sequences in *D. radiodurans* Yrn1 that are important for Rsr binding (Fig. 1A, box). As this motif resembles the GGUCCGA that is contacted by *X. laevis* Ro60 (Stein et al. 2005), we consider it likely that it is involved in Rsr recognition. Although the vertebrate motif can base-pair with conserved 3' residues to form a bulged helix, Ro60 primarily interacts with the 5' strand of the RNA (Stein et al. 2005), suggesting helix formation may not be needed for Ro60 recognition.

Surprisingly, the longest blocks of conserved sequences were located in the middle of the putative YrIA RNAs (Fig. 1C,D). In the MFOLD-predicted secondary structures, these sequences fold into two stem-loops that are nearly invariant between the putative YrIA RNAs (Fig. 1B–D, called T and D). Although not conserved in primary sequence, the nucleotides between these two conserved stem-loops can fold to form a third stem-loop (Fig. 1, AS). Many of the RNAs were also predicted to contain a fourth, less-conserved stem-loop upstream of stem-loop T (Fig. 1C,D, called V). Notably, the conserved “T” stem-loop was also present in *D. radiodurans* Yrn1 (Fig. 1A).

Inspection of the two most conserved stem-loops revealed similarities to the D- and T-arms of tRNAs (Fig. 2). The resemblance was most evident when *S. Typhimurium* YrIA or its putative orthologs were oriented such that the AS stem-loop between the “T” and “D” stem-loops of YrIA corresponds to the tRNA acceptor stem and the V region corresponds to the tRNA variable arm (Fig. 2A). In this

FIGURE 1. Newly identified YrIA-like RNAs. (A) *D. radiodurans* Y RNA. Regions important for Rsr and PNPase binding are indicated (Chen et al. 2013). (Box) conserved sequences required for binding of Ro60 to *X. laevis* and *D. radiodurans* Y RNAs. Sequences that may correspond to the GNCGAAN₀₋₁G motif in YrIA RNAs are in red, as are sequences within the potential "T" stem-loop. (B) *S. Typhimurium* YrIA and YrIB. Nucleotides that are identical in six of the seven aligned sequences (D) are red. For clarity, only the GNCGAAN₀₋₁G motif and nucleotides within and adjacent to the four stem-loops described in the text are colored. (C) Putative YrIA RNAs from *P. fulva* and *R. etli*. Nucleotides were colored as in B. Because the 5' and 3' ends of these RNAs have not been determined, they are indicated as dashed lines. Structures were predicted using Mfold (Zuker 2003) and RNAfold (Gruber et al. 2008), and adjusted manually to maximize structural homology with other YrIA RNAs. (D) Sequences of representative YrIA-like RNAs identified by iterative blast searches from *P. fulva* 12-X, *R. etli*, *Gordonia polyisoprenivorans*, *Streptosporangium roseum*, *Mycobacterium smegmatis*, and Mycobacteriophage ET08 were aligned with YrIA using Clustal Omega (Goujon et al. 2010; Sievers et al. 2011) (Supplemental text 1) and aligned further manually. Nucleotides that are identical in six of the seven sequences are red, while positions in which six of the seven sequences are either pyrimidines (Y) or purines (R) are blue. The "D" and "T" arms are shaded light blue, while the "V" and "AS" arms are in gray. The potential Rsr binding site is shaded pink.

orientation, many conserved nucleotides in YrIA RNAs correspond to invariant nucleotides in tRNA (Fig. 2B,C). Notably, at least seven tertiary interactions found in tRNA could potentially form in most YrIA orthologs (Fig. 2C,D). Importantly, for one tertiary interaction, the Levitt base pair between nucleotides 15 and 48 that stabilizes the tRNA L-shape (Levitt 1969), the various YrIA RNAs contain compen-

satory changes to allow formation of either a G15:C48 or A15:U8 base pair (Figs. 1D, 2C). Similar tRNA-like domains were present in the YrIA RNAs identified by Infernal (Supplemental Fig. S1). Moreover, although a full "D" arm is not evident in *D. radiodurans* Yrn1, this RNA can be modeled to contain some D-loop features (Fig. 2E). When modeled in this orientation, *D. radiodurans* Yrn1 contains a potential "anticodon

FIGURE 2. YrIA RNAs contain a tRNA-like domain. (A) *S. Typhimurium* YrIA drawn to resemble the tRNA cloverleaf. Nucleotides shown to be modified *in vivo* are indicated, as are the enzymes that carry out modification. (B) A generic tRNA. Nucleotides that are invariant, or nearly invariant, in tRNA are red, while nucleotides that are usually pyrimidine (Y) or purine (R) are blue (Dirheimer et al. 1995). The positions of the ψ 55 and dihydrouridine modifications are indicated. (C) The YrIA tRNA-like domain. Nucleotides that are conserved in YrIA RNAs (Fig. 1D) are represented in red, while conserved purines and pyrimidines are blue. In most YrIA RNAs, the conserved YUAY (nucleotides 22–25 in *S. Typhimurium*, panel A) is predicted to begin the V loop. The proposed secondary structure is supported by enzymatic probing (Supplemental Fig. S2). (D) *Escherichia coli* tRNA-Ala-GCA. Tertiary interactions are depicted by lines connecting the involved bases. Nucleotides that are conserved in YrIA orthologs are colored as in (C). All seven tertiary interactions shown can potentially form in YrIA RNA. (E) *D. radiodurans* Yrn1 drawn to illustrate similarities with the tRNA cloverleaf. The modified uridine that may correspond to ψ 55 is indicated. (F) Total RNA from *S. Typhimurium* strains SL1344 and LT2 was subjected to CMC modification and primer extension to detect pseudouridines in YrIA RNA (lanes 5–8). RNA extracted from *S. Typhimurium* LT2 carrying a mutation in the TruB catalytic domain was also assayed (lanes 9,10). To generate markers, primer extension was carried out on *S. Typhimurium* LT2 RNA in the presence of dideoxy nucleotides (lanes 1–4). (G) *D. radiodurans* RNA was subjected to primer extension as in (E) to detect pseudouridines in Yrn1 (lanes 5,6). Lanes 1–4, dideoxy sequencing. (H) RNA from *S. Typhimurium* LT2 was incubated with alkali for the indicated times and subjected to primer extension to detect dihydrouridines. A strain carrying a mutation that inactivates DusA catalytic activity was also assayed.

arm” that resembles tRNAs in containing a 5-bp stem (Fig. 2E, AC); however the “anticodon loop” contains 6 nt, rather than the usual 7, and a V arm is not present.

Bacterial Y RNAs contain nucleotide modifications characteristic of tRNAs

As a test of our model that some bacterial Y RNAs resemble tRNAs, we determined whether these RNAs contain a characteristic nucleotide modification, the pseudouridine at position 55 (ψ 55) in the T-loop of nearly all tRNAs (Fig. 2B; Bjork et al. 1987). Total RNA from *S. Typhimurium* and *D. radiodurans* was incubated with *N*-cyclohexyl-*N*- β -(4-methylmorpholinium)-ethylcarbodiimide *p*-tosylate (CMC), which reacts irreversibly with pseudouridines. Afterward, primer extension was performed because reverse transcrip-

tase halts at the nucleotide 3' to the modification (Bakin and Ofengand 1993). In both YrIA and Yrn1, a strong block to primer extension was detected corresponding to modification at the predicted site (Fig. 2F, lanes 6 and 8; Fig. 2G, lane 6). Pseudouridylation at this site was not detected when *S. Typhimurium* carried a mutation (D48C) that disrupts catalysis by TruB, the enzyme responsible for ψ 55 in tRNA (Fig. 2F, lane 10; Gutsell et al. 2000). We also detected pseudouridine at nucleotide 23 in YrIA from the virulent strain *S. Typhimurium* SL1344 (Fig. 2F, lane 6), but not in YrIA from the attenuated LT2 strain (lane 8).

We also tested whether YrIA and Yrn1 contain dihydrouridine (D), which is present in one or more conserved positions in all tRNA D-loops (Fig. 2B; Phizicky and Hopper 2010). Using a primer extension assay based on the finding that mild alkaline hydrolysis opens the dihydrouracil ring

and blocks primer extension (Xing et al. 2004), we detected dihydrouridine in the “D loop” of *S. Typhimurium* YrIA, but not that of *D. radiodurans* Yrn1 (Fig. 2H, lanes 6–8 and data not shown). Importantly, a mutation that inactivates *S. Typhimurium* DusA, the enzyme that modifies D20a in the *E. coli* tRNA^{fMet} D-loop (Bishop et al. 2002; Savage et al. 2006), eliminates dihydrouridine formation in YrIA (K140A, analogous to *E. coli* K153A) (Fig. 2H, lanes 10–12).

To determine whether the predicted tRNA-like structures form in vitro, we carried out enzymatic probing on Y RNAs synthesized with T7 RNA polymerase. Using two RNases that preferentially cleave single-stranded RNA, T1 (which cleaves after G) and T2 (which cleaves after all four nucleotides), as well as V1, which cleaves double-stranded or stacked nucleotides, we obtained cleavage patterns consistent with the proposed secondary structures (Supplemental Fig. S2). In keeping with findings that *X. laevis* Ro60 contacts conserved bases in the 5' strand of a helix formed by base-pairing the 5' and 3' ends (Stein et al. 2005), incubation of *D. radiodurans* Rsr with Yrn1 resulted in protection of the analogous nucleotides from all three nucleases (Supplemental Fig. S2; nucleotides G7, G8, C9, G11, and A12). In addition, many nucleotides within this stem showed enhanced V1 cleavage, suggesting Rsr binding stabilizes its formation (Supplemental Fig. S2D).

M. smegmatis YrIA RNA can be cleaved by RNase P and undergo CCA addition

To confirm that potential YrIA RNAs identified through homology searching existed in vivo, we examined *M. smegmatis*. We chose this organism because the putative RNA is not under control of the RtcR transcriptional activator, a feature that prevents its transcription during normal growth in *S. Typhimurium* and possibly other bacteria (Chen et al. 2013; Das and Shuman 2013). In *M. smegmatis*, the predicted YrIA is 5' of the Ro60 ortholog, within the annotated N terminus of the *MSMEI_1161* locus (Fig. 3A).

To determine whether the YrIA RNA was bound by *M. smegmatis* Rsr, we fused

three copies of Flag to Rsr and expressed it under control of the tetracycline-inducible TetRO promoter (Williams et al. 2010). Immunoprecipitation with anti-Flag, followed by Northern blotting with a probe complementary to the YrIA RNA 3' end, revealed a band of the expected ~147 nt (Fig. 3B, lane 5). However, the probe also detected a major band at ~65 nt, far shorter than expected (Fig. 3B, lane 5).

FIGURE 3. *Mycobacterium smegmatis* YrIA contains a tRNA-like module that can undergo RNase P cleavage and CCA addition. (A) Map of the *M. smegmatis* *rsr* locus. (B,C) Lysates from *M. smegmatis* expressing Flag₃-Rsr on a plasmid, or the empty vector, were subjected to immunoprecipitation using anti-Flag. YrIA and YrIB were detected by Northern blotting. In B, the oligonucleotide probes were complementary to sequences in the 3' (lanes 1–5) or 5' half of the RNA (lanes 6–9). (D,E) Predicted secondary structures of YrIA and YrIB. The structure of the YrIA “acceptor stem” after cleavage and CCA addition is indicated by the arrow in D. Conserved nucleotides within the GNCGAAN₀₋₁G motif and the tRNA-like domain are colored red as in Figure 1. (F) 5'-labeled *M. smegmatis* YrIA was incubated with RNase P RNA under conditions that support cleavage. At the indicated times, aliquots were removed and fractionated in denaturing gels (lanes 3–7). (Arrow) Cleavage product. Lanes 1 and 2, alkaline hydrolysis and T1 ribonuclease ladders.

Reprobing to detect the 5' portion of YrlA revealed another prominent ~82-nt band in the immunoprecipitate (Fig. 3B, lane 9). By synthesizing cDNAs, we discovered that the 65 and 82 nt RNAs consisted of the 3' and 5' halves of YrlA, respectively, and that the 65 nt RNA corresponds to the precise species expected if YrlA undergoes cleavage by RNase P, the endoribonuclease that matures tRNA 5' ends (Fig. 4D; Kazantsev and Pace 2006). Moreover, the 82 nt RNA contains an additional CA, such that this RNA resembles tRNA in containing a CCA tail (Fig. 3D). Reprobing the blot with oligonucleotides complementary to sequences between YrlA and Rsr revealed a second Rsr-bound RNA that we call YrlB (Fig. 3C). Both RNAs were encoded upstream of Rsr and processed from a single precursor. Notably, YrlB also contains a possible T-arm (Fig. 3E).

To test if *M. smegmatis* YrlA could be an RNase P substrate, we incubated in vitro synthesized RNA with the catalytic subunit of *E. coli* RNase P under conditions that promote cleavage (Guerrier-Takada and Altman 1984). As expected if YrlA forms a tRNA-like structure, M1 RNA cleaved the RNA at the predicted site (Fig. 3F, lanes 5–7, arrow). We have not detected similar cleavage of *D. radiodurans* Yrn1 or *S. Typhimurium* YrlA either in vivo or in vitro (data not shown), most likely because only *M. smegmatis* YrlA contains a seven base pair “acceptor stem,” a major determinant of RNase P cleavage (Altman et al. 1993). Nonetheless, the results that

M. smegmatis YrlA can be cleaved by RNase P in vitro and undergoes cleavage and CCA addition in vivo, and that YrlB also contains a T-arm, support our hypothesis that some bacterial Y RNAs contain a domain resembling tRNA.

D. radiodurans encodes at least one additional Y RNA

The finding that *S. Typhimurium* and *M. smegmatis* each contain at least two Y RNAs prompted us to reexamine *D. radiodurans*. Previously, we identified four small RNAs, designated a, b, c and d, that were encoded upstream of Rsr and up-regulated after ultraviolet (UV) irradiation (Fig. 4A; Chen et al. 2000). Because RNA c (here called Yrn1) was the major Rsr-bound RNA and could be folded to resemble metazoan Y RNAs, we designated this RNA as a Y RNA (Chen et al. 2000). In contrast, RNAs a, b, and d bound Rsr less strongly and their predicted structures were less similar to other Y RNAs (Supplemental Fig. S3; Chen et al. 2000), making it unclear whether they interact with Rsr in the same way as canonical Y RNAs. However, both RNA a and RNA b contain “T arms” resembling that of Yrn1 and YrlA, although the T arm of RNA a lacks the U corresponding to Ψ55 in tRNA (Supplemental Fig. S3).

Notably, during in vivo crosslinking of Rsr to RNA targets (Wurtmann and Wolin 2010), we identified another ncRNA encoded upstream of Yrn1 (RNA e) (Fig. 4A,B). Because this new RNA (unlike RNAs a, b, and d) contains a long stem, formed by base-pairing the 5' and 3' termini, that includes sequences important for binding of *X. laevis* Ro60 and *D. radiodurans* Rsr to Y RNAs (Fig. 4B, box; Green et al. 1998; Stein et al. 2005; Chen et al. 2013), we named this RNA Yrn2. Northern blotting revealed that Yrn2 is synthesized as a polycistronic precursor together with tRNA^{Asp} and Yrn1 (Fig. 4C, lanes 3 and 6; Fig. 4D, lanes 2 and 5). Similar to Yrn1 (Chen et al. 2000), Yrn2 is up-regulated after UV irradiation and requires Rsr for stable accumulation (Fig. 4D, lanes 4–6). Thus, all three characterized bacterial species contain at least two Y RNAs, one of which contains a module resembling tRNA. Additionally, at least one RNA in each species folds to form a long hairpin with a small pyrimidine-rich loop (Supplemental Fig. S4, *D. radiodurans* Yrn2, *M. smegmatis* YrlB, *S. Typhimurium* YrlB). As in mammalian cells (Wolin et al. 2013), these other Y RNAs may allow Ro60 to form ribonucleoprotein complexes with a wider range of components.

Functional implications

Our discovery that bacterial Y RNAs contain a domain resembling tRNA both expands the inventory of tRNA mimics and raises the question of how this module contributes to YrlA and Yrn1 function. Since in both *D. radiodurans* and *S. Typhimurium*, these ncRNAs are complexed with Rsr and the PNPase exoribonuclease (Chen et al. 2013), one possibility is that the extremely stable tRNA fold protects the ncRNAs from endonucleolytic nicks that could serve as entry

FIGURE 4. *D. radiodurans* contains at least one additional Y RNA. (A) The *rsr* locus. (B) Structure of Yrn2 predicted by Mfold. The boxed sequence, which is conserved in animal cell Y RNAs, is important for binding of Rsr to Yrn1 (Chen et al. 2013). (C,D) Wild-type and Δrsr strains were either unirradiated or irradiated with UV light as described (Chen et al. 2000). At the indicated times, aliquots were removed and the extracted RNAs subjected to Northern analysis to detect either Yrn1 (C) or Yrn2 (D).

points for PNPase and other exoribonucleases. If *M. smegmatis* YrIA RNA functions similarly, CCA addition (and possibly also aminoacylation) may protect the RNase P-cleaved RNA, as mature tRNAs are not normally substrates for bacterial exoribonucleases (Li et al. 2002). In this scenario, differences between bacterial and animal cell nucleases and/or the greater subcellular compartmentalization of animal cells may obviate the need for a tRNA-like module, as this domain is not apparent in identified metazoan Y RNAs (Supplemental Fig. S4 and our observations). A general role in protecting bacterial Y RNA from nucleases would also be consistent with findings that features of the tRNA-like module differ between YrIA RNAs and Yrn1, but would not account for the observation that sequences within both the D and T stems that are not involved in maintaining tRNA tertiary structure (Dirheimer et al. 1995) are nearly invariant in YrIA RNAs (Figs. 1, 2).

A second possibility is that the tRNA module is important for interaction of these RNAs with other components, such as PNPase or ribosomes. Although biochemical and structural experiments suggest that the S1/KH domains of PNPase bind the end of Yrn1 containing the T arm (Chen et al. 2013), experiments in which we deleted the T arm from *D. radiodurans* Yrn1 did not reveal significant differences in complex assembly (X Chen and SL Wolin, unpubl.). Additionally, although *D. radiodurans* Rsr sediments with partially degraded ribosomal subunits during prolonged growth in stationary phase, this association does not require Yrn1 RNA (Wurtmann and Wolin 2010). Thus, while we favor the idea that the tRNA-like module is involved in protein–RNA or RNA–RNA interactions, we do not yet know the identity of the potential interactors.

To date, the best-characterized ncRNA that resembles tRNA is tmRNA, which rescues stalled ribosomes by functioning as both a tRNA and an mRNA (Moore and Sauer 2007; Keiler and Feaga 2014). Although tmRNA and YrIA RNAs each contain a pseudouridine-containing T arm, tmRNA also contains an acceptor stem that undergoes aminoacylation, allowing it to bind the translation elongation factor EF-Tu, enter ribosomes with the SmB protein and act as an acceptor for the stalled polypeptide (Moore and Sauer 2007; Keiler and Feaga 2014). Although *M. smegmatis* YrIA, which undergoes RNase P cleavage and CCA addition, could potentially be aminoacylated, we have not detected cleavage of *S. Typhimurium* YrIA or *D. radiodurans* Yrn1 at the analogous site, making it unlikely that the ability to undergo aminoacylation is a general feature of these RNAs. However, it is possible that all three Y RNAs interact with ribosomes, translation factors, or other tRNA-interacting proteins to influence cell metabolism.

Consistent with a separate evolutionary lineage, both the Ro60-binding and tRNA-like modules of *D. radiodurans* Yrn1 differ from those of YrIA RNAs. The Ro60-binding modules of the *D. radiodurans* RNAs more closely resemble metazoan Y RNAs in that both strands of the helix are re-

tained, while only the 5' strand is recognizable in the *S. Typhimurium* and *M. smegmatis* RNAs (Supplemental Fig. S4). Also, while both Yrn1 and YrIA RNAs contain domains resembling tRNAs, the YrIA domain mimics the tRNA D and T arms and acceptor stem, as DusA and TruB modify the expected nucleotides in *S. Typhimurium* YrIA, and RNase P and the CCA-adding enzyme recognize *M. smegmatis* YrIA. In contrast, we did not detect dihydrouridine in the Yrn1 “D loop,” making it unclear if this stem–loop forms and/or resembles a D arm in vivo. The orientation of the predicted T and D arms with respect to the RNA 5' ends also differs between YrIA RNAs and Yrn1, as does whether the stem–loop between these arms occupies the position of the tRNA acceptor stem or anticodon arm (Fig. 2, Supplemental Fig. S4). Thus, while both YrIA and Yrn1 RNAs may have originated from tRNAs, they appear to have evolved independently.

Although our studies identified potential Y RNAs in >250 bacteria and phages, we are certain that more RNAs remain to be found. Because our characterization of Ro60-associated RNAs in all three bacteria identified RNAs that eluded bioinformatic discovery, similar immunoprecipitation experiments will likely identify additional Y RNAs in other species. As there were also Ro60-containing bacteria for which we failed to identify a strong YrIA candidate (Supplemental Table S1), we expect that Y RNAs with sequence and structural variations will be uncovered in additional searches. Conversely, some predicted RNAs, despite their homology with characterized YrIA RNAs, may not be expressed or may only be expressed under specific stress conditions. We also consider it likely that our definition of Y RNAs (which currently does not include the three other ncRNAs encoded near *D. radiodurans* Rsr) will expand as more RNAs are characterized.

Finally, we note that although Y RNAs were first identified in human cells, the functions of these RNAs have been best characterized in a single bacterium. Our identification of these RNAs in a wide range of bacteria, including many that are far more amenable to genetic manipulation than *D. radiodurans*, lays the groundwork for a host of studies that should vastly expand our understanding of these long enigmatic ncRNAs.

MATERIALS AND METHODS

Bacterial strains and growth conditions

D. radiodurans R1 was grown at 30°C in TGY (0.8% tryptone, 0.1% glucose, 0.4% yeast extract). *S. Typhimurium* LT2 was grown at 37°C in LB medium. *M. smegmatis* MC2 155 was grown at 37°C in Middlebrook 7H9 liquid media supplemented with 10% albumin-dextrose complex (ADC), 0.05% Tween 80, 0.1 M CaCl₂, 100 µg/mL carbenicillin, and 100 µg/mL cycloheximide. To construct the D48C mutation in *S. Typhimurium* LT2 TruB, the 5' portion of *truB*, with ~160 bp of 5' flanking sequence, was amplified from genomic DNA using primers 5'-GCTTGCTGGGTAAAGCGATG-

3' and 5'-CAACATGCCGGTCGCCAGCGGGCACAGCGCGCCGGTATGCCCCGGCGCGGTTG-3'. This mutated fragment was joined with the 3' portion of *truB* and 215 bp of 3' flanking sequence amplified from genomic DNA using primers 5'-CCCGCTGGCGACCGCATGTTG-3' and 5'-GTGTGCTTTGCGTCACGACCG-3'. The resulting DNA was inserted into the *Sma* I site of pSB890 (Palmer et al. 1998). Subsequent genetic manipulations were as described (Chen et al. 2013). A similar strategy was used to generate the K140A mutation in *DusA*.

To construct the *M. smegmatis* strain carrying Flag₃-Rsr, *rsr* (*MSMEI_1162*) was amplified from genomic DNA using 5'-GTGGACATCCTCAAGACCATTTC-3' and 5'-CGGGAAGCTTCTAGATGTGCGCCGCGTGAG-3', fused to three copies of Flag and cloned into the *Bam*HI/*Hind*III sites of pKW08-Lx (Williams et al. 2010). The resulting plasmid pKW08-Flag-MSRsr1 (called pFlag₃-Rsr in Fig. 4) was transformed into *M. smegmatis*, generating a strain that expressed Flag-tagged Rsr under control of the TetRO promoter. Genetic manipulations were as described (van Kessel and Hatfull 2008).

Detection of modified nucleotides

Pseudouridine mapping was performed essentially as described (Ofengand et al. 2001). Briefly, total RNA was incubated in 167 mM CMC in BEU buffer (50 mM Bicine, pH 8.5, 4 mM EDTA, 7 M urea) at 37°C for 20 min. After precipitating with ethanol and washing with 70% ethanol, the pellet was dissolved in 50 mM sodium carbonate buffer (pH 10.4), 2 mM EDTA and incubated at 37°C for 4 h. The modified RNA was subjected to primer extension using SuperScript III Reverse Transcriptase (Invitrogen) and 5' labeled oligonucleotide primers 5'-AGGGTGGCGACAAGTTTATGTG-3' (*S. Typhimurium* YrIA) and 5'-AGTGCTCTGGACAAGGGTTCGGG-3' (*D. radiodurans* Yrn1). The resulting cDNAs were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol and fractionated in 8% polyacrylamide/8.3 M urea gels. Dihydrouridines were detected as described (Xing et al. 2004). Total RNA was incubated with 0.1 N KOH at 37°C for 2–30 min, and neutralized by adding 5× First-Strand Buffer (Invitrogen). The RNA was then mapped by primer extension as described above.

Northern blotting

Total RNA was isolated from bacteria using hot acid phenol as described (Chen et al. 2000). Northern blotting was as described (Chen et al. 2007). Oligonucleotide probes were as follows: 5'-GCTCGGTGATGTGGGCGTGTGAGGTAT-3' (*M. smegmatis* YrIA-3'), 5'-GACCATTCATTAGAAGTGGTAA-3' (*M. smegmatis* YrIA-5'), 5'-AGTCGAACCTTGAAGTCGCCAGATA-3' (*M. smegmatis* YrIB), 5'-CACCGCTTCCGCCCATTTGAGGTTGG-3' (*D. radiodurans* Yrn1), 5'-ATGTTTTGTTCTACTGGGAAAGTAAATAA-3' (*D. radiodurans* Yrn2).

RNA structure probing

After using PCR to place *S. Typhimurium* YrIA and *D. radiodurans* Yrn1 RNA sequences behind the T7 promoter, RNAs were transcribed with T7 polymerase. For enzymatic probing, 5'-labeled RNA was first refolded by heating to 65°C for 3 min in 20 mM

HEPES (pH 7.5), 50 mM NaCl and 5 mM MgCl₂, followed by slow cooling to room temperature. For YrIA, 23 fmol RNA was incubated in the same buffer with 1.25 units of RNase T1 (Sigma), 0.32 units RNase T2 (Invitrogen), or 0.00037 units of RNase V1 for 10 min. For Yrn1, 2.2 pmol of *D. radiodurans* Rsr (purified from baculovirus [Chen et al. 2013]) was mixed with 23 fmol refolded RNA in 20 mM HEPES pH 7.5, 50 mM NaCl, and 5 mM MgCl₂. After incubating at room temperature for 30 min and on ice for 30 min, RNases were added as described above and the reactions incubated on ice for 10 min. Afterward, RNAs were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol and fractionated in 8% polyacrylamide/8.3 M urea gels.

Identification of *M. smegmatis* Y RNAs

M. smegmatis carrying pKW08-Flag-MSRsr1 or the parent pKW08-Lx vector were grown to mid-log phase (~1 OD₆₀₀) and Rsr expression induced by adding 50 ng/mL of tetracycline to the media for 2.5 h. After harvesting, cells were lysed using a French press in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 5% glycerol, 1 mM MgCl₂, 2 mM β-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1.25 mM vanadyl ribonucleoside complexes, and 1× protease inhibitor cocktail (Roche Applied Science). Anti-Flag immunoprecipitations were as described (Chen et al. 2013). For cDNA synthesis, RNAs were ligated to RNA linkers L5 (5'-OH AGGGA GGACGAUGCGG 3'-OH) and L3 (5'-P GTGTCACTCACTTCCA GCGG-3'-puromycin), reverse transcribed with DNA oligomer P3 (5'-CCGCTGGAAGTGACTGACAC-3') and amplified with P3 and P5 (5'-AGGGAGGACGATGCGG-3') (Ule et al. 2005). RNA ends after cleavage and CCA addition were determined by amplifying with 5'-GAGCTCGGTGATGTGGGCGTGTG-3' and P5 (to obtain the 5' end of the YrIA 65 nt fragment) and with 5'-CACTTCTAATGGAATGGTCGC-3' and P3 (to obtain the 3' end of the 82 nt fragment).

RNase P cleavage of *M. smegmatis* YrIA

DNA containing YrIA behind the T7 promoter was amplified from genomic DNA using 5'-CATGACTAGTAATACGACTCACTATA GGGCGCTTGTTGATGTGAGCCG-3' and 5'-GAGCTCGGTGATGTGGGCG-3'. YrIA RNA was synthesized from this template using T7 RNA polymerase (Promega) according to the manufacturer's instructions. RNase P M1 RNA was synthesized with T7 RNA polymerase using plasmid pJA2' (a gift of Dr. S. Altman, Yale University) that was linearized with *Fok* I. The RNase P assay was as described (Guerrier-Takada and Altman 1984). Briefly, 187 nM of M1 RNA and 1 nM of 5' ³²P-labeled YrIA RNA were each suspended separately in RNase P cleavage buffer (50 mM Tris-HCl, pH 7.5, 100 mM NH₄Cl, 100 mM MgCl₂), incubated at 55°C for 5 min, and slow-cooled to 37°C. The two RNAs, together with 0.7 units/μL RNase-OUT RNase inhibitor, were then combined and incubated at 37°C.

Bioinformatic analyses

Following initial primary sequence homology searches using BLASTN, the sequences in Figure 1D were aligned using Clustal Omega (Goujon et al. 2010; Sievers et al. 2011) and the alignment used to build a consensus secondary structure model using

Infernal (version 1.1) (Nawrocki and Eddy 2013) (Supplemental text 1). After collecting genome sequences for bacteria that were annotated in Genbank as possessing a TROVE-domain protein, we discarded redundant sequences and genome fragments that contained <400 bp flanking the presumptive Ro60. This yielded sequences from 303 bacterial species, 119 of which were complete genomes. Because some genomes encode two TROVE-domain proteins, a total of 307 possible Ro60 orthologs were present. Although our initial search identified YrIA RNAs in most searched phage genomes (Supplemental Table S2) relatively few YrIA RNAs were identified in cyanobacteria and δ -proteobacteria, and some of these RNAs contained a longer “acceptor stem” (e.g., *Stanieria cyanosphaera*) (Supplemental Fig. S1). To identify additional RNAs, a second covariance model was built that included these sequences (Supplemental text 2). Potential YrIA RNAs found using this second search with *E*-value <0.00005 are summarized in Supplemental Table S1. As dimeric tRNAs could potentially score as false-positives (with the T stem of the first tRNA and the D stem of the second tRNA appearing as a YrIA RNA-related sequence), all sequences that overlapped annotated tandem tRNA genes were discarded. To have the highest confidence data set, only YrIA-related sequences that were both the best matches to the Infernal model in the genome or partial genome search and were located within 4 kb of the Ro60 ortholog were reported. In those cases where there were multiple YrIA-like sequences with *E*-values <0.00005 within 4 kb of Rsr, all sequences were reported. In this way, 355 putative YrIAs were identified in 254 bacteria and 22 bacteriophages (Supplemental Tables S1 and S2).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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