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A screening system for artificial small RNAs that inhibit the growth of *Escherichia coli*

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We have developed a screening system for artificial small RNAs (sRNAs) that inhibit the growth of *Escherichia coli*. In this system, we used a plasmid library to express artificial sRNAs (approximately 200 bases long) containing 60 bases of random nucleotide sequence. The induced expression of the known rydB sRNA in the system reduced the amount of its possible target mRNA, rpoS, supporting the reliability of the method. To isolate clones of sRNAs that inhibited the growth of *E. coli*, we used two successive screening steps: (i) colony size selection on plates and (ii) monitoring *E. coli* growth in a 96-well plate format. As a result, 83 artificial sRNAs were identified that showed a range of inhibitory effects on bacterial growth. We also introduced nucleotide replacements into one of the highly inhibitory sRNA clones, H12, which partially abolished the inhibition of bacterial growth, suggesting that bacterial growth was inhibited in a sequence-specific manner.

Keywords: bacterial growth/biotechnology/*Escherichia coli*/screening system/small RNA.

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; sRNA, small RNA; CRISPR, clustered regularly interspaced short palindromic repeat.

Recently, there have been many reports that bacterial genomes encode a huge number of small non-coding RNAs (sRNAs) [reviewed by Waters *et al.* (*1*)]. In *Escherichia coli*, some of these sRNAs have been identified with several approaches, including bioinformatics (*2–4*), tiling array analysis (*5–7*) and cDNA cloning (*8–10*). Interestingly, the expression patterns of many sRNAs change during bacterial growth or in response to medium conditions (*7*, *8*). Furthermore, some mRNAs contain regulatory RNA sequences called 'riboswitches', predominantly located in the 5′-untranslated region, that control target translation (*11*). It has also been reported that clustered regularly interspaced short palindromic repeats (CRISPRs) function as a prokaryotic silencing system. These sequences have been found in ~40% of bacterial and 90% of archaeal genomes (*12*). A number of sRNAs are expressed from CRISPR loci and their sequences show similarity to those of prokaryotic phages, viruses and transposons, indicating that these sRNAs function as a self-defense system (*13*). Taken together, these findings indicate that sRNAs are important regulators in prokaryotes. Therefore, we hypothesized that it may be possible to control bacterial gene expression or even growth using artificial sRNAs.

We developed a screening system to isolate artificial sRNAs that inhibit the growth of *E. coli*. We used a T7-promoter-driven library of expression plasmids, with each plasmid encoding an artificial sRNA with 60 random nucleotides in length. We used two successive screening steps: (i) colony size selection on plates and (ii) monitoring bacterial growth in a 96-well plate format. In this way, we identified 83 artificial sRNAs that showed a range of inhibitory effects on bacterial growth. We also discuss the reliability of the screening system and possible applications of this system.

Materials and Methods

Construction of an expression plasmid library of artificial sRNAs

We first prepared the insert DNA fragments by PCR amplification of 60-bp synthetic DNA fragments with random sequences (Ram-60) using KOD Dash enzyme (Toyobo Biochemicals, Osaka, Japan). PCR was performed for 28 cycles of 94°C (30 s), 50°C (2 s) and 72°C (10 s) with the specific primers, RS and RA. The amplified fragments contained an XbaI restriction enzyme site, three stop codons, a 60-bp random sequence and one NotI restriction enzyme site (Fig. 1A). We subcloned the insert into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible pET-28 b expression vector (Novagen, Madison, WI, USA) and designated the resulting construct pASR, for artificial small RNAs. The library constructed (~10^6 colony-forming units per microgram vector DNA) was used to screen the sRNA-expressing clones. The oligonucleotides used are summarized in Supplementary Table SII.

Expression and screening of artificial sRNAs that inhibit the growth of E. coli

Escherichia coli strain HMS174(DE3) (Novagen) was transformed with the expression plasmids. The transformants were grown on Luria–Bertani (LB) agar plates containing 30 μg/ml kanamycin for 15 h at 37°C. Single colonies picked from the LB plates were used to inoculate 200 μl of LB medium containing 30 μg/ml kanamycin in a 96-well plate (TPP, Trasadingen, Switzerland). The growth of each *E. coli* culture at 37°C with slight agitation, with or without 40 μM IPTG, was monitored directly at 600 nm on a spectrophotometer (SpectraMax Plus 384; Molecular Devices, Inc., Sunnyvale, CA, USA) (Fig. 1B). After the preparation of the plasmids, the nucleotide sequence of each DNA insert was determined.

RNA preparation and northern blot hybridization

A single colony of *E. coli* strain HMS174(DE3) carrying the appropriate expression plasmid was used to inoculate 10 ml of LB broth

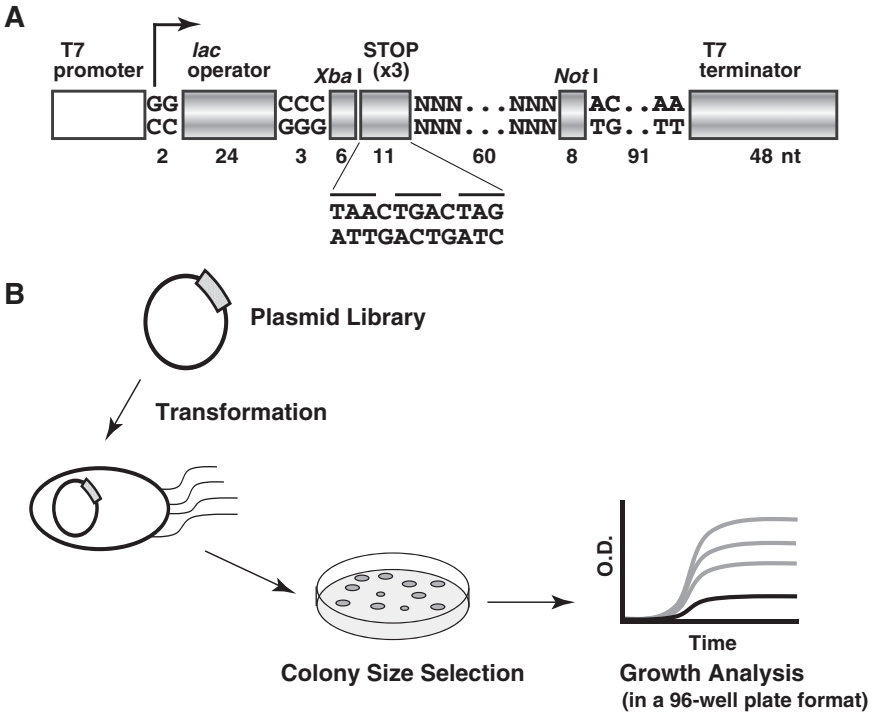

Fig. 1 Screening system for artificial small RNAs (sRNAs) that inhibit the growth of *E. coli*. (A) Structure of the inserted region of the artificial sRNA expression plasmids, pASR. Regulatory sequences and restriction enzyme sites are boxed. The STOP region contains three successive translation termination signals; 'N' indicates a random nucleotide sequence. The numbers indicate the nucleotide length of each region. (B) Strategy for screening the artificial sRNAs.

containing 30 μg/ml kanamycin. After overnight incubation at 37°C, the culture was diluted (1 : 9) with fresh LB medium containing 30 μg/ml kanamycin and then recultured under the same conditions to an optical density at 600 nm (OD_{600}) of 0.65. The expression of an artificial sRNA was induced with 40 μM IPTG at 37°C for 1 h (*E. coli* was also cultured at 37°C for 1 h without IPTG as a control). These cultured cells were harvested, immediately treated with RNAprotect Bacteria Reagent (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol and stored at −80°C until RNA extraction.

The total RNA was extracted with the RNeasy Midi Kit (Qiagen), with a slight modification. Instead of using an RNeasy Midi column, we used phenol–chloroform extraction to efficiently collect the complete set of RNAs, including the low-molecular weight RNAs. For the analysis of the small RNAs (20–500 bases), the RNA (10 μg per lane) was separated on a denaturing 6% polyacrylamide gel containing 8 M urea and transferred onto Hybond-N^+ membrane (GE Healthcare, Piscataway, NJ, USA) by electroblotting. For the analysis of larger RNAs (0.5–10 kb), RNA (4.0 μg per lane) was separated on a denaturing 1% agarose gel and transferred onto Hybond-N^+ membrane by capillary blotting overnight. The 3′-ends of specific oligonucleotide probes were labelled with the Biotin 3′-end DNA Labeling Kit (Pierce Biotechnology, Rockford, IL, USA). The membranes were hybridized with these probes in ULTRAhyb-Oligo Hybridization Buffer (Ambion, Austin, TX, USA) at 50°C for the artificial sRNAs (AS-lacO probe), 42°C for the rpoS mRNA (AS-rpoS probe) and 65°C for 5 S rRNA (AS-5 S rRNA probe). The washing temperature was the same as each hybridization temperature. The non-isotopic blots were visualized with the BrightStar BioDetect Kit (Ambion) using ECF™ Substrate (GE Healthcare). The images were captured with a Molecular Imager FX Pro (Bio-Rad Laboratories, Hercules, CA, USA). The oligonucleotides used are summarized in Supplementary Table SII.

Results and Discussion

A screening system for artificial sRNAs that inhibit the growth of E. coli

To generate artificial sRNAs, we used an IPTG-inducible expression plasmid, pET-28 and constructed a library of plasmids containing 85-bp XbaI–NotI fragment inserts, each comprising 60 bases of random nucleotide sequence. The structure of the insert region is shown in Fig. 1A. Since this system is under the control of an IPTG-inducible T7 RNA polymerase promoter, and this promoter is sometimes leaky in the absence of T7 RNA polymerase, we chose a plasmid containing the *lac* operator sequence to reduce background expression. This plasmid also encodes the T7 terminator sequence. Transcripts derived from this expression plasmid were ~200 bp long. We deleted the original Shine–Dalgarno sequence from the plasmid and added three consecutive stop codons immediately before the random sequence to prevent the translation of the artificial sRNAs into polypeptides. In this way, the expression of the artificial sRNAs from the pASR plasmid was tightly controlled.

Figure 1B shows our screening strategy for artificial sRNAs that inhibit *E. coli* growth (we isolated no clones that enhanced the growth rate). We applied two successive screening steps: (i) colony size selection on plates and (ii) monitoring the growth of *E. coli* in a 96-well plate format. First, we introduced the pASR library into competent *E. coli* cells, strain HMS174(DE3). This *E. coli* strain expresses T7 RNA polymerase when the inducer IPTG is added to the medium or agar. We tested a series of IPTG concentrations and found that 40 μM was the lowest concentration that adequately induced sRNA expression in our system (data not shown). In the first screening step, we used plates with a 9 cm diameter so that the sizes of 100–1000 colonies could be distinguished per plate. Generally, smaller colonies contained a plasmid

that strongly inhibited the growth of *E. coli* (data not shown). Then, in the second step, the resulting clones, with a variety of colony sizes, were further screened by monitoring their growth in a 96-well plate format. We directly monitored *E. coli* growth at OD_{600} using a high-throughput microplate spectrophotometer at 37°C with slight agitation.

Totally, 24 examples of changes in *E. coli* growth induced by artificial sRNAs are shown in Fig. 2. Without IPTG induction, all the colonies grew with similar growth curves. However, in the presence of the inducer IPTG, a range of growth inhibition was observed, especially after 6 h of culture. Then, we selected 83 sRNA plasmids that inhibited *E. coli* growth and determined their nucleotide sequences (Supplementary Table SI). Although about 86% of the random sequence region in the 83 selected clones was exactly 60 nt in size, as expected, 12 clones had nucleotide deletions or insertions (the average length of the sRNAs in the 83 plasmids was 62 nt; the range was 32–158 nt). These may have resulted from aberrant PCR amplification, because the synthetic template DNA contained 60 random nucleotide sequences and it is possible that some template DNA may have hybridized with each other during the PCR reaction. However, we confirmed that all these clones had a region of three consecutive stop codons immediately before the random sequence to prevent the translation of the artificial sRNAs into polypeptides. We classified these selected sRNAs into three groups based on their inhibitory effects on *E. coli* growth: class I (maximum OD_{600} 0.2–0.4; clones H1–H24), class II (maximum OD_{600} 0.4–0.7; clones M1–M41) and class III (maximum OD_{600} 0.7–0.95; clones L1–L18).

To confirm the expression of the artificial sRNAs, we randomly selected six clones (H1, H12, M13, M18, L1 and L8) and examined their transcripts by northern blot hybridization (Fig. 3). Major transcripts of around 200 bases long were detected in an IPTG-dependent manner. The transcript sizes were consistent with the length calculated from the first nucleotide at the transcription start site to the last nucleotide before the T7 terminator (Fig. 1A). Two read-through transcripts were also observed among all the clones examined. Interestingly, there were small clone-specific bands at around 20–100 bases, suggesting that some sRNAs possessed their own RNA-processing or RNA-degradation pathway.

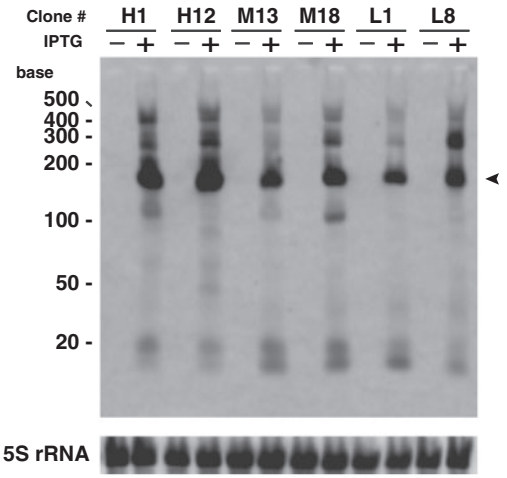

Fig. 3 Northern blot analysis of artificial sRNA expression in *E. coli*. *Escherichia coli* containing each plasmid (pASR-H1, -H12, -M13, -M18, -L1 or -L8) were grown to an optical density at 600 nm of 0.65 in LB medium containing 30 μg/ml kanamycin at 37°C. (+), The expression of artificial sRNAs was induced in the presence of 40 μM IPTG at 37°C for 1 h. (−), *E. coli* was also cultured at 37°C for 1 h without IPTG, as the control. Total RNAs were prepared from these cells and separated on a denaturing 6% polyacrylamide gel containing 8 M urea. The AS-lacO probe [antisense oligonucleotide corresponding to the *lac* operator region (Fig. 1) of the plasmid] was used for sRNA detection. The arrowhead indicates the positions of major transcripts. 5 S rRNA was used as the loading control. Similar results were obtained in at least two independent experiments.

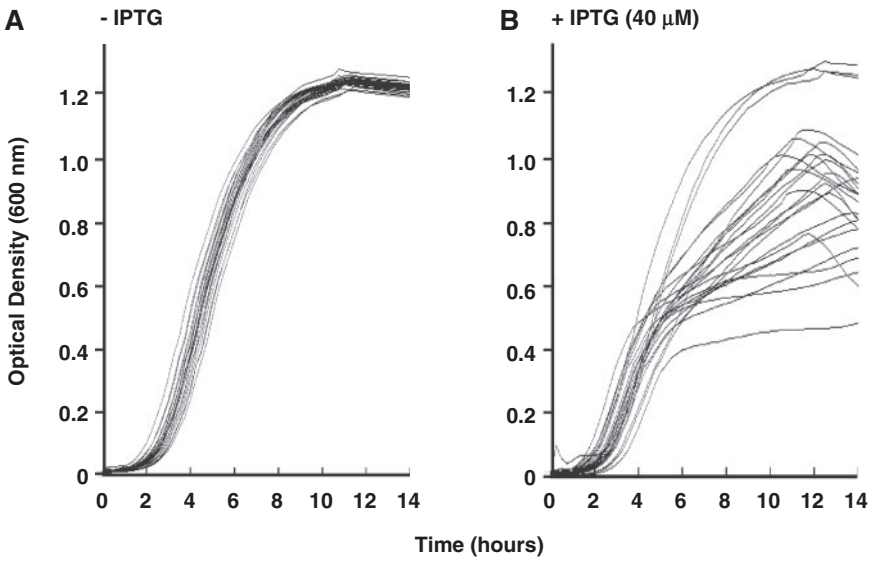

Fig. 2 Total of 24 examples of changes in *E. coli* growth induced by artificial sRNAs. Single colonies of *E. coli* containing each pASR plasmid were inoculated into 200 μl of LB medium in 96-well plates and incubated at 37°C without IPTG (–IPTG; A) or in the presence of 40 μM IPTG (+IPTG; B). Cell growth was monitored by scanning the optical density at 600 nm.

Reliability of the screening system and the possible mechanisms of growth inhibition by artificial sRNAs

Does the inhibitory effect on *E. coli* growth really depend on the DNA insert in our system? To answer this question, we recloned some selected DNA inserts into a new but the same expression vector and compared the maximum OD_{600} values for *E. coli* expressing the original insert and the recloned insert. We thus showed that the inhibitory effects did indeed depend on the plasmids used and there were no significant differences in the growth rates of the original and the recloned inserts (data not shown).

Next, we examined the effect of the induction of a known sRNA on its reported target mRNA using our expression plasmid pASR system. For this purpose, we used rydB sRNA. The rydB sRNA is ~60 bases long and its overexpression in stationary phase in LB medium reduced the expression of the rpoS–lacZ fusion to 40% (*7*). We induced the expression of rydB sRNA in stationary phase in LB medium using the pASR plasmid. Figure 4A shows that an artificial transcript-encoding rydB sRNA was expressed in a similar way to the six sRNA examples shown in Fig. 3. We also observed that the expression of the *rpoS* gene was reduced after the overexpression of the artificial sRNA that included rydB (Fig. 4B). The normalized ratio (rpoS/rRNAs) of the reduction, calculated with an image analyzer, was 0.41. This result supports the previous finding (*7*) and also suggests that the reduction in rpoS expression is, at least in part, regulated by its mRNA level. Therefore, we concluded that the pASR system is also useful for the analysis of known sRNA functions.

Finally, we made mutant sRNAs to show that the inhibition of *E. coli* growth in our system is actually mediated by the sequence of the artificial sRNA. For this purpose, we introduced some nucleotide replacements into one of the inhibitory sRNA clones, H12, making two mutant clones, H12-M1 and H12-M2 (Fig. 5A). The positions and the 4 nt replaced in each sRNA were randomly selected. We tested the effects of these sRNAs on the growth of *E. coli* (Fig. 5B), after first confirming that the original clone, H12-WT, exhibited strong inhibition of *E. coli* growth. Compared with the no-IPTG (–IPTG) condition, the effect of the sRNA derived from the H12-WT clone (+IPTG condition) was observed after 4 h of cultivation and the OD_{600} remained stable at a low level (0.24–0.27) for ~10 h. This suggested that the *E. coli* growth in the presence of IPTG was bimodal, showing two-step growth curve: inhibition of the growth was evident only around 4 h after IPTG induction. Although the H12-M1 clone showed a growth pattern quite similar to that of the H12-WT clone, the H12-M2 clone partially relieved the inhibition of bacterial growth and its OD_{600} increased to around 0.35. Further, in order to analyse the expression of H12-WT, H12-M1 and H12-M2 sRNAs during the growth inhibition, we conducted northern blot analysis and showed that there were no significant differences among their transcript levels (data not shown). These data suggest that the inhibition of *E. coli* growth is sRNA sequence specific and that the mutated nucleotide(s) were partially involved in the inhibition, although we do not know which size of sRNAs [either expected size, read-through or processed transcript(s)] are involved in this growth inhibition. In conclusion, all these results suggest that the growth of *E. coli* was effectively controlled by sRNAs, with the degree of inhibition depending on the sRNA sequence expressed.

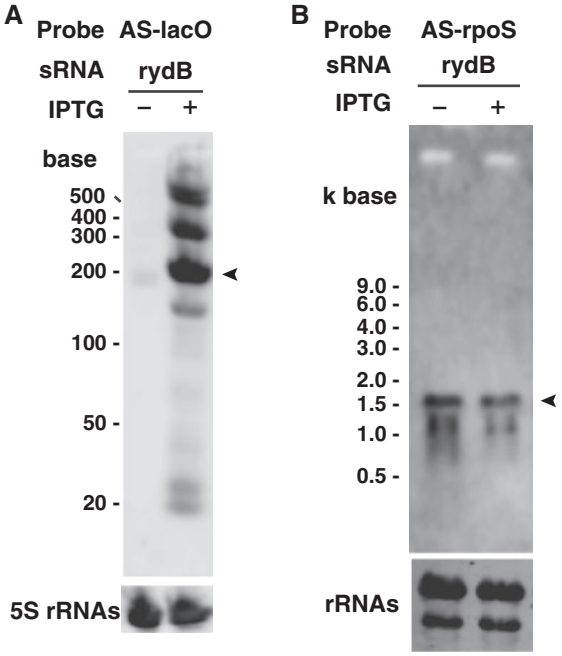

Fig. 4 rydB sRNA expression in the pASR plasmid system and its effect on *rpoS* gene expression. (A) northern blot analysis of rydB sRNA expression in *E. coli*. *Escherichia coli* containing the pASR–rydB plasmid was grown to an optical density at 600 nm of 0.65 in LB medium containing 30 μg/ml kanamycin at 37°C. (+), The expression of rydB sRNA was induced in the presence of 40 μM IPTG at 37°C for 1 h. (–), *E. coli* was also cultured at 37°C for 1 h without IPTG as the control. Total RNAs were prepared from these cells and separated on a denaturing 6% polyacrylamide gel containing 8 M urea. The AS-lacO probe was used for sRNA detection. An arrowhead indicates the position of the major transcript. 5 S rRNA was used as the loading control. (B) The same total RNAs prepared as in (A) are separated on a denaturing 1% agarose gel. The AS-rpoS probe was used for northern blot hybridization and the arrowhead indicates the position of the *rpoS* transcript. The 16 S and 23 S rRNAs stained with SYBR Green II (Molecular Probes, Inc., Eugene, Oregon, USA) were used as the loading controls.

We then investigated the underlying mechanisms of *E. coli* growth inhibition by each artificial sRNA. It has previously been reported that endogenous bacterial sRNAs play important regulatory roles in a variety of cellular processes: (i) antisense gene regulation; (ii) binding to either specific RNAs or proteins; and (iii) binding to small chemicals in the case of riboswitches (*1*, *11*). Using TargetRNA, a bioinformatics tool for identifying sRNA targets (*14*), we identified potential target mRNAs for some of our candidate sRNAs (data not shown). However, in general, we did not detect any rules (for example, in terms of the GC content or RNA secondary structure) governing the nucleotide sequences of either the strongly or weakly inhibitory clones. It is likely that there are

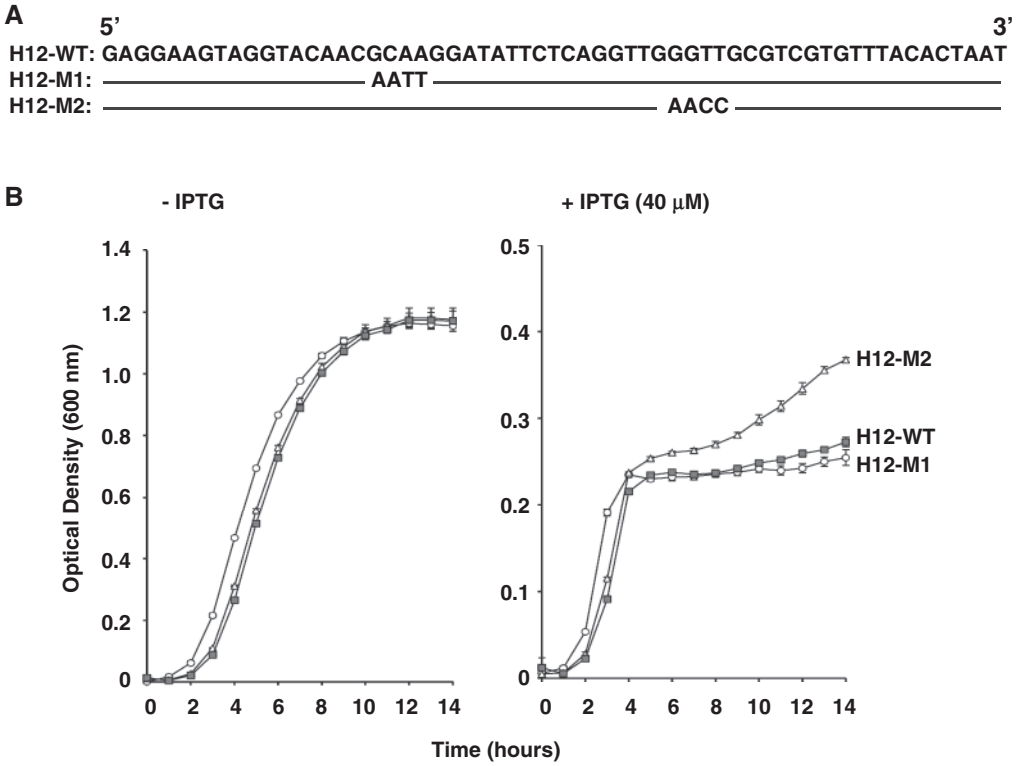

Fig. 5 Mutation analysis of H12 sRNA. (A) Nucleotide sequence of the insert in the artificial sRNA expression plasmid pASR–H12 and its mutants. In the mutants, the nucleotide sequences that were replaced between H12-WT are shown. (B) Growth curve of *E. coli* expressing either the pASR–H12 plasmid or its mutants. Single colonies of *E. coli* containing each pASR plasmid (H12-WT, H12-M1 or H12-M2) were inoculated into 200 μl of LB medium in 96-well plates and incubated at 37°C without IPTG or in the presence of 40 μM IPTG. Cell growth was monitored by scanning the optical density at 600 nm. The data represent the means and standard deviations of three separate experiments.

many inhibitory mechanisms underlying the action of each clone.

In this study, we have demonstrated that artificial sRNAs can provide a unique tool to control bacterial growth. This may be applied not only to *E. coli*, but also to other bacteria, including pathogenic bacteria such as *Mycobacterium tuberculosis*, *Yersinia pestis* and *Vibrio cholerae*.

Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of interest

None declared.

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