Paired termini stabilize antisense RNAs and enhance conditional gene silencing in *Escherichia coli*

Nobutaka Nakashima\(^1,2\), Tomohiro Tamura\(^2,3\) and Liam Good\(^1,*\)

\(^1\)Department of Cell and Molecular Biology, Programme for Genomics and Bioinformatics, Karolinska Institute, Berzelius väg 35, 171 77 Stockholm, Sweden, \(^2\)Research Institute of Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, 062-8517 Sapporo, Japan and \(^3\)Laboratory of Molecular Environmental Microbiology, Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, 060-8589 Sapporo, Japan

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ABSTRACT

Reliable methods for conditional gene silencing in bacteria have been elusive. To improve silencing by expressed antisense RNAs (asRNAs), we systematically altered several design parameters and targeted multiple reporter and essential genes in *Escherichia coli*. A paired termini (PT) design, where flanking inverted repeats create paired dsRNA termini, proved effective. PTasRNAs targeted against the ackA gene within the acetate kinase-phosphotransacetylase operon (ackA-pta) triggered target mRNA decay and a 78% reduction in AckA activity with high genetic penetrance. PTasRNAs are abundant and stable and function through an RNase III independent mechanism that requires a large stoichiometric excess of asRNA. Conditional ackA silencing reduced carbon flux to acetate and increased heterologous gene expression. The PT design also improved silencing of the essential fabI gene. Full anti-fabI PTasRNA induction prevented growth and partial induction sensitized cells to a FabI inhibitor. PTasRNAs have potential for functional genomics, antimicrobial discovery and metabolic flux control.

INTRODUCTION

Advanced genetics and metabolic engineering require reliable conditional gene control. Unfortunately, gene disruption techniques remain difficult, applicable to a restricted set of species and strains and are particularly problematic in cases where the target gene is growth critical. In many eukaryotic systems, antisense and RNAi-mediated gene silencing is popular, whereas in bacteria RNAi mechanisms are absent and antisense technology is less developed and applied. Expressed antisense RNAs (asRNAs) appear to be suitable for conditional gene silencing in diverse bacteria (1,2) and for large scale applications. Indeed, asRNA can repress translation in diverse bacteria (3–8), and library screens have revealed effective antisense transcripts and putative new essential genes (9,10) and inhibitors (11). The expressed asRNA method may be more efficient in Gram-positive bacteria such as *Staphylococcus aureus* (5,9–11) and *Clostridium acetobutylicum* (7,12) relative to Gram-negative bacteria, such as *Escherichia coli*, but reports of mixed results (13) suggest that design improvements are needed. Also, bacteria have endogenous asRNA regulators (14) and cell permeable modified antisense agents are efficient inhibitors (15,16). Therefore, achieving improved antisense silencing in *E.coli* appears to be largely a technical challenge.

Here we found that a simple paired-termini (PT) design stabilizes asRNA cassettes and enhances conditional gene silencing in *E.coli*. Two well-characterized and tractable genes were targeted using a range of design variants. The results show that PTasRNAs are stabilized, accumulate following induction and provide reliable gene inhibition that is sufficient to alter growth phenotypes. The approach is compatible with comprehensive forward and reverse RNA-level genetics of both essential and non-essential bacterial genes and there are potential applications in pharmaceutical discovery and metabolic engineering.

MATERIALS AND METHODS

Culture conditions and general techniques

Luria–Bertani (LB) media are comprised of 1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl. M9 media comprises 17 mg/ml Na\(_2\)HPO\(_4\) 12H\(_2\)O, 3 mg/ml KH\(_2\)PO\(_4\), 0.5 mg/ml NaCl, 1 mg/ml NH\(_4\)Cl, 2 mM MgSO\(_4\), 0.1 mM CaCl\(_2\), 50 mg/ml leucine [TOP10 (Invitrogen Corp.) is a leucine auxotrophic strain]. *E.coli* TOP10 strain was used as a host cell throughout the experiments and grown in LB media at 37°C if not otherwise specified. M9 media with 2% glucose or with 2 ml sodium acetate or M9Z media (M9 media plus 0.1% NZ-amine) with 2% glucose was used. Antibiotics were included to maintain

*To whom correspondence should be addressed. Tel: +46 8 5248 6385, Fax: +46 8 32 39 50; Email: liam.good@ki.se

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plasmids at the following concentrations; chloramphenicol, 34 μg/ml; kanamycin, 20 μg/ml; ampicillin, 70 μg/ml for LB media or 35 μg/ml for M9-based media. For induction, transformants pre-grown overnight in the absence of the inducer were diluted 1:400 with the media containing one of the inducers and grown to logarithmic phase (absorbance at 550 nm = 0.6–0.8). As an inducer, 0.2% of arabinose or 1 mM of IPTG in final concentration was used if not otherwise specified. In the absence of inducer, no inhibition of target gene expression was observed in all experiments unless the constitutive promoter was used (data not shown).

Plasmid constructs and further information are described in Supplementary Materials and Methods, Figure S1 and Table S1.

Cell extracts in phosphate-buffered saline were prepared using Silica beads (0.1 mm diameter, www.biospec.com) and a Beadbeater 3110BX (www.biospec.com). Protein concentration was estimated by Bradford-assay (Bio-Rad laboratories Inc.). β-Galactosidase activity was measured as described Ref. (16). All P-values are two tailed and were calculated at http://faculty.vassar.edu/lowry/VassarStats.html.

Extracellular acetate concentration was measured with an enzymatic test kit (Boehringer-Mannheim, Darmstadt, Germany) according to the manufacturer’s instruction.

**AckA assay**

Cell extracts (20 μl, typically 1 mg/ml of protein concentration) were mixed with 60 μl of reaction buffer [200 mM Tris–HCl (pH 8.0), 10 mM ATP, 267 mM potassium acetate, 10 mM MgCl2, 6% hydroxylamine hydrochloride (neutralized with KOH before using)] and incubated for 60 min at 37°C (17). The reaction was stopped by the addition of 60 μl of 10% trichloroacetic acid followed by the addition of 10 μl of 2.5% FeCl3 (dissolved in 2N HCl). After the debris was removed by brief centrifugation, 120 μl of the mixture was transferred to the 96-well plate (FALCON 3072, Becton Dickinson Labware Co.) and absorbance of red color was measured at 540 nm (A540) using VERSAmax spectrophotometer (Molecular Devices Corporation, CA, USA). As a control, the same reaction was performed with PBS substituted for cell extract (A540). A540 values were subtracted from A1540 values (A540 – A0540 = A540) and activity was calculated as A540/mg protein/ml (units).

**Pta assay**

The reaction mixture (180 μl) was prepared in the 96-well plate and contained 200 mM Tris–HCl (pH 8.0), 5 mM MgCl2, 9.8 mM NAD+*, 3.75 mM CoA, 15 mM malic acid, 20 mM acetyl phosphate (lithium potassium salt), 2.3 U/µl citrate synthase, 3.3 U/µl malate dehydrogenase and cell extract (typically, 0.05 mg/ml of protein concentration) at 37°C (18). Absorbance of NADH was read at 340 nm each minute and the slope of the conversion curve was used to calculate the specific activity/mg protein/min/ml.

**GFP assay**

To measure green fluorescent protein (GFP) fluorescence in liquid media, transformants were cultured in a flask containing 12 ml M9Z glucose media and IPTG was added at culture density 0.6–0.7. GFP fluorescence was monitored with a NOVostar fluorescence/luminescence reader (BMG Labtechnologies) with 180 μl cell culture placed in a 96-well plate (COSTAR 3632, Corning Inc.), using 390 nm excitation and 520 nm emission wavelengths. GFP fluorescence on solid media was detected on inverted plates using a Typhoon 9400 (Amersham Biosciences) with 488 excitation and 520 emission filters.

**RNA analysis by northern blot**

Total RNAs were purified by the hot acid-phenol method (19). To determine RNA half-lives, cells were treated with 250 μg/ml of rifampicin [stock solution was dissolved with dimethyl sulfoxide (DMSO), 50 mg/ml] and RNA degradation was stopped immediately by the addition of an ethanol–phenol solution (20). Northern blots were carried out using AlkPhosDirect Labelling and Detection System (Amersham Biosciences) according to the manufacturers’ instructions. For detection of ackA asRNAs, ECF substrate (Amersham Biosciences) was used, whereas for ackA-pta mRNA, the chemiluminescent CDP-star substrate (Amersham Biosciences) was used. Typically, 25 μg/ml of total RNAs were loaded for each lane and ribosomal RNA bands were visualized by ethidium bromide staining followed by scanning with a Typhoon 9400 (excitation and emission wavelengths were 532 and 610 nm, respectively). Band densities were estimated with ImageJ software (http://rsb.info.nih.gov/ij/). A double-stranded DNA probe for ackA mRNA (probe A, 1.2 kb) was prepared by PCR using sSN17, sSN6 and pHN551 (see Supplementary Materials and Methods for details). Sequences of single-stranded oligonucleotide probes used for detection of ackA asRNAs were ATAGGTACTTCCATGTCGAGTAAGTTAGTACTGTTCTGAACTGCGGTAAGTTAGTACTGGTTCTGAACGTGGGTAGTTC (probe B) and CCGGTTCTGCCTTATTAATCTGTATCGGCTGAABAATTCTCCTCCTACCC (probe C).

Lengths of asRNAs are predicted from transcription start site of the Ppad or Pter to the end of the stem–loop structure of the rrnB terminator. Predicted asRNA lengths are: ackA asRNA without PT, 454 bp; ackA-PT1 asRNA, 479 bp; ackA-PT2 asRNA, 495 bp; ackA-PT3 asRNA, 514 bp; ackA-PT4 asRNA, 476 bp; ackA-PT5 asRNA, 276 bp; ackA-PT6 asRNA, 336 bp; ackA-PT7 asRNA, 499 bp; ackA-PT7Δ asRNA, 460 bp.

**Zone of inhibition assay on agar plates**

Mixtures consisting of 8 ml LB-1.1% agar (autoclaved and cooled to 40°C), 0.04 mM IPTG and 50 μl of an overnight culture of cells harboring the fabH-PT7 asRNA plasmid or the control PT7 plasmid were solidified in petri dishes and 2.5 μl of inhibitor solution was applied to plate surfaces. The plates were incubated for 14 h, stained by adding 1.8 ml of acridine orange solution (60 μg/ml) and scanned with a Typhoon 9400 scanner (excitation and emission wavelengths were 488 and 526 nm, respectively). Inhibitors were dissolved in 10% DMSO. DMSO alone was not inhibitory and we conducted the same experiments in the absence of IPTG and confirmed there were no changes in zone sizes for the control strains (data not shown).
RESULTS

asRNA expression and silencing of ackA-lacZ

To assess silencing by expressed RNA, we first targeted ackA within the ackA-pta operon as an example of a metabolic gene that is experimentally tractable and an interesting candidate for conditional control in industrial applications (21). AckA is central to carbon flux to and from acetate. In aerobic industrial fermentations, overflow metabolism to acetate reduces growth and inhibits macromolecule biosynthesis and product yields (22). The ackA-pta operon was targeted previously using standard asRNA strategies with modest effects (\(\leq 20\%\) inhibition) (3). To ease comparison of asRNA design parameters (Figure 1), we constructed a reporter plasmid containing ackA-lacZ translational fusion, where expression is driven by the native ackA promoter (Figure 2A). An ackA asRNA-expressing plasmid was constructed, containing the arabinose-inducible bad promoter (\(P_{bad}\)), araC and rrnB terminator. The reporter plasmid and asRNA-expressing plasmid carry different replication origins and selection markers, allowing stable maintenance of co-transformants (for details, see Supplementary Figure S1).

To benchmark our analyses to standard asRNA designs, we expressed a previously described 147 base ackA asRNA complementary to the 5'-untranslated region (5'-UTR) and start codon region (3), without flanking sequence modification. This construct inhibited expression only 15%, similar to the results reported earlier (3) and not significantly different than control levels (\(P = 0.17\)). In addition, flanking sequences that are reported to stabilize RNAs were added to the ackA asRNA sequence (6), including the 5'-UTR sequence of ompA mRNA (121 bp) (23–25), and the 3' end of DsrA RNA (67 bp) (23). However, these flanking sequences did not improve inhibition (data not shown). Therefore, the existing asRNA designs did not provide sufficient silencing in these assays.

Improved gene silencing using PTasRNAs

An alternative strategy to stabilize asRNAs is to pair the termini using flanking inverted repeats to create a hairpin structure with the antisense sequence within a large loop (Figure 1). To test this design, we compared the efficacy of \(P_{bad}\)-driven ackA asRNA cassettes (147 bp, shown as type L in Figure 2) flanked by PT consisting of 21–57 bp. The PT contains a high GC content to strengthen pairing and resist double-stranded RNA specific RNase degradation (26) (Figure 1, PT1-3). Addition of PT to asRNA improved silencing (\(P < 0.01\)) and appeared to improve with PT length (Figure 2B). However, we suspect that plasmid stability is
compromised by the longest PT, as the transformation efficiencies for PT3-containing-plasmids was lower than observed for other plasmids (data not shown) and long inverted repeats may cause plasmid loss.

To test the effects of neighboring sequences and structures, we compared several additional design variants. First, the 5’ leader sequence was removed from PT2 (PT4), and this showed no improvement ($P = 0.49$). Second, the 3’ extension sequence was removed from PT4 (PT5), and this even reduced inhibition ($P < 0.01$) (Figure 2B). Third, the PT structure was expressed adjacent to the antisense cassette (PT6), and this construct was not inhibitory. Also, to ensure that silencing is gene selective and dependent on an antisense sequence, we included empty vector controls and PT4-bla asRNA (complementary to the bla gene) as an unrelated control, and observed no inhibition (Figure 2B, shown in ‘-‘ and ‘-*‘). Therefore, a 38 base PTasRNA design is advantageous over the standard design.

To assess whether antisense cassette size affects inhibition, we compared the 147 base asRNA (type L) with a shorter 29 base asRNA (type S). Both versions were inhibitory (Figure 2C), indicating that the PT design works with different sized asRNA cassettes.

As a further comparison to reported silencing technology, we expressed a ‘parallel complementary RNA’ designed to express a parallel sequence of the ackA gene; the sequence

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### Figure 2. Silencing of plasmid-encoded ackA-lacZ by PT asRNAs driven by weak ($P_{bad}$) and strong ($P_{trc}$) promoters. (A) Schematic of the ackA-lacZ fusion mRNA, two types of ackA asRNAs (type L and type S) and an ackA parallel complementary RNA (type P). (B) Silencing by asRNAs with various PT and flanking structures. β-galactosidase activities were determined in the presence of arabinose. Cells were co-transformed with the ackA-lacZ reporter and ackA antisense plasmids. PT type (1–6, see also Figure 1) and absence (–) or presence (L) of ackA asRNA are shown at bottom. The ‘-‘ with an asterisk is an unrelated bla asRNA (191 bp) control. (C) Silencing by long and short asRNA cassettes as well as a parallel complementary RNA cassette. (D) Silencing by asRNAs driven by the $P_{lac}$ β-galactosidase activities are shown as in (B), except that gray and open bars indicate β-galactosidase activities in the absence and presence of IPTG, respectively. Note that PT4-asRNA driven by the $P_{lac}$ is transcribed constitutively because the lac operator is absent. Average values are from triplicate experiments with std values indicated.
of the ackA asRNA is 5′-AUCAAUUAUAGGUACUUCGAGUAGUA-3′, while the sequence of the parallel complementary RNA is 5′-AUGAGCGUACCUGAUCAGG-AUAUUACCAGA-3′ (shown as type P in Figure 2A). Tchurikov and co-workers reported that the inhibition efficiency of parallel complementary RNAs exceeds asRNAs when targeted to the lon mRNA (8). However, we observed no inhibition with a parallel complementary RNA targeted to the ackA mRNA (Figure 2C). We used the 147 base asRNA for further experiments.

To further improve inhibition efficiency, the 38 base PT design (PT4) was altered by replacing Pbad and araC with PTRC. The PTRC is stronger than Pbad (27) and we expected the PTRC construct to express more asRNAs than Pbad. Consistent with this we observed improved inhibition (Figure 2D). This result suggests that asRNA abundance is an important parameter. To provide IPTG-inducible control to the PTRC construct, the lac operator sequence (and also lacP) was inserted (PT7) and we observed inducible ackA inhibition (Figure 2D). Finally, to confirm the need for PT in the PT7 construct, the downstream portion of the PT7 duplex was deleted (PT7Δ), and this construct was less effective. While less efficient than the intact PT7 construct, ackA-PT7Δ asRNA expressed from Prc was almost as effective as ackA-PT4 asRNA expressed from Pbad. This may reflect the fact that Pbad is weaker than Prc or that Pbad produces an inhomogeneous culture, containing subpopulations of differentially induced cells, due to aspects of the arabinose uptake system (28).

To test the level of genetic penetrance at the colony and cell level, another reporter plasmid containing an ackA-DsRed fusion (red fluorescent protein) (29) was constructed and targeted with the ackA-PT7 asRNA. Following ackA-PT7 asRNA expression, red fluorescence on plates was uniformly reduced in all colonies examined (Supplementary Figure S2) and fluorescence microscopy indicated that most cells expressed almost no red fluorescence (Supplementary Figure S2). Also, upon withdrawal of the inducer, red fluorescence was restored to control levels (Supplementary Figure S2). These results indicate that PTasRNA silencing is penetrant and reversible.

### asRNA inhibition of the chromosomal ackA-pta operon

To test whether the effects observed with reporter constructs are observed when targeting a chromosomal gene, the effects of asRNAs with and without PT on the native ackA-pta expression were investigated by directly measuring AckA and Pta activities (Figure 3). Again, the best ackA inhibition efficiency was seen when ackA-PT7 asRNA was expressed from the PTRC (Figure 3C); 78% of AckA activity was eliminated relative to the activity of the cell expressing PT7 with no asRNA sequence. Because the pta gene is co-transcribed with ackA, Pta activity was measured following ackA-PT7 asRNA induction, and was reduced by 48% (Figure 3D), indicating inhibition of the whole operon. Similar downstream gene inhibition occurs when lacZ is inhibited within the lacZYA operon (30), and this may reflect expression coordination mechanisms within operons (12).

### Target and antisense RNA abundance and stability

When asRNAs hybridize with target mRNAs and inhibit gene expression, the mechanism of inhibition could be repression of translation initiation or triggered mRNA decay (5,11). To learn more about silencing by PTasRNAs, we probed ackA-pta mRNA abundance. Total RNAs were purified from cells grown in the presence or absence of inducers and were subjected to northern analysis (Figure 4). The asRNAs that reduced gene expression at the protein level also reduced mRNA abundance (Figure 4B, the result with probe A). The near parallel silencing pattern suggests that triggered mRNA decay is the major mechanism of antisense inhibition in this case. Residual AckA-Pta activities could be due to long half lives for these proteins following efficient mRNA silencing.

If the PTasRNAs are stabilized and expressed in large quantities, asRNA levels in cells should increase with PT addition. Therefore, we probed asRNAs in all ackA-asRNA

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**Figure 3.** Inhibition of ackA-pta expression by asRNAs. (A) Schematic maps of the ackA-pta operon mRNA and the ackA asRNA are shown. (B) An illustration of central carbon flux. Acetyl-P is acetyl phosphate. (C) Silencing of AckA in the absence (a gray bar) and presence (open bars) of inducer. Cells were transformed with ackA antisense plasmids as indicated. Activity values are the average of three replicates with std values indicated. (D) Pta activity values as shown for AckA.
structures on for points following rifampicin addition and analyzed using probe B. Note that used as loading controls. (respectively. The rRNA bands stained with ethidium bromide (EtBr) were three probes (Probe A–C) used for hybridization. (maps of the ackA-pta operon mRNA (left) and ackA asRNAs (right) with three probes (Probe A–C) used for hybridization. (B) Effect of PT and control structures on ackA mRNA and asRNA abundance. Probes A, B and C target ackA mRNA, the ackA asRNA sequence and the downstream sequence of PT, respectively. The rRNA bands stained with ethidium bromide (EtBr) were used as loading controls. (C) Effect of PT and control structures on asRNA half life. Total RNA was purified from cells harvested at the indicated time points following rifampicin addition and analyzed using probe B. Note that for ackA-PT4 and ackA-PT7 asRNAs the amount of total RNA loaded was only diluted 10-fold to enable quantification of the abundant asRNAs. Relative band intensities were plotted in a log–linear format and half-lives were estimated from line slopes.

expressing strains using asRNA probes B and C (Figure 4A). The probe B result (Figure 4B) shows that the amount of ackA-PT1 asRNA expressed from the $P_{bad}$ was higher than ackA asRNA without PT (~2-fold) expressed from the $P_{bad}$. Also, the amounts of ackA-PT4 asRNA expressed from the $P_{bad}$ and ackA-PT7 asRNA expressed from the $P_{rec}$ were higher than ackA asRNA without PT (~15- and 24-fold, respectively). We quantified ackA mRNA and asRNA band intensities and confirmed a negative correlation between asRNA abundance and residual mRNA levels ($R^2 = 0.912$; $P < 0.0001$, see Supplementary Figure S3). These results further support the idea that high asRNA abundance is needed for efficient silencing.

The major bands for ackA-PT1 asRNA, ackA-PT4 asRNA and ackA-PT7 asRNA (lower bands, shown as 3′ truncated asRNA) migrated faster than expected. Predicted RNA lengths for ackA asRNA without PT, ackA-PT1 asRNA, ackA-PT4 asRNA and ackA-PT7 asRNA were 454, 479, 495 and 499 bp, respectively, but the major bands for PT-containing-asRNAs appeared to be ~250 bp. Furthermore, the major bands were not detected by probe C (Figure 4B, the lowest panel). These results indicate that ackA PTasRNAs lack 3′ end single stranded sequences, possibility due to premature termination following transcription of the PT sequences or rapid degradation of the 3′ sequences up to the paired structure. Given the lack of signals in these lanes when using probe C and the similarity between PT and Rho-independent terminators, we favor premature termination (Figure 4B, the lowest panel). Also, similar premature termination has been reported for ribozyme sequence flanked by inverted repeats (31).

Next, we determined half-lives of ackA asRNAs by stopping de novo transcription in growing cells with rifampicin. Quantification of northern band intensities (Figure 4C) showed that half-lives were prolonged by the addition of PT. Therefore, PTasRNAs are abundant and stable, and this may explain their improved efficacy. The half-life for ackA-PT7 asRNA expressed from the $P_{rec}$ was twice that of ackA-PT4 asRNA expressed from the $P_{bad}$ although the PT structures differ only slightly.

To test whether the PT duplex or asRNA/mRNA duplex are cleaved by the double strand selective RNase III, the northern analyses were repeated in RNase III minus cells and in the same cells expressing plasmid-encoded RNase III. Expression of RNase III did not alter PTasRNA mobility or abundance and did not alter silencing (Supplementary Figure S4), indicating an RNase III independent silencing mechanism.

**Effect of ackA-PTasRNAs on acetate metabolism**

An ackA-pta deletion mutant produces less acetate than wild-type cells due to limited carbon flux to acetate (32). To test whether ackA-PTasRNA induction reduces acetate production, we measured acetate concentrations in liquid minimal media (M9) containing a high amount of glucose. The ackA-PT7 asRNA expressed from the $P_{rec}$ was used because this construct showed the best efficiency in the earlier assays (Figure 3C). Following ackA-PT7 asRNA induction, acetate levels were reduced ~30% (Figure 5). Note that *E.coli* carries at least two pathways for producing acetate (AckA-Pta and PoxB pathways) (33). Therefore, acetate levels are expected to be only partially reduced with inhibition of AckA activity, as observed here.

AckA is needed for *E.coli* to use acetate as a carbon source at high acetate concentrations (acetate scavenging) (34,35). To test whether ackA-PT7 asRNA induction prevents growth on acetate, growth on minimal media containing either glucose or acetate as a primary carbon source was evaluated. Then, cells expressing the ackA-PT7 asRNA were grown on acetate media, colony formation was abolished completely (Figure 6). *E.coli* carries at least two pathways for scavenging acetate (AckA-Pta and Acs pathways) (33), and the present results are consistent with reports that AckA-Pta is needed for acetate scavenging (34). Essentially identical results were obtained using TOP10, wild-type K12 and BL21(DE3) (a derivative of *E.coli* B strain) strains (Figure 6), indicating that the asRNA method works well in several *E.coli* strains. Therefore, with high induction,
ackA-asRNA effects can be sufficient to mimic the phenotypes of the null mutant.

Effect of ackA-PTasRNAs on heterologous protein expression

Reduced acetate production is advantageous in several industrial applications. For example, ackA-pta negative strains show improved heterologous protein production (32), but suffer from slow growth rates. Conditional ackA-pta inhibition should permit rapid growth but enable carbon to be redirected from acetate at a late growth stage to enhance heterologous protein production. To test this possibility, we introduced a GFP plasmid (model of a heterologous protein) into cells containing a constitutive or inducible ackA-PT asRNAs plasmid. The results shown in Figure 7A indicate that final GFP fluorescence was enhanced 1.9-fold using ackA-PT7 and 2.5-fold using ackA-PT4 asRNA. Also, spot application of IPTG on cells grown on M9Z glucose plates showed increased green fluorescence when ackA-PT7 asRNA was co-expressed with GFP (Figure 7B).

Effect of fabI asRNAs on cell growth and triclosan sensitivity

To ask whether PTasRNAs are suitable for studies of genes essential for growth, we targeted the fabI gene encoding enoyl-ACP reductase, which is essential for fatty acid biosynthesis and vegetative growth (36) (Figure 8A). The results (Figure 8B) indicate that growth inhibition was observed only when cells harboring the fabI-PT7 asRNA plasmid were plated onto 1 mM IPTG-containing media. We also expressed fabI asRNAs lacking PT from Pbad and no inhibition was observed (data not shown). Again, the inhibition efficiency of PT7-asRNA expressed from Pind was higher than when using the standard design, indicating that this PT design provides a reliable strategy to inhibit target gene expression.

If the growth inhibitory fabI asRNAs are fabI selective, partial inhibition should sensitize cells to the FabI inhibitor, triclosan (37,38), but not to inhibitors that target other cellular processes. To evaluate inhibitor sensitivity, we used a zone of inhibition assay (11) and fabI expression was partially silenced using fabI-PT7 asRNA induced with 0.04 mM of IPTG (Figure 8C). On plates containing cells expressing fabI-PT7 asRNA, the zone of clearing for triclosan was enlarged relative to cells expressing control PT7 RNA without an antisense insert, indicating sensitization. Zone sizes were not altered for the unrelated inhibitors, trimethoprim (inhibitor of dihydrofolate reductase type I) and rifampicin (inhibitor for RNA polymerase β subunit) (Figure 8C). Therefore, cells expressing fabI-PT7 asRNA are selectively sensitized to triclosan.

DISCUSSION

PT asRNAs provide improved gene silencing tools for E.coli. Bacterial RNAs are typically short lived, and there is evidence that half-life is an important factor in asRNA efficacy (2,6) and that a molar excess of asRNAs (and ribozymes) is required for efficient inhibition (4). An excess relative abundance should enable asRNAs to efficiently out-compete ribosomes at target RNAs. Therefore, the improved efficacy of PTasRNAs appears to be due to improved stability, which raises asRNA abundance. Also, it is possible that termini pairing aids target recognition by constraining the folded RNA. Finally, following mRNA recognition, PTasRNAs may trigger intrinsic decay mechanisms (39), which are apparently independent of RNase III, but may involve RNase E, or other RNases.

PTasRNAs are effective, yet we suspect that further improvements are possible. For example, target site position and length issues are not well addressed here, nor in the literature, although it is clear that target selection is an important factor. Most natural asRNAs target the translation

Figure 5. Effect of ackA asRNA on acetate production. (A) Culture turbidity (upper panels, absorbance at 550 nm) and extra-cellular acetate concentrations (lower panels) for each transformant are shown. Cells were grown in M9 plus glucose media with or without IPTG at 37°C. Values are the average of triplicate experiments with std values indicated. (B) AckA activities in cultures harvested during the logarithmic phase of growth in the absence (gray bars) and presence (open bars) of IPTG. Note that M9 plus glucose media was used in this experiment.
initiation region (40, 41), and we suspect that this region is a reasonable choice for most genes, but other susceptible regions exist (11). Successful asRNA cassettes as short as 12mer have been reported (6); however, in the absence of knowledge about target accessibility, we favor larger antisense cassettes, which are more likely to contain structures that efficiently seed hybridization. Indeed, we found that the inhibition efficiency of a 147 base cassette exceeded that of a 29 base cassette (Figure 2C). Further comparisons are needed on this issue, and target accessibility prediction tools (12, 42) may be helpful. Also, \textit{RecA}/\textit{C0} strains showed more stable inhibition (data not shown) and we observed improved results in solid media relative to liquid media, possibly due to plasmid stability problems in liquid culture. Plasmid stability is a common problem, and plasmids containing growth inhibitory sequences and long inverted repeats may be unstable (43). Therefore, PT length should be the minimum that provides asRNA stability without compromising plasmid stability. Therefore, while the PT design proved effective against all genes and in all strains tested, it is clear that plasmid stability requires attention.

The effects of PTasRNAs may reflect the action of certain natural RNAs. For example, natural asRNAs are also stabilized by paired sequences, and in the case of the FinP natural asRNA, stability and target interaction is promoted by the RNA chaperone FinO recognition of paired sequences (44). Also, the K1 plasmid encoded Hok toxin mRNA is processed into a stable isoform with perfectly matched PT (45). Therefore, PTasRNA effects may reflect endogenous mechanisms for stabilizing RNA and promoting interactions.
The PTasRNA design provides a framework to optimize and apply asRNA gene silencing in many applications. Forward and reverse RNA level genetics can be considered (46), and there are potential applications in pharmaceutical discovery and metabolic engineering. For example, improved conditional redirection of metabolic flux could enhance metabolic engineering, and here we show that ackA asRNA induction in mid-growth phase increases heterologous gene expression. Also, silencing of essential genes can aid discovery and metabolic engineering, and here we show that PT design and information about the stability and RNase activities (48,49) should be considered, but we suspect that the PT design and information about the stability and abundance of asRNAs will enable improved silencing.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR online.

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