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RNase III Controls *mltD* mRNA Degradation in *Escherichia coli*

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Abstract RNase III is a double-stranded RNA-specific endoribonuclease that processes and degrades numerous mRNA molecules in *Escherichia coli*. A previous genome-wide analysis of *E. coli* transcripts showed that steady-state levels of *mltD* mRNA, which encodes membrane-bound lytic murein transglycosylase D, was most affected by changes in cellular concentration of RNase III. Consistent with this observation, in vitro and in vivo analyses of *mltD* mRNA revealed RNase III cleavage sites in the coding region of *mltD* mRNA. Introduction of a nucleotide substitution at the identified RNase III cleavage sites inhibited RNase III cleavage activity on *mltD* mRNA, resulting in, consequently, approximately two-fold increase in the steady-state level of the mRNA. These findings reveal an RNase III-mediated regulatory pathway that modulates *mltD* expression in *E. coli*.

Introduction

The RNase III family of enzymes is one of the key ribonuclease families that determine mRNA stability in both prokaryotes and eukaryotes. RNase III is encoded by the *rnc* gene in *Escherichia coli* and was initially characterized as playing key role in rRNA processing [3]. A recent study that utilized genome-wide analysis of *E. coli* transcripts showed that the abundance of a few hundred mRNA species could be regulated by RNase III [11], indicating its active role in mRNA degradation and processing in *E. coli*.

Among the potential RNase III targets identified by genome-wide analysis, steady-state levels of *mltD* mRNA were most greatly affected by changes in the cellular concentration of RNase III [11]. The *mltD* gene encodes for a membrane-bound lytic murein transglycosylase D (*mltD*), which was initially discovered by comparative analysis on the basis of the structure of the 70 kDa soluble lytic transglycosylase (Slt70) protein [6, 13]. Lytic transglycosylases are a large family of the peptidoglycan degrading enzymes that include glucosaminidases, muramidases, and amidases [2, 6, 14]. While other enzymes that belong to the lytic transglycosylase family have been relatively well studied, the physiological role and regulation of the *mltD* gene have not been characterized. In this study, we investigated the functional role of RNase III activity on *mltD* expression in *E. coli*.

Materials and Methods

Strains and Plasmids

The *E. coli* strain MG1655 *rnc-14*::ΔTn*10* was constructed by P1 transduction of *rnc-14*::ΔTn*10* allele from the *E. coli* strain HT115 [12]. MG1655*mltD* was constructed by deleting the *mltD* open reading frame in the MG1655 genomic DNA using a procedure described by Datsenko and Wanner [4]. PCR primers used in these experiments were *mltD*-5′-UTR-P1 (5′-ATCGGTGCCTTTTTATTATC TGGTTTGTCAGTGTAGGCTGGAGCTGCTTC-3′) and *mltD*-3′-UTR-P2 (5′-GTCTTTTAAGCAACTATTGACA CACACATGCATATGAATATCCTCCTTA-3′). To construct pCAT924-*mltD*, a DNA fragment containing the *mltD* gene was amplified using the PCR primers, *mltD*

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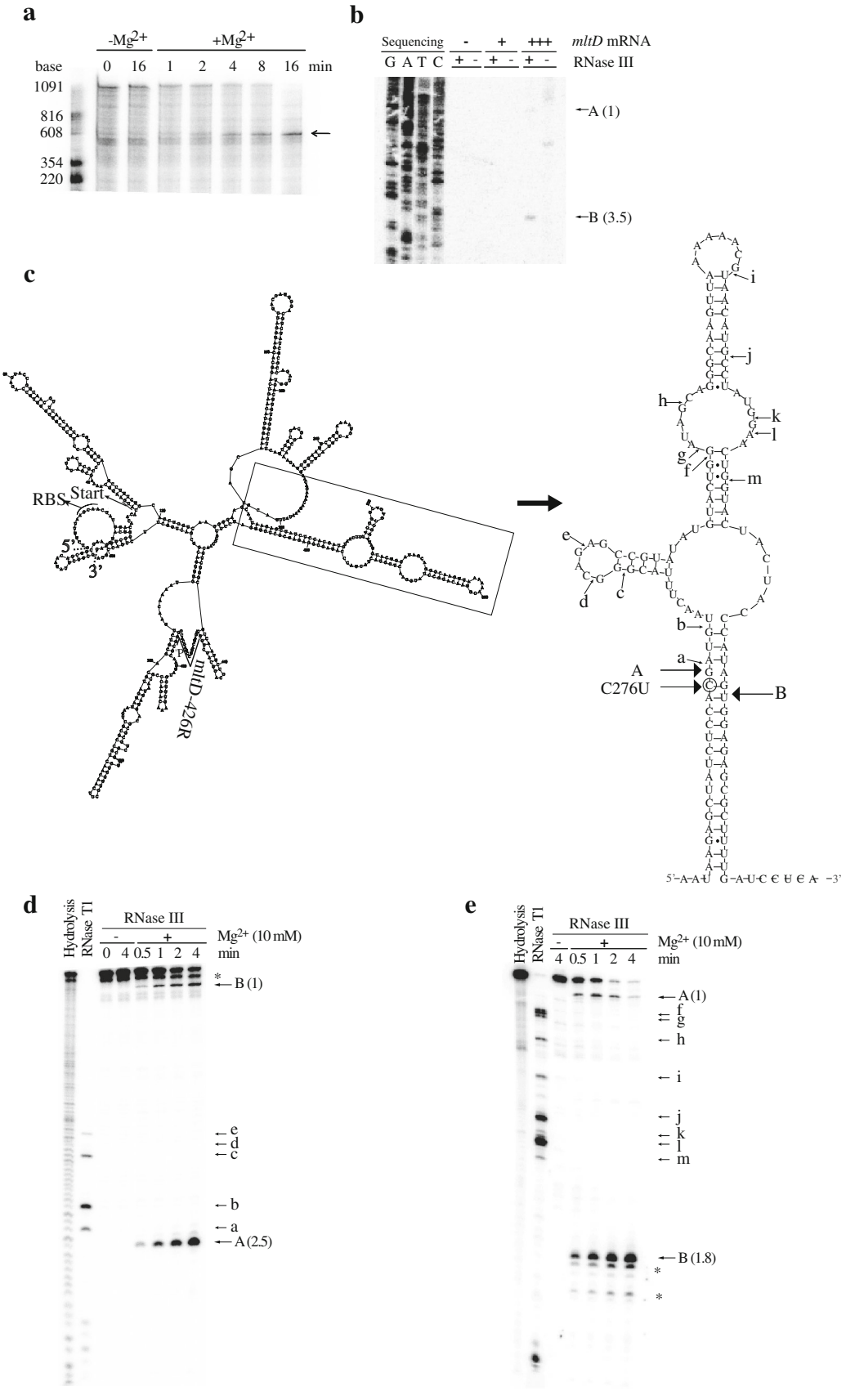
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◀ **Fig. 1** Identification of RNase III cleavage sites in *mltD* mRNA in vitro and in vivo. **a** In vitro cleavage of the full-length *mltD* RNA. One picomole of 5′-^{32}P-end-labeled *mltD* transcript was incubated with 2.4 pmol of purified RNase III in a cleavage buffer with (III + Mg^{2+}) or without $MgCl_2$ (III). Samples were withdrawn at the indicated time intervals and separated on 8 % polyacrylamide gels containing 8 M urea. The size of the cleavage product was estimated using size markers generated by internally labeled transcripts. **b** Primer extension analysis of *mltD* mRNA synthesized in vivo. Total RNA was prepared from MG1655 and MG1655*rnc-14::ΔTn10* that endogenously (total 100 μg) or exogenously (pCAT924-*mltD*) overexpressed (total 50 μg) *mltD* mRNA and were hybridized with a 5′-end–labeled primer (*mltD*-426R). Synthesized cDNA products were analyzed on a 10 % polyacrylamide gel. Sequencing *ladders* were produced using the same primer used in cDNA synthesis and PCR DNA encompassing the *mltD* gene as a template. −, no expression; +, endogenous expression; +++, overexpression. **c** The predicted secondary structure of *mltD* mRNA. The secondary structure was deduced using the M-fold program [16]. The model hairpin RNA used for in vitro cleavage assays in **d**, **e** is shown in the *right panel*. The position of C276U mutation used in Fig. 2 is indicated. **d, e** In vitro cleavage of the model *mltD* hairpin RNA. One picomole of 5′-(D) or 3′-(E) ^{32}P-end-labeled *mltD* model hairpin were incubated with 0.9 pmol of purified RNase III in a cleavage buffer with (III + Mg^{2+}) or without $MgCl_2$ (III). Samples were withdrawn at the indicated time intervals and separated on 10 % polyacrylamide gels containing 8 M urea. Cleavage products (*A* and *B*) were identified using size markers generated by alkaline hydrolysis (Hydrolysis) and predicted secondary structure of the hairpin was confirmed by analyzing the cleavage patterns of the model hairpin RNA after RNase T1 digestion. RNase T1 cleavage sites are indicated in **c**, **d, e** with *arrows* labeled with *a–m*.The relative amounts of cleaved products *A* and *B* are indicated in the parentheses. Other minor cleavage products are indicated with *asterisks* in **d** might have been produced from RNase III digestion of RNA transcripts containing an incomplete 3′- or 5′-end

pCAT924 F (5′-ATGCGGCCGCGTCTTTTAAGCAAC TATTGAC-3′) and *mltD* pCAT924 R (5′-ATGCGGCCG CTCAGGAATCTGGCATGTTGTT-3′). The products were then cloned into the *Not*I site in pCAT924 [1], to generate the pCAT924-*mltD* construct. To construct the pCAT924-*mltD*-C276U plasmid, DNA fragments containing nucleotide substitution at the cleavage region of *mltD* mRNA were amplified using the overlap extension PCR method, digested with *Not*I, and subcloned into the *Not*I site of the pCAT924-*mltD* construct. The PCR primers used were *mltD*-C276U-R (5′-TCTGCCCGTAAAGTTA CATCATGGAGATAGCTCTTATTGCG-3′), *mltD* +277F (5′-GATGTAACTTTACGGGCAGA-3′), *mltD* pCAT924 F, and *mltD* pCAT924 R.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed and analyzed as previously described [15]. Total RNA was isolated using RNeasy miniprep kit (Qiagen) from MG1655 cells harboring pCAT924-*mltD* or pCAT924-*mltD*-C276U grown in LB containing ampicillin (100 μg/ml) at 37 °C to OD_{600} = 0.6. Synthesis of *mltD* and *rnc* cDNA was performed using PrimeScript 1st strand cDNA synthesis kit system for RT-PCR (Takara), 1 μg of total RNA, and *mltD* and *rnc* specific primers as per the manufacturer's instructions. The primers used for RT-PCR were 5′-AT GAAGGCAAAAGCGATATT-3′ and 5′-TCAGGAATCT GGCATGTTGT-3′ for *mltD*, and 5′-TGTGGATGAAGG CGATATGA-3′ and 5′-CGAGAATTGACTCACGAC GA-3′ for *rnc*.

In vitro Cleavage and Primer Extension Analysis

His-tagged RNase III purification and cleavage assays were performed as previously described [1]. The procedure for synthesis of 3′/5′-end-labeled transcripts was performed as previously described [9]. In vitro cleavage assay was performed and analyzed as previously described [7]. The following primers were used to synthesize transcripts: *mltD*-T7 (5′-TAATACGACTCACTATAGAAACTTTC TTGTCATCGGCTC-3′) and *mltD*-R (5′-TCAGGAATCT GGCATGTTGT-3′) for synthesis of full length transcript, and *mltD*-short T7 (5′-CTTAATACGACTCACTATAGG GAATAAGAGCTATCTCCACGA-3′) and *mltD*-1/3 R (5′-TGAGGATCAAAAGCGCTCTC-3′) for synthesis of model hairpin transcript. Primer extension analysis was performed using 5′-^{32}P-labeled primer, *mltD*-426R (5′-CC CCGTGCTCGGAATGATCT-3′).

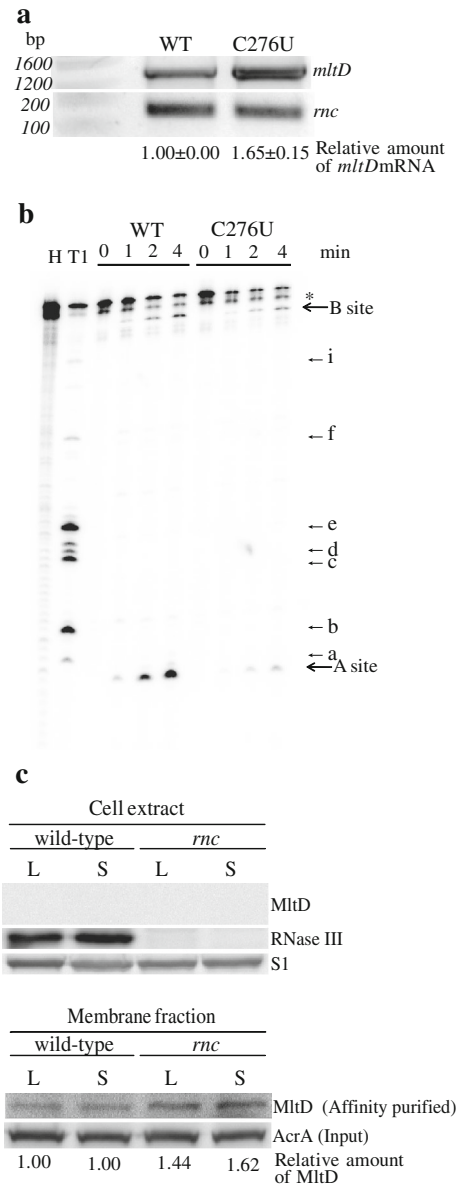
Analysis of *mltD* Protein

The procedures for western blot analysis, preparation of membrane fraction of *E. coli* cells, and nickel extraction was performed as previously described [8, 10].

Results

Identification of RNase III Cleavage Sites in *mltD* mRNA

Our previous microarray results identified about 100 genes that are downregulated by RNase III [11]. Among these, *mltD* mRNA levels were most significantly affected by changes in cellular RNase III concentration [11]. This observation prompted us to examine the basis of the correlation between steady-state *mltD* mRNA levels and RNase III concentrations. First, we tested whether *cis*-acting elements that are responsive to RNase III are present in *mltD* mRNA by performing an RNase III cleavage assay using a synthetic *mltD* transcript. As shown in Fig. 1a, RNase III cleavage reaction with a 5′-^{32}P-end-labeled *mltD* transcript generated an RNA product of which size is between 354 and 608 nt. We were able to detect only one



◀ **Fig. 2** Inhibition of RNase III cleavage of *mltD* mRNA by introduction of a nucleotide substitution at the cleavage site. **a** Effect of a nucleotide substitution at the RNase III cleavage region on *mltD* mRNA decay. The plasmid pCAT924-*mltD*-C276U expresses *mltD* mRNA containing a nucleotide substitution (C276U) at the RNase III cleavage region. MG1655 harboring either the pCAT924-*mltD* or pCAT924-*mltD*-C276U plasmids were grown in LB containing ampicillin (100 μg/ml) at 37 °C to $OD_{600} = 0.6$ and total RNA samples were prepared from the cultures. Steady-state levels of *mltD* mRNA were assessed using semi-quantitative RT-PCR. **b** Effects of the C276U mutation on RNase III cleavage activity on *mltD* mRNA in vitro. 5′-^{32}P-end-labeled *mltD* model hairpin and that containing the C276U mutation were used for in vitro RNase III cleavage assays and analyzed in the same way described in the legend to Fig. 1**d**, **e**. H, Hydrolysis; T1, RNase T1. RNase T1 cleavage sites identified in Fig. 1**c**, **d**, **e** are indicated with *arrows* labeled with *a–f* and *i*. **c** Western blot analysis of *mltD* protein. MG1655 and MG1655*rnc-14::ΔTn10* harboring pCAT924-*mltD* were grown in LB medium at 37 °C. The cultures were taken in the mid-log ($OD_{600} = 0.7$) and the stationary ($OD_{600} = 5.7$) phase for preparation of cell extract and membrane fraction. *mltD* protein was isolated by affinity chromatography using Ni^{2+} column and analyzed by immunoblottin using anti-His antibody. *AcrA* and *S1* proteins were used to provide internal standards to evaluate the amount of membrane fraction and cell extract in each lane, respectively. *AcrA* protein was also used to validate fractionation of membrane. *L* and *S* stand for log and stationary phase, respectively

cleavage product. There are a few explanations for this observation. RNA fragments generated by RNase III cleavage at sites located to the either 5′- or 3′-terminus may have similar sizes, which cannot be resolved in the gel. Another possibility is that RNase III cleavages at both sites occurred rapidly and, consequently, the 5′-end labeled cleavage product generated by RNase III cleavage at the site located to the 5′-terminus of the transcript accumulated. Next, to identify RNase III cleavage sites in *mltD* mRNA, we performed primer extension experiments using 5′-^{32}P-end-labeled primer (*mltD*-426R) that were designed to bind to a region downstream of the RNase III cleavage site as deduced from the RNase III cleavage assay shown in Fig. 1a. Total RNA purified from wild-type and *rnc*-deleted cells that endogenously express *mltD* mRNA was used for the primer extension experiments. Unfortunately, we were not able to detect any cDNA bands (Fig. 1b). It is likely that cleavage products of RNase III were not detected by primer extension analysis because they were rapidly degraded by other ribonucleases following the RNase III cleavage. This phenomenon has been reported for *bdm* mRNA [11]. It is also possible that expression levels of *mltD* mRNA are not high enough to be detected by primer extension analysis. Therefore, we reasoned that we might be able to detect cDNA products from RNase III cleavage products of *mltD* mRNA when it is overexpressed. For this reason, we cloned the *mltD* gene into a multicopy number plasmid pCAT924 that expresses the *mltD* protein with a C-terminal hexahistidine under the control of a constitutive trp^c promoter [5]. The resulting plasmid, pCAT924-*mltD*, was introduced into wild-type and *rnc*-deleted cells, and total RNA was prepared from these strains for primer extension experiments. We observed two distinct cDNA bands that were only present in the lanes loaded with cDNA products from the reaction containing total RNA prepared from wild-type *E. coli* cells that overexpressed *mltD* mRNA (Fig. 1b). These cDNA bands corresponded to sites that were positioned in the double-stranded region of the *mltD* mRNA coding sequence (Fig. 1c). RNase III cleavage at these sites was predicted to produce products with an overhang of two nucleotides at the 3′-end, which is characteristic of RNase III cleavage products. These sites were designated as cleavage sites A and B.

mltD mRNA cleavage by RNase III at sites A and B were further demonstrated biochemically using an in vitro synthesized model hairpin RNA (Fig. 1c, right panel) and purified RNase III. The model hairpin RNA has a nucleotide sequence between +258 and +392 nt from the *mltD* start codon and which contains RNase III cleavage sites A and B in the *mltD* mRNA. RNase III cleavage of a 5′-^{32}P-end-labeled model hairpin RNA in vitro generated one major and one minor cleavage product, the lengths of which corresponded to cleavage sites A and B, respectively (Fig. 1d). The radioactivity in the cleavage product at site A was ~2.5 times higher than that at site B. The cleavage product at site A appeared to be more abundant since the model hairpin was labeled at the 5′-end and the cleavage product at site A accumulated during the cleavage reaction. The results from RNase III cleavage assays with 3′-^{32}P-end-labeled model hairpin RNA confirmed cleavage sites A and B, and 1.8 times more accumulation of the cleavage product at site B (Fig. 1e), supporting that RNase III does not differentially cleave sites A and B. These results also explain why the amount of cDNA product corresponding to A site is about one-third of the amount of that corresponding to B site in the primer extension analysis of *mltD* mRNA in vivo (Fig. 1b).

RNase III Cleavage at A and B Sites Regulates *mltD* Expression

To test whether RNase III cleavage at A and B sites regulates *mltD* degradation, we introduced the nucleotide substitution, C276U, on the strand facing the cleavage site B in the *mltD* over-expression plasmid (pCAT924-*mltD*). The C276U mutation was chosen to avoid possible effects of a nucleotide substitution on other than RNase III cleavage activity. This mutation does not alter either the *mltD* mRNA's secondary structure or subsequent amino acid sequence. Wild-type and mutant *mltD* mRNA was overexpressed in an *mltD*-null *E. coli* strain and *mltD* mRNA steady-state levels and RNase III cleavage specificity were investigated. The steady-state levels of the mutant *mltD* mRNA were 1.7 times greater than wild-type levels (Fig. 2a), indicating that RNase III cleavage activity on sites A and B influences *mltD* mRNA degradation. This conclusion was further supported by experimental results showing that RNase III was not able to efficiently cleave an in vitro synthesized model hairpin RNA containing the C276U mutation (Fig. 2b).

Next, we tested whether RNase III-mediated *mltD* mRNA degradation consequently affects levels of *mltD* protein. Wild-type and *rnc*-deleted strains were transformed with pCAT924-*mltD* and total protein was obtained from cultures of the resulting transformants in mid-log and early stationary phase. Total protein preparations were separated by SDS-PAGE gels and subjected to western blot analysis using an anti-His-tag antibody. However, we were not able to detect *mltD* protein in cellular extract despite it being overexpressed (Fig. 2c). Considering that the coding region of *mltD* mRNA folds into a stem-loop structure that contains the RNase III A and B cleavage sites, we conclude that *mltD* is not efficiently expressed because it has a weak ribosome binding site (Fig. 1c). For this reason, we affinity-purified and analyzed *mltD* from membrane fractions of cell extracts using a Ni^{2+} column. Our results indicate that *mltD* expression levels are increased by an approximately 1.5-fold in the *rnc*-deleted strain as compared to the wild-type strain (Fig. 2c). This suggests that *mltD* mRNA degradation by RNase III significantly contributes to the levels of *mltD* protein.

Discussion

We investigated the functional role of RNase III in the post-transcriptional regulation of *mltD* expression in *E. coli* cells and demonstrated that RNase III cleavage in the coding region of *mltD* mRNA contributes to the degradation of *mltD* mRNA, which, consequently, affects *mltD* protein levels (Figs. 1, 2). RNase III–mediated cleavage of *mltD* mRNA was inhibited by a nucleotide substitution at the cleavage site in the *mltD* mRNA, further demonstrating that RNase III cleavage largely contributes to *mltD* degradation (Fig. 2a, b). Sequence analysis of the *mltD* gene indicated that it is likely expressed from the sigma factor 70 promoter and that *mltD* mRNA appears to be inefficiently translated because the 5′-UTR does not contain a strong ribosome binding site (Fig. 1c). This notion is also supported by our western blot analysis of *mltD* in cells harboring the pCAT924-*mltD* plasmid (Fig. 2c). Such inefficient translation of *mltD* mRNA by ribosomes would generate regions of the mRNA that are freely folded into secondary structures, some of which allow for RNase III interaction.

A detailed characterization of the physiological roles of *mltD* and mechanisms that modulate RNase III activity on *mltD* mRNA will provide clues as to why *mltD* expression requires posttranscriptional regulation by RNase III.

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