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A novel fluorescent reporter system for monitoring and identifying RNase III activity and its target RNAs

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Bacteriophage vectors for achieving single-copy gene expression linked to a colorigenic reporter assay have been used successfully for genetic screening applications. However, the limited number of cloning sites in these vectors, combined with the requirement for *lac*- strains and the time- and/or media-dependence of the chemical-based colorimetric reaction, have limited the range of applications for these vectors. An alternative approach using a fluorescent reporter gene such as green fluorescent protein (GFP) or GFP derivatives could overcome some of these technical issues and facilitate real-time monitoring of promoter and/or protein activity. Here, we report the development of a novel translational bacteriophage fusion vector encoding enhanced GFP (eGFP) that can be incorporated into the chromosome as a single-copy gene. We identified a *Bacillus* promoter (BP) that is stably expressed in *Escherichia coli* and drives ~6-fold more expression of eGFP than the T7 promoter in the absence of inducer. Incorporating this BP and RNase III target signals into a single system enabled clear detection of the absence or downregulation of RNase III activity in vivo, thereby establishing a system for screening and identifying novel RNase III targets in a matter of days. An RNase III target signal identified in this manner was confirmed by post-transcriptional analysis. We anticipate that this novel translational fusion vector will be used extensively to study activity of both interesting RNases and related complex or to identify or validate targets of RNases that are otherwise difficult to study due to their sensitivity to environmental stresses and/or autoregulatory processes.

Introduction

Studies of gene regulation typically utilize reporter proteins with an assayable activity for the identification of promoter(s) and the analysis of gene expression in various organisms.[1-3] Bacteriophage vectors for single-copy gene expression have been used successfully for colorigenic assays of reporter gene expression.[4-6] Expression from these vectors is very easy to quantify: the assays are insensitive to certain environmental factors and typically have relatively high sensitivity and low background. In addition, many hypersensitive detection systems and antibodies are available for detecting and monitoring gene expression. There are, however, several limitations to this type of system: (1) a limited number of cloning sites in the vector; (2) the requirement for *lac*- test strains for the colorigenic assay; and (3) the chemical reactions required for detecting gene expression and developing the color read-out are time- and media-dependent. An alternative system to colorigenic reporters is needed to overcome all of these limitations.

Fluorescent proteins have been available as reporter genes for analyzing gene expression in both bacterial and eukaryotic systems.[7,8] These include the original member of this group of proteins, green fluorescent protein (GFP), which was obtained from *Aqua victoria*, as well as several of its derivatives, including enhanced GFP (eGFP) and GFP*uv*, which have improved detection characteristics.[9,10] Moreover, color derivatives of GFP, such as blue fluorescent protein (BFP), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), have been developed and are also widely available.[11] Unlike β-galactosidase or similar enzyme-based reporter systems, detection of a fluorescent protein is dependent primarily on its level of expression and is not dependent on activity. Signals are thus free from many constraints that would affect enzyme performance. Furthermore, these proteins are highly stable, even if cell viability is compromised.

RNase activity is very sensitive to environmental conditions[12] and autoregulation,[13,14] which makes the precise monitoring of in vivo RNase activity challenging. Moreover, most validated RNase mutant strains available for analyzing RNase activity are in *lac*+ background, which requires genetic manipulation to be useful for the colorigenic assays. A fluorescent reporter system, on the other hand, would facilitate monitoring of RNase activity and identification of novel regulators without the need for chemical

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reactions to detect the incorporation of cleavable sequences into the vector.

Here, we have developed a bacteriophage translational fusion vector encoding a fluorescent protein as a reporter gene. This system enabled us to efficiently monitor promoter and enzyme activities in vivo, as well as genetic screening of potential RNase III targets. We identified an interspecies-specific *Bacillus* promoter (BP) that is stably and stress-insensitively expressed in *Escherichia coli* and drives the expression of a reporter gene more efficiently than the T7 promoter in the absence of inducer. By combining the BP with RNase III target signals, we established and validated an integrated system for monitoring the absence or down-regulation of RNase III activity in vivo and identifying a novel target of RNase III from an *E. coli* chromosomal DNA library. An RNase III target signal identified in this manner was independently confirmed as a post-transcriptional regulatory target of RNase III. The system and strategies presented here will greatly expand both the number and types of promoters and genes that can be analyzed by reporter gene assays, particularly those proteins whose activity is sensitive to environmental conditions and/or under strict autoregulation.

Results

Construction of translational fusion reporter plasmid. We constructed a translational fusion reporter system using pRS1553[4] as the plasmid backbone. First, nucleotides between positions +132 and +137 of pRS1553 were mutated to create a *Bam*HI site. Second, the ribosomal binding site (RBS) and the first seven codons of the LacZ were eliminated by *Bam*HI digestion. The vector was then re-ligated to generate pRSK1. To create a universal translational fusion vector for reporter assays, pRSK1 was further modified by insertion of two multiple cloning sites (for promoter analysis, MCS1: *Eco*RI-*Sma*I-*Sac*I and for protein function analysis, MCS2: *Nco*I-*Kpn*I-*Xba*I-*Xho*I-*Hin*dIII-*Bam*HI) flanking a set of RBS (modified from Thomas et al.)[15] between the *Eco*RI and *Bam*HI sites of pRSK1. The resultant vector was termed pRSK2. The advantages of pRSK2 included the incorporation of two MCSs (nine cloning sites) compared with pRS1553, in which only three cloning sites for promoter analysis are available (**Fig. 1**).

Although *lacZ* expression vectors and chromosomal fusions are widely used and our system is amenable to *lacZ* reporter gene analysis, introduction of a fluorescent reporter gene into pRSK1 or pRSK2 could potentially be more useful for the analysis of promoter activity as it would eliminate the need for chemical reactions. eGFP is expressed as a soluble fluorescent protein with high quantum yield in *E. coli*. The eGFP open reading frame with a termination codon was inserted into the *Hin*dIII/ *Bam*HI site of pRSK2, generating pRSK3 (**Fig. 1D**).

Screening and analysis of an interspecies promoter from *Bacillus*. Environmental stress can be problematic in developing a universal vector for reporter gene analysis. For example, a sudden increase in reporter gene expression due to bacterial growth can mask subtle changes in a gene of interest. To identify promoter sequences that were less environmentally sensitive yet still exhibited strong activity, we focused on promoter regions from a non-host bacterium, *B. subtilis*. To identify novel promoters of *B. subtilis* that were active in *E. coli*, we used a bidirectional promoter-trap system, pBGR1[16] to screen a *B. subtilis* promoter library. By monitoring the reporter expressions, three independent sets of *E. coli* transformants that were higher than the control vector were screened (approximately 1×10^7 clones). Twelve clones (BGR series) were selected as positive, and, of these, four (BGR1, 2, 4 and 12) remained positive after plasmid isolation and re-transformation (**Fig. 2A**). The four clones were sequenced, and three of them (BGR2, BGR4 and BGR12) contained the same insertion (**Fig. 2B**). A BLAST search revealed that the insertion corresponded to part of the intergenic region between *ywcA* and *gspA* located 84 bp upstream of *gspA* in *Bacillus amyloliquefaciens* strain FZB42 (**Fig. 2C**). The database did not contain the complete *B. subtilis* GB03 genome sequence, but the corresponding region of GB03 partially matched that of FZB42. BGR2 was selected for further analysis because it generated the strongest fluorescent signal; the promoter was termed BP as an acronym for *Bacillus* promoter.

To determine the utility of pRSK3 for promoter analysis, we incorporated the bacteriophage T7 promoter or BP into either the *Eco*RI/*Nco*I or *Eco*RI/*Sac*I sites of pRSK3 to generate vectors with either an original or synthetic RBS, respectively (**Fig. 3A**). To assess the relative activity of the two promoters, expression of eGFP from transformants of *E. coli* BL21 (DE3) with pRSK3, pRSK3-T7, or pRSK3-BP was evaluated with or without inducer (**Fig. 3B**). The eGFP expression in cells of pRSK3 was approximately 7% of that seen with pRSK3-BP in the presence and absence of IPTG. Interestingly, the promoter activity of BP was about 6-fold higher than that of T7 in the absence of IPTG, and about 2-fold lower than T7 in the presence of IPTG. Moreover, BP did not affected by cold-stress responses (**Fig. 3C**), suggesting that non-species-specific promoters would be useful for constitutive gene expression of genes of interest in a target species without perturbed by chemicals and stresses. Therefore, identification of other interspecies-specific promoters that have yet to be identified will be interesting future works.

Monitoring RNase III activity. We further applied our system to monitor RNase activity. Many currently available reporter genes for RNase activity consist of the autoregulatory regions of RNase itself fused to *lacZ* (e.g., *rne::lacZ* or *rnc'-'lacZ*),[13,14] making us difficult to evaluate the effect of cellular factors on RNase activity. To circumvent these limitations, we incorporated the known RNase III cleavage site of *E. coli bdm* (the entire open reading frame without the stop codon)[14] into the *Xba*I/*Xho*I site of pRSK3-BP. Expression of pRSK3-BP-*bdm* in an *rnc14* mutant strain resulted in fluorescence signals that were about 6-fold stronger than in the *rnc*+ strain (**Fig. 4A**). We further showed that the eGFP expression from pRSK3-BP-*bdm* (A157U), a *bdm* site mutant that lowered RNase III cleavage activity,[17] in wild-type (*rnc*+) was increased ~20% (**Fig. 4B**), confirming that the our system could monitor the mutation affecting the RNase III activity. Moreover, overexpression of a known RNase III activity regulator, YmdB,[18] resulted in only 2.6-fold increase in eGFP expression in the ectopic expression of YmdB (+IPTG) compared

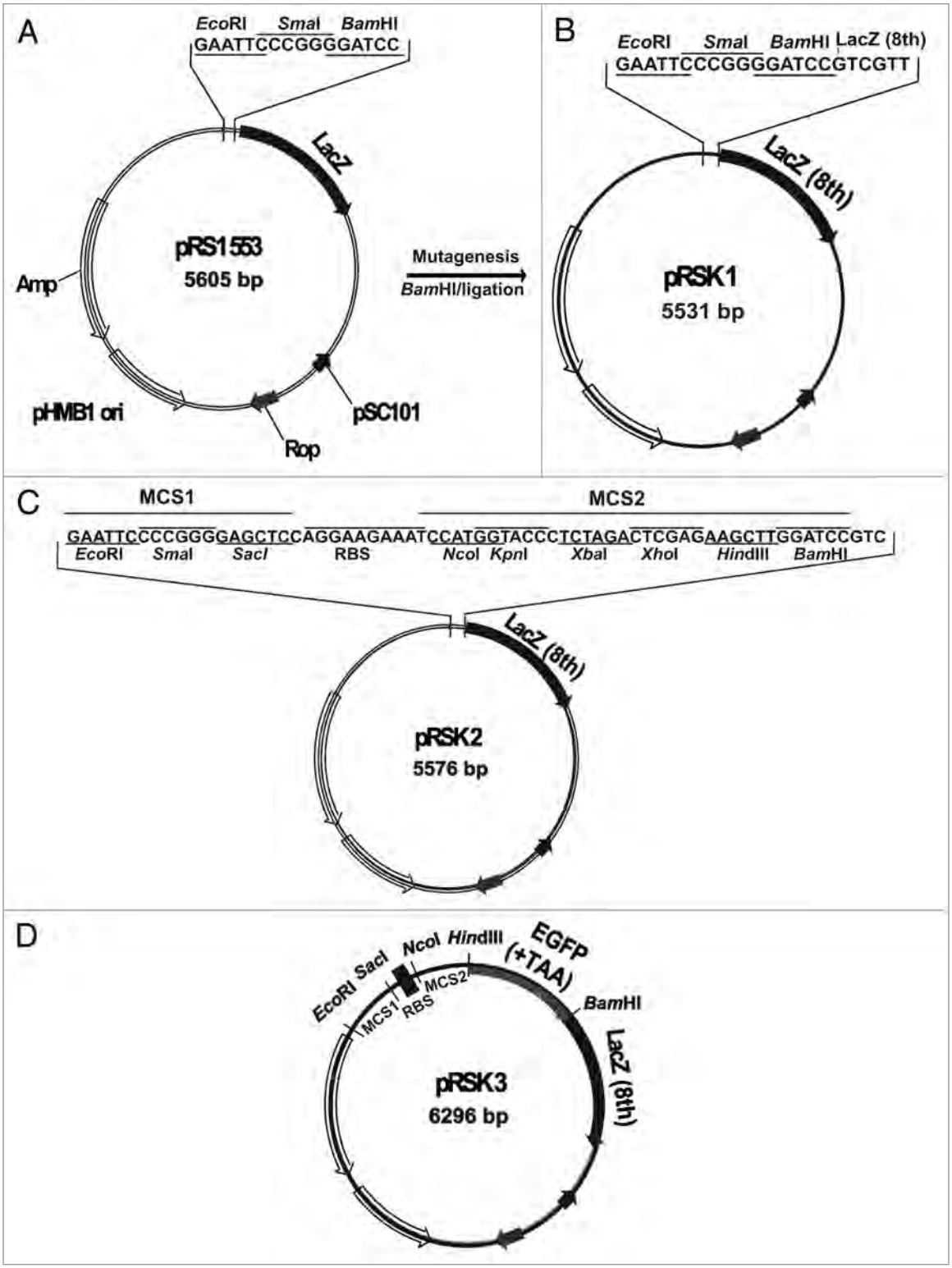
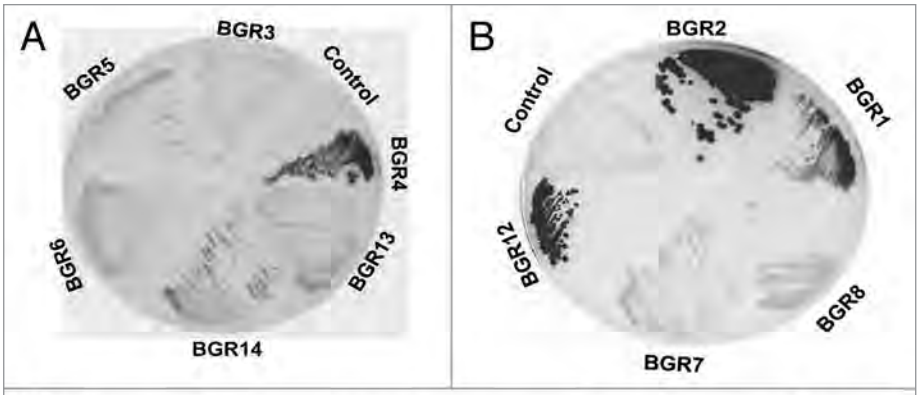


Figure 1. Schematic representation of plasmids and sequences used in this study. Plasmid maps of (A) pRS1553, (B) pRSK1, (C) pRSK2 and (D) pRSK3 are shown. Unique restriction enzyme sites are indicated. RBS, ribosome binding site; MCS1 and MCS2, multiple cloning sites 1 and 2, respectively. MCS1 can be used for promoter analysis, while MCS2 is mainly used for analysis of protein function.

with cells that did not express YmdB (-IPTG) (**Fig. 4C**). These results indicated that the fluorescent reporter system can detect subtle (approximately 2~3-fold) changes in RNase III activity in vivo and may be appropriate for the identification of novel RNase III regulators that are activated in response to specific cellular stresses.



C

Clone	Insert size (bp)	Adjacent genes	Regions	Direction
BGR1	439	*ytsJ<>dnaE*	**2754857-2755295**	<<<
BGR2	139	*ywcA<>gspA*	**3669223-3669301**	<<<
BGR4	129	*ywcA<>gspA*	**3669223-3669301**	<<<
BGR12	177	*ywcA<>gspA*	**3669223-3669301**	<<<

D

BP (139 bp)

GGATC TTACA CATTT TTTCT GCGGG AATGT GCTGT TTGCG
ATTTG ATATC TTTTG CATTC TCTGT TAAAA TTCAT TTCAT
AGCTA ATGAT TCTGA CAATA AAAAG GAGCA TGACG CCATT
GACGA ATCGC AACGA TGCC

Figure 2. Fluorescence screening of a *Bacillus* promoter library. Screening of a *Bacillus* pBGR1-promoter library using (A) GFP or (B) DsRed as reporters. (C) DNA sequencing of selected positive clones using the KSKRI21 primer. Regions and directions in bold indicate NCBI database matches with *B. amyloliquefaciens* FZB42 and promoter orientation of adjacent genes, respectively. (D) DNA sequence of *Bacillus* promoter (BP). Underlined sequences indicated regions described in (C).

Stability and real-time monitoring function of fluorescent reporter system. We tested eGFP expression from pRSK3-BP by stress conditions such as cold stress to eliminate the copy number variation from plasmid. We found that eGFP expression from BP was not fluctuated by cold stress (**Fig. 4D**). This suggests that eGFP expression from BP is highly useful for a good reporter system for the activity of RNase III in that condition. Therefore, this prompted us to use pRSK3-BP-*bdm* for real-time monitoring system for RNase III activity. To show the usefulness, we monitored the presence or absence of RNase III activity and identified that eGFP from pRSK3-BP-*bdm* in *rnc14* expressed more than in *rnc*+ under cold stress from hours to days without any perturbations. These indicate that our system could sensitively monitor in real-time for the difference of RNase III activity under environmental stresses.

Screening of novel *E. coli* RNase III targets. To identify novel RNase targets using our fluorescent reporter systems, we first generated an RNase III target library by *Nco*I digestion of *E. coli* chromosomal DNA. Analysis of the number of *Nco*I recognition sites in *E. coli* K12 MG1655 chromosomal DNA revealed 612 cleavage sites. After complete digestion with *Nco*I, fragments in the 0.1–1 kbp range were eluted from an agarose gel and then ligated into *Nco*I-linearized pRSK3-BP. The library was used to transform an *rnc*+ strain and we screened approximately 10,000 colonies from three independent sets of transformants. Non-fluorescent colonies were selected, and then the plasmids were isolated and used to re-transform an *rnc14* mutant strain. A total of eight colonies were selected that exhibited stronger fluorescence compared with pRSK3 transformants (data not shown). Sequencing revealed that all of the clones contained part of the coding region of *hemL* (eight hits; +314 to +431 refer to start codon as +1) of *E. coli*, which encodes glutamate-1-semialdehyde aminomutase.[19] Interestingly, this region has never been reported as a target of RNase III. Quantitatively, expression of eGFP from a plasmid containing the *hemL* fragment was about 2-fold higher in the *rnc14* background compared with *rnc*+ (**Fig. 5A**). Supportingly, the in vitro *hemL* transcript (+301~+457) was directly cleaved by RNase III with multiple sites (**Fig. 5B-C**) (structure predicted by RNADraw, www.rnadraw.com).[20] Further examination of the steady-state levels of *hemL* by qRT-PCR using three different pairs of primers in an *rnc*+ or *rnc14* background showed the levels of *hemL* RNA were higher 2-fold in *rnc14* compared with *rnc*+ (**Fig. 5D**). Additionally, the amount of intact *hemL* transcript in vivo was 2.5-fold higher in *rnc14* cells compared with *rnc*+ by primer extension analysis (**Fig. 5E, F**). All data support that *hemL* is a newly identified RNase III-dependent target. Therefore, we proved that our fluorescent reporter is a sensitive enough for monitoring the weak dependency (~2-fold of difference) of cellular transcript on RNase III in less than 2 days. This may be useful for identifying RNase III dependent targets that are affected by multiple ribonucleases.

Discussion

The novel translational fluorescent reporter system described herein was developed with several unique features compared with currently available enzyme-based reporter gene systems. First, this new system contains nine unique cloning sites upstream to eGFP coding region, resulted in the largest number of cloning sites for such kind of vectors. Second, the vector can be used for making single-copy chromosomal fusions because the original features of pRS415[5] for generating chromosomal translational *lacZ* fusion genes by homologous recombination were retained.

pRS1553 has been widely used as a promoter-less transcriptional fusion vector for promoter analysis using LacZ expression as a read-out. While it is useful, background expression of LacZ makes it difficult to monitor slight changes in promoter and terminator activity and to differentiate protein activities, i.e.,

RNase activity, in vivo. Although several translational fusion vectors are available for bacterial systems such as pRS415, the large size and small number of cloning sites limit their uses. The current system overcomes these issues and represents an improvement in efficiency for reporter gene systems.

RNases are key factors in the regulation of gene expression. Thus, the ability to control RNase activity, and hence gene expression, efficiently could serve as a means to manipulate cellular pathways. However, RNase activity is highly dynamic and is regulated by a number of *trans*-acting regulators, including YmdB, RraA, RraB, as well as other as-yet uncharacterized proteins.[17,18,21,22] How broadly the known *trans*-acting regulators participate in RNA cleavage or decay and whether the regulator-target pairs differ depending on environmental stress are open questions. For example, osmotic regulation of *bdm* mRNA levels in *E. coli* is partially RNase III-dependent,[17] but the involvement of YmdB, a regulator of RNase III in response to cold stress,[18] in this process has yet to be fully elucidated. Moreover, the level of YmdB is not affected by osmotic stress, suggesting that there may be other stress-dependent or target-dependent *trans*-acting regulator(s) of RNase III.[17,18] In the current study, the incomplete inactivation of *bdm* by YmdB overexpression was identified (**Fig. 4**), supporting the above hypothesis. Thus, the vector system developed herein is an appropriate system for screening and identifying such regulators of RNase III.

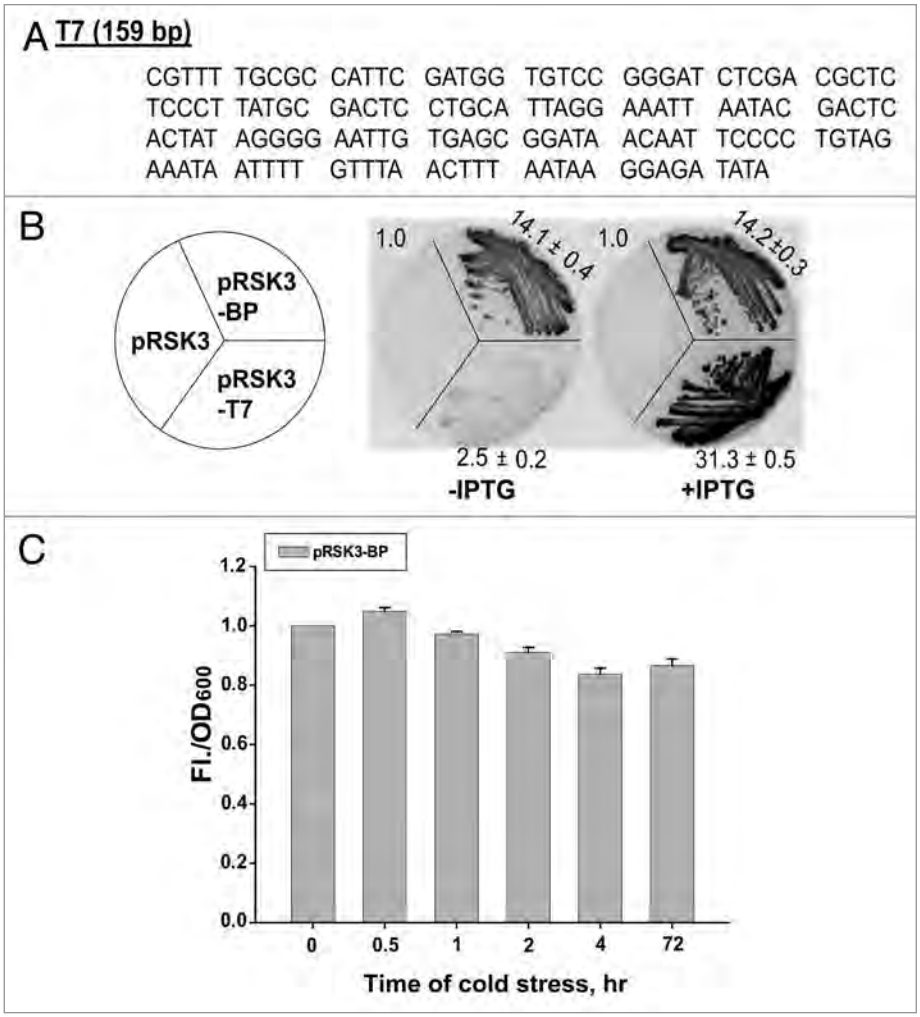


Figure 3. Characteristics of BP in *E. coli*. (A) Sequence of the T7 promoter. The exact insert *Eco*RI/*Nco*I for T7 is indicated. (B) Comparison of activity between BP and T7 promoter. Transformants of pRSK3 (control), pRSK3-BP or pRSK3-T7 in *E. coli* BL21 (DE3) were re-streaked onto LB/Amp plates with or without IPTG (1 mM). The relative intensities of eGFP were determined from independent colonies (n = 3) were measured by LAS-3000 and shown. (C) Effect of cold-stress on BP activity. Cells (pRSK3-BP in BW25113) were grown to OD_{600} = 0.5 at 37°C and moved the culture to 4°C for the cold-stress. The fluorescence/OD_{600} (Fl./OD_{600}) values after specific time of cold-stress were measured. Relative values to that of without cold-stress were shown from the average of three independent experiments.

Most of currently available RNase mutant strains are temperature sensitive, do not have λ prophages and are *lacZ*+ [i.e., RNase III mutant HT115: W3110 *rnc14::Tn10*[23]; RNase P mutant-NHY322: UY211 Δ(*proB lac*), *ara, gyrA, thi, zic-501::Tn10, rnpA*-].[24,25] Thus, the precise determination of promoter or RNase activity by assaying LacZ activity is problematic due to the high basal level of β-galactosidase. Therefore, additional genetic modifications are needed. The fluorescent reporter system described in the current study overcomes these problems.

Several attempts to identify RNase targets using microarrays have been reported.[17,26,27] For RNase III, many putative targets that are affected by the level of RNase III have been identified by microarray analysis;[17] however, with the exception of *bdm*, these targets have not been validated and the validation process is still challenging. The mRNA levels of *hemL* we identified as an RNase III target in this study differed by only about 2-fold in an *rnc*+ compared with *rnc14* background. Importantly, this difference might not have been detected using an enzyme-based colorimetric assay. We also found that the difference of cleavage site between in vivo and in vitro transcripts of *hemL*. It might be originated from either structure difference or the RNA with 5' ends cleaved by RNase III in vitro might be the intermediate product in vivo.

Using our system, even the library was biased both in terms of the number and type of sequences represented we could identify a hidden RNase III target. Further identification of RNase III targets by screenings with our system will enable us to unravel the role of RNase III in the turnover of unidentified cellular transcripts. This in turn will lead to a better understanding of the

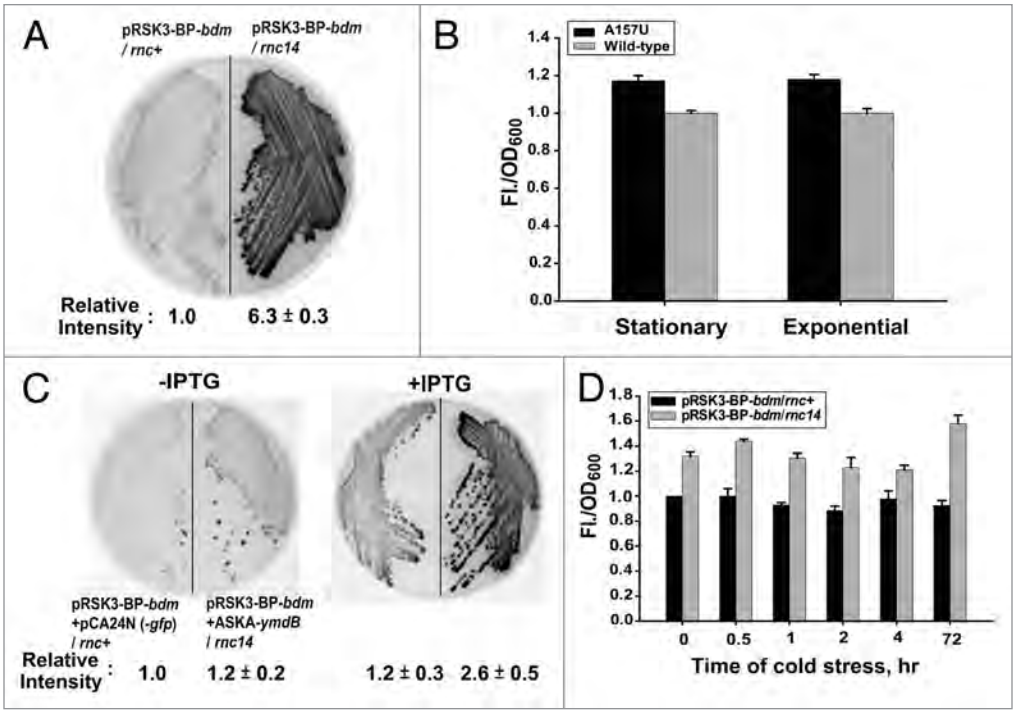


Figure 4. Regulation of *bdm* by RNase III. (A) Effect of RNase III on *bdm*-eGFP expression. pRSK3-BP-*bdm* transformants of BW25113 (*rnc*+) or BW25113 (*rnc14*) were re-streaked onto an LB/Amp plate. (B) Effect of RNase III cleavage site mutation (A157U) in *bdm* on eGFP expression. eGFP expression from either wild-type or A157U of *bdm* in exponential (OD_{600} = 0.5) or stationary (OD_{600} = 3.0) phase growth was measured. The relative values of fluorescence/OD_{600} (Fl./OD_{600}) were shown. (C) Effect of YmdB-mediated downregulation of RNase III on *bdm*-eGFP expression. Co-transformants of BW25113 (*rnc*+) containing pRSK3-BP-*bdm* with either pCA24N (-*gfp* version) or ASKA-*ymdB* were re-streaked onto LB/Amp/Cm plates with or without 1 mM IPTG. Image analysis of (A) and (C) was performed using an LAS-3000 image analyzer. Relative intensity represents an averaged level of eGFP expression from five different colonies and one of the representative data was shown. (D) Monitoring RNase III activity variation in real-time. pRSK3-BP-*bdm* in *rnc*+ or *rnc14* were grown to OD_{600} = 0.5 and transferred to 4°C. The relative values of Fl./OD_{600} after specific time of cold-stress were shown from three independent experiments.

role of RNase III in stress-responses, and serve as a foundation for characterizing the activity and regulation of other RNases, such as RNase P and PNPase, whose regulators have yet to be identified.

Moreover, it has been known that sRNA-mRNA interactions are important for the regulation of gene expression.[28] Our system will be useful for the assays by incorporating target mRNA libraries for the screening of sRNA-dependent mRNA targets, making us to identify the good candidates for sRNA-mediated gene regulation.

In conclusion, the fluorescence reporter system developed in the current study represents an improvement over current colorimetric systems for monitoring RNase activity and identifying target RNAs. The potential of this system lies in the ability to measure slight changes in protein activity or sRNA-mRNA interactions in response to environmental signals with lower background and a less environmentally sensitive promoter and powerful fluorescence detection.

Materials and Methods

Strains, oligonucleotides, chemicals and enzymes. The parental bacterial strains used in this study were *E. coli* K12 strains DH5α, BL21 (DE3) and BW25113.[29] Gram-positive *Bacillus subtilis* strain GB03[30] was used for the preparation of the promoter library. An RNase III mutant strain (*rnc14*) in the BW25113 background was derived from strain HT115[23] by general P1 transduction.[31] Plasmids used in this study are listed in **Table 1**. Oligonucleotides were synthesized by Bioneer Inc. and are listed in **Table 2**. Isolation of plasmid DNA (Bioneer, Inc.), restriction enzyme digestion (Fermentas) and ligation with T4 DNA ligase (LigaFast Rapid DNA Ligation System; Promega) were performed according to the manufacturer's protocols. All chemicals, including isopropyl β-D-1-thiogalactopyranoside (IPTG), were purchased from Sigma-Aldrich Inc. AccuPower Pfu PCR Premix (Bioneer) was used to amplify genes for cloning purposes. Colony PCR was performed using an EmeraldAmp GT PCR Master Mix (Takara).

Construction of libraries. Chromosomal DNA from *B. subtilis* strain GB03 was prepared using a G-Spin Genomic DNA extraction kit (Intron). A GB03 promoter library was prepared by partial digestion of the chromosomal DNA with *Sau*3AI followed by agarose gel electrophoresis and elution of products approximately 0.3–0.5 kbp in size from the gel. Digested products were ligated into *Bam*HI-linearized pBGR1.[16] An RNase III target library was prepared as follows. Genomic DNA from *E. coli* BW25113 was digested to completion with *Nco*I, and fragments in the 0.1–1.0 kbp range were eluted following agarose

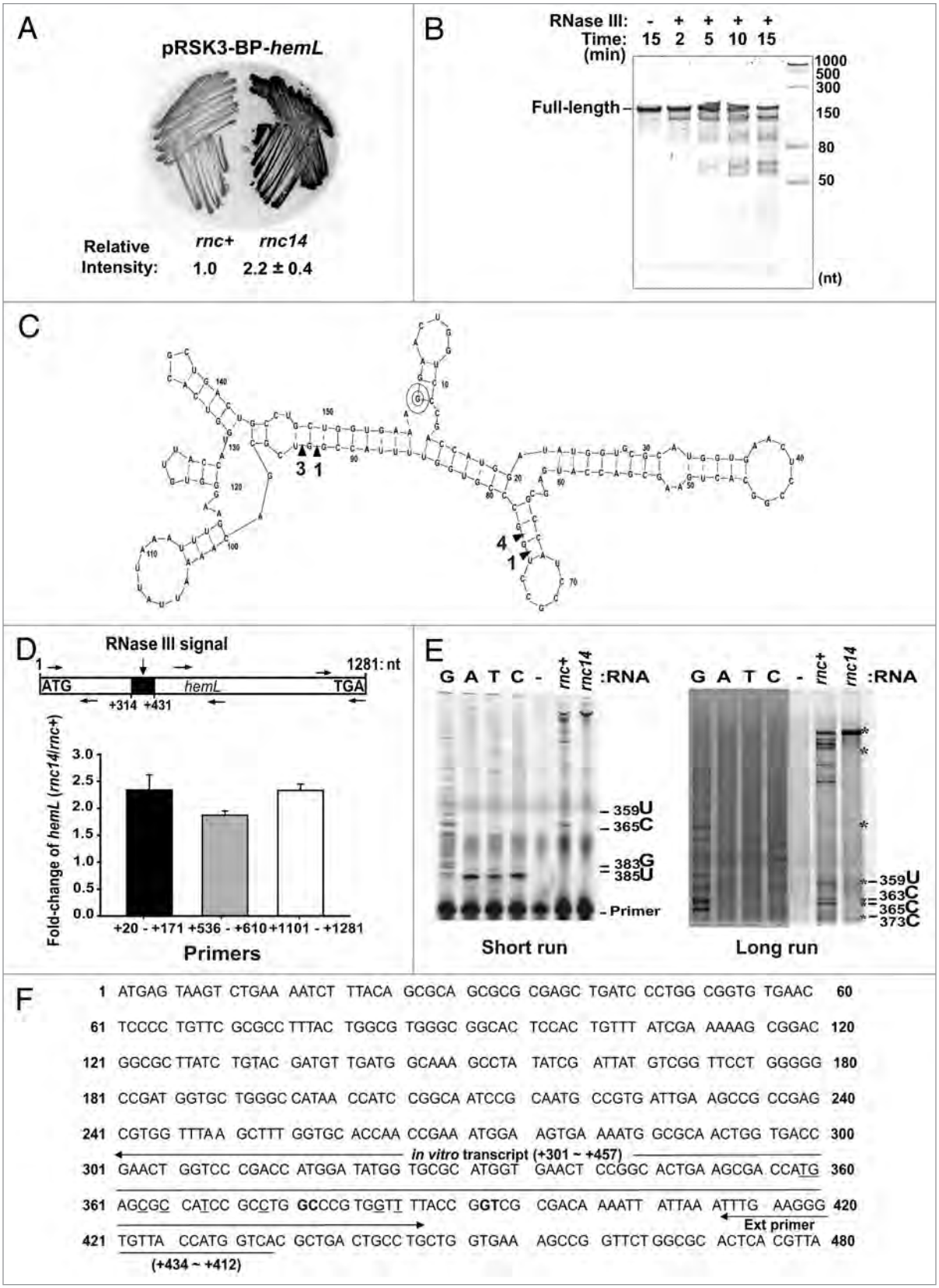


Figure 5. For figure legend, see page 8.

Figure 5. RNase III-dependent modulation of *hemL* transcript. (A) Expression of eGFP from pRSK3-BP-*hemL* in an *rnc*+ or *rnc14* background. Expression was 2.2-fold higher in the *rnc14* background compared with *rnc*+; colonies were quantified using an LAS-3000 image analyzer. (B) In vitro cleavage assays of *hemL* transcript by RNase III. *hemL* transcript (+301~+457) was incubated with RNase III by time-course manner and run on 10% PAGE (+7M Urea) gel. Full-length indicates the intact transcript. Size marker (ssRNA marker, New England Biolabs) were run together and indicated by size (nt). (C) Predicted RNA secondary structure of the *hemL* region of pRSK3-BP-*hemL* (+314 ~+431) with cleavage sites. RNA structure was predicted by RNADraw and cleavage sites determined by 5' RACE (9 colonies) were indicated by arrows. The characters in bold indicated the numbers of sequenced colonies having the position as 5' end. (D) qRT-PCR analysis of *hemL*. qRT-PCR was performed using isogenic strains of those described in (A) and the indicated sets of primers (+20~+171, +536~+610 and +1101~+1281) (arrows). The fold-change of *hemL* expression represents an average value (n = 3) from qRT-PCR analysis in *rnc14* and *rnc*+ for each primer set normalized to 16S rRNA. (E) Primer extension analysis of native *hemL* transcript. Asterisks (*) indicate the RNase III-dependent cleavage sites. The amount of intact *hemL* transcript was 2.3-fold higher in *rnc14* cells. Samples run on 8% PAGE (+7M Urea) gel were shown after short or long time electrophoresis. (F) DNA sequences of partial *hemL* coding region were indicated. Underlined sequences indicate the 5' ends of in vitro cleavage products by 5' RACE, while bold sequences show the in vivo RNase III-dependent sites.

Table 1. Plasmids used in this study

Plasmid	Description	References
pRS1553	Low-copy transcriptional *lacZ* fusion	4
pRSK1	RBS was removed from pRS1553 and re-annealed by *Bam*HI	This study
pRSK2	MCS1 and MCS2 in pRSK1	This study
pRSK3	pRSK2, eGFP	This study
pRSK3-BP	BP in pRSK3	This study
pRSK3-T7	T7 in pRSK3	This study
pBGR1	Promoter library of GB03	16
pSuper-retro-neo-gfp	Template for eGFP amplification	Oligoengine, Inc.
pRSF-1b	Template for T7 promoter region	Novagen
pCA24N (-*gfp*)	*gfp* region was eliminated by *Bam*HI cut and self-ligation	This study
ASKA-*ymdB*	ASKA library	33
ASKA-*hemL*	ASKA library	33

gel electrophoresis. Digested products were ligated into *Nco*I-linearized pRSK3-BP.

Mutagenesis and DNA sequencing. Mutagenesis of pRSK3-BP-*bdm* (A157 to U157) were performed by KOD-Plus Mutagenesis Kit (Toyobo) with mutagenic primers. Sequences of pRS1553 and pRSK1–2 or pRSK3 series of constructs were confirmed by DNA sequencing (SolGent Inc.) using the sequencing primers KSKRI11 or KSKRI12, respectively. Other vectors were sequenced with the appropriate primers.

Fluorescence detection. Expression of fluorescent eGFP in bacterial colonies was detected by a luminescent image analyzer, LAS-3000 (Fujifilm). Image exposure time was 0.25 sec and images were quantified using Multi Gauge software (ver 2.0) (Fujifilm). Each experiment was performed at least in triplicate. Expression of eGFP in culture was measured by 2104 EnVision multilabel plate readers (PerkinElmer) and analyzed by Wallac Envision manager 1.12.

RNA isolation and quantitative (q)PCR. *E. coli* strain BW25113 or BW25113 (*rnc14*) was grown to an OD_{600} of 0.8 and then total RNA was extracted using an RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. The quantity of extracted RNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The integrity of the samples was assessed by 1.2% agarose gel electrophoresis and visualization under UV light. cDNA synthesis (20 μl reaction) was performed with total RNA (1 μg) and random hexamer primer as the priming oligo using an ReverTraAce qPCR RT Kit (Toyobo), according to the manufacturer's instructions. The thermocycling parameters were as follows: 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec and elongation at 72°C for 30 sec. Amplification was followed by melting curve analysis at 65°C to 95°C, with step increases of 0.5°C every 5 sec. Melting curve analysis was performed with 1 μL of the cDNA mixture using an IQ SYBR Green Supermix Kit and CFX96 real-time PCR detection system (Bio-Rad). The gene-specific primer sets for 16S rRNA and *hemL* mRNA were designed by PrimerQuest (www.idtdna.com). The baseline and threshold values were automatically determined using CFX manager software (ver. 2.0, Bio-Rad), and -ΔΔCT calculations were done using Microsoft Excel.

In vitro cleavage assays and 5' RACE. For the preparation of RNA substrate, *hemL* regions of +301~+457 were PCR amplified and the PCR fragment was further used as a template for in vitro transcription with T7 RiboMax kit (Promega) as described in the protocol. The RNA in size 157 nt was cleaned up by RNeasy MinElute Cleanup kit (Qiagen) and used further. RNase III cleavage activity was performed as described previously[18] except that RNA substrate (250 ng/reaction) was incubated with ShortCut RNase III (0.01 U) in 20 μL of reaction buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 0.1 mM DTT, 10 mM $MgCl_2$) at 37°C for 2 to 15 min. The reactions were stopped with 2x RNA loading dye containing 20 mM EDTA. The products were electrophoresed by 10% PAGE gel (+7 M urea) and stained with RNA stain gel II (Invitrogen). The 5' ends of cleavage products were determined by 5' RACE analysis as described[32] with minor modifications using SMARTScribe Reverse Transcriptase (Clontech) with random hexamer as a priming primer followed by PCR amplification with primers TSO[32] and KSKRI27 with EmeraldAmp® GT PCR Master Mix, and pGEM-T-easy vector (Promega) cloning.

Primer extension analyses. To identify the 5' ends of *hemL* transcripts, primer extension analysis was performed according to the protocols of SuperScript® III Reverse Transcriptase (Invitrogen) with 55°C for 1.5 h. To this end, cells of *rnc*+ or *rnc14* containing ASKA-*hemL* were grown to OD_{600} of 0.2 and induced *hemL* expression by 0.5 mM IPTG to OD_{600} of 0.8,

Table 2. Oligonucleotides used in this study

Oligo Name	Sequence (5' to 3')	Purpose
KSKRI1	GACCA TGATT ACGGA TTC**AG GA**TCC GTCGT TTACA ACGTC G	Mutagenesis for *Bam*HI of pRS1553
KSKRI2	CGACG TTGTA AACG ACGG**A TCC**TG AATCC GTAAT CATGG TC	Mutagenesis for *Bam*HI of pRS1553
KSKRI3	AATTC CCCGG GGAGC TCAGG AAGAA ATTCC ATGGT ACCC T CTAGA CTCGA GAAGC TTG	pRSK2 insert_for
KSKRI4	GATCC AAGCT TCTCG AGTCT AGAGG GTACC ATGGA ATTTC TTCCT GAGCT CCCCG GGG	pRSK2 insert_rev
KSKRI5	CCAAG CTTAT GGTGA GCAAG GGCG	pRSK3 EGFP insert (for)
KSKRI6	CGGGA TCCTT ACTTG TACAG CTCGT CC	pRSK3 EGFP insert (rev)
KSKRI7	CGGAA TTCGG ATCTT ACACA TTTTT TCT	pRSK3-BP insert (for)
KSKRI8	GCGTG AGCTC GGCAT CGTTG CGATT CGT	pRSK3-BP insert (rev)
KSKRI9	CGGAA TTCGG TTTTG CGCCA T TCGA	pRSK3-T7 insert (for)
KSKRI10	TACCC ACGTG ATGGT GGTGG TGATG	pRSK3-T7 insert (rev)
KSKRI11	CTGCG CAACT GTTGG GAAGG GCG	pRS1553 and pRSK1–2 sequencing primer
KSKRI12	CGTCC ATGCC GAGAG TG	pRSK3 series sequencing primer
KSKRI13	CTGAA AATCT TTACA GCGCA	*hemL* qRT-PCR (for)-set1
KSKRI14	CCGAC ATAAT CGATA TAGGC	*hemL* qRT-PCR (rev)-set1
KSKRI15	GTACT TATAA TGATC TGGCT TCT	*hemL* qRT-PCR (for)-set2
KSKRI16	CGACG ATAAT ACAGG CAAT	*hemL* qRT-PCR (rev)-set2
KSKRI17	CTATC AGGAT GTGAT GGCCT	*hemL* qRT-PCR (for)-set3
KSKRI18	TCACA ACTTC GCAAA CACCC G	*hemL* qRT-PCR (rev)-set3
KSKRI19	GCTAC AATGG CGCAT ACAAA	16S qRT-PCR (for)
KSKRI20	TTCAT GGAGT CGAGT TGCAG	16S qRT-PCR (rev)
KSKRI21	TGAAC TCGGT GATGA CGTTC	pBGR1-sequencing primer
KSKRI22	GTAAAACGACGGCCAGT	M13F (-20) for RACE sequencing
KSKRI23	GCTCTAGAATGTTTACTTATTATCAGGCAG	
KSKRI24	GCCCTCGAGAAGCGAGGGTGCTGGCCACT	
KSKRI25	GATAA CGACA TCCTC **T**GTGA TATCT ACCAG C	Mutagenesis of A157U of *bdm*
KSKRI26	GCTGG TAGAT ATCAC **A**GAGG ATGTC GTTAT C	Mutagenesis of A157U of *bdm*
KSKRI27	TTTCA CCAGC AGGCA G	5' RACE primer (rev)

[1]Mutagenic regions were indicated in bold. [2]Underlined sequences indicate the restriction enzyme sites.

followed by RNA isolation using RNeasy kit. Total cellular RNA (100 μg) was used for primer extension with 20 pmol of 5'-Cy5 labeled extension primer (+434~+412: 5'-Cy5-ACT GGT ACC ATT GTG GGA AGT TT-3'; Bioneer) with sequencing ladders reaction of plasmid ASKA-*hemL* by AccuPower DNA sequencing kit (Bioneer). Reaction products were electrophoresed by 8% PAGE (+7 M Urea) gel and imaged by Typhoon 9410 (GE Healthcare) (emission filter was set up at 580 nm and detector at 488 nm).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Hughes KT, Maloy SR. Use of operon and gene fusions to study gene regulation in Salmonella. Methods Enzymol 2007; 421:140-58; PMID:17352921; http://dx.doi.org/10.1016/S0076-6879(06)21013-9.
2. Silhavy TJ, Berman ML, Enquist LW. Experiments with gene fusions. NY, Cold Spring Harbor: Cold Spring Harbor Laboratory 1984.
3. Silhavy TJ, Beckwith JR. Uses of lac fusions for the study of biological problems. Microbiol Rev 1985; 49:398-418; PMID:3005818.
4. Pepe CM, Suzuki C, Laurie C, Simons RW. Regulation of the "tetCD" genes of transposon Tn10. J Mol Biol 1997; 270:14-25; PMID:9231897; http://dx.doi.org/10.1006/jmbi.1997.1094.
5. Podkovyrov SM, Larson TJ. A new vector-host system for construction of lacZ transcriptional fusions where only low-level gene expression is desirable. Gene 1995; 156:151-2; PMID:7737510; http://dx.doi.org/10.1016/0378-1119(95)00053-9.
6. Simons RW, Houman F, Kleckner N. Improved single and multicopy lac-based cloning vectors for protein and operon fusions. Gene 1987; 53:85-96; PMID:3596251; http://dx.doi.org/10.1016/0378-1119(87)90095-3.
7. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 1994; 263:802-5; PMID:8303295; http://dx.doi.org/10.1126/science.8303295.

8. Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. Primary structure of the Aequorea victoria green-fluorescent protein. Gene 1992; 111:229-33; PMID:1347277; http://dx.doi.org/10.1016/0378-1119(92)90691-H.
9. Cormack BP, Valdivia RH, Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 1996; 173(1 Spec No):33-8; PMID:8707053; http://dx.doi.org/10.1016/0378-1119(95)00685-0.
10. Crameri A, Whitehorn EA, Tate E, Stemmer WP. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat Biotechnol 1996; 14:315-9; PMID:9630892; http://dx.doi.org/10.1038/nbt0396-315.
11. Tsien RY. The green fluorescent protein. Annu Rev Biochem 1998; 67:509-44; PMID:9759496; http://dx.doi.org/10.1146/annurev.biochem.67.1.509.
12. Chen C, Deutscher MP. Elevation of RNase R in response to multiple stress conditions. J Biol Chem 2005; 280:34393-6; PMID:16135521; http://dx.doi.org/10.1074/jbc.C500333200.
13. Jain C, Deana A, Belasco JG. Consequences of RNase E scarcity in Escherichia coli. Mol Microbiol 2002; 43:1053-64; PMID:11929550; http://dx.doi.org/10.1046/j.1365-2958.2002.02808.x.
14. Matsunaga J, Dyer M, Simons EL, Simons RW. Expression and regulation of the rnc and pdxJ operons of Escherichia coli. Mol Microbiol 1996; 22:977-89; PMID:8971718; http://dx.doi.org/10.1046/j.1365-2958.1996.01529.x.
15. Thomas DY, Dubuc G, Narang SE. Escherichia coli plasmid vectors containing synthetic translational initiation sequences and ribosome binding sites fused with the lacZ gene. Gene 1982; 19:211-9; PMID:6293930; http://dx.doi.org/10.1016/0378-1119(82)90008-7.
16. Han SS, Lee JY, Kim WH, Shin HJ, Kim GJ. Screening of promoters from metagenomic DNA and their use for the construction of expression vectors. J Microbiol Biotechnol 2008; 18:1634-40; PMID:18955811.
17. Sim SH, Yeom JH, Shin C, Song WS, Shin E, Kim HM, et al. Escherichia coli ribonuclease III activity is downregulated by osmotic stress: consequences for the degradation of bdm mRNA in biofilm formation. Mol Microbiol 2010; 75:413-25; PMID:19943899; http://dx.doi.org/10.1111/j.1365-2958.2009.06986.x.
18. Kim KS, Manasherob R, Cohen SN. YmdB: a stress-responsive ribonuclease-binding regulator of E. coli RNase III activity. Genes Dev 2008; 22:3497-508; PMID:19141481; http://dx.doi.org/10.1101/gad.1729508.
19. Ilag LL, Jahn D, Eggertsson G, Söll D. The Escherichia coli hemL gene encodes glutamate 1-semialdehyde aminotransferase. J Bacteriol 1991; 173:3408-13; PMID:2045363.
20. Matzura O, Wennborg A. RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. Comput Appl Biosci 1996; 12:247-9; PMID:8872395.
21. Lee K, Zhan X, Gao J, Qiu J, Feng Y, Meganathan R, et al. RraA. a protein inhibitor of RNase E activity that globally modulates RNA abundance in E. coli. Cell 2003; 114:623-34; PMID:13678585; http://dx.doi.org/10.1016/j.cell.2003.08.003.
22. Zhou L, Zhao M, Wolf RZ, Graham DE, Georgiou G. Transcriptional regulation of the Escherichia coli gene rraB, encoding a protein inhibitor of RNase E. J Bacteriol 2009; 191:6665-74; PMID:19717586; http://dx.doi.org/10.1128/JB.00344-09.
23. Takiff HE, Chen SM, Court DL. Genetic analysis of the rnc operon of Escherichia coli. J Bacteriol 1989; 171:2581-90; PMID:2540151.
24. Kirsebom LA, Isaksson LA. Involvement of ribosomal protein L7/L12 in control of translational accuracy. Proc Natl Acad Sci USA 1985; 82:717-21; PMID:3883346; http://dx.doi.org/10.1073/pnas.82.3.717.
25. Kirsebom LA, Baer MF, Altman S. Differential effects of mutations in the protein and RNA moieties of RNase P on the efficiency of suppression by various tRNA suppressors. J Mol Biol 1988; 204:879-88; PMID:2464697; http://dx.doi.org/10.1016/0022-2836(88)90048-4.
26. Lee K, Bernstein JA, Cohen SN. RNase G complementation of rne null mutation identifies functional interrelationships with RNase E in Escherichia coli. Mol Microbiol 2002; 43:1445-56; PMID:11952897; http://dx.doi.org/10.1046/j.1365-2958.2002.02848.x.
27. Li Y, Altman S. A specific endoribonuclease, RNase P, affects gene expression of polycistronic operon mRNAs. Proc Natl Acad Sci USA 2003; 100:13213-8; PMID:14585931; http://dx.doi.org/10.1073/pnas.2235589100.
28. Beisel CL, Storz G. Base pairing small RNAs and their roles in global regulatory networks. FEMS Microbiol Rev 2010; 34:866-82; PMID:20662934.
29. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci USA 2000; 97:6640-5; PMID:10829079; http://dx.doi.org/10.1073/pnas.120163297.
30. Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Paré PW. Bacterial volatiles induce systemic resistance in Arabidopsis. Plant Physiol 2004; 134:1017-26; PMID:14976231; http://dx.doi.org/10.1104/pp.103.026583.
31. Miller JH. A short course in bacterial genetics. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press 1992.
32. Pinto FL, Lindblad P. A guide for in-house design of template-switch-based 5' rapid amplification of cDNA ends systems. Anal Biochem 2010; 397:227-32; PMID:19837043; http://dx.doi.org/10.1016/j.ab.2009.10.022.
33. Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, et al. Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. DNA Res 2005; 12:291-9; PMID:16769691; http://dx.doi.org/10.1093/dnares/dsi012.