


**ORIGINAL RESEARCH**

# Characterization of a *Deinococcus radiodurans* MazF: A UACA-specific RNA endoribonuclease

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**Abstract**

Microbes are known to withstand environmental stresses by using chromosomal toxin–antitoxin systems. MazEF is one of the most extensively studied toxin–antitoxin systems. In stressful environments, MazF toxins modulate translation by cleaving single-stranded RNAs in a sequence-specific fashion. Previously, a chromosomal gene located at DR0417 in *Deinococcus radiodurans* was predicted to code for a MazF endoribonuclease (MazF<sub>DR0417</sub>); however, its function remains unclear. In the present study, we characterized the molecular function of MazF<sub>DR0417</sub>. Analysis of MazF<sub>DR0417</sub>-cleaved RNA sites using modified massively parallel sequencing revealed a unique 4-nt motif, UACA, as a potential cleavage pattern. The activity of MazF<sub>DR0417</sub> was also assessed in a real-time fluorometric assay, which revealed that MazF<sub>DR0417</sub> strictly recognizes the unique tetrad UACA. This sequence specificity may allow *D. radiodurans* to alter its translation profile and survive under stressful conditions.

**KEYWORDS***Deinococcus radiodurans*, MazEF, sequence specificity, toxin–antitoxin system

## 1 | INTRODUCTION

Toxin–antitoxin (TA) systems are common in the bacterial and archaeal kingdoms (Pandey & Gerdes, 2005). They are typically encoded by operons comprising the genes of a stable toxin that disrupts cellular activities and an unstable antitoxin that alleviates the toxin's effect. In response to stress, antitoxins are rapidly degraded because of their labile nature, thus freeing the corresponding toxins (Page & Peti, 2016). These toxins then arrest prokaryotic growth in many different ways; for example, they can impede DNA replication, cell wall synthesis, translation, cell division, and ATP production (Schuster & Bertram, 2013). Among these mechanisms, translation inhibition caused by intracellular RNA digestion is the most common (Yamaguchi & Inouye, 2011).

The MazEF family, which is comprised of MazE antitoxin and MazF toxin, is one such representative TA system (Aizenman,

Engelberg-Kulka, & Glaser, 1996). In *Escherichia coli*, MazE forms a heterohexamer with MazF and inactivates the activity of MazF as a sequence-specific endoribonuclease (Kamada, Hanaoka, & Burley, 2003). Once cells encounter specific stresses, however, unstable MazE is preferentially degraded by ClpAP protease, liberating the MazF toxin endoribonuclease (Aizenman et al., 1996; Hazan, Sat, & Engelberg-Kulka, 2004). The released MazF then alters translation globally by cutting cellular RNAs at ACA sites (Amitai, Kolodkin-Gal, Hananya-Meltabashi, Sacher, & Engelberg-Kulka, 2009; Sauert et al., 2016; Vesper et al., 2011; Zhang et al., 2003). Although the functions of prokaryotic MazF toxins remain unclear, they are thought to have various biological roles such as in virulence (Rothenbacher et al., 2012; Tiwari et al., 2015; Zhu et al., 2009), phage defense systems (Alawneh, Qi, Yonesaki, & Otsuka, 2016; Hazan & Engelberg-Kulka, 2004), persister generation (Tripathi, Dewan, Siddique, & Varadarajan, 2014), and

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programmed cell death (Nariya & Inouye, 2008); an important characteristic of MazF endoribonucleases that may contribute to these physiological functions is their sequence-specificities. In most cases, MazF toxins require strict sequences for RNA cleavages, which are typically 3–7-nucleotide (nt) motifs (Miyamoto, Kato, Sekiguchi, Tsuneda & Noda, 2016a; Miyamoto, Yokota, Tsuneda, Noda et al. 2016b; Nariya & Inouye, 2008; Rothenbacher et al., 2012; Schifano et al., 2014; Schuster et al., 2013; Verma & Bhatnagar, 2014; Yamaguchi & Inouye, 2011; Yamaguchi, Nariya, Park, & Inouye, 2012; Zhang et al., 2003; Zhu et al., 2006, 2008, 2009). Thus, microbes may reprogram their translation by shutting down most translation processes or by eliminating specific transcripts to cope with unfavorable surroundings.

*Deinococcus radiodurans* is a Gram-positive bacterium that inhabits a variety of environments. It is remarkably resistant to different types of stress, such as desiccation, oxidative stress, DNA damage, ionizing radiation, and ultraviolet radiation (Makarova et al., 2001). Previously, computational analysis predicted that a chromosomal gene, located at DR0417 in *Deinococcus radiodurans*, codes for a MazF toxin (MazF<sub>DR0417</sub>) (Chopra, Saumitra, Pathak, Bhatnagar, & Bhatnagar, 2013; Pandey & Gerdes, 2005), suggesting that *D. radiodurans* utilizes MazF<sub>DR0417</sub> as a posttranscriptional regulator and regulates its translation under stressful conditions. In the present study, we found that MazF<sub>DR0417</sub> is a toxin endoribonuclease and constitutes an authentic TA system together with its cognate antitoxin MazE, encoded by the locus DR0416 (MazE<sub>DR0416</sub>). Analysis of MazF<sub>DR0417</sub>-cleaved RNA products using modified massively parallel sequencing revealed that MazF<sub>DR0417</sub> cleaves RNA specifically at a unique 4-nt motif, UACA. The indispensability of the tetrad for effective RNA cleavage was also demonstrated using a fluorescent quenching approach. The results indicate that MazF<sub>DR0417</sub> may play a role in adaptation to stressful environments by promoting the selective degradation of intracellular RNAs.

## 2 | MATERIALS AND METHODS

### 2.1 | Plasmids and oligonucleotides

The expression vector pET21c was purchased from Takara Bio Service (Shiga, Japan). The pET19b expression vector encoding *mazE*<sub>DR0416</sub>, whose codon usage was optimized for recombinant protein expression in *E. coli*, was purchased from GenScript Japan (Tokyo, Japan). Double-stranded DNA fragments, including the *mazF*<sub>DR0417</sub> DNA sequence flanked by *Bam*HI/*Eco*RI sites, were purchased from Life Technologies Japan Ltd. (Tokyo, Japan). The pMX-T vector encoding *D. radiodurans mazEF* genes was purchased from Life Technologies Japan Ltd. Fluorescently modified oligonucleotides were purchased from Japan Bio Services (Saitama, Japan). The chemically synthesized tRNA<sup>Val</sup> oligonucleotide was purchased from Gene Design (Osaka, Japan).

### 2.2 | Plasmid construction

Double-stranded DNA fragment encoding *mazF*<sub>DR0417</sub> gene was digested with *Eco*RI and *Bam*HI (Toyobo, Osaka, Japan) and purified

using a MinElute PCR purification kit (Qiagen, Hilden, Germany). Likewise, the pMX-T plasmid encoding *D. radiodurans mazEF* genes (*mazEF*<sub>DR0416DR0417</sub>) was digested with *Bam*HI (New England Biolabs, Ipswich, MA, USA) and cleaned with a QIAquick PCR purification kit (Qiagen). The linearized pMX-T plasmid was then digested with *Eco*RI (Takara), and the DNA fragment containing *mazEF*<sub>DR0416DR0417</sub> genes was recovered by using a QIAquick gel extraction kit (Qiagen). These DNA fragments were then ligated into the corresponding pET21c multiple cloning site using a DNA ligation kit (Takara), producing pET21c-*mazF*<sub>DR0417</sub> and pET21c-*mazEF*<sub>DR0416DR0417</sub>, and they were introduced into the *E. coli* strain DH5 $\alpha$  (Nippon Gene, Tokyo, Japan). pET21c-*mazF*<sub>DR0417</sub> and pET21c-*mazEF*<sub>DR0416DR0417</sub> were extracted using a QIAprep Spin Miniprep Kit (Qiagen) and the sequences were validated using an AB 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol.

### 2.3 | Growth effect of MazE<sub>DR0416</sub> and MazF<sub>DR0417</sub>

The *E. coli* cells harboring pET21c empty vector, pET21c encoding *D. radiodurans mazF* or *mazEF* were cultivated at 37°C for 12 h in liquid LB medium supplemented with 100  $\mu$ g/mL ampicillin. Turbid overnight cultures were then streaked onto the LB plates containing 100  $\mu$ g/mL ampicillin and 0.2% glucose with or without 25  $\mu$ mol/L IPTG at 37°C.

### 2.4 | Protein expression

pET19b-*mazE*<sub>DR0416</sub> was introduced into the *E. coli* strain BL21 (DE3) (BioDynamics Laboratory Inc., Tokyo, Japan), whereas pET21c-*mazF*<sub>DR0417</sub> was introduced into the strain BL21 (DE3) (Nippon Gene) using the heat-shock method. The *E. coli* cells harboring pET19b-*mazE*<sub>DR0416</sub> or pET21c-*mazF*<sub>DR0417</sub> were grown overnight in liquid LB medium supplemented with 100  $\mu$ g/mL ampicillin at 37°C. These cells were inoculated into 1 L LB medium containing 100  $\mu$ g/mL ampicillin. One millimolar IPTG was added to induce MazE<sub>DR0416</sub> and MazF<sub>DR0417</sub> when the OD<sub>600</sub> reached 0.8 and 3.0, respectively. The cells were harvested by centrifugation at 7,000g after 5 and 3.5 h of incubation for MazE<sub>DR0416</sub> and MazF<sub>DR0417</sub>, respectively.

### 2.5 | Purification of MazE<sub>DR0416</sub>

*Escherichia coli* cells containing MazE<sub>DR0416</sub> were thawed on ice and suspended in 18 mL of 6 mol/L urea buffer (10 mmol/L sodium phosphate buffer (pH 8.0), 150 mmol/L NaCl, 0.025% Triton X-100, 6 mol/L urea, 2.5 mmol/L  $\beta$ -mercaptoethanol, and 20 mmol/L imidazole). The suspended cells were then incubated on ice for 5 min in the presence of 0.09 mg/mL lysozyme. The cells were lysed by sonication and collected by centrifuging at 7000g. The supernatant was then filtered through a 0.45- $\mu$ m membrane (Millex, Darmstadt, Germany) and applied to a 1-mL His-Trap FF column (GE Healthcare, Little Chalfont, UK). Urea was then removed by gradually substituting the 6 mol/L urea buffer with binding buffer (20 mmol/L sodium phosphate buffer (pH 8.0), 300 mmol/L NaCl, 0.05% Triton X-100, 5 mmol/L  $\beta$ -mercaptoethanol, and 40 mmol/L imidazole) using an

AKTA pure 25 (GE Healthcare). The following program was used for this procedure: flow rate, 1 mL/min; linear elution gradient, 20 column volumes (cv). Subsequently, the column was washed with 32 cv of binding buffer. Deca-histidine-tagged MazE<sub>DR0416</sub> was selectively eluted by increasing the concentration of elution buffer (20 mmol/L sodium phosphate buffer (pH 8.0), 300 mmol/L NaCl, 0.05% Triton X-100, 5 mmol/L β-mercaptoethanol, and 500 mmol/L imidazole) using the following program: flow rate, 1 mL/min; linear elution gradient, 20 cv; fraction size, 0.5 mL. Molecular weight and purity were confirmed by the Agilent 2200 TapeStation P200 ScreenTape Assay (Agilent Technologies, Santa Clara, CA, USA). Protein concentration was determined using the Qubit Protein Assay Kit (Life Technologies, Carlsbad, CA, USA).

## 2.6 | Purification of MazF<sub>DR0417</sub>

The recombinant MazF<sub>DR0417</sub> was purified as described previously (Miyamoto et al. 2016a, 2016b), with slight modifications. *Escherichia coli* cells containing MazF<sub>DR0417</sub> were thawed on ice and suspended in 32 mL of binding buffer (20 mmol/L sodium phosphate buffer (pH 8.0), 300 mmol/L NaCl, 5 mmol/L β-mercaptoethanol, and 50 mmol/L imidazole). The cells were lysed by sonication and collected by centrifuging at 7000 g. The supernatant was then filtered through a 0.45-μm membrane (Millex) and applied to a 1-mL His-Trap FF crude column (GE Healthcare). Nonspecifically bound proteins were removed by washing with 32 cv of binding buffer using AKTA pure 25 (GE Healthcare). Hexa-histidine-tagged MazF<sub>DR0417</sub> was selectively eluted by gradually increasing the elution buffer concentration using the following program: flow rate, 1 mL/min; linear elution gradient, 20 cv; fraction size, 0.5 mL. The composition of the elution buffer was as follows: 20 mmol/L sodium phosphate buffer (pH 8.0), 300 mmol/L NaCl, 5 mmol/L β-mercaptoethanol, and 500 mmol/L imidazole. Molecular weight and purity were confirmed by the Agilent 2200 TapeStation P200 ScreenTape Assay (Agilent Technologies). Protein concentration was determined using the Qubit Protein Assay Kit (Life Technologies).

## 2.7 | Enzymatic activity of MazE<sub>DR0416</sub> and MazF<sub>DR0417</sub>

Synthetic RNA constructs were prepared as described in our previous study (Miyamoto et al. 2016a). One hundred nanograms of RNA 500-2 was incubated with 1.2, 6, or 30 pmol of MazF<sub>DR0417</sub> at 37°C for 2 h in MazF reaction buffer (20 mmol/L Tris-HCl (pH 8.0), 1 mmol/L dithiothreitol, 0.01% Triton X-100, and 4 U of recombinant RNase inhibitor (Takara)) in a 50-μL reaction volume. As a control reaction, 30 pmol of MazF<sub>DR0417</sub> was preincubated with 300 pmol of MazE<sub>DR0416</sub> at room temperature for 10 min, and this mixture was incubated with 100 ng of RNA 500-2 at 37°C for 2 h in MazF reaction buffer in a final volume of 50 μL. These RNAs were purified with RNA Clean and Concentrator™-5 (Zymo Research, Orange, CA, USA). Next, gel loading buffer II (Ambion, Austin, TX, USA) was added to each sample. The samples were incubated at 95°C for 5 min and then separated

on a 10% polyacrylamide gel containing 7 mol/L urea. The RNA was stained using SYBR Gold (Life Technologies) and then detected using a Typhoon 9210 imager (GE Healthcare).

## 2.8 | Cleavage sequence identification

A sequencing library was constructed as described in our previous study (Miyamoto et al. 2016a), with slight modifications. Briefly, five RNA mixtures were incubated with 1.5 μg of MazF<sub>DR0417</sub> at 37°C for 30 min in MazF reaction buffer in a 20-μL reaction volume. Phosphorylation, barcode ligation, and sequencing were performed as described previously (Miyamoto et al. 2016a). The sequence data were analyzed with CLC Genomics 7.5.1., using the same parameters outlined in our previous work (Miyamoto et al. 2016a). Relative coverage increase, which is defined as the coverage at the (n + 1)<sup>th</sup> position divided by the coverage at the n<sup>th</sup> position, was calculated for all reference samples. Nucleotide positions with coverage <500 were excluded from analysis. Of these nucleotide positions, those showing the overall top 15 and top 25 relative coverage increases were selected. The sequences five-base pairs upstream and downstream of these positions were extracted and aligned using WebLogo (Crooks, Hon, Chandonia, & Brenner, 2004). These sequence data have been submitted to the DDBJ database under the accession number DRA004579.

## 2.9 | Fluorometric analysis

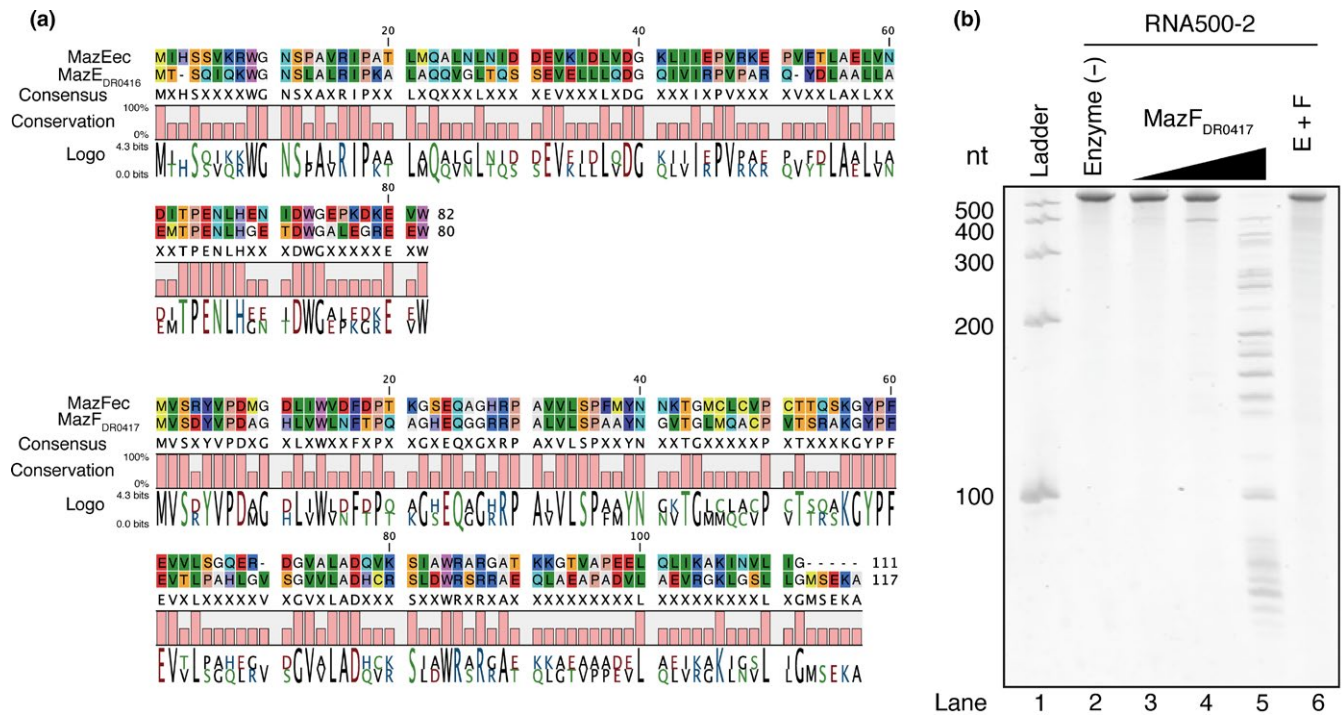
Fluorometric analysis was performed as described previously (Miyamoto et al., 2016a, 2016b). Fifteen or 600 ng of MazF<sub>DR0417</sub> was incubated with 10 pmol of fluorescently labeled oligonucleotides in MazF reaction buffer in a total volume of 20 μL. For control reactions, the oligonucleotides were also treated with 100 ng of RNase A (Novagen, Madison, WI, USA) in MazF reaction buffer in a final volume of 20 μL. All reactions were conducted at 37°C in triplicate. The fluorescence intensity was recorded every 1 min using a Light Cycler 480 system (Roche, Basel, Switzerland) with 483 nm excitation and 533 nm detection filters.

## 2.10 | tRNA cleavage

Five picomoles of chemically synthesized tRNA<sup>Val</sup> was incubated with 0.1, 0.9, or 8.1 pmol of MazF<sub>DR0417</sub> at 37°C for 30 min in MazF reaction buffer in a 20-μL reaction volume. Gel loading buffer II (Ambion) was added to each sample. These samples were incubated at 95°C for 5 min and then separated on a 10% polyacrylamide gel containing 7 mol/L urea. The RNA was stained using SYBR Gold (Life Technologies) and then detected using a Typhoon 9210 imager (GE Healthcare).

## 2.11 | Accession numbers

The GenBank accession numbers are as follows: MazE<sub>DR0416</sub> (NP\_294139), MazF<sub>DR0417</sub> (NP\_294140), tRNA<sup>Val</sup> (AE000513), and



**FIGURE 1** MazF endoribonuclease conserved in *D. radiodurans*. (a) Pairwise alignment of *E. coli* MazE (MazEec) and *D. radiodurans* MazE (MazE<sub>DR0416</sub>) (upper panel); pairwise alignment of *E. coli* MazF (MazFec) and *D. radiodurans* MazF (MazF<sub>DR0417</sub>) (lower panel). (b) MazF<sub>DR0417</sub>-mediated RNA cleavage. A 533-nt artificially designed RNA (RNA 500-2) was incubated with 1.2, 6, and 30 pmol of MazF<sub>DR0417</sub>. The rightmost lane included 30 pmol of MazF<sub>DR0417</sub> preincubated with 300 pmol of MazE<sub>DR0416</sub>

artificially designed RNAs 500-2 (AB610940), 1000-1 (AB610944), 1000-2 (AB610945), 1000-3 (AB610946), 1000-4 (AB610947), and 1000-5 (AB610948).

### 3 | RESULTS

#### 3.1 | MazE<sub>DR0416</sub> and MazF<sub>DR0417</sub> constitute a TA system

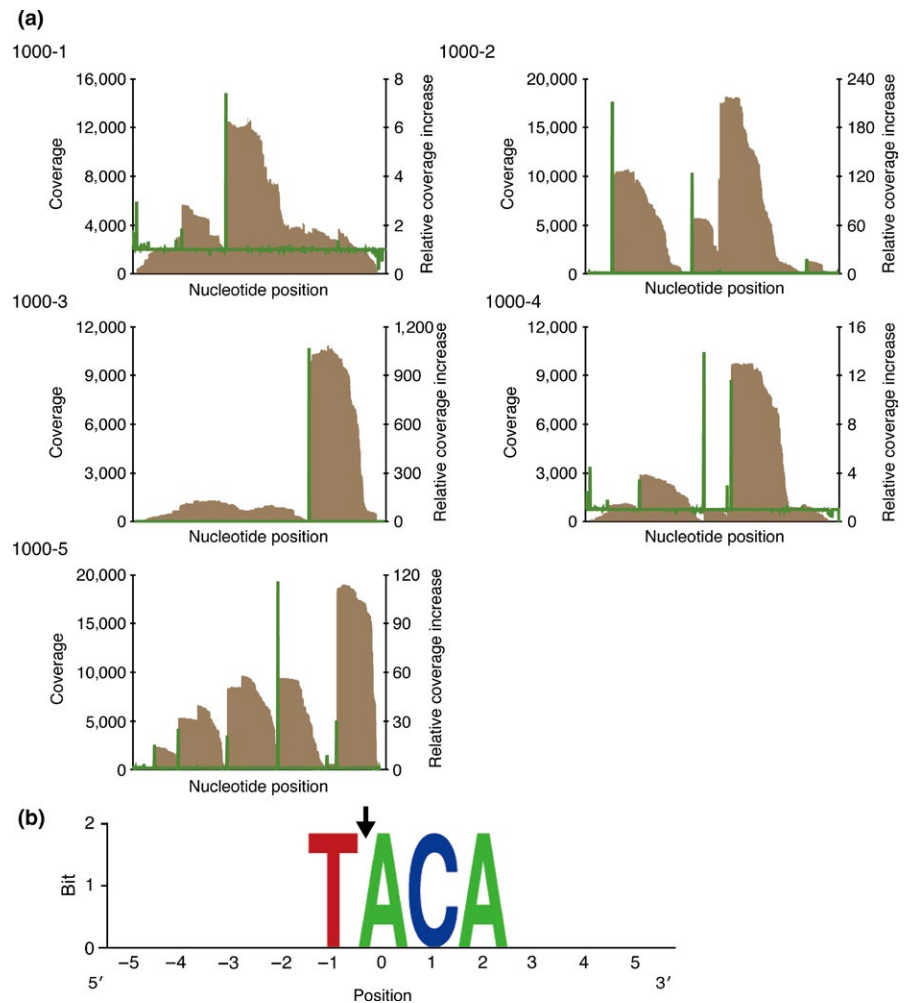
Previously, the genes located at DR0416 and DR0417 were presumed to encode a TA pair, the antitoxin MazE (MazE<sub>DR0416</sub>) and the toxin MazF (MazF<sub>DR0417</sub>) (Figure S1a), which shows 42.7% and 43.6% similarity to *E. coli* MazE and MazF, respectively (Figure 1a) (Chopra et al., 2013; Pandey & Gerdes, 2005). To briefly assess whether these components constitute a genuine TA system, we first cloned these genes into IPTG inducible vectors and expressed them in *E. coli*. As shown in Figure S1b, *E. coli* growth was inhibited when MazF<sub>DR0417</sub> was expressed. In contrast, the cell growth was restored in the case of MazE<sub>DR0416</sub> coexpression (Figure S1b, right panel). Unexpectedly, we observed MazF<sub>DR0417</sub>-mediated growth inhibition even in the absence of IPTG (Figure S1b, middle panel); this would be probably because the 'leaky' expression of MazF<sub>DR0417</sub> is toxic to the cells. We next purified the recombinant proteins to study the cleavage activity of MazF<sub>DR0417</sub>. When we investigated the purity and molecular weight of the protein by gel electrophoresis, a single peak was observed (Figure S1c, lower panel), indicating that we had obtained highly purified MazF<sub>DR0417</sub>. We next coincubated MazF<sub>DR0417</sub> with substrate RNA

(RNA 500-2), and dose-dependent RNA fragmentation was observed (Figure 1b). Furthermore, the banding patterns of cleaved RNA were unique in length (Figure 1b, lane 5), indicating that MazF<sub>DR0417</sub> is a functional toxin endoribonuclease possessing sequence specificity. Next, we purified MazE<sub>DR0416</sub> (Figure S1c, upper panel) and examined the effect of MazE<sub>DR0416</sub> on quenching of the enzymatic activity of MazF<sub>DR0417</sub>. As expected, MazF<sub>DR0417</sub>-catalyzed cleavage was abolished by preincubation with MazE<sub>DR0416</sub> (Figure 1b, lane 6). Therefore, MazE<sub>DR0416</sub> and MazF<sub>DR0417</sub> constitute an authentic TA system.

#### 3.2 | MazF<sub>DR0417</sub> recognizes specific 4-nt motifs

After confirming that *mazF*<sub>DR0417</sub> encodes an endoribonuclease, we investigated its sequence specificity using a modified RNA-seq approach recently developed in our laboratory (Miyamoto et al., 2016a). In this approach, five synthetic RNA constructs (1000-1, 1000-2, 1000-3, 1000-4, and 1000-5) were digested with MazF<sub>DR0417</sub>, and the fragmented RNA products harboring the cleavage sites at their 5'-ends were preferentially detected by identifying the nucleotide positions with increased coverage (Figure 2a), followed by extraction of the sequences located five bases upstream and five bases downstream of the identified nucleotides (Table S1).

As shown in Figure 2b, a strong consensus 4-nt motif, TACA, was observed in the analysis of the overall top 15 sequences (Table S1), suggesting that UACA is the major target of MazF<sub>DR0417</sub>. However, RNA 500-2 was degraded into many fragments (Figure 1b, lane 5), despite that it contains only two UACA sites (Table S2). Thus, we speculated



**FIGURE 2** Analysis of the cleavage sequence of MazF<sub>DR0417</sub>. (a) Graph of the coverage (brown bar) and relative coverage increase (green line). (b) Conserved sequences around the nucleotide positions with increased coverage. The nucleotide position with significant increases in coverage was set to zero. The black arrow indicates the cleavage position

that MazF<sub>DR0417</sub>, similar to other MazF homologues (Miyamoto et al., 2016b; Park, Yamaguchi, & Inouye, 2011; Schifano et al., 2014; Verma & Bhatnagar, 2014; Zhu et al., 2009), possesses suboptimal cleavage sequences with relatively weak cleavage affinities. When we next analyzed the overall top 25 sequences, UACC, UACU, and AACA were additionally detected (Table S1 and Figure S2), indicating that MazF<sub>DR0417</sub> recognizes these specific sequences with lower affinities. In all reference samples, coverage increased at the second adenine (UACA, UACC, UACU, and AACA) (Table S1). Accordingly, MazF<sub>DR0417</sub> was likely to recognize these tetrads and cleave RNAs immediately upstream of the adenine (Figures 2b and S2).

### 3.3 | MazF<sub>DR0417</sub> cleaves specific tetrads in a strict manner

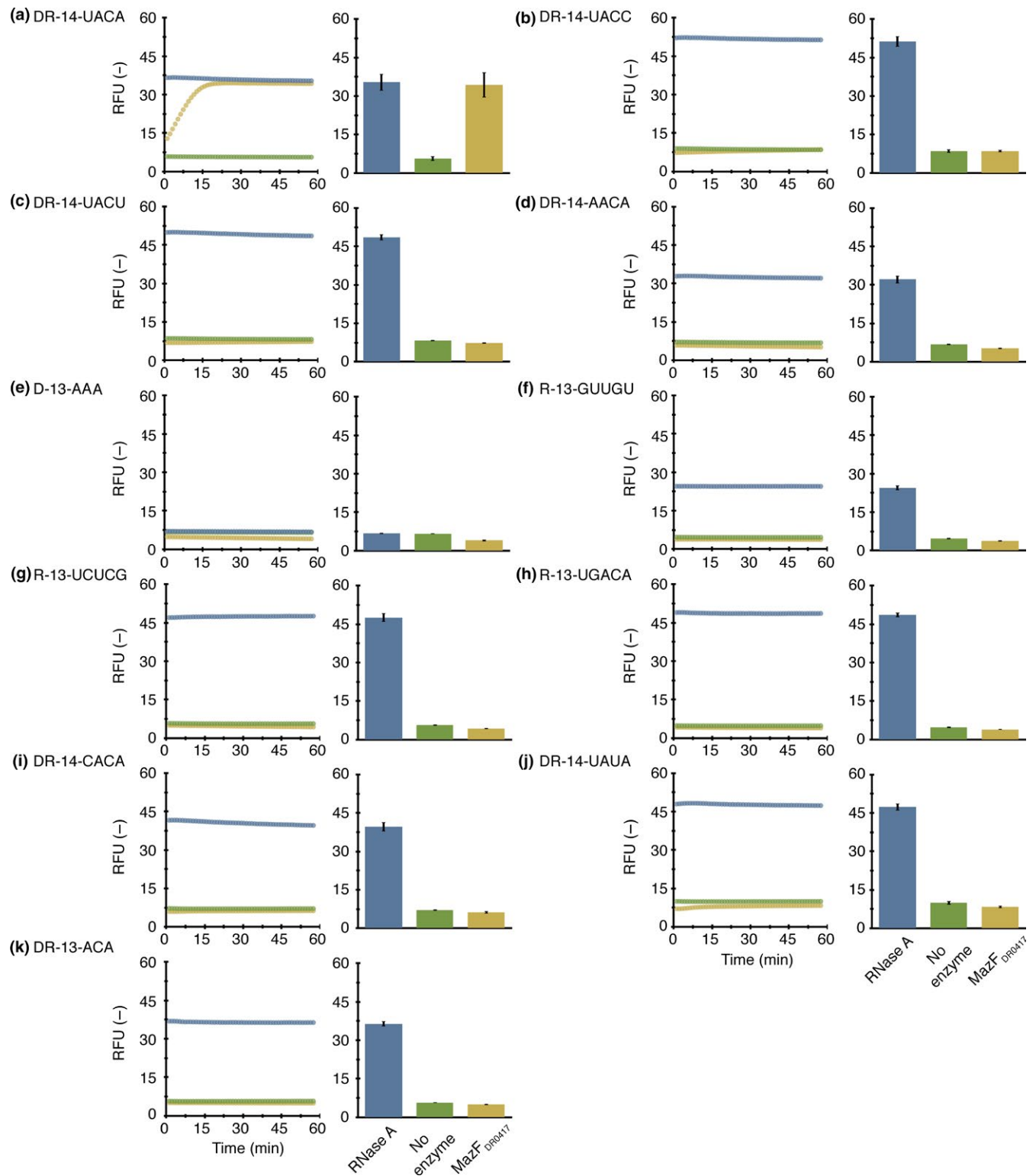
To further confirm that MazF<sub>DR0417</sub> is a sequence-specific endoribonuclease, we utilized a fluorescence quenching technique. Briefly, short oligonucleotides tagged with 6-carboxyfluorescein and black hole quencher-1 at the 5'-end and 3'-end, respectively, were treated with endoribonucleases. Because the two dyes were tethered by DNA and/or RNA nucleotides, the fluorescence of 6-carboxyfluorescein was typically quenched. However, as oligonucleotides were cut and unbound,

**TABLE 1** Sequences of fluorescently labeled oligonucleotides

Name	Sequence (5' to 3') <sup>a</sup>
DR-14-UACA	AAAAAU <u>UACA</u> AAAAA
DR-14-UACC	AAAAAU <u>UACC</u> AAAAA
DR-14-UACU	AAAAAU <u>UACU</u> AAAAA
DR-14-AACA	AAAAAA <u>ACA</u> AAAAA
D-13-AAA	AAAAAAAAAAAAA
R-13-GUUGU	<u>GUUGU</u> CAUGCCGG
R-13-UCUCG	<u>UCUCG</u> GUGCGUUG
R-13-UGACA	<u>UGACA</u> CGAACC GC
DR-14-CACA	AAAAA <u>CACA</u> AAAAA
DR-14-UAUA	AAAAAU <u>UAUA</u> AAAAA
DR-13-ACA	AAAAAA <u>ACA</u> AAAAA

<sup>a</sup>Underlined letters represent RNA nucleotides, whereas other letters represent DNA nucleotides.

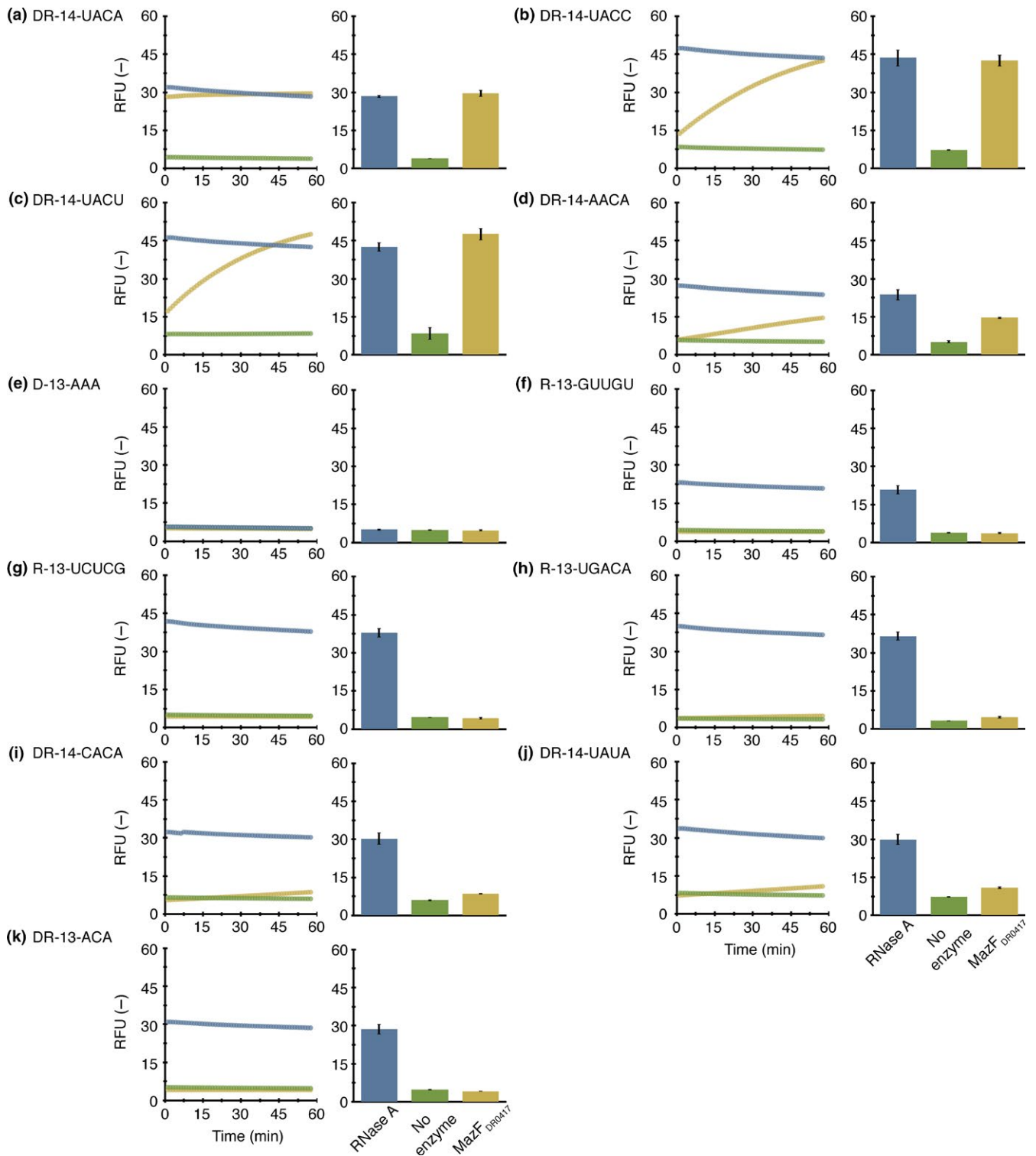
their fluorescence intensities increased because of the increasing amount of unquenched 6-carboxyfluorescein (Miyamoto et al., 2016a, 2016b; Wang & Hergenrother, 2007). We evaluated the cleavage specificity of MazF<sub>DR0417</sub> using the oligonucleotides listed in Table 1.



**FIGURE 3** Sequence-specific RNA cleavage with a small amount of MazF<sub>DR0417</sub>. Fifteen nanograms of MazF<sub>DR0417</sub> (yellow) was incubated with 10 pmol of fluorescently modified oligonucleotides: (a) DR-14-UACA, (b) DR-14-UACC, (c) DR-14-UACU, (d) DR-14-AACA, (e) D-13-AAA, (f) R-13-GUUGU, (g) R-13-UCUCG, (h) R-13-UGACA, (i) DR-14-CACA, (j) DR-14-UAUA, and (k) DR-13-ACA. In the control reactions, fluorescence intensities in the presence of 100 ng of RNase A (blue) and absence of enzymes (green) at each time point (left) and the end point (right) were measured

To examine whether UACA and three other sequences (UACC, UACU, and AACA) are the determinants of MazF<sub>DR0417</sub>-mediated RNA cleavage, we synthesized DNA-RNA chimeric oligonucleotides including corresponding RNA tetrads (DR-14-UACA, DR-14-UACC,

DR-14-UACU, and DR-14-AACA). Consistent with the RNA-seq results, DR-14-UACA was cleaved (Figures 3a and 4a). Meanwhile, the three other chimeric oligonucleotides (DR-14-UACC, DR-14-UACU, and DR-14-AACA) were cleaved less effectively, as cleavage



**FIGURE 4** Sequence-specific RNA cleavage with an excessive amount of MazF<sub>DR0417</sub>. Six hundred nanograms of MazF<sub>DR0417</sub> (yellow) was incubated with 10 pmol of fluorescently modified oligonucleotides: (a) DR-14-UACA, (b) DR-14-UACC, (c) DR-14-UACU, (d) DR-14-AACA, (e) D-13-AAA, (f) R-13-GUUGU, (g) R-13-UCUCG, (h) R-13-UGACA, (i) DR-14-CACA, (j) DR-14-UAUA, and (k) DR-13-ACA. In the control reactions, fluorescence intensities in the presence of 100 ng of RNase A (blue) and absence of enzymes (green) at each time point (left) and the end point (right) were measured

was not observed until an excessive amount of MazF<sub>DR0417</sub> was added (Figures 3b–d and 4b–d). These results reinforce that UACA is the major cleavage site of MazF<sub>DR0417</sub>.

Notably, there was no increase in fluorescence intensity in a DNA oligonucleotide (D-13-AAA) associated with MazF<sub>DR0417</sub> treatment (Figures 3e and 4e). Furthermore, none of the three RNA

oligonucleotides (R-13-GUUGU, R-13-UCUCG, and R-13-UGACA), the internal sequences of which were identical to that of the RNA substrate (RNA 1000-4) used in the RNA-seq, were cleaved by MazF<sub>DR0417</sub> (Figures 3f–h and 4f–h). Because these oligonucleotides are devoid of specific tetrads, these results support the sequence specificity of MazF<sub>DR0417</sub>.

Finally, to investigate whether MazF<sub>DR0417</sub> recognition is strict, we prepared three mutated oligonucleotides (DR-14-CACA, DR-14-UAUA, and DR-13-ACA). Because the RNA-seq results suggested that MazF<sub>DR0417</sub> cleaves RNAs at U<sup>^</sup>ACA (where <sup>^</sup> denotes the position of the cleavage), we altered the bases preceding and following the second adenine to the pyrimidine bases C and U, respectively (DR-14-CACA and DR-14-UAUA). In addition, an oligonucleotide whose first uracil was removed from the tetrad was synthesized (DR-13-ACA). In all cases, we observed complete blockage (Figures 3i–k) or reduction in cleavage (Figures 4i–k), demonstrating that the recognition of MazF<sub>DR0417</sub> is specific to some tetrads. From these data, we concluded that MazF<sub>DR0417</sub> is a four-base cutter whose prime target is UACA.

## 4 | DISCUSSION

TA systems are common in prokaryotic chromosomes and plasmids and are frequently found in multiple loci in the same organism (Chopra et al., 2013; Pandey & Gerdes, 2005; Sevin & Barloy-Hubler, 2007). They are activated under stressful conditions and they enhance bacterial stress resistance. Depending on the antitoxin nature and its manner of neutralizing toxin activity, TA systems are currently classified into six distinct classes (Page & Peti, 2016). Among these, the type II TA system, where a protein antitoxin inhibits the activity of its cognate protein toxin by forming a toxin-antitoxin complex, is one of the most widely studied classes (Schuster & Bertram, 2013). Based on the similarities in the toxin sequences, these systems are further divided into several families (i.e., MazEF, VapBC, and HigBA) (Leplae et al., 2011).

In the current study, we demonstrated that a *D. radiodurans* chromosomal TA pair encoded by the DR0416 and DR0417 loci forms a canonical type II MazEF system (Figures 1b and S1b). Furthermore, we revealed that the MazF toxin (MazF<sub>DR0417</sub>) functions as a 4-nt specific cutter and strictly recognizes the UACA tetrad (Figures 2–4). To the best of our knowledge, this is the first MazF that specifically recognizes the U<sup>^</sup>ACA sequence (Masuda & Inouye, 2017; Schifano & Woychik, 2017). Recently, Zorzini et al., (2016) determined the crystal structure of *E. coli* MazF (MazFec) in complex with the substrate analogue d(A<sup>1</sup>U<sup>2</sup>A<sup>3</sup>C<sup>4</sup>A<sup>5</sup>U<sup>6</sup>A<sup>7</sup>), and reinforced the notion that MazFec recognizes ACA triplet strictly and that MazFec cleaves the substrate at the position of <sup>^</sup>ACA and A<sup>^</sup>CA (Miyamoto et al., 2016a; Vesper et al., 2011; Zhang, Zhang, Hara, Kato, & Inouye, 2005a; Zhang et al., 2003). They mentioned that the MazFec recognition site could be divided into two different regions: first, the one where dU<sup>2</sup> is located, which is called the upstream binding site; and second, the one that accommodates d(A<sup>3</sup>C<sup>4</sup>A<sup>5</sup>U<sup>6</sup>), which is called the downstream binding groove. In

the upstream region, they observed a prominent cavity, and they reasoned that both purine and pyrimidine bases could be accommodated in the space; thus, any base could be located at the position of one-base upstream of the first A (<sup>^</sup>ACA). Furthermore, they mentioned that MazFec possesses two distinct positions of the cleavage sites (<sup>^</sup>ACA and A<sup>^</sup>CA), as the lack of hydrogen bonds between dU<sup>6</sup> in the downstream binding groove and the MazFec recognition site reduces the specificity of the cleavage position. Taken together, MazF<sub>DR0417</sub> is distinct from MazFec in the following two points: first, it recognizes the unprecedented tetrad, UACA; indeed it does not cleave ACA (Figures 3k and 4k). Second, MazF<sub>DR0417</sub> cleaves U<sup>^</sup>ACA (Figure 2b).

It was predicted that the gene encoded at DR0662 also codes for a MazF toxin (MazF<sub>DR0662</sub>) (Pandey & Gerdes, 2005). Given that these two MazF endoribonucleases show only 22.8% similarity (Figure S3), it is not surprising that they recognize distinct sequences. In fact, although the cleavage sequence and recognition length of MazF<sub>DR0662</sub> remain unknown, Shimada, Takayama, Asada, & Kato, (2011) previously suggested that purified MazF<sub>DR0662</sub> cleaved the RNA oligonucleotide lacking the UACA tetrad at the UU<sup>^</sup>CCUUU site. Therefore, the difference in cleavage specificity may be beneficial for *D. radiodurans* to enrich specific transcripts to withstand certain environmental stresses.

Sequence-specific toxin endoribonucleases have been proposed to target messenger RNAs selectively, resulting in protein-mediated RNA interference; therefore, these endoribonucleases were also referred to as “mRNA interferases” (Christensen-Dalsgaard & Gerdes, 2008; McKenzie et al., 2012; Zhang, Yamaguchi, & Inouye, 2009; Zhang et al., 2005a; Zhang, Zhu, Zhang, & Inouye, 2005b; Zhang et al., 2003; Zhu et al., 2006). However, recent studies documented that some of these endoribonucleases also target transfer RNA, enabling cells to alter their translation using a different mechanism (Cruz et al., 2015; Schifano et al., 2016). In these reports, single-stranded regions of tRNAs were cleaved by endoribonucleases, thereby blocking protein synthesis. Analysis of tRNA sequences of *D. radiodurans* revealed that the UACA sequence was located within the anticodon stem-loop of tRNA<sup>Val</sup> (Figure S4a and Table S2). Thus, MazF<sub>DR0417</sub> may also inhibit translation indirectly by inactivating the function of tRNA<sup>Val</sup> as an adaptor molecule; indeed, MazF<sub>DR0417</sub> halved the chemically synthesized tRNA<sup>Val</sup> *in vitro* (Figure S4b). Future studies are necessary to validate whether native tRNA<sup>Val</sup> is a genuine target of this enzyme.

In summary, our data showed that MazF<sub>DR0417</sub> is a functional endoribonuclease and recognizes a unique tetrad, UACA. These data indicate that this enzyme enables *D. radiodurans* to acclimate to environmental changes through direct and/or indirect growth modulation.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.



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