

# Conditional gene silencing of multiple genes with antisense RNAs and generation of a mutator strain of *Escherichia coli*

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## ABSTRACT

In this study, we describe a method of simultaneous conditional gene silencing of up to four genes in *Escherichia coli* by using antisense RNAs. We used antisense RNAs with paired termini, which carried flanking inverted repeats to create paired double-stranded RNA termini; these RNAs have been proven to have high silencing efficacy. To express antisense RNAs, we constructed four IPTG-inducible vectors carrying different but compatible replication origins. When the *lacZ* antisense RNA was expressed using these vectors, *lacZ* expression was successfully silenced by all the vectors, but the expression level of the antisense RNA and silencing efficacy differed depending on the used vectors. All the vectors were co-transformable; the antisense RNAs against *lacZ*, *ackA*, *pta* and *pepN* were co-expressed, and silencing of all the target genes was confirmed. Furthermore, when antisense RNAs were targeted to the mutator genes *mutS*, *mutD* (*dnaQ*) and *ndk*, which are involved in DNA replication or DNA mismatch repair, spontaneous mutation frequencies increased over 2000-fold. The resulting mutator strain is useful for random mutagenesis of plasmids. The method provides a robust tool for investigating functional relationships between multiple genes or altering cell phenotypes for biotechnological and industrial applications.

## INTRODUCTION

Robust gene disruption and silencing methods are required to characterize genes with unknown functions or to engineer cells so that can express desired traits. Disruption of a particular gene on a chromosome is a

suitable technique for ensuring the complete absence of its function; however, this is possible with a limited set of species and strains and is particularly problematic when the gene is crucial for growth. On the other hand, gene silencing is applicable even when the targeted genes are essential for growth; moreover, conditional or reversible gene silencing can be achieved easily. Antisense RNA- and RNA interference (RNAi)-mediated gene silencing are popular techniques used in eukaryotes; however, RNAi mechanism is absent in bacteria, and hence, single-stranded antisense RNAs (asRNAs) expressed from expression vectors are frequently used for gene silencing (1). The expressed asRNAs are typically designed to hybridize the sequences flanking the ribosome-binding site (RBS) and the start codon of the target mRNAs. They prevent ribosomes from recognizing the RBS, and thus inhibit translation (2–4).

There are many examples of the application of expressed asRNAs in diverse bacteria (4–8). For example, asRNA expression library screens in *Staphylococcus aureus* have revealed putative new essential genes and growth inhibitors (5), and in the acetone-producing bacterium *Clostridium acetobutylicum*, the acetone fermentation pathway has been investigated by the silencing of individual genes (9). However, there are also many reports that state that low silencing efficacy hampers phenotypic or molecular analysis, particularly in *Escherichia coli* (1,10).

To overcome the low silencing efficacy in *E. coli*, we recently developed a new IPTG-inducible expression vector that expresses asRNAs with paired termini (PT), wherein flanking inverted repeats create paired double-stranded RNA termini (Figure 1) (4). The PTasRNA has a much higher silencing efficacy than conventional asRNAs because of better RNA stability, which increases RNA abundance in cells. In fact, the PTasRNA against the *fabI* gene, a gene essential for growth, inhibited cell growth, while a conventional asRNA did not (4).

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In our previous study, only one PTasRNA expression vector was constructed; however, in theory, multiple asRNAs can be expressed simultaneously in cells to target multiple genes. In this study, we constructed four PTasRNA expression vectors for *E. coli*. All these vectors were IPTG-inducible, compatible and co-transformable. Therefore, up to four genes could be silenced in parallel. We demonstrated parallel silencing of a functionally unrelated gene set (*lacZ*, *ackA*, *pta* and *pepN*) and a related gene set (*mutS*, *mutD* and *ndk*) and obtained expected phenotypes. Silencing of the latter gene set resulted in the generation of a mutator strain of *E. coli* that was useful for random mutagenesis. Multiple gene silencing mechanism is useful for investigating gene function and gene interactions or for cell engineering.

## MATERIALS AND METHODS

### *E. coli* strains, plasmids and general techniques

The *E. coli* MG1655 strain was used as a host for expressing PTasRNAs throughout the study. The DH5 $\alpha$  strain was used for only plasmid preparation. Both the strains were always cultured in Luria Broth (LB; 1% bacto tryptone, 0.5% bacto-yeast extract and 1% NaCl) in the presence or absence of appropriate antibiotics at 37°C. The antibiotics used to select transformants were apramycin (35  $\mu\text{g ml}^{-1}$ ), chloramphenicol (24  $\mu\text{g ml}^{-1}$ ), kanamycin (15  $\mu\text{g ml}^{-1}$ ) and ampicillin (50  $\mu\text{g ml}^{-1}$ ). The plasmids used in this study are listed in Table 1. Transformation was carried out by the standard chemical method, and co-transformation with multiple plasmids was carried out by introducing two or more plasmids simultaneously. Cell density was measured at 600 nm in 200  $\mu\text{l}$  of the cultures in a 96-well plate (Nalge Nunc International, Denmark; product no. 269620) by using the Safire microplate reader (Tecan, Switzerland) and the LS-PLATEmanager 2004 data analysis program (Wako Pure Chemicals Co., Japan). PCR was performed using *Pfu* turbo polymerase (Stratagene, La Jolla, CA) or KOD-plus polymerase (Toyobo, Japan). When phosphate groups were required at the 5'-end of the PCR fragments for subsequent ligation, T4 polynucleotide kinase (Toyobo) was used to phosphorylate the oligonucleotides.

### Construction of plasmids

The oligonucleotides that were used to amplify asRNA sequences from the genomic DNA of the MG1655 strain by PCR are shown in Table 2. All the PCR fragments were digested with NcoI and XhoI and cloned into the NcoI-XhoI moiety of the plasmids pHN1009, pHN678, pHN1242, pHN1257 and/or pHN1270 (Figure 1 and Table 1, and see below for the construction procedure).

To amplify the ColE1 and ampicillin-resistance gene sequences, PCR was performed using the primers (TTT GCTAGCTCTTTTGTATTTTCTAAATACATTC and AAAGGTACCATTATGCATGCCGCTTCGCCTTCGCGC) and pBAD/HisA (Invitrogen Corp., Carlsbad, CA) as a template. The amplified fragment was digested with NheI and KpnI and ligated to the fragment containing the *lacI<sup>f</sup>* and IPTG-inducible promoter sequences.

The latter fragment was obtained by performing PCR using the primers (TTTGCTAGCAGAAGCATTGGTG CACCGTGCAG and AAAGGTACCGTGGAGGTAA TAATTGACGATATGA) and pHN678 (4) as a template, followed by digestion with NheI and KpnI. The resulting plasmid was named as pHN1009.

The pSC101 replication origin and apramycin-resistance gene were amplified by PCR by using a primer set (TTCATGCATTGACAGTAAGACGGGTA AGCC and TAAGCTAGCTATGGACAGTTTTCCCT TTGATATGTA) and pMW118 (Nippon Gene, Japan) as a template, and by using a primer set (TAGGCTAG CGATCCATGCCGTATTTGCAGCAGTG and TTTA TGCATTGGTACCAAACGGCATTGAGCGTCAGCA TATCATC) and pUC57-aac [custom-synthesized by Genscript Corp. (Piscataway, NJ) on the basis of a DNA sequence with a GenBank accession number X01385] as a template, respectively. Both the PCR fragments were digested with NsiI and NheI and ligated, thereby yielding pHN1104. To remove the unwanted SpeI and XhoI sites in pHN1104, PCR-based site-directed mutagenesis was performed, and the sequences of both the sites were modified from ACTAGT and CTCGAG to TC TAGT and CTCGAA, respectively. The mutagenized plasmid was named as pHN1114. A 1.9-kb fragment was excised from pHN1009 by digestion with KpnI and NheI and cloned into the KpnI-NsiI moiety of pHN1114 (NheI and NsiI sites were blunted using T4 DNA polymerase prior to ligation), thereby yielding pHN1237. A 1.4-kb fragment carrying the kanamycin-resistance gene was excised from pHN267 (11) by digestion with KpnI and SpeI and was inserted into the KpnI-NheI moiety of pHN1237, thereby yielding pHN1242. The copy-number mutant of pHN1242 was generated by inverse PCR using 5'-phosphorylated primers (AAGGCTTTAAGATTTTC CAGTGGAC and TAACCAAAGGATTCCTGATTTCC A) and named as pHN1257.

In order to amplify the RK2 origin sequence, PCR was performed using two 5'-phosphorylated primers (TCTCG CTAGCTTTCTTCAGGGCCGACAATC and AGGCC TCCGGGAGGGTTTCGAGAAGGGG) and pCF430 [National BioResource Project (NIG, Japan); *E. coli*] as a template. A fragment containing the *lacI<sup>f</sup>*, IPTG-inducible promoter and apramycin-resistance gene was PCR-amplified using two primers (TAGGCTAGCGATCCAT GCCGTATTTGCAGCAGTG and TGCTAACTTACA TTAATTGCGTTGC) and pHN1237 as a template. These PCR fragments were ligated, thereby yielding pHN1270.

### Protein assays

For expressing the *lacZ*, *ackA*, *pta* and *pepN* PTasRNAs and measuring their protein activities, transformants with PTasRNA expression vector(s) were pre-grown overnight in the absence of IPTG, diluted 1:400 with fresh media containing 1 mM IPTG, and cultured from mid- to late-logarithmic phase. Cells were disrupted in phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) using glass beads and a Multi-beads shocker (Yasui Kikai, Japan) at 4°C, and the

cell extracts were prepared by centrifugation at  $20\,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The total protein concentration was determined using the Bio-Rad Bradford assay kit (Bio-Rad, Hercules, CA).

The LacZ activity was measured in a microplate well (Nalge Nunc International; product no. 269620) at room temperature. The reaction mixture ( $180\ \mu\text{l}$  per well) was composed of 0.14 M NaCl, 2.7 mM KCl, 10.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{MgSO}_4$ , 50 mM 2-mercaptoethanol,  $0.4\ \text{mg ml}^{-1}$  *o*-nitrophenyl- $\beta$ -galactoside and cell extract. The absorbance was read at 420 nm/min using the Safire reader, and the slope was used to calculate the specific activity ( $\text{mg protein}^{-1}\ \text{min}^{-1}$ ).

For the AckA assay, each reaction mixture ( $180\ \mu\text{l}$ ) was prepared in a UV-transparent microplate well (Costar, NY; product no. 3635) that contained 200 mM Tris-HCl (pH 7.3), 200 mM potassium acetate, 1.5 mM ATP, 2 mM  $\text{MgCl}_2$ , 6.4 mM phospho(enol)pyruvate, 0.8 mM NADH, 2 mM dithiothreitol, 3.9 U pyruvate kinase, 9.5 U lactic dehydrogenase and cell extract (typically,  $0.8\ \mu\text{g protein}$ ) at room temperature. The NADH absorbance was read at 340 nm/min using the Safire reader, and the slope of the conversion curve was used to calculate the specific activity ( $\text{mg protein}^{-1}\ \text{min}^{-1}$ ). The Pta activity was measured as was done in the AckA assay by using a reaction mixture ( $180\ \mu\text{l}$ ) containing 200 mM Tris-HCl (pH 8.0), 5 mM  $\text{MgCl}_2$ , 15 mM malic acid, 10 mM  $\text{NAD}^+$ , 3.8 mM CoA, 20 mM acetyl-phosphate (lithium-potassium salt), 2.5 U citrate synthase, 64 U malate dehydrogenase and cell extract (typically,  $4\ \mu\text{g protein}$ ).

For the PepN assay, each reaction mixture ( $50\ \mu\text{l}$ ) was prepared in a black microplate well (Costar; product no. 3915) and contained 0.5 mM fluorogenic suc-LLVY-AMC substrate (Bachem, Germany) and cell extract (typically,  $50\ \mu\text{g protein}$ ) at room temperature. Fluorescence was read per minute using the Safire reader, and the slope of the fluorescence intensity was used to calculate the specific activity ( $\text{mg protein}^{-1}\ \text{min}^{-1}$ ). The settings were as follows: excitation, 380 nm; emission, 460 nm; excitation and emission bandwidths, 12 nm; gain, 177; number of flashes, 10; Z-position  $11019\ \mu\text{m}$  and integration time,  $500\ \mu\text{s}$ .

#### Estimation of the plasmid copy number

The plasmid copy number per cell was estimated by the agarose gel staining method (12), with modifications. Each plasmid was digested with EcoRV and XhoI to yield two bands and subjected to agarose gel electrophoresis. Three different volumes of each plasmid were loaded onto the gel, thus ensuring linearity of the band intensities. The resulting six bands were used for estimating the band intensities per kilobase for each plasmid per experiment. A Molecular-imager FX Pro (Bio-Rad) was used for visualizing the bands and their densities were densitometrically estimated using ImageJ (<http://rsb.info.nih.gov/ij/>).

#### RNA techniques

The total RNA was purified using the hot acid-phenol method (13), and in the case of real-time quantitative

RT-PCR assays, the purified RNA was treated with DNase I and further purified with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) to remove the genomic DNA completely.

For northern blot analysis, typically  $20\ \mu\text{g}$  of the total RNA was loaded in each lane, and ribosomal RNA bands were visualized by ethidium bromide staining and scanned by the Molecular-imager FX Pro. Two 5'-end-biotin-labelled oligonucleotides (GGAAACAGCTATGACCATGATTACGGATTCACTGGCCGTCGTTTTACA and GGTTATTTATGACTCAACAGCCACAAGCCAAATACCGTCACGATTATC) were synthesized and used as probes for *lacZ* and *pepN* PTasRNAs respectively; the probes were detected on the membranes by streptavidin-Alexa Fluor 680 conjugate (Invitrogen Corp.) and Odyssey infrared-imaging system (LI-COR Biosciences, Lincoln, NE) according to the manufacturer's instructions.

A real-time quantitative RT-PCR assay was performed using the One Step SYBR PrimeScript RT-PCR Kit II (Takara Bio Co., Japan) and the Mx3000P Real-Time PCR System. Results were analyzed with MxPro-Mx 3000P software (Stratagene). As a normalizer gene, 16S rRNA was amplified with primers (TTGCTCATTGACGTTACCCG, ACGCCAGTAATTCCGATTA) (14), and the *lacZ* mRNA was amplified with primers (GAG AATCCGACGGTTGTGA, GTTGCACCACAGATGAAACG) that were designed not to amplify the *lacZ* PTasRNA.

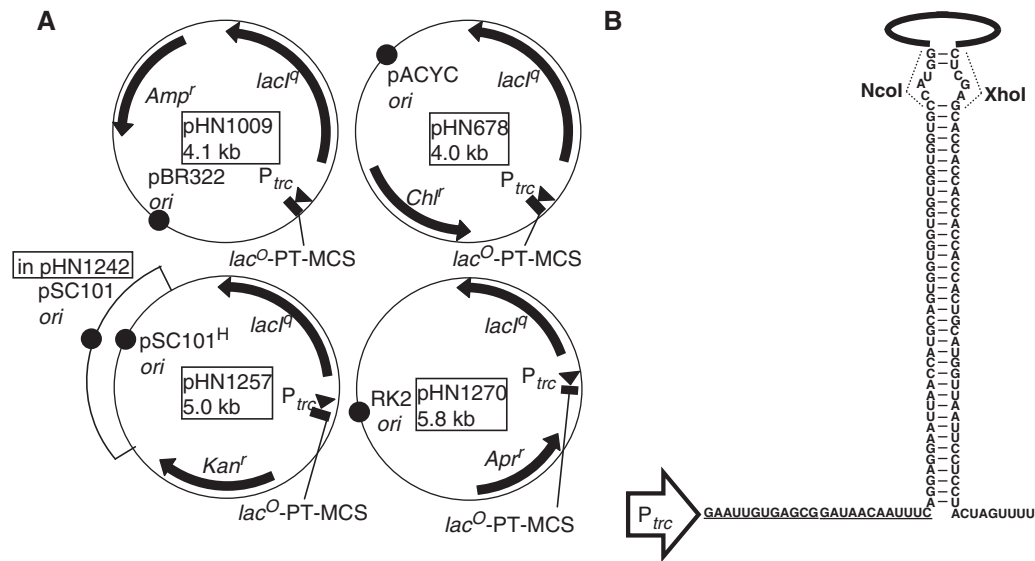
#### Estimation of mutation frequency

Transformants with PTasRNA expression vector(s) were pre-grown overnight in the absence of IPTG, diluted  $1:2 \times 10^7$  with fresh media containing or not containing 1 mM IPTG, and grown overnight. Appropriately diluted cultures were plated onto LB, LB plus  $100\ \mu\text{g ml}^{-1}$  rifampicin, and LB plus  $40\ \mu\text{g ml}^{-1}$  nalidixic acid plates, and the colonies that appeared after 15–18 h of incubation were counted. For one experiment of each transformant, six replicates were analyzed and the median was obtained. It is reported elsewhere that the median is more appropriate than the mean in this experiment (known as the Luria-Delbrück experiment or the fluctuation test), because the results are sensitive to occasional "jackpot" cultures; the median is not affected by extreme scores (15). For each transformant, mean of medians from sets of three experiments was calculated.

## RESULTS

#### Construction of PTasRNA expression vectors

We constructed four types of PTasRNA expressing vectors as shown in Figure 1A and Table 1. All the vectors harbored the *trc* promoter ( $P_{trc}$ ), *lac* operator sequence (*lac<sup>o</sup>*) and *lac* repressor gene (*lacI<sup>q</sup>*); hence, PTasRNA expression was IPTG inducible. Throughout the study, asRNAs containing PT composed of 38 base pairs (Figure 1B) were employed because such PT are known to have the best silencing efficacy (4). Each asRNA sequence comprises of 101–152 nucleotides (Table 2), was placed between the NcoI and XhoI restriction sites,



**Figure 1.** PTasRNA expression vectors and expressed PTasRNA. (A) Arrows indicate open reading frames or promoters, and circles indicate *oris*. MCS, multiple cloning site; *Amp<sup>r</sup>*, ampicillin-resistance gene; *Chl<sup>r</sup>*, chloramphenicol-resistance gene; *Kan<sup>r</sup>*, kanamycin-resistance gene and *Apr<sup>r</sup>*, apramycin-resistance gene. (B) Structure of a PTasRNA from its 5'-end. The curved line indicates the asRNA sequence (or a MCS control sequence in the case of an empty vector). The residues that have been underlined are transcribed from the *lac<sup>o</sup>* sequence.

**Table 1.** Plasmids used

Plasmid name	Relevant characteristics	Source
(General cloning vector)		
pACYC184	pACYC <i>ori</i> , <i>Chl<sup>r</sup></i> , <i>Tet<sup>r</sup></i>	(45)
(Empty vector)		
pHN1009	pBR322 <i>ori</i> , <i>Amp<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-MCS</i>	This study
pHN678	pACYC <i>ori</i> , <i>Chl<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-MCS</i>	(4)
pHN1242	pSC101 <i>ori</i> , <i>Kan<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-MCS</i>	This study
pHN1257	pSC101 <sup>H</sup> <i>ori</i> , <i>Kan<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-MCS</i>	This study
pHN1270	RK2 <i>ori</i> , <i>Apr<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-MCS</i>	This study
(PTasRNA expression vector)		
pHN1244	pBR322 <i>ori</i> , <i>Amp<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-lacZ</i> asRNA	This study
pHN1246	pACYC <i>ori</i> , <i>Chl<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-lacZ</i> asRNA	This study
pHN1249	pSC101 <i>ori</i> , <i>Kan<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-lacZ</i> asRNA	This study
pHN1259	pSC101 <sup>H</sup> <i>ori</i> , <i>Kan<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-lacZ</i> asRNA	This study
pHN1274	RK2 <i>ori</i> , <i>Apr<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-lacZ</i> asRNA	This study
pHN1258	pACYC <i>ori</i> , <i>Chl<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-pepN</i> asRNA	This study
pHN1275	RK2 <i>ori</i> , <i>Apr<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-pepN</i> asRNA	This study
pHN1245	pBR322 <i>ori</i> , <i>Amp<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-ackA</i> asRNA	This study
pHN1260	pSC101 <sup>H</sup> <i>ori</i> , <i>Kan<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-pta</i> asRNA	This study
pHN1251	pACYC <i>ori</i> , <i>Chl<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-mutS</i> asRNA	This study
pHN1295	pSC101 <sup>H</sup> <i>ori</i> , <i>Kan<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-mutD</i> asRNA	This study
pHN1300	RK2 <i>ori</i> , <i>Apr<sup>r</sup></i> , <i>lacI<sup>q</sup></i> , <i>P<sub>trc</sub></i> , <i>lac<sup>o</sup>-PT-ndk</i> asRNA	This study

and carried an anti-RBS sequence corresponding to each mRNA.

pHN1009, pHN678, pHN1257 and pHN1270 (Figure 1A) were co-transformable and could be stably maintained in a single cell in any combination because each harbored a different antibiotic resistance marker and plasmid replication origin (*ori*). pHN1242 (Figure 1A) is identical to pHN1257, except that the former harbors the pSC101 *ori*, which is the wild-type *ori* of pSC101. The pSC101<sup>H</sup> *ori* has a single-nucleotide substitution in the pSC101 *ori*, and this substitution is known to elevate the plasmid copy number to ~4-fold

(16). The largest difference among these vectors is the plasmid copy number, which is attributable to the *ori*, and the reported copy number of each *ori* per cell is as follows: pBR322, 50–70 (17); pACYC, 20–30 (17); pSC101, 6–12 (16,17); pSC101<sup>H</sup>, 30 (16) and RK2, 6–10 (18).

### Single-gene silencing and effect of copy number

Before studying multiple-gene silencing, we first validated the abovementioned vectors by single-gene silencing. We subcloned the *lacZ* asRNA sequence (Figure 2A)

into the vectors so that the *lacZ* PTasRNA is expressed in the presence of IPTG. As controls, transformants with empty vectors were also used, and the silencing level of each *lacZ*-PTasRNA-expressing vector was evaluated relative to the LacZ activity of the corresponding empty vector (Figure 2B). We chose this comparison method because LacZ activities were different among transformants with empty vectors, possibly due to different *lacI*<sup>H</sup> gene dosage or the effect of used antibiotics.

When the pBR322- or pSC101<sup>H</sup>-*ori*-based vector (high-copy vector) was used, over 85% of LacZ activity was lost. On the other hand, when the pACYC-, pSC101- or RK2-*ori*-based vector (medium- or low-copy vector) was used, 62–76% of LacZ activity was lost. We experimentally confirmed the plasmid copy number of the vectors (Table 3) and observed that these values were consistent with the ones reported earlier except for the pSC101<sup>H</sup>-*ori*-based vector; the observed value was higher than expected.

**Table 2.** Oligonucleotides used for asRNA amplification

Target gene	Sequence	Length of asRNA sequence (bp)
<i>lacZ</i>	gcactcgaggctttacactttatgcttcc tcccatggttgaataaacgacggccagtgaatc	119
<i>pepN</i>	cgctcgaggaatagactgaacaccagactc taccatggaatccggcgacgataatcg	102
<i>ackA</i>	aactcgagctacgctctatggctccctgac caccatggtactcttaccatttactgcatc	147
<i>pta</i>	ccctcgaggcgtaacgaaaggataaac ttccatggcagcgatcacccaaggctg	101
<i>mutS</i>	aactcgagccatcacccccattaatcagg ggccatgggcttcagcctgagatactgct	110
<i>mutD</i>	tgctcgagcatctctggtagacttctgta tcccatggcctcatagtgcgaccaatc	152
<i>ndk</i>	ccctcgagctcattataatccgcccattct gtccatggctaccggcttcggtttgatgat	115

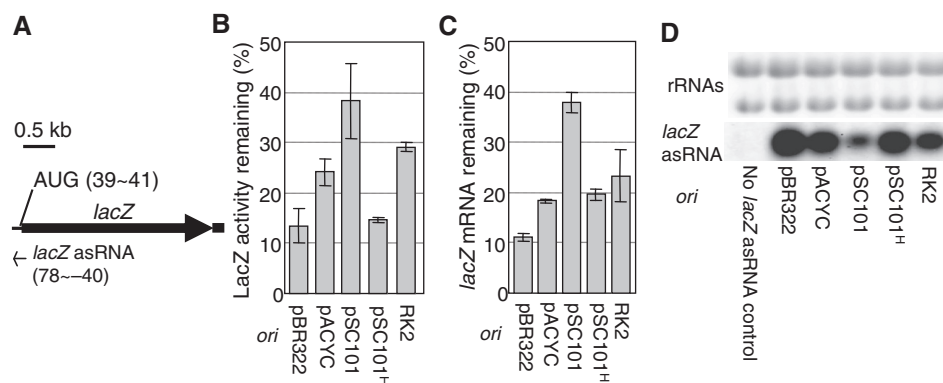
Although the copy number of the RK2-*ori*-based vector was almost the same as that of the pSC101-*ori*-based vector (Table 3), the silencing level was different between these vectors (Figure 2B). In addition, a similar vector dependent silencing pattern was observed when a gene other than *lacZ* was targeted (see Supplementary Figure S2). To further validate the vectors, we detected the *lacZ* mRNA and the *lacZ* PTasRNA with real-time quantitative RT-PCR and northern blot analyses, respectively (Figure 2C and D). As expected, in general, when the high-copy vectors were used, higher amounts of the *lacZ* PTasRNA were expressed and lower *lacZ* mRNAs remained. Also, the remaining LacZ activities (Figure 2B) and the *lacZ* mRNAs (Figure 2C) were well-correlated, but in the case of pSC101<sup>H</sup>-*ori*-based vector, the *lacZ* mRNA level was slightly high compared to the LacZ level. From these results, we conclude that the plasmid copy number greatly influences expression of PTasRNAs and silencing efficacy, but also other factors such as plasmid stability and/or overall plasmid structure are important.

Growth rates of all the transformants, including the control transformant, in the presence of IPTG were indistinguishable (data not shown), indicating that the expression of the *lacZ* PTasRNA has no off-target effect on other genes crucial for growth.

**Table 3.** Plasmid copy number

<i>ori</i>	pBR322	pACYC	pSC101	pSC101 <sup>H</sup>	RK2
Relative copy no. <sup>a</sup>	2.5 ± 0.3	1.1 ± 0.2	0.56 ± 0.04	2.0 ± 0.1	0.59 ± 0.05

<sup>a</sup>Plasmid copy number per cell relative to that of pACYC184, and averaged values from triplicate experiments are shown with SD. Plasmid names used are (from left to right) pHN1244, pHN1246, pHN1249, pHN1259 and pHN1274.

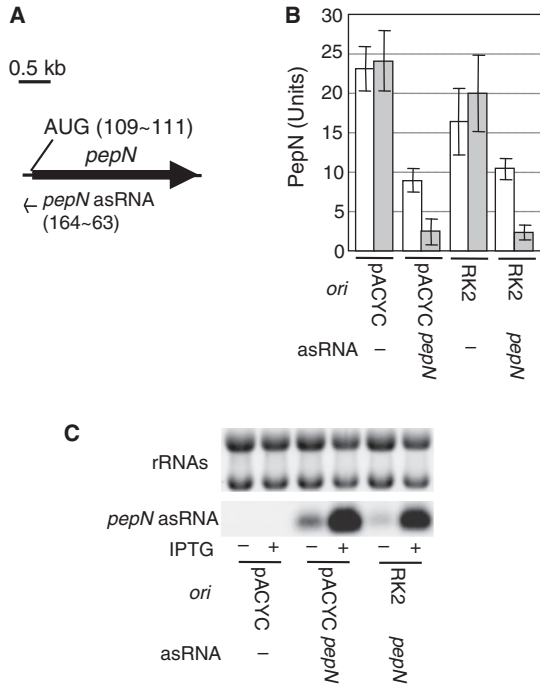


**Figure 2.** Silencing of *lacZ* with PTasRNAs. (A) Schematic maps of the *lacZ* mRNA and the *lacZ* asRNA sequence. (B) Cells were transformed with five *lacZ*-PTasRNA-expressing vectors (pHN1244, pHN1246, pHN1249, pHN1259 and pHN1274) and five empty vectors (pHN1009, pHN678, pHN1242, pHN1257 and pHN1270) and cultured in the presence of IPTG. Each LacZ activity remaining was calculated as; LacZ activity of a transformant with a *lacZ*-PTasRNA-expressing vector (Units)/LacZ activity of a transformant with a corresponding empty vector (Units). Absolute LacZ activities of all transformants are shown in Figure S1. The values are represented as the mean ± standard deviation (SD) of three replicates. (C) The *lacZ* mRNAs remaining after silencing were quantified with real-time quantitative RT-PCR. Each value was calculated in the same way as in the panel (B). (D) The *lacZ* PTasRNA was detected by northern blot (lower panel). The rRNA bands stained with ethidium bromide (upper panel) were used as loading controls. As the no *lacZ* asRNA control, the transformant with pACYC184 was used.

Using the pACYC- or RK2-ori-based vector, another reporter gene, *pepN* (encoding an aminopeptidase) (19), was targeted in the same way as *lacZ* was (Figure 3A), and 90 and 88% of the PepN activity was reduced, respectively (Figure 3B). Therefore, the RK2-ori-based vector exerts sufficient silencing efficacy when the target gene is susceptible to PTasRNA. However, when transformants harboring the *pepN*-PTasRNA-expressing vector were cultured without IPTG as controls, significant reduction of

PepN activity was observed (30–60%, Figure 3B). When other genes were targeted, no or little silencing was observed in the absence of IPTG (4) (also see below). These results indicate that in the absence of IPTG, the basal expression level of  $P_{trc}$  affects the expression of target genes only when they are PTasRNA-sensitive. There are many reports indicating that expression from  $P_{trc}$  is leaky (20), and we experimentally confirmed significant expression of the *pepN* PTasRNA in the absence of IPTG (Figure 3C). If basal expression from  $P_{trc}$  is problematic, the promoter should be changed to tightly-regulated ones. For example, we have reported a PTasRNA-expressing vector from which expression is induced with L-arabinose and is less leaky (pHN649, see Supplementary Figure S3) (4). Indeed, when *pepN* was targeted using this vector, reduction of PepN activity was observed only in the presence of L-arabinose (see Supplementary Figure S3).

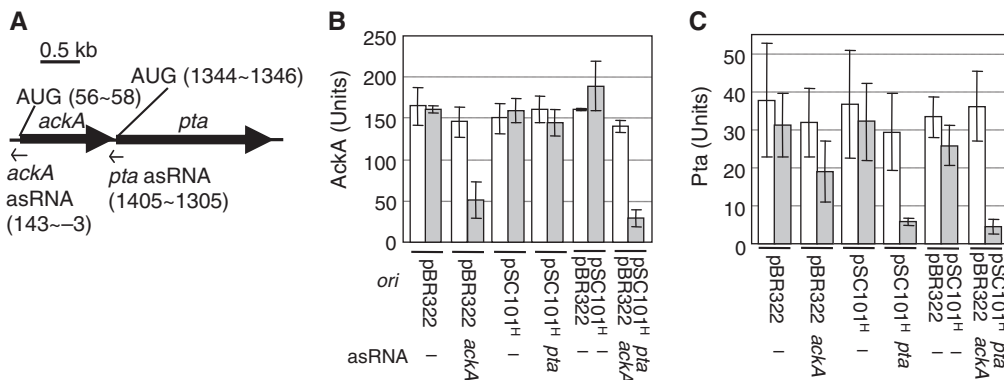
The growth rates of all these transformants in the presence of IPTG were indistinguishable (data not shown), again indicating that no critical off-target genes were affected.



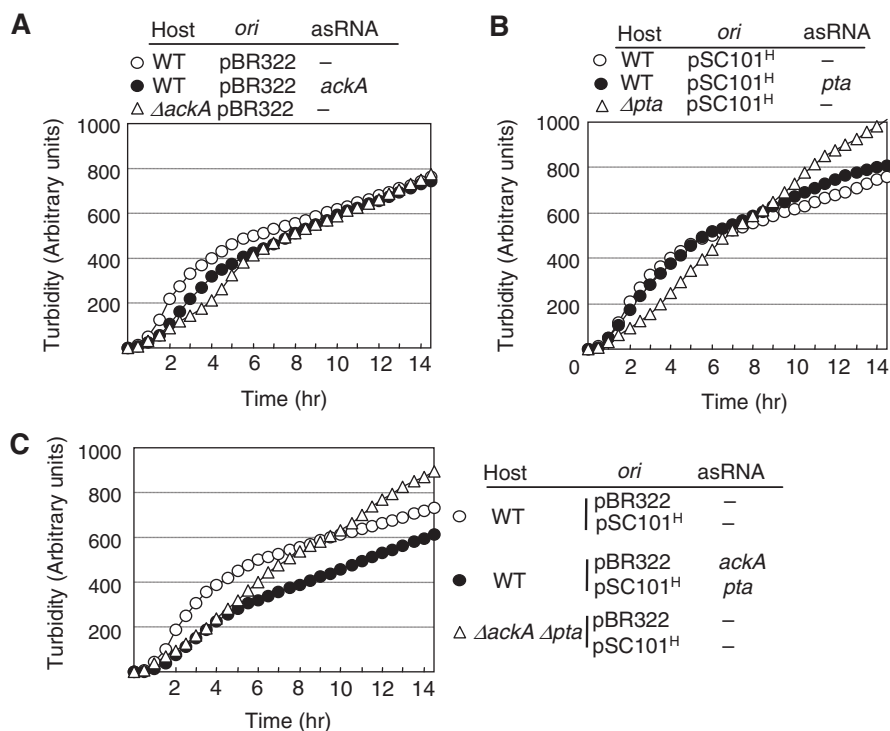
**Figure 3.** Silencing of *pepN* (A) Schematic maps of the *pepN* mRNA and *pepN* asRNA sequences. (B) Values of PepN activity. Open and gray bars are results with the absence and presence of IPTG, respectively. The names of the plasmids used are (from left to right) pHN678, pHN1258, pHN1270 and pHN1275. (C) The *pepN* PTasRNA was detected by northern blot.

### Double silencing within an operon

To experimentally confirm multiple-gene silencing, the *ackA-pta* operon was chosen as the next target of PTasRNAs (Figure 4A) because the enzymatic activities of AckA and Pta can be easily measured. The *ackA* and *pta* genes encode acetate kinase and phosphotransacetylase respectively, and both are involved in acetate metabolism (21). The *ackA* PTasRNA was expressed from the pBR322-ori-based vector, and the *pta* PTasRNA was expressed from the pSC101<sup>H</sup>-ori-based vector. Silencing efficacy was determined using single-transformants of each PTasRNA-expressing vector and using co-transformants of both the vectors. The result (Figure 4B and C) indicated that double silencing is possible by co-transformation, and the level of silencing of both the genes in the co-transformant was the same or higher than that in each single-transformant.



**Figure 4.** Double silencing of the *ackA* and *pta* reporter genes. (A) Schematic maps of the *ackA-pta* mRNA and *ackA* and *pta* asRNA sequences. (B) For the AckA assay, cells were transformed with plasmids carrying the indicated features and cultured in the absence (open bars) or presence (gray bars) of IPTG. The names of the plasmids used are (from left to right) pHN1009, pHN1245, pHN1257, pHN1260, both pHN1009 and pHN1257, and both pHN1245 and pHN1260. The activity values are represented as the mean  $\pm$  SD of at least three replicates. (C) Values of Pta activity in the identical transformants.



**Figure 5.** Comparison of the growth rates between *ackA-pta* silenced and disrupted cells. (A–C) Growth curves of the indicated transformants are shown. Wild-type (WT) and disrupted (*ΔackA*, *Δpta* and *ΔackA Δpta*) cells were transformed with the indicated PTasRNA vectors and cultured in presence of IPTG. Used plasmids are the same as shown in Figure 4.

The growth rates of these transformants were compared to those of corresponding disruptants (schematic maps of the disruptants are shown in Figure S4). Cells expressing the *ackA* PTasRNA grew slower than wild-type cells but faster than *ΔackA* cells (Figure 5A). Cells expressing the *pta* PTasRNA grew similarly to wild-type cells, while *Δpta* cells grew slowly but to high cell densities compared to wild-type cells (Figure 5B). Similar growth phenotypes of *pta* mutants have been reported by other groups (22,23). Cells expressing both the *ackA* and *pta* PTasRNAs grew poorly, but *ΔackA Δpta* cells grew to high cell densities as *Δpta* cells (Figure 5C). We assume that these discrepancies in the growth rates are unrelated to off-target effects of the PTasRNAs because growth retardation caused by expressing *ackA* and *pta* PTasRNAs was recovered by overexpressing the *ackA* and *pta* genes (Figure S5). Instead, residual AckA and/or Pta activities in PTasRNA-expressing cells might affect the growth rate, or because AckA activity in *Δpta* cells and Pta activity in *ΔackA* cells were reduced to 69 and 62% respectively (see Supplementary Figure S4), imbalance between AckA and Pta in the disruptants might have impact on the growth rates.

### Quadruple silencing

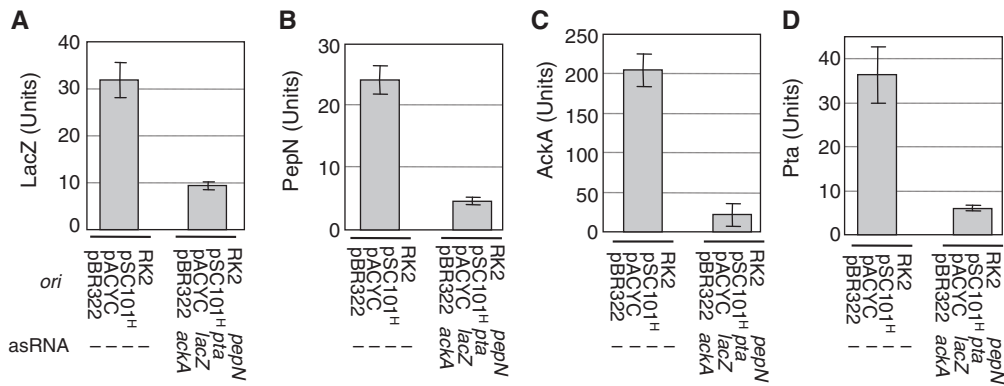
Next, we wanted to determine whether the simultaneous co-transformation with the four vectors and quadruple silencing are possible. The *lacZ*, *pepN*, *ackA* and *pta* PTasRNAs were expressed from the pACYC-, RK2-, pBR322- and pSC101<sup>H</sup>-*ori*-based vectors, respectively.

Silencing efficacy in a quadruple transformant of these vectors was evaluated by measuring LacZ, PepN, AckA and Pta activities, and the activities were compared to those of a control transformant with four empty vectors (Figure 6). We confirmed that all the genes were silenced, and the silencing efficacies were comparable to those of their respective single transformants (Figures 2B, 3B, 4B and 4C). From these results, we conclude that simultaneous silencing of up to four genes is possible.

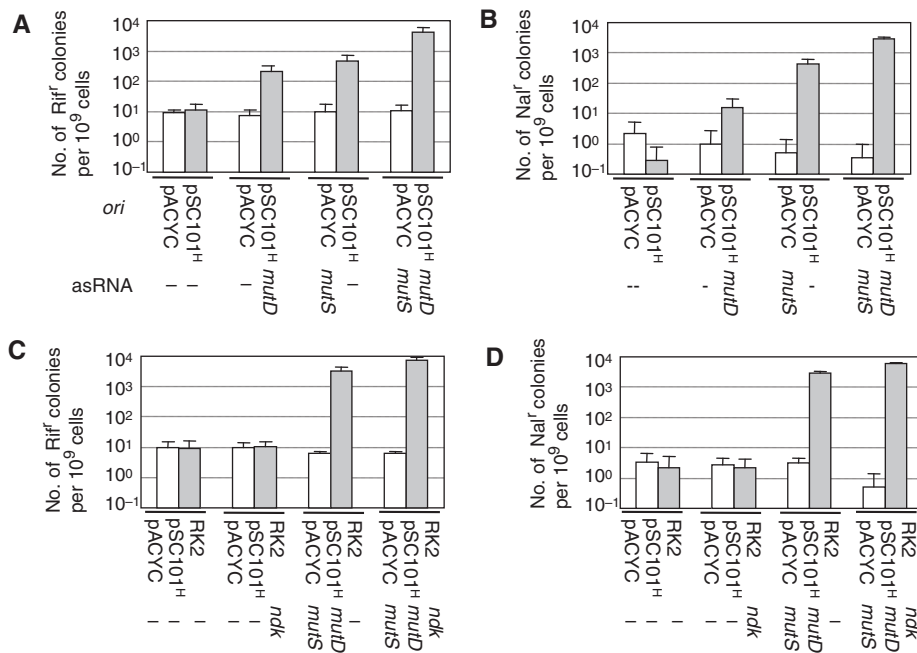
### Application of the multiple-gene silencing system

A multiple-gene silencing method will enable the investigation of the functional relationships among genes. In order to determine the functional relationship among related genes, we selected the mutator genes *mutS*, *mutD* (*dnaQ*) and *ndk* as targets of the PTasRNA. The *mutS* gene product is involved in DNA mismatch repair (24,25), *mutD* encodes DNA polymerase  $\epsilon$  subunit (3'-5' exonuclease) (26–28), and *ndk* encodes nucleotide diphosphate kinase (Ndk) (29). The Ndk protein is involved in the maintenance of deoxyribonucleotide triphosphate (dNTP) pools, and an *ndk*-deficient strain has an imbalanced dNTP pool (29). *E. coli* strains with defects in any of these genes tend to have elevated spontaneous mutation frequency (30).

The *mutS*, *mutD* and *ndk* asRNA sequences were subcloned into the pACYC-, pSC101<sup>H</sup>- and RK2-*ori*-based vectors, respectively, and the PTasRNAs were expressed in various combinations (Figure 7). When any of the PTasRNAs directed against these genes was expressed,



**Figure 6.** Quadruple silencing of *lacZ*, *pepN*, *ackA* and *pta* genes. (A) For the LacZ assay, cells were co-transformed with four empty vectors (left bar; pHN1009, pHN678, pHN1257 and pHN1270) or four PTasRNA vectors (right bar; pHN1245, pHN1246, pHN1260 and pHN1275) and cultured in the presence of IPTG. The activity values are represented as the mean  $\pm$  SD of three replicates. (B–D) Values of the PepN, AckA and Pta activities in the identical transformants.



**Figure 7.** Multiple silencing of the mutator genes, *mutS*, *mutD* and *ndk*. (A) Cells were co-transformed with two vectors carrying the indicated features and cultured in the absence (open bars) or presence (gray bars) of IPTG. Rif<sup>r</sup> means rifampicin resistance. The values are the mean  $\pm$  SD of three replicates. (B) Result of the same experiment as in (A) is shown except that Nal<sup>r</sup> means nalidixic acid resistance. (C and D) Cells were co-transformed with three vectors carrying the indicated features; the other experimental conditions are the same as those used for cells shown in (A) and (B).

there was an increase in the spontaneous mutation frequency, which was determined by the rate of appearance of rifampicin- (Rif<sup>r</sup>) and nalidixic acid (Nal<sup>r</sup>)-resistant colonies (Figure 7). The mutation frequency increased with the number of silenced genes; the highest frequency was observed when three genes were silenced, and it was over 2000-fold higher than that of the control. The effect of the *ndk* PTasRNA was observed to a limited extent compared to that of the *mutS* or *mutD* PTasRNA. This finding is consistent with the results of the previous study which indicated that *ndk* is a modest base substitution mutator and its phenotype is strengthened in the *mutS* background (29). Taken together, the results suggest

that silencing of multiple mutator genes causes additive or synergistic increase of spontaneous mutation frequency, thereby generating a mutator strain.

Till date, many mutator genes have been identified (30), and therefore further silencing of fourth gene is possible. However, we think that this mutator strain can be used for preparing plasmid libraries that harbor random mutations (see ‘Discussion’ section for details). Given that most popular cloning vectors have pBR322 *ori* (or closely related pUC *ori*) and the ampicillin-resistance gene, we think that it is better to avoid using the pBR322-*ori*-based vector (pHN1009, Figure 1) for silencing of mutator genes.



## DISCUSSION

The advantages of the gene-silencing method used in this study are inducibility, portability, high throughput and multiplicity of the targeted genes. The inducibility and portability enable the application of this method to genes essential for growth and to any *E. coli* strain. The high throughput and multiplicity are important features for genome-wide analyses, systems biology or gene-network analyses. It is noteworthy that this is one of the first examples of multiple-gene silencing in bacteria. In this study, we showed simultaneous gene silencing of up to four genes; however, we assume that there is requirement for silencing more genes simultaneously. The easiest solution for this is to sub-clone multiple asRNA sequences into one vector. Alternatively, it should also be possible to integrate PTasRNA expression cassettes into chromosomes by using transposons or site-directed recombination methods, although in this case, the portability of PTasRNAs is lost. Another possibility is to add synthetic antisense agents (antisense oligonucleotides or such analogues) into the culture media (31); however, this method is costly and is not applicable in the case of large-scale cultures. Taken together, it seems that the most feasible strategy is to develop gene-silencing methods that can complement those available for gene knock-out. It should also be noted that several methods have been developed for achieving homologous recombination and gene disruption without leaving transformation markers on chromosomes (32,33).

Expressed asRNAs have been shown to function in diverse bacteria, and thus far, the asRNAs developed in bacteria are mostly targeted against the RBS and start-codon region (3). This suggests that asRNAs inhibit gene expression by steric hindrance of ribosomes (3). However, in general, the methods for developing asRNAs in bacteria are more difficult than those for developing asRNAs in eukaryotes. This is thought to be due to the coupling of transcription and translation processes in bacteria. Bacterial ribosomes can initiate translation even when nascent mRNAs are being transcribed because bacteria do not have a nuclear membrane that sequesters ribosomes from the transcription machineries (2). Consequently, there is little time for bacterial asRNAs to access target mRNAs, and indeed, it has been reported that the efficacy of asRNAs increases when translation is decoupled from transcription using mutant ribosomes with slow translation rates (2). In order to overcome this coupling problem in wild-type cells, bacterial asRNAs usually have to be in excess quantities compared to that of the target mRNAs (2,4). Our PTasRNA design provides one advantage: the PT portion stabilizes asRNAs, thus resulting in higher accumulation of PTasRNAs (4). Increasing the plasmid copy number is another option that has been shown in Figure 2.

As shown in Figures 2B and 3B, the sensitivity of target mRNAs to PTasRNA silencing varies. We assume that the sensitivity is determined by many factors such as mRNA turnover rate, steady-state mRNA expression level, effectiveness of the mRNA translation initiation and accessibility of the PTasRNA to the mRNA.

Among these factors, accessibility appears to be very important on the basis of the following observation: when *lacZ* was targeted by several versions of *lacZ* PTasRNAs, which were of similar length and carried the anti-RBS sequences but were targeted at slightly different positions of the *lacZ* mRNA, the silencing efficacy clearly differed among the different versions (see Supplementary Figure S6). Probably, the mRNA secondary structure affects the accessibility of PTasRNAs. However, there could be other possible reasons, and further studies need to be performed for comparing the efficacy of the different versions, and the use of tools for predicting target-site accessibility (34) may be helpful.

Recently, it has been shown that many bacteria have natural small RNAs that act as asRNAs and inhibit function of specific mRNAs (35,36). Stability and function of such RNAs are frequently governed by RNA chaperons, e.g. Hfq (35,36). Exploiting such endogenous mechanisms but not using the artificial PT design shown here is an alternate choice for efficient silencing. However, it should be noted that over-expressed natural small RNAs may compete endogenous mechanisms (e.g. competes for Hfq binding) and may cause unwanted phenotypes non-specifically.

Triple silencing of mutator genes and generation of a mutator strain have been demonstrated in this study. Mutator strains are useful for introducing random mutations in plasmids. The most popular mutator strain is *E. coli* XL1-Red (Stratagene), which has mutations in *mutS*, *mut*, and *mutT* loci and has a 5000-fold higher mutation frequency than the wild type (37,38). Therefore, the XL1-Red strain is used to generate plasmid libraries harboring random mutations. However, the growth of the XL1-Red strain is very slow, and maintaining it in a stable state is problematic. Our mutator strain is more advantageous than the XL1-Red strain because the mutation frequency can be adjusted by adding or removing the plasmid and maintenance is simple. Camps and co-workers recently reported an alternative method to create mutator strains. They employed a highly error-prone DNA polymerase I coded on a plasmid (39). The most advantageous feature of their method is that the mutations occurring on plasmids is 400-fold higher than those on chromosomes, thus ensuring genetic stability of the host strain. It might be interesting to investigate the combination of our method with theirs for introduction of more mutations.

We believe that there are other possible applications of this method because most cellular phenotypes are governed by multiple genes; hence, obtaining a desired phenotype often requires simultaneous manipulation of multiple genes. For example, while producing industrially valuable metabolic intermediates such as succinate, it is necessary to disrupt or silence multiple genes involved in central carbon flux (40,41). The central carbon flux is composed of complex gene networks, and recent advances in systems biology make it possible to predict the number of genes that have to be disrupted or silenced for optimized carbon flux. Our method is appropriate for this purpose because it has a higher throughput than gene disruption method and many silencing combinations can be easily

tested. In addition, silencing of essential genes can lead to the discovery of new antimicrobials, which are in great demand (42,43), as demonstrated by the recent discovery of platensimycin (44). Our genetic studies performed at RNA level will assist researches in the identification of gene functions and cell engineering.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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