MazF Endoribonucleolytic Toxin Conserved in Nitrospira Specifically Cleave the AACU, AACG, and AAUU Motifs

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Abstract: MazF is an endoribonucleolytic toxin that cleaves intracellular RNAs in sequence-specific manners. It is liberated in bacterial cells in response to environmental changes and is suggested to contribute to bacterial survival by inducing translational regulation. Thus, determining the cleavage specificity provides insights into the physiological functions of MazF orthologues. Nitrospira, detected in a wide range of environments, is thought to have evolved the ability to cope with their surroundings. To investigate the molecular mechanism of its environmental adaption, a MazF module from Nitrospira strain ND1, which was isolated from the activated sludge of a wastewater treatment plant, is examined in this study. By combining a massive parallel sequencing method and fluorometric assay, we detected that this functional RNA-cleaving toxin specifically recognizes the AACU, AACG, and AAUU motifs. Additionally, statistical analysis suggested that this enzyme regulates various specific functions in order to resist environmental stresses.

Keywords: Nitrospira; toxin-antitoxin systems; MazF; sequence-specificity; RNase

Key Contribution: A MazF module from Nitrospira strain ND1 was found to be a functional RNA-cleaving toxin and its specific cleavage sites were identified.

1. Introduction

Toxin-antitoxin (TA) systems are involved in bacterial survival by regulating cell growth under various environmental stresses [1–3]. Since its discovery, dozens of TA systems have been detected on prokaryotic chromosomes and plasmids, and they have been categorized into six types [3–10]. The most common type II TA systems consist of a pair of co-regulated proteins: a toxin that inhibits cell growth by suppressing essential cellular processes and an antitoxin that acts as its cognate inhibitor [3,5]. Normally, the two proteins form a stable toxin-antitoxin complex, but when cells are exposed to environmental stresses, proteases are induced and preferential degradation of antitoxins liberate toxins that inhibit cell growth [1–3,5].
MazEF is an archetypical pair of type II TA systems that encodes an endoribonucleolytic toxin, MazF and its cognate antitoxin MazE [11,12]. The *Escherichia coli* MazF is known to contribute to cellular survival in high temperature, starvation, and antibiotics conditions [13] by cleaving intracellular RNAs in an ACA-specific manner and inducing translational regulation [14,15]. However, the physiological roles of MazF enzymes in other species remain largely unclear because their recognition sequences are different among MazF homologues [16]. Depending on the cleavage specificity, MazF may either generally degrade intracellular RNAs to reduce overall protein synthesis [11,14] or severely inhibit the expression of specific genes [17]. Thus, determining the recognition sequence and its abundance in the transcripts is the first step to predicting the physiological functions of MazF orthologues.

Previously, we detected the recognition sequences of two MazF enzymes conserved in *Nitrosomonas europaea*, a representative ammonia-oxidizing bacterium, and performed statistical analysis to predict their main regulation targets [18,19]. The results suggested that one of the MazF enzymes improves heavy metal resistance by leaving the related transcripts uncleaved [18], while another downregulates cellular activities by digesting mRNAs essential to ammonia oxidation and carbon fixation [19]. As the other crucial contributor to complete nitrification, the environmental adaption of nitrite-oxidizing bacteria (NOB) is also of interest. Among all known NOB, the genus *Nitrospira* has been recognized as the most diverse and widespread [20]. Through recent genome-based studies, *Nitrospira* has been found to possess great metabolic versatility and is thought to have evolved the ability to cope with a wide range of environmental stresses [21,22].

In this study, we focused on a MazF module from *Nitrospira* strain ND1 isolated from the activated sludge of a wastewater treatment plant [23] and characterized by whole genome sequence analysis [24]. The specific cleavage sites of the endoribonuclease were detected by using a combination of massive parallel sequencing and fluorometric analysis. Based on the results, the main regulation targets were predicted.

2. Results

2.1. Identifying the TA System in *Nitrospira* Strain ND1

Using RASTA-Bacteria (Rapid Automated Scan for Toxins and Antitoxins in Bacteria), an automated method for identifying TA modules [25], we predicted RNA cleaving TA systems such as MazEF, VapBC, and HigBA in *Nitrospira* strain ND1 genome. Because MazF is known to target mRNA, in addition to tRNA and rRNA [16], MazF may have a broader effect on protein expression as compared to other TA systems. In this study, we focused on the genes located at NSND_50520 and NSND_50521, which are predicted to encode a MazEF pair (hereafter designated as MazF-nd1 and MazE-nd1, respectively) (Figure 1a). To determine whether MazF-nd1 encodes a functional toxin, we expressed MazF-nd1 in *E. coli* and observed clear growth inhibition (Figure S1). Thus, we subsequently obtained both recombinant proteins (Figure 1b) and treated them with synthesized RNA (Figures 1c,d). In reactions in which MazF-nd1 was incubated with the RNA, dose-dependent RNA fragmentation was observed, indicating that MazF-nd1 functions as an endoribonuclease (Figure 1c, lanes 3–5). Because the cleavage pattern was different from that of *E. coli* MazF (MazF-ec), MazF-nd1 is presumed to recognize unique RNA motifs other than ACA (Figure 1c, lanes 7–9). Moreover, when MazE-nd1 was added to the MazF-nd1 cleaving reaction, RNA degradation was inhibited in a MazE-nd1 concentration-dependent manner (Figure 1d, lanes 3–5). Taken together, these results demonstrated that MazE-nd1 and MazF-nd1 constitute an authentic MazEF system.
Figure 1. (a) Genetic context of MazEF-nd1. Locus tags with sizes and distance are shown; (b) molecular weight and purity of obtained MazE-nd1 and MazF-nd1, whose theoretical molecular weights are 10.8 and 13.4 kDa, respectively. Lanes 1 and 3, ladder; lane 2, MazE-nd1; lane 4, MazF-nd1; (c) enzymatic activity of MazF-nd1. Synthesized 533-nt RNA (500-2) was used as a substrate. Lane 1, ladder; lanes 2 and 6, control reactions with no enzymes; lanes 3–5, 0.373, 3.73, and 18.6 pmol of MazF-nd1, respectively, was added; lanes 7–9, 0.250, 1.25, and 6.25 U of E. coli MazF (MazF-ec), respectively, was added for comparison; (d) enzymatic activity of MazE-nd1. Synthesized 533-nt RNA (500-2) was used as a substrate. Lane 1, control reaction with no enzyme; lane 2, 5 pmol of MazF-nd1 was added; lanes 3–5, 1, 5, and 25 pmol of MazE-nd1, respectively, was added with 5 pmol of MazF-nd1; lane 6, 25 pmol of MazE-nd1 was added; lane 7, ladder.

2.2. Estimating MazF-nd1 Cleavage Sites

An RNA-sequencing approach, previously developed in our laboratory [26], was used to detect the candidate recognition sequences of MazF-nd1. Five synthetic RNAs (1000-1, 1000-2, 1000-3, 1000-4, and 1000-5) were digested by MazF-nd1 and a unique 45-nucleotide barcode RNA was ligated to the digested 5′-end of the fragments. The MazF-cleaved sites were detected by specifically mapping the barcode-ligated reads and identifying the nucleotide positions with high relative coverage increases; the value was defined as the coverage of the position divided by the coverage of a former position (Figure 2a). We selected 50 nucleotides showing the largest relative coverage increases (Table S1). These nucleotides and their nearby sequences were analyzed to determine the nucleotide frequency at each position using WebLogo [27]. Four-base motifs, AAUU and AACU, were distinctively extracted as potential recognition sequences (Figure 2b). Furthermore, MazF-nd1 is likely to cleave those sequences between the two adenines because the coverage increased significantly at the second A-residue (Figure 2b).
2.3. MazF-nd1 Recognizes RNA at AACU, AACG, and AAUU Sequences

In the 50 sequences selected from our sequencing results (Table S1), only 9 and 15 sequences contained AAUU and AACU around the most increased adenine, respectively, while the remaining sequences contained AAUU-or-AACU-like motifs that differed in either the first or last base, such as AAUC and UACU. Since some MazF homologues were found to possess more than one cleavage sequence with different affinities [28,29], we performed a fluorometric assay [30] using a set of short DNA/RNA chimeric oligonucleotides, which does not form a strong secondary structure, to determine the main recognition sequences from among the candidate sequences listed in Table 4. Because both ends of these probes were tagged with a pair of dyes where fluorescence resonance energy transfer occurs, fluorescence intensity increases when the oligonucleotides are cleaved, and the two dyes become separated. As predicted from the RNA-sequencing results, the fluorescence intensity rapidly increased when the probes containing AAUU and AACU were treated with MazF-nd1 (Figure S2a,c). The reaction of the AACG-containing probe showed a similar increase (Figure S2d). The fluorescence intensities of other reactions were also increased to different degrees (Figure S2b,d), suggesting that MazF-nd1 possesses numbers of suboptimal cleavage sequences. The probes were cleaved by MazF-nd1 specifically at the RNA motifs because changes in fluorescence intensity was not detected when probes containing only DNA or RNA without any of the potential cleavage sequences were used as the substrates (Figure S2e).
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To compare all reactions quantitatively, we first converted the fluorescence data into percentages by considering the average fluorescence intensity of RNase-treated reactions as 100% and those of reactions with no enzyme as zero. Next, the percentage fluorescence intensity of each reaction was fitted to an integrated rate equation to calculate the initial reaction velocities (Figure 3). As shown in Table 1, reactions with AACU, AACG, and AAUU sequences showed higher initial reaction velocities than reactions with other similar sequences by at least fivefold. Taken together, our results clearly demonstrate that MazF-nd1 is an active ribonuclease that cleaves RNA in a sequence-specific manner at AACU, AACG, and AAUU.

![Figure 3. Percentage fluorescence intensity fitted to an integrated rate equation.](image)

**Table 1.** Initial reaction velocity with each oligonucleotide.

| Sequence 1 | Occurrence in Substrate RNA 2 | Initial Reaction Velocity 3 | $F_{\text{max}}$ 3 | $k$ 3 |
|------------|------------------------------|----------------------------|---------------------|-------|
| AACU       | 17                           | 6.421                      | 85.67               | $7.50 \times 10^{-2}$ |
| AACG       | 24                           | 5.669                      | 84.23               | $6.73 \times 10^{-2}$ |
| AAUU       | 12                           | 5.652                      | 84.36               | $6.70 \times 10^{-2}$ |
| AAUG       | 21                           | 0.998                      | 74.66               | $1.34 \times 10^{-2}$ |
| UACU       | 32                           | 0.701                      | 58.59               | $1.20 \times 10^{-2}$ |
| UAUU       | 11                           | 0.697                      | 62.44               | $1.12 \times 10^{-2}$ |
| AAUC       | 28                           | 0.649                      | 75.43               | $8.61 \times 10^{-3}$ |
| AAUA       | 20                           | 0.509                      | 59.29               | $8.58 \times 10^{-3}$ |
| AACA       | 21                           | 0.427                      | 53.82               | $7.93 \times 10^{-3}$ |
| CAUU       | 20                           | 0.423                      | 53.20               | $7.95 \times 10^{-3}$ |
| GACU       | 15                           | 0.421                      | 65.96               | $6.38 \times 10^{-3}$ |
| AACG       | 28                           | 0.410                      | 49.96               | $8.21 \times 10^{-3}$ |
| CACU       | 17                           | 0.398                      | 58.92               | $6.75 \times 10^{-3}$ |
| GAUU       | 24                           | 0.390                      | 63.03               | $6.19 \times 10^{-3}$ |

1 Underlined quartets were detected in the RNA-sequencing (see Discussion). 2 Number of sequences contained in the five substrate RNAs (1000-1 to 1000-5) that are used in the RNA-sequencing. 3 Initial reaction velocity are calculated by multiplying $F_{\text{max}}$ with $k$ (see Materials and Methods).
2.4. Analysis of Intracellular Targets of MazF-nd1

Determining the recognition sequence of MazF enabled estimation of its intracellular targets and the molecular behavior of Nitrospira strain ND1 under environmental stresses. Accordingly, we evaluated the potential effects of MazF-nd1 on Nitrospira strain ND1 based on the probability of the AACU, AACG, and AAUU sequences existing in all 4624 coding sequences (CDS) (Table S2). As described previously [31], the parameter $P$ shows a smaller value when $K$, which is the actual number of the recognition sequences in a gene, is significantly larger than $E$, which is the mathematically calculated number. To estimate the intracellular targets of MazF-nd1, 25 protein-coding sequences with the smallest $P$ values were analyzed (Table 2). Although 9 of the 25 genes were not annotated for Nitrospira, some of the listed genes encode notable proteins. For example, FumC, ranked third, is recognized as a tricarboxylic acid (TCA) cycle enzyme stimulated by iron limitation [32,33], and proteins of TonB-dependent transporter, which is a well-known iron transporter, were ranked 11, 17, and 25 [34]. Because iron is an important cofactor for nitrite oxidoreductase, a key enzyme in NOB that oxidizes nitrite to nitrate and shuttles electrons into the respiratory chain [20], downregulation of the genes encoding TonB-dependent receptor may cause inhibition of overall cellular processes.

Transcripts without recognition sequences are considered to be tolerant to MazF [28,31]. Thus, we extracted genes lacking MazF-nd1-recognition sequences. Here, 201 CDS were detected but only 18 were annotated (Table 3). Interestingly, a mercuric transport protein, extracted as a MazF-tolerant gene in N. europaea, one of the most well-investigated nitrifiers [18], was detected. These results suggest that the two MazF orthologues from different nitrifiers may commonly improve heavy metal resistance.

### Table 2. Top 25 MazF-nd1-sensitive genes in Nitrospira strain ND1 genome.

| Rank | Locus Tag | Product                                      | Length (bp) | $K$   | $E$  | $P$  |
|------|-----------|----------------------------------------------|-------------|-------|------|------|
| 1    | 60124     | Protein of unknown function                   | 1980        | 38    | 15.66| $1.11 \times 10^{-6}$ |
| 2    | 50240     | Conserved protein of unknown function         | 3540        | 44    | 22.66| $4.30 \times 10^{-5}$ |
| 3    | 50464     | FumC: fumarate hydratase (fumarase C), aerobic class II | 1455        | 29    | 12.96| $7.75 \times 10^{-5}$ |
| 4    | 62020     | Conserved protein of unknown function         | 3270        | 43    | 23.02| $1.20 \times 10^{-4}$ |
| 5    | 60419     | IspU: undecaprenyl pyrophosphate synthase     | 786         | 16    | 5.99 | $4.71 \times 10^{-4}$ |
| 6    | 63509     | Conserved protein of unknown function         | 5319        | 53    | 32.37| $5.16 \times 10^{-4}$ |
| 7    | 50535     | Sensory response regulator with diguanylate cyclase domain | 2127        | 32    | 16.78| $5.75 \times 10^{-4}$ |
| 8    | 61480     | Conserved protein of unknown function         | 2316        | 37    | 20.91| $8.77 \times 10^{-4}$ |
| 9    | 63396     | YaeT: putative outer membrane protein assembly factor | 2325        | 36    | 20.29| $9.74 \times 10^{-4}$ |
| 10   | 62925     | Sigma-54 dependent transcriptional regulator (Modular protein) | 2601        | 33    | 18.10| $1.00 \times 10^{-3}$ |
| 11   | 61386     | TonB-dependent siderophore receptor           | 2334        | 34    | 19.27| $1.45 \times 10^{-3}$ |
| 12   | 62179     | Conserved exported protein of unknown function | 507         | 11    | 3.69 | $1.45 \times 10^{-3}$ |
13  50325  FlgG: flagellar component of cell-distal portion of basal-body rod  792  16  6.71  1.50 \times 10^{-3} \\
14  50347  Protein of unknown function  5361  59  38.96  1.59 \times 10^{-3} \\
15  63512  Conserved protein of unknown function  3252  31  17.19  1.67 \times 10^{-3} \\
16  60586  Exported protein of unknown function  1371  23  11.52  1.77 \times 10^{-3} \\
17  61395  Putative TonB-dependent receptor  2262  35  20.30  1.80 \times 10^{-3} \\
18  60858  ThiI: putative tRNA sulfurtransferase  1179  16  6.90  2.10 \times 10^{-3} \\
19  61921  Putative regulatory protein, MerR family, cobalamin B12-binding efflux transporter, outer membrane factor lipoprotein  1521  23  11.69  2.33 \times 10^{-3} \\
20  63116  HyfR: hydrogenase-4 transcriptional activator  2043  29  16.12  2.50 \times 10^{-3} \\
21  62910  Putative di-haem cytochrome c  2076  28  15.46  2.86 \times 10^{-3} \\
22  63252  RhlE: ATP-dependent RNA helicase  1353  18  8.49  2.89 \times 10^{-3} \\
23  61398  Putative TonB-dependent receptor  2325  35  20.92  2.89 \times 10^{-3} \\

1 \ K \text{ represents the actual number of the recognition sequences in a gene.}  \\
2 \ E \text{ represents the mathematically calculated number of the recognition sequences in a gene.}  \\
3 \ P \text{ is the parameter used to estimate the intracellular targets of MazF-nd1 (see Materials and Methods).}

Table 3. Eighteen annotated MazF-nd1-tolerant genes in *Nitrospira* strain ND1 genome.

| Locus Tag | Product | Length (bp) |
|-----------|---------|-------------|
| 50353     | FlgG: flagellar protein | 372         |
| 50758     | Putative nucleotidyltransferase (fragment) | 183         |
| 50957     | Insertion element ISR1 uncharacterized 10 kDa protein A3 (fragment) | 159         |
| 60210     | TadA: tRNA-specific adenosine deaminase | 489         |
| 60360     | RcnA: putative nickel/cobalt efflux system | 708         |
| 60698     | AcyP: acylphosphatase | 348         |
| 60976     | Bfr: bacterioferritin, iron storage and detoxification protein | 480         |
| 60984     | MreD: putative cell shape-determining protein | 501         |
| 61420     | Nqo: NADH-quinone oxidoreductase chain 10 | 513         |
| 61675     | MerT: mercuric transport protein | 381         |
| 61748     | Putative membrane protein insertion efficiency factor (modular protein) | 264         |
| 62137     | K⁺-transporting ATPase, F subunit (fragment) | 90          |
| 62252     | RbfA: ribosome-binding factor A | 399         |
| 62630     | Cytochrome bd-type quinol oxidase, subunit 2 | 1017        |
| 62661     | AtpE: ATP synthase subunit c | 231         |
| 62937     | YitW: MIP18 family protein | 324         |
| 63168     | TatA: Sec-independent protein translocase protein | 294         |
| 63277     | Sulfate-binding protein (fragment) | 492         |

3. Discussion

Nitrifying bacteria are generally sensitive to environmental fluctuations and easily enter a dormant state. Previously, we showed the MazF endoribonucleases (referred to as MazFNE1181 and MazFne1 in the previous papers) in *N. europaea* are functional growth regulators, and their sequence-specificities may allow *N. europaea* to alter its translation profile and survive under certain stressful conditions.
conditions [18,19]. MazF-nd1 shares only 34.2% and 23.5% identities with MazFne1B1 and MazFne1,
respectively (Figure S3). Thus, we hypothesized that MazF-nd1 might possess a unique cleavage
sequence and could have unique physiological roles.

In the present study, we employed a combination of massive parallel sequencing and
fluorometric assays to determine the cleavage sequences of MazF-nd1. Although sequence logo
analysis predicted that AACU and AAUU are the main targets (Figure 2b), six other sequences were
also detected through massive parallel sequencing (Table S1 and Table 1). Hence, we compared the
initial reaction velocities quantitatively using fluorometric assays, and revealed that AACC also
serves as a determinant of MazF-nd1, in addition to AACU and AAUU (Table 1 and Figure 3). The
reason why the AACC motif was not recovered by sequencing logo analysis remains to be fully
clarified but one possible explanation is that MazF-mediated-RNA-cleavage may have been hindered
by secondary structures in the long substrate RNAs used in the sequencing-based assay [35]. It is
further important to recognize that the magnitude of relative coverage increase may not always
 correlate with MazF cleavage-activity, because the value is simply defined as the coverage of the
position divided by the coverage of a former position (see Materials and Methods).

It is well known that MazF homologues are widespread across the bacterial and archaebal
domains [36]. They cleave single-stranded RNAs at specific three to seven base motifs, thereby
modulating translation through degradation of intracellular RNA pools or silencing of specific
transcripts (Table S3) [11,15,17–19,26,28,29,31,37–45]. Here, we demonstrated that MazF-nd1 cuts
AACU, AACC, and AAUU motifs. To the best of our knowledge, MazF-nd1 is the first
endoribonuclease that strongly recognizes the three specific tetrads. Interestingly, Legionella
pneumophila MazF (MazF-lp) also cleaves AACU [39], despite the fact that it only shows 36.0% identity (Figure S4), and the reason for this remains to be elucidated.

Bacteria such as N. europaea, Deinococcus radiodurans, and Mycobacterium tuberculosis harbor
multiple MazF endoribonucleases with different sequence-specificities [16,18,19,29,41–44],
suggesting functional diversity of MazF homologues even within a single bacterial species or
genome. Notably, RASTA-Bacteria predicted that the gene encoded at NSND_63228 in Nitrospira
strain ND1 genome also codes for a MazF toxin (MazF-nd2). Given that the two MazF proteins share
only 24.1% identity (Figure S5), it appears that they possess distinct RNA cleavage specificities and
physiological roles. Here, we have failed to construct an expression vector encoding the mazF-nd2
gene (stop codons were repeatedly inserted into its open reading frame, probably due to the high
toxicity of MazF-nd2; data not shown). As such, it could be hypothesized that Nitrospira strain ND1
might utilize both MazEF pairs depending on the surroundings, where MazF-nd1 could be involved
in reversible growth arrest rather than programmed cell death. Further studies are warranted to
understand how the MazEF pairs regulate translation and benefit Nitrospira under stressful situations.

4. Conclusion

In conclusion, we found that a MazF module in the Nitrospira strain ND1 is a functional RNA-
cleaving toxin that specifically recognizes the AACU, AACC, and AAUU motifs. We predicted its
intracellular targets; our results suggest that this enzyme regulates various specific functions to resist
environmental stresses. Our findings provide a foundation for further studies of the environmental
adaptation of Nitrospira.

5. Materials and Methods

5.1. Plasmids, RNA, and Oligonucleotides

Plasmids pET21a-mazE-nd1 and pET21a-mazF-nd1 were purchased from GenScript (Tokyo,
Japan). The mazE-nd1 and mazF-nd1 genes were codon-optimized to improve their translation
efficiency in E. coli. Synthetic RNAs 500-2, 1000-1, 1000-2, 1000-3, 1000-4, and 1000-5 were prepared
as described in a previous study [26]. The 45-nt barcode RNA (GCUGA UGGCG AUGAA UGAAC
ACUGC GUUUG CUGGC UUGA UGAAA) was purchased from Japan Bio Services (Saitama,
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Japan). Fluorescence-labeled oligonucleotides, shown in Table 4, were purchased from Japan Bio Services and BEX (Tokyo, Japan).

Table 4. Oligonucleotide sequences used in fluorometric assay.

| Name     | Sequence                  |
|----------|---------------------------|
| DR-14-AAUU | aaaaaAAUaaaaa            |
| DR-14-UAUU | aaaaaUAUaaaaa            |
| DR-14-GAUU | aaaaaGAUaaaaa            |
| DR-14-CAUU | aaaaaCAUaaaaa            |
| DR-14-AAUA | aaaaaAAUaaaaa            |
| DR-14-AAUG | aaaaaAAUGaaaaa           |
| DR-14-AAUC | aaaaaAAUCaaaaa           |
| DR-14-ACGU | aaaaaACGUaaaaa           |
| DR-14-CACU | aaaaaCACUaaaaa           |
| R-13-GUUGU | GUUGUCAUGCCG             |

1 All sequences are shown from 5′ to 3′. Uppercase letters represent RNA nucleotides, while lowercase letters represent DNA nucleotides. For all oligonucleotides, 6-carboxyfluorescein was attached at the 5′-end and black hole quencher-1 was attached at the 3′-end.

5.2. In Vivo Toxicity of MazF-nd1

The pET21a-mazF-nd1 and pET21c plasmids were introduced into E. coli BL21 (DE3) (BioDynamics Laboratory, Tokyo, Japan), which were then cultivated at 37 °C overnight on LB plates containing 100 µg/mL ampicillin. These cells were then pre-cultivated for 12 h in LB medium containing 100 µg/mL ampicillin. The pre-cultivated cells were streaked on LB plates containing 100 µg/mL ampicillin with or without 100 µM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and were incubated at 37 °C overnight.

5.3. Protein Expression

The pET21a-mazE-nd1 and pET21a-mazF-nd1 plasmids were introduced into E. coli BL21 (DE3) (BioDynamics Laboratory) and were cultivated at 37 °C overnight on LB plates containing 100 µg/mL ampicillin. These cells were then pre-cultivated overnight in LB medium containing 100 µg/mL ampicillin and inoculated into 1 L of ampicillin supplemented LB medium. One millimolar of IPTG was added to induce MazE-nd1 and MazF-nd1 when the OD600 values exceeded 5.0. After 3.5 h of incubation, the cells were harvested by centrifugation at 9200 × g and then stored at −80 °C until use.

5.4. Protein Purification

E. coli cells containing MazE-nd1 and MazF-nd1 were thawed on ice and suspended in 18 mL of binding buffer (20 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl, 0.05% Triton X-100, 5 mM β-mercaptoethanol, and 40 mM imidazole). The cells were lysed by sonication and collected by centrifugation at 8900 × g. The supernatant was then filtered through a 0.45-µm membrane (Millex, Darmstadt, Germany) and applied to 1 mL His-Trap FF column (GE Healthcare, Little Chalfont, UK). Non-specifically bound proteins were removed by washing with 32 column volumes (cv) of binding buffer using AKTA pure 25 (GE Healthcare). Hexa-histidine-tagged MazE-nd1 and MazF-nd1 were selectively eluted by increasing the concentration of elution buffer (20 mM sodium phosphate buffer.
(pH 8.0), 300 mM NaCl, 0.05% Triton X-100, 5 mM β-mercaptoethanol, and 500 mM 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 performing sodium dodecyl sulfate polyacrylamide gel electrophoresis and protein concentration was determined using a Protein Assay (Bio-Rad, Hercules, CA, USA).

5.5. Enzymatic Activity of MazF-nd1 and MazE-nd1

To test the enzymatic activity of MazF-nd1, 0.373, 3.73, or 18.6 pmol of MazF-nd1 or 0.250, 1.25, or 6.25 U of E. coli MazF (Takara, Shiga, Japan) was added to 200 ng of RNA 500-2. Five pmol of MazF-nd1 was first pre-incubated with 1, 5, or 25 pmol of MazE-nd1 at 25 °C for 15 min. As a control reaction, 5 pmol of MazF-nd1 and 25 pmol of MazE-nd1 was pre-incubated solely at 25 °C for 15 min. Next, 50 ng of RNA 500-2 was added to each sample. All samples were incubated at 37 °C for 1 h in a total of 30 µL of MazF reaction buffer (20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 0.01% Triton X-100, and 4 U of recombinant RNase inhibitor (Takara)). RNAs were purified with RNA Clean and Concentrator™-5 (Zymo Research, Irvine, CA, USA) and then incubated with gel loading buffer II (Ambion, Austin, TX, USA) at 95 °C for 5 min. Samples were separated on 10% polyacrylamide gel containing 7 M urea. RNA was stained using SYBR Gold (Life Technologies, Carlsbad, CA, USA) and detected using a Typhoon 9210 imager (GE Healthcare).

5.6. Cleavage Sequence Identification

The cleavage sequence of MazF-nd1 was identified using a previously developed sequencing method [26]. In this study, 300 ng of MazF-nd1 and a 1.5 µg of mixture of five synthetic RNAs—1000-1, 1000-2, 1000-3, 1000-4, and 1000-5 were incubated at 37 °C for 1 h in MazF reaction buffer. Sequencing was performed using the MiSeq platform (Illumina, San Diego, CA, USA), and data were analyzed using CLC Genomics 10.1.1 (Qiagen, Venlo, The Netherlands). Barcode-ligated reads were extracted as describe in a previous report [26], and were mapped against the references using the following parameters: match score, mismatch cost, insertion cost, and deletion cost equal 3; length and similarity fraction both equal 1. Nucleotide positions with coverage less than 5000 were excluded from analysis, and 50 nucleotide positions showing the highest relative coverage increases, which is defined as the coverage of the position divided by the coverage of a former position, were selected. Sequences from 5 bp upstream to 5 bp downstream of these positions were extracted and aligned using WebLogo [27]. The sequencing dataset was deposited into the DDBJ Sequence Read Archive under the accession number DRA008257.

5.7. Fluorometric Detection of MazF-nd1 Activity

Forty picomoles of MazF-nd1 was added to 10 pmol of fluorescent-labeled oligonucleotides in a total volume of 20 µL. Additionally, a reaction with 100 ng of RNase A (Novagen, Darmstadt, Germany) was also performed as a positive control. All samples were incubated at 37 °C in MazF reaction buffer. Fluorescence intensity was recorded every 80 s using a Light Cycler 480 system (Roche, Basel, Switzerland) with 483 nm excitation and 533 nm detection filters. All data were collected in triplicate and the average was calculated.

The fluorescence intensity was converted into a percentage by considering the average fluorescence intensity of RNase-treated reactions as 100% and that of reactions with no enzyme as zero. The percentage fluorescence intensity of each reaction was fitted to the integrated rate equation (Equation 1):

\[ F(t) = F_{\text{max}}[1 - \exp(-kt)] \]  

where \( F(t) \) was the fluorescence intensity at time \( t \); \( F_{\text{max}} \) was the presumed maximum fluorescence intensity; and \( k \) was the observed rate constant. The cleavage activity of MazF-nd1 for each sequence was calculated as the initial reaction velocity by multiplying \( F_{\text{max}} \) by \( k \) following the derivative (Equation 2):
\[ F'(0) = F_{\text{max}} k \]  

Data analysis was conducted with KaleidaGraph 4.5.0 (Synergy Software, Reading, PA, USA).

5.8. Analyzing the Frequency of MazF-nd1 Cleavage Sites in Nitrospira Strain ND1 Genome

All 4624 protein-coding sequences from Nitrospira strain ND1 were obtained from the NCBI database as of 18 March 2019. According to a previous study [31], \( P \) was determined using the following equation (Equation 3):

\[ P = \sum_{i=k}^{L-3} p^i (1 - p)^{L-3-i} \frac{(L - 3)!}{i!(L - 3 - i)!} \]  

In this equation, \( p \) is the probability of either AACU, AACG, or AAUU appearing in a Nitrospira gene, which was calculated as the sum of (percentage of A) \(^2 \times\) (percentage of C) \(\times\) (percentage of U + percentage of G) and (percentage of A) \(^2 \times\) (percentage of U) \(^2\). \( L \) represents the length of the CDS, \( K \) is the actual number of recognition sequences in the CDS, and \( E \) is the expected number of recognition sequences in the CDS, calculated as \( p(L - 3) \).

5.9. Accession Numbers

The GenBank accession numbers were as follows: Nitrospira strain ND1 (FWEX01000010-FWEX01000006, MazEF-nd1 encoded in FWEX01000005), mazF-nd1 (SLM42134), mazE-nd1 (SLM42135), 500-2 (AB610940), 1000-1 (AB610944), 1000-2 (AB610945), 1000-3 (AB610946), 1000-4 (AB610947), and 1000-5 (AB610948).

Supplementary Materials: The following are available online at www.mdpi.com/2072-6651/12/5/287/s1: Figure S1: Toxicity of MazF-nd1, Figure S2: Sequence-specific RNA cleavage with MazF-nd1, Figure S3: Pairwise alignment of the MazF sequences, Figure S4: Pairwise alignment of MazF-nd1 and MazF-lp, Table S1: Fifty most frequent nucleotide sequences selected from all five references, Table S2: Analysis of intracellular targets of MazF-nd1, Table S3: Cleavage specificities of MazF homologues.

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