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***Bacillus subtilis* MazF-bs (EndoA) is a UACAU-specific mRNA interferase**

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Abstract

MazF is an mRNA interferase which cleaves mRNAs at a specific sequence. Here, we show that in contrast to MazF-ec from *Escherichia coli*, which specifically cleaves ACA sequences, MazF-bs from *Bacillus subtilis* is an mRNA interferase that specifically cleaves a five-base sequence, UACAU. MazF homologues widely prevailing in Gram-positive bacteria were found to be highly homologous to MazF-bs, suggesting that they may also have similar cleavage specificity. This cleavage site is over-represented in the *B. subtilis* genes associated with biosynthesis of secondary metabolites, suggesting that MazF-bs may be involved in the regulation of the production of secondary metabolites.

Keywords

MazF; EndoA; YdcE; Toxin-antitoxin system; *Bacillus subtilis*; mRNA interferase

1. Introduction

MazF is an mRNA interferase originally discovered in *Escherichia coli* as a toxin that specifically cleaves intracellular mRNAs at ACA sequences [1]. In normally growing cells, MazF-ec (MazF from *E. coli*) forms a stable complex with its cognate antitoxin, MazE-ec, however, under stress conditions, unstable MazE-ec is preferentially degraded to release free MazF-ec in the cells, which then cleaves cellular mRNAs to inhibit protein synthesis, leading to growth arrest [2]. Since the discovery of MazF-ec, a number of MazF homologues and other mRNA interferases with different mRNA cleavage specificities have been found [3]. *Mycobacterium tuberculosis* has at least seven MazF homologues (MazF-mt1 to -mt7), four of which (MazF-mt1, -mt3, -mt4, and -mt6) cause cell growth arrest when induced in *E. coli*. MazF-mt1 and MazF-mt6 cleave mRNAs at UAC triplet sequences and at U-rich regions, respectively [4]. Furthermore, MazF-mt3 cleaves RNAs at UU[^]CCU or CU[^]CCU, while MazF-mt7 cleaves at U[^]CGCU ([^] indicates the cleavage site) [5]. These pentad sequences are significantly underrepresented in several genes, including members of the PE and PPE families, large families of proteins that play a role in *tuberculosis* immunity and pathogenesis. It has also been demonstrated that a MazF homologue from *Myxococcus*

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xanthus (MazF-mx) is essential for the programmed cell death of this developmental bacterium during the fruiting body formation [6]. MazF-ec has been shown to be toxic when induced in mammalian cells, causing Bak-dependent programmed cell death [7]. Recently, we reported that CD4⁺ cells carrying the *E. coli mazF* gene under the Tar promoter from HIV-1 acquires resistance to HIV-1 infection [8].

Staphylococcus aureus contains one MazF homologue, MazF-sa, which has been shown to cleave mRNA at VUUV' (V and V' are A, C, or G) [9,10]. However, MazF-sa was found to have more stringent cleavage specificity and cleaves mRNAs at UACAU sequences. The 3.5 kb MS2 phage RNA was used as a substrate. As MazF and its homologues cleave single-stranded RNAs and the MS2 phage RNA contains few secondary structures, the reaction was carried out in the presence of CspA, an RNA chaperone, which prevents the formation of secondary structures in RNA [11]. *Bacillus subtilis* also contains a MazF homologue, MazF-bs (EndoA) [12]. The crystal structure of MazF-bs has been determined [13], which is very similar to those of *E. coli* MazF-ec [14] and Kid [15] proteins. As seen from the crystal structure, MazF-bs forms a homo-dimer with each monomer containing two α -helices and seven β -strands.

The consensus cleavage sequence for MazF-bs was determined previously to be UAC [12,16] with the use of a 375-base RNA containing the *thrS* leader region [16]. The identity and homology between MazF-bs and MazF-sa are 62.8 and 79.3%, respectively, while those between MazF-bs and MazF-ec are only 18.3 and 40.5%, respectively. Notably, MazF-sa specifically cleaves RNAs at a pentad sequence, UACAU, and its induction in *E. coli* only slows the growth as opposed to the complete inhibition of cell growth caused by MazF-ec (data not shown). Previously it has been shown that a 5-base cutter such as MazF-mt3 could be expressed in *E. coli* without co-expression of its cognate antitoxin since a 5-base cutter is much less toxic than a 3-base cutter [5]. Therefore, here, we reexamine the cleavage site specificity of MazF-bs and how the MazF-bs cleavage site is represented in some specific groups of genes in *B. subtilis*.

2. Materials and Methods

2.1 Bacterial strains and plasmids

E. coli BL21 (DE3) was used. The *mazF-bs* (*endoA*) and *mazE-bs* (*endoAI*) genes in the *mazEF* operon were separately amplified by PCR using the *B. subtilis* 168 genomic DNA as a template and cloned into pColdIII (TaKaRa Bio) and pET21c (Novagen), respectively with primers (MazF-bs forward: TATACATATGATTGTGAAACGC and MazF-bs Reverse (His₆: TATAGAATTCCTTAATGATGATGATGATGATGAAAATCAATGAGTGC). Subsequently, the *mazF-bs* gene was cloned into pBAD33.

2.2 Protein purification

To purify C-terminal His₆-tagged MazF-bs, *pCold-mazF-bs* was introduced into *E. coli* BL21(DE3). The expression of MazF-bs was induced by treatment with 0.5 mM isopropyl- β -D-1-thiogalactoside (IPTG) for 3 h at 37°C. MazF-bs was purified with Ni-NTA agarose (Qiagen) following the manufacturer's protocol.

2.3 Primer extension

For primer extension analysis of mRNA cleavage sites *in vitro*, 0.8 μ g phage MS2 RNA (Roche) was incubated with and without purified MazF-bs protein in the presence and absence of CspA protein (32 μ g) at 37°C for 5 min in a reaction mixture (10 μ l) containing 10 mM Tris-HCl (pH 8.0) and 0.5 μ l RNase inhibitor (Roche). Primer extension was carried out at 47°C for 1 h as described previously [17]. The reactions were stopped by sequence

loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol EF). The samples were incubated at 90°C for 5 min prior to electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

2.4 Analysis of MazF-bs cleavage sites in *B. subtilis* genome

The genomic sequence of *B. subtilis* 168 was retrieved from NCBI RefSeq (accession no. NC_000964) and all coding sequences (CDS) were extracted from the record. The nucleotide composition of each CDS was calculated by using Perl script.

3. Results

3.1 MazF-bs has high homology to MazF homologues in Gram-positive bacteria

Using BLAST search, we found that MazF-bs has significant identity (62.8 to 94%) and similarity (79.3 to 96.6%) to MazF homologues from various Gram-positive bacteria (Fig. 1A). However, MazF-bs has low identity (19.2 to 37.3%) and similarity (38.3 to 55.9%) to MazF homologues from Gram-negative bacteria (Fig. 1B). Although *Clostridium botulinum* and *C. difficile* are located far from *B. subtilis* in the 16S rRNA phylogenetic tree (Supplementary Fig. 1A), these MazF homologues show high identity (66.4 and 69.2%, respectively) and similarity (83.2 and 81.7%, respectively) to MazF-bs (Fig. 1A) and are located close to MazF-bs in the protein phylogenetic tree (Supplementary Fig. 1B).

To examine if MazF homologues exist in other Gram-positive bacteria, we used BLASTCLUST (BLAST score-based single-linkage clustering), which is part of the NCBI BLAST package. This program clusters proteins based on pairwise matches found by using the BLAST algorithm. To date, predicted TA systems are collected in the TADB database (total of 20816 protein sequences) [18] and we used this database for running BLASTCLUST. Minimum length coverage and similarity threshold in BLASTCLUST used were 0.8 and 60%, respectively. A cluster of proteins including MazF-bs had a total of 102 toxins from 99 strains of which two strains were Gram-negative strains and all other 97 strains were Gram-positive bacteria (data not shown). This result showed that MazF-bs homologues are mainly located in Gram-positive bacteria and MazF-bs homologues in Gram-positive bacteria have wide-ranging distribution in Gram-positive bacteria.

3.2 MazF-bs is toxic in *E. coli* and is neutralized by MazE-bs

The *mazF-bs* gene was cloned in the pBAD33 plasmid which was designated as pBAD-*mazF-bs*. The *mazE-ec* and *mazE-bs* genes were cloned in pET21c (Novagen) and designated as pET21c-*mazE-bs* or pET21c-*mazE-ec*, respectively, as described in Materials and Methods. *E. coli* BL21 (DE3) cells were co-transformed with these plasmids to express the toxin (MazF-bs), antitoxin (MazE-bs and MazE-ec), or both of them together (MazF-bs with MazE-bs or MazF-bs with MazE-ec). Co-transformants of *mazF-bs* with *mazE-bs* formed colonies in the presence of 0.05 mM IPTG, without 0.1% arabinose wherein only MazE-bs was induced (Fig. 2A). However, when only MazF-bs was induced in the presence of 0.1% arabinose (Fig. 2B) or when MazF-bs was induced with MazE-ec, (Fig. 2C), no colonies were formed. The data indicated that MazE-ec cannot neutralize MazF-bs toxicity in *E. coli* although MazF-bs toxicity can be neutralized by MazE-bs (Fig. 2C).

We also examined the effect of MazF-bs induction in a liquid culture on cell growth. MazF-bs expression was induced with 0.1 mM IPTG. As shown in Fig. 2D, growth inhibition was observed only after 2 h induction but cell growth was not completely inhibited, which is in a sharp contrast to the induction of MazF-ec, which inhibits cell growth within 30 min after induction [1]. There was a difference in the effects of toxicity exerted by MazF-bs and MazF-ec on plates. Cells expressing MazF-bs were able to form colonies in the presence of

0.05% arabinose, while cells expressing MazF-ec were not able to form colonies in the presence of 0.05% arabinose, indicating that MazF-bs is less toxic than MazF-ec upon heterologous expression in *E. coli* (data not shown).

3.3 MazF-bs specifically cleaves RNA at UACAU

MazF-bs was purified to determine its cleavage specificity. For this purpose, 3.5-kb phage MS2 RNA was used as a substrate. After incubating MS2 RNA with and without purified MazF-bs, the products were analyzed by primer extension as described previously [17]. Through these experiments, a total of twelve cleavage sites were identified as listed in Table 1. Six of them consisted of a consensus sequence, UACAU (Fig. 3). The remaining six cleavage sites contained a base substitution either at the second base or the fourth base in the consensus sequence. Among the sites with the single base substitutions, two UCCAU and one UACUU showed band intensity stronger than the other three sequences but not as strong as at the consensus sequences. Notably, VACAU or UCAV (where V=G, A, and C) were not cleaved even in the presence of CspA (Supplementary Fig. 2). These results indicate that MazF-bs cleaves RNA with a high specificity for UACAU.

MS2 RNA was unable to cover all four nucleotides at positions -1 and +5 in the consensus sequence (first U residues is designated as +1). All six UACAU sequences found in MS2 RNA have only U or C at position -1 and C, A, or G at position +5. Therefore, four oligoRNAs were synthesized to cover the entire sequence of MS2 RNA. RNA1 (5'-AGAUCUACAUAUGAA-3' [control]), RNA2 (5'-AGAUGUACAUAUGAA-3'), RNA3 (5'-AGAUAUACAUAUGAA-3'), RNA4 (5'-AGAUCUACAUUUGAA-3') (bases at position -1 and +5 are underlined), RNA2 and RNA3 have G and A in position -1 and RNA4 has U residue at position +5. However, the replacement of residues at position -1 or +5 did not affect the cleavage activity of MazF-bs in comparison with the control RNA (RNA1) and MS2 RNA experiments (Fig. 4). These results indicate that MazF-bs cleaves RNA at a five-base sequence, UACAU.

3.4 Significant abundance of the pentad sequence in the genes involved in secondary metabolite biosynthesis, transport and catabolism in *B. subtilis*

We analyzed the number of the pentad sequence in every ORF on the *B. subtilis* genome (4,176 ORFs) (accession no. NC_000964) with the use of a Perl script in descending order. As shown in Table 2, ten of the top twenty genes containing the pentad sequence are involved in secondary metabolite biosynthesis, transport and catabolism according to the COG database [19]. In the *S. aureus* genome, the genes which contain significantly abundant MazF-sa cleavage sites have been identified to be related to pathogenic factors [11], however, none of the top ten UACAU-rich genes in *S. aureus* were found to exist in the *B. subtilis* genome, because *B. subtilis* is not pathogen.

4. Discussion

We showed that MazF from *B. subtilis* has high homology with MazF homologues widely found in Gram-positive bacteria, including pathogenic Gram-positive bacteria such as *B. cereus* causing food poisonings [20], *B. anthracis* causing anthrax, a virulent and highly contagious disease and *S. aureus* causing invasive infections such as pneumonia, osteomyelitis, and endocarditis [21]. MazF-bs likely plays a similar role in cell physiology as other MazF homologues in other Gram-positive bacteria.

Using the BLASTCLUST program, it was found that MazF-bs is widely prevailing Gram-positive bacteria; a total of 102 MazF homologues from 99 strains (61 species), of which 97 strains are Gram-positive bacteria. TA systems are proposed to have invaded the bacterial

genomes using horizontal gene transfer [22], and the 99 strains mentioned above are erratically distributed in the phylogenetic tree based on 16S rRNA. Nevertheless, MazF-bs homologues appear to be preferentially located in Gram-positive bacteria. For example, *S. aureus* and *Halothermothrix orenii* are located far from *B. subtilis* in the phylogenetic tree, but both MazF-sa and MazF-ho have significantly high homology with MazF-bs (Supplementary Fig 1B). Interestingly, the protein multiple alignment of MazF homologues in the 99 strains described above shows high variations in their C-terminal regions. It remains to be elucidated if the C-terminal regions have a specific function for each strain during evolution. The BLASTCLUST analysis showed that there are a total of 27 MazF-ec homologues, which are only from 6 different species. Furthermore, out of the 27 MazF-ec homologues, 22 are located in different *E. coli* strains. Thus, it appears that MazF-bs homologues are widely prevailing in various Gram-positive bacteria, while the distribution of MazF-ec homologues is more limited to a very few bacterial species.

Although there is a very high structural similarity between MazF-bs and MazF-ec [12], MazE-ec could not neutralize MazF-bs toxicity, suggesting that each antitoxin has been co-evolved with its cognate toxin. MazE antitoxins of MazF homologues shown in Fig. 1A have high homology to MazE-bs but not to MazE-ec although MazEs in Gram-negative bacteria are not preserved (data not shown). It was confirmed that MazF-bs is neutralized by MazE-bs by using two different induction systems in *E. coli* as reported before using *Pspac-ydcDE* to induce both proteins [12].

As shown in Fig. 2D, MazF-bs expression did not severely inhibit cell growth. Since MazF-ec cleaving RNAs at ACA sequences completely inhibits cell growth 30 min after induction, our data well correspond to the result that MazF-bs cleaves RNAs at a specific five-base sequence rather than a three-base sequence. As a result, MazF-bs alone was well expressed in *E. coli* without its cognate antitoxin, MazE-bs. Statistically a unique pentad sequence can be found only once in every 1,024-base sequence, provided that the RNA contains equal numbers of bases and has random sequence. Therefore, MS2 RNA consisting of 3,569 nucleotides is expected to contain at least three pentad sequences and actually six consensus cleavage sites, UACAU were found in the RNA. From the analysis of minor cleavage sites, we concluded that MazF-bs RNA cleavage sites are neither XACAU nor UACAX (where X=G, A, and C). MazF-sa and MazF-bs were found to share the same pentad cleavage specificity, suggesting that other MazF homologues from Gram-positive bacteria listed in Fig. 1 may also have the same cleavage specificity.

B. subtilis produces more than twenty four different antibiotics [23]. Among them, nine genes involved in the synthesis of three antibiotics are major targets for MazF-bs (Table 2). Surfactin is one of the 24 antibiotics produced by *B. subtilis* [24] and plipastatin is a lipopeptide antifungal antibiotic [25]. The *urfAB* and *urfAA* genes encode surfactin synthetases, which are essential for biofilm formation [26]. It was also shown that surfactin can act as a signal molecule to induce biofilm formation similar to a quorum sensing inducer [27]. These results suggest that MazF-bs may be involved in regulation of biofilm formation. Although the biological function of polyketide and plipastatin antibiotics is not well understood in *B. subtilis*, polyketide has been shown to play a role in self-defense, aggression and communication [28]. In *S. aureus*, the mRNA for SraP contains an unusually large number of a pentad sequence, UACAU (a total of 43), the specific cleavage site for MazF-sa [11]. SraP is homologous to GspB of *S. gordonii* which is a glycol protein which enhances cell communication between the pathogen and human platelets [29]. It is interesting to further investigate if MazF homologues in Gram-positive bacteria may play an important role in cell-cell communication and biofilm formation. The actual roles and functions of MazF-bs in *B. subtilis* remain to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- MazF-bs from *Bacillus subtilis* is an mRNA interferase.
- MazF-bs specifically cleaves a five-base sequence, UACAU.
- Many MazF homologues in Gram-positive bacteria were highly homologous to MazF-bs.
- UACAU is highly represented in *B. subtilis* genes for the biosynthesis of secondary metabolites.

Fig. 1.

Sequence alignments of MazF-bs with other MazF homologues in Gram-positive bacteria (A) and in bacteria which RNA cleavage specificities of mazF type RNA interferases are known (B). (bs: *Bacillus subtilis*, ba: *Bacillus anthracis*, bc: *Bacillus cereus*, lm: *Listeria monocytogenes*, da: *Dethiobacter alkaliphilus*, ho: *Haloferoxanthus orenii*, tt: *Thermoanaerobacter tengcongensis*, cd: *Clostridium difficile*, cp: *Clostridium perfringens*, cb: *Clostridium botulinum*, c: *Carnobacterium sp.*, sa: *Staphylococcus aureus*, ec: *Escherichia coli*, mt: *Mycobacterium tuberculosis*, and mx: *Myxococcus xanthus*). White and black boxes indicate β -sheets and α -helices on the basis of the secondary structures of MazF-bs, respectively.

Fig. 2.

Induction of MazF-bs and MazE-bs in *E. coli*. *E. coli* BL21(DE3) was transformed using pBAD33-*mazF-bs* together with pET21c-*mazE-bs* or pET21c-*mazE-ec*. Transformed cells were then grown on M9 plates containing 0.2% glycerol and casamino acids with 0.05 mM IPTG (A), 0.1% arabinose (B), and 0.05 mM IPTG plus 0.1% arabinose (C). The plates were incubated at 37°C for 18 h. (D) growth curves of *E. coli* BL21 (DE3) cells harboring pColdIII-*mazF-bs*. The cells were grown in M9-glucose liquid medium at 37°C in the presence (closed circles) or absence (open circles) of 0.1 mM IPTG. After 2 hours, the cell culture was diluted 10-fold by M9-glucose liquid medium in the presence (closed circles) or absence (open circles) of 0.1 mM IPTG.

Fig. 3.

Primer extension analysis of MazF-bs cleavage sites in MS2 RNA. CspA was added in all reactions (A to F) except for lanes 6 and 8 in A and B. Cleavage sites indicated by arrows on the RNA sequences were determined using the RNA ladder shown on the left. Consensus sequence, UACAU, was underlined.

Fig. 4.

In vitro cleavage of the four different oligoRNAs with MazF-bs. Each oligoRNA was incubated without or with MazF-bs for 5 min. Black arrow indicates the bands which show the full sizes of oligoRNA (15 bases). Each RNA ladder was prepared by the treatment of sodium hydroxide in all kinds of RNA oligos. L is RNA ladder.

Table 1

Cleavage sites of MazF-bs in MS2 RNA

Cleavage site
With U[^]ACAU
UGACU U [^] ACAU CGAAG
GGUUU U [^] ACAU AAACG
GCUCC U [^] ACAU GUCAG
UUUCU U [^] ACAU GACAA
CGUUU U [^] ACAU CAAGA
GUCGC U [^] ACAU AGCGU
With sequence differing from U[^]ACAU by 1 base
GAUGG U [^] CCAU ACCUU
GUGGU U [^] CCAU ACUGG
CGUCG U [^] ACCU UAGCU
UUGCU U [^] ACUU AAGGG
ACUAC U [^] ACGU AGUCA
GUUCG U [^] ACUU AAAUA

Table 2

Top 20 genes including the high number of UACAU that is the consensus sequence of MazF-bs in the *B. subtilis* genome. COG (Clusters of Orthologous Groups of proteins) is a database delineated by comparing protein sequences encoded in complete genomes, representing major phylogenetic lineages [19].

Rank	#UACAU	Length (bp)	PID	Gene	COG	Product
1	17	16467	255767399	<i>pksN</i>	COG3321Q	polyketide synthase of type I
2	16	12789	255767398	<i>pksM</i>	COG3321Q	polyketide synthase
3	15	15132	255767396	<i>pksJ</i>	COG3321Q	polyketide synthase of type I
4	12	10752	255767106	<i>surfAB</i>	COG1020Q	surfatin synthetase
5	12	10764	255767105	<i>surfAA</i>	COG1020Q	surfatin synthetase
6	12	13617	255767397	<i>pksL</i>	COG3321Q	polyketide synthase of type I
7	11	3255	50812254	<i>nrpEB</i>	COG0209F	phage SPbeta phage ribonucleoside-diphosphate reductase, alpha subunit
8	11	7668	16078893	<i>ppsC</i>	COG1020Q	plipastatin synthetase
9	11	10812	255767428	<i>ppsD</i>	COG1020Q	plipastatin synthetase
10	9	2520	16079163	<i>yonO</i>	-	conserved hypothetical protein; phage SPbeta
11	9	7686	255767429	<i>ppsA</i>	COG1020Q	plipastatin synthetase
12	8	1218	255767470	<i>yonJ</i>	-	conserved hypothetical protein; phage SPbeta
13	7	3606	16078957	<i>yobI</i>	-	putative NTPase with transmembrane helices
14	7	7005	255767831	<i>wapA</i>	COG3209M	cell wall-associated protein precursor
15	7	7137	255767732	<i>dhbF</i>	COG1020Q	siderophore 2,3- dihydroxybenzoateglycine- threonine trimeric ester bacillibactin synthetase
16	6	588	16079041	<i>yonM</i>	COG3331R	hypothetical protein; SPbeta phage
17	6	1038	16079717	<i>yrdP</i>	COG2072P	putative oxidoreductase
18	6	1947	255767270	<i>manR</i>	COG3711K	transcriptional antiterminator
19	6	1995	16077106	<i>metS</i>	COG0143J	methionyl-tRNA synthetase
20	6	2874	16080569	<i>avrA</i>	COG0178L	exonuclease ABC subunit A