RESEARCH LETTER

A genome-wide inducible phenotypic screen identifies antisense RNA constructs silencing *Escherichia coli* essential genes

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Abstract

Regulated antisense RNA (asRNA) expression has been employed successfully in Gram-positive bacteria for genome-wide essential gene identification and drug target determination. However, there have been no published reports describing the application of asRNA gene silencing for comprehensive analyses of essential genes in Gram-negative bacteria. In this study, we report the first genome-wide identification of asRNA constructs for essential genes in *Escherichia coli*. We screened 250 000 library transformants for conditional growth inhibitory recombinant clones from two shotgun genomic libraries of *E. coli* using a paired-termini expression vector (pHN678). After sequencing plasmid inserts of 675 confirmed inducer sensitive cell clones, we identified 152 separate asRNA constructs of which 134 inserts came from essential genes, while 18 originated from nonessential genes (but share operons with essential genes). Among the 79 individual essential genes silenced by these asRNA constructs, 61 genes (77%) engage in processes related to protein synthesis. The cell-based assays of an asRNA clone targeting *fusA* (encoding elongation factor G) showed that the induced cells were sensitized 12-fold to fusidic acid, a known specific inhibitor. Our results demonstrate the utility of the paired-termini expression vector and feasibility of large-scale gene silencing in *E. coli* using regulated asRNA expression.

Introduction

During the past few decades, bacterial pathogens have become increasingly resistant to antibiotics, limiting treatment options for infections caused by drug-resistant bacterial pathogens (Boucher *et al.*, 2009). As we face growing antibiotic resistance, the development of novel antibiotics continues to stagnate. Therefore, there is an urgent need for the discovery of new antibacterial agents to target drug-resistant bacteria, especially Gram-negative pathogens (Boucher *et al.*, 2009).

Regulated antisense RNA (asRNA) expression has been used effectively to study gene functions in different bacterial systems, including *Streptococcus mutans* (Wang & Kuramitsu, 2005), *Staphylococcus aureus* (Ji *et al.*, 2001; Forsyth *et al.*, 2002), and *Escherichia coli* (Nakashima & Tamura, 2009). By blocking the expression of its target gene, an asRNA increases the sensitivity of bacteria only to specific inhibitors for a protein encoded by that target gene (Forsyth *et al.*, 2002; Young *et al.*, 2006). This differential sensitivity screening assay has been used to validate mechanisms of action for known antibiotics (Forsyth *et al.*, 2002; Ji *et al.*, 2004) and to discover novel antibacterial inhibitors (Young *et al.*, 2006; Wang *et al.*, 2007). Furthermore, hundreds of *S. aureus* asRNA strains have been configured into a TargetArray, which was employed to study mechanisms of action of antibacterial inhibitors (Donald *et al.*, 2009; Xu *et al.*, 2010). Thus, regulated asRNA expression has a great potential for antibiotic drug discovery.

However, the regulated asRNA approach has seen limited success in Gram-negative bacteria, including *E. coli*.

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There have been no published reports describing the adoption of the regulated asRNA approach for comprehensive genome-wide essential gene determination and/or silencing in Gram-negative bacteria. It has been recognized that asRNA-mediated down-regulation of gene expression in *E. coli* is inefficient for reasons not yet clearly understood (Wagner & Flardh, 2002). Attempts to improve the efficiency were rather frustrating initially (Engdahl *et al.*, 2001). Several years ago, a series of expression vectors were designed such that expressed asRNA molecules have paired-termini to enhance their stability and hence gene knock-down efficiency in *E. coli* (Nakashima *et al.*, 2006). In this report, we present a first genome-wide attempt to obtain cell growth inhibitory *E. coli* asRNA constructs through phenotypic screening two shotgun genomic libraries based on a paired-termini expression vector, pHN678 (Nakashima *et al.*, 2006). Our results will stimulate further studies of gene functions, coordinated gene expression on operons and interactions of cellular processes via regulated asRNA in *E. coli*. Furthermore, the collection of the *E. coli* asRNA clones generated using this approach will be a valuable tool in the antibiotic drug discovery, especially for therapeutics targeting Gram-negative bacterial pathogens.

Materials and methods

Construction of random genomic libraries

Genomic DNA was extracted from *E. coli* MG1655 cells (American Type Culture Collection, Manassas, VA) using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) followed by partial digest with Sau3AI or CviKI-1 (NEB, Ipswich, MA). The resulting DNA fragments (200–800 bp) were purified from agarose gels using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA). Plasmid vector was digested with BamHI (if Sau3AI was used to digest the genomic DNA) or SnaBI (if CviKI-1 was used), dephosphorylated using Antarctic Phosphatase (NEB) and then ligated with the inserts using the T4 DNA ligase (Life Technologies, Carlsbad, CA). Ligation mixtures were transformed into *E. coli* DH5α competent cells (Life Technologies) and plated onto LB agar plates plus 34 μg mL^{-1} chloramphenicol. Cloning efficiency of the pHN678 library was determined by colony PCR using the following primers: 5′-CGACATCATAACGGTTCTGGCAAAT-3′ (forward) and 5′-GACCGCTTCTGCGTTCTGATTT-3′ (reverse) (Eurofins MWG Operon, Huntsville, AL). The primers were designed so that in the absence of an insert, a 290-bp band should be detected by PCR. Any band larger than this size would indicate the presence of a cloned DNA.

Inducible phenotypic screening for inducer sensitive clones

Colonies from the random genomic libraries were individually picked with sterile tooth picks, inoculated into wells of 96-well microplates (Corning #3370; Fisher, Pittsburgh, PA) containing LB broth plus chloramphenicol, and grown overnight at 37 °C for 16 h. Each 96-well microplate was then replica plated onto two sets of Nunc's Omni Trays (Rochester, NY) using a 96-pin replicator (V&P Scientific, San Diego, CA). Both trays contained LB agar plus chloramphenicol, with one of them supplemented with 1 mM IPTG (inducing plate). A positive cell clone (PT18, targeting *rplF* and *rpsH* genes) was included in each microplate as a positive control. Inducer sensitive clones were identified via growth defects (lethal or defective growth) present only on the inducing plates. The inducer sensitivity of these clones was confirmed again prior to plasmid insert sequencing. Each inducer sensitive clone was given a clone number beginning with a prefix PT because the paired-termini vector pHN678 was used. The clone names of Library C clones are affixed with a letter 'C' to differentiate from those from the Sau3AI digested library.

Insert DNA sequencing and bioinformatics analysis

Plasmids were isolated from confirmed inducer sensitive clones and sequenced at Eton Bioscience Inc. (San Diego, CA) to determine the DNA sequences of the inserts and their orientations. The DNA sequences were then compared with the annotated genomic sequence of *E. coli* MG1655 (GenBank accession number NC_000913) to determine the origin of DNA inserts and their orientation using NCBI BLAST. The essentiality of the corresponding target gene was determined based the Profiling of *E. coli* Chromosome (PEC) database (http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp). The operon structure for relevant genes targeted by asRNA was obtained from the REGULONDB (http://regulondb.ccg.unam.mx/) to determine whether other essential genes are present in the targeted operon.

Selective asRNA cell sensitization

To quantitatively measure the IPTG-induced growth inhibition in *E. coli* asRNA cell clones (e.g. *fusA* cell clone, PT44), seven-point IPTG dose–response curves were obtained as described previously (Xu *et al.*, 2006). To determine the initial inducer conditions appropriate for sensitizing asRNA cell clones, IPTG concentrations causing between 70% and 80% cell growth inhibition for asRNA clones were determined. One asRNA clone (PT44) targeting *fusA* gene (which encodes elongation factor G)

was studied in more detail to demonstrate selective cell sensitization. Specifically, an exponential growth culture of PT44 was inoculated into fresh LB broth plus chloramphenicol and appropriate IPTG concentrations (and no IPTG control). The inoculum was combined in a microplate with seven-point serial dilutions of fusidic acid, a known inhibitor of elongation factor G, and cell growth in each well of the microplate was monitored as described previously (Xu *et al.*, 2006). Fusidic acid dose–response curves of cell growth under various inducer treatments were generated using the 8-h time-point optical density data and graphed using Prism software (GraphPad, San Diego, CA). Once the optimal IPTG concentration was obtained, additional cell-based assays were performed against a panel of known antibiotics of different chemical classes to obtain fold sensitization values [the ratio between the IC_{50} value (the concentration at which cell growth inhibited 50% compared with control) under noninduced condition and that of induced condition].

Results

Construction of random genomic libraries using shotgun cloning

Based on the result of a time-course Sau3AI digestion (Fig. S1a, Supporting Information), the optimal partial digestion time was 4 h to generate DNA fragments of appropriate size for library construction. Ligation mixtures were transformed into *E. coli* DH5α competent cells. Insert cloning efficiency analysis (Fig. S1b) indicated that the cloning efficiency for this library was 92%. To increase the randomness of the genomic DNA generated, an alternative genomic library was constructed using a blunt-end producing restriction endonuclease (CviKI-1). The cloning efficiency of the CviKI-1-based library (termed Library C) was 90%.

Screening for inducer sensitive clones

To screen for inducible growth inhibitory recombinant clones, transformants were grown overnight with chloramphenicol in the presence or absence of inducing IPTG. An example of screening plates and sensitive clones is shown (Fig. S1c). A total of 1500 confirmed IPTG-sensitive clones were obtained from screening 250 000 individual transformants. Only 675 of the 1500 confirmed clones were sequenced. An example of inducer-dependent inhibition of growth of asRNA clone PT113 is shown in Fig. S1d.

AS constructs targeting *E. coli* essential genes

Plasmid DNAs from a total of 675 confirmed inducer sensitive clones were sequenced. It was determined that enough clones were analyzed because more analysis leads to identification of duplicates, suggesting that the phenotypic screening process under the condition scheme is approaching saturation. Among the sequenced clones, 134 separate clones contained insert DNA sequences derived from and in antisense orientation to known essential genes based on PEC database (Table 1). For most of the essential genes targeted by asRNAs, multiple gene silencing asRNA constructs were discovered, with *rplF* gene (encoding 50S ribosomal subunit protein L6) being 'hit' the most (17 times) (Table 1). Because many essential genes engaging in a cellular process are usually clustered in an operon, many essential operons are targeted by a multitude of asRNAs, especially the operons for ribosomal protein genes. For example, *rplN* operon that contains 11 essential genes was 'hit' by 17 unique asRNAs (Fig. 1a, with two asRNAs not shown owing to space limit). On an individual gene level, four unique asRNAs were found to target *fusA* gene (Fig. 1b), while another four to target *rpoC* gene (Fig. 1d).

While plasmids of the majority of the remaining confirmed inducer sensitive clones were found to contain insert sequences corresponding to nonessential genes (data not shown), 18 separate asRNA constructs were identified to have derived their inserts from nonessential genes which share operons with known essential genes (Fig. 1b–d; Table 1). For example, five asRNA constructs, originated from nonessential genes (*priB* and *rplI*) of the *rpsF* operon, can individually inhibit cell growth upon induction (Fig. 1c). Similarly, each of the two asRNA constructs, originated from the nonessential *rplK* and/or *rplA* genes, inhibits cell growth when induced by IPTG (Fig. 1d). These results strongly suggest that induced asRNA silences gene expression at the operon level. Interestingly, an asRNA construct (PT32) targets both *tufA* and *tufB* genes because the asRNA complements mRNA regions of both genes 100% (Fig. 1b). Additionally, another asRNA construct (PT323) derived its sequences from the *tufB* gene located on an unlinked operon which also has two essential genes (*glyT* and *thrU*) (Fig. 1b). While PT32 could derive its sequences from either *tufA* or *tufB* because of identical sequences in the relevant regions, PT323 shares 100% homology with *tufB* gene but has one mismatch nucleotide with *tufA* sequence (data not shown). Combining essential genes directly targeted with those indirectly targeted (via operon effects) by asRNA constructs, a total of 79 essential genes can be silenced or knocked down by 152 separate asRNA constructs (Table 1 and Table S1). It is noteworthy that among these 79 essential genes, 61 (77%) are involved in processes related to protein synthesis (Table S1).

Table 1. *Escherichia coli* genes targeted by asRNA constructs, other essential genes present on the same operons and insert DNA genome coordinates

Gene	Product encoded*	Other essential genes within operon†	asRNA clone insert coordinates,‡ clones and clone types
accC	Acetyl-CoA carboxylase, biotin carboxylase subunit	*accB*	(3404969–3405089): PT312
acpP	Acyl carrier protein (ACP)	*fabD, fabG*	(1150883–1150973): PT1192C, PT1193C
bamA	Outer membrane protein assembly factor, forms pores; required for OM biogenesis; in BamABCD OM protein complex	*lpxD, fabZ, lpxA, lpxB*	(2242734–2242870): PT164
fusA	Protein chain elongation factor EF-G, GTP-binding	*rpsL, rpsG*	(3470872–3471062): PT3, PT43, PT499 (3469579–3469688): PT4, PT144, PT212, PT220, PT221, PT226 (3470419–3470651): PT44, PT313 (3470656–3470867): PT257
glnS	Glutamyl-tRNA synthetase	None	(705881–706317): PT200 (705276–705473): PT418
gltX	Glutamyl-tRNA synthetase	None	(2518066–2518186): PT311, PT319, PT320, PT375, PT475, PT497
infB	Fused protein chain initiation factor 2, IF2:membrane protein/conserved protein	*nusA*	(3311410–3311552): PT129
ispB	Octaprenyl diphosphate synthase	None	(3332571–3332694): PT157
leuS	Leucyl-tRNA synthetase	*nadD, holA, lptE*	(673825–674065): PT7, PT46
parE	DNA topoisomerase IV, subunit B	None	(3172272–3172325): PT1589C
pheS	Phenylalanine tRNA synthetase, alpha subunit	*thrS, infC, rplT, pheM, pheT*	(1796927–1797014): PT1157C, PT1173C, PT1561C
pheT	Phenylalanine tRNA synthetase, beta subunit	*thrS, infC, rplT, pheS, pheM*	(1794440–1794587): PT79, PT335, PT368 (1794205–1794396): PT153 (1794205–1794357): PT412 (1794809–1794914): PT1108C, PT1261C (1794112–1794204): PT422 (1795187–1795315): PT1608C
prfA	Peptide chain release factor RF-1	*hemA, prmC*	(1264626–1264716): PT69, PT123, PT147, PT316 (1264548–1264716): PT381
rplB	50S ribosomal subunit protein L2	*rpsJ, rplC, rplD, rplW, rpsS, rplV, rpsC, rplP, rpmC, rpsQ*	(3449247–3449437): PT1316C
rplC	50S ribosomal subunit protein L3	*rpsJ, rplD, rplW, rplB, rpsS, rplV, rpsC, rplP, rpmC, rpsQ*	(3450082–3450342): PT117 (3450200–3450427): PT1363C
rplD	50S ribosomal subunit protein L4	*rpsJ, rplC, rplW, rplB, rpsS, rplV, rpsC, rplP, rpmC, rpsQ*	(3450082–3450342): PT117 (3450200–3450427): PT1363C (3449574–3449772): PT1578C (3449913–3450111): PT1603C
rplE	50S ribosomal subunit protein L5	*rplN, rplX, rpsN, rpsE, rpsH, rplF, rplR, rpmD, rplO, secY*	(3445153–3445259): PT121 (3444964–3445113): PT1254C (3444903–3444963): PT1297C
rplF	50S ribosomal subunit protein L6	*rplN, rplX, rplE, rpsE, rpsH, rpsN, rplR, rpmD, rplO, secY*	(3444089–3444227): PT18, PT35, PT263, PT265, PT266, PT267, PT268, PT269, PT272, PT275, PT276, PT277, PT355, PT362, PT365 (3443744–3444084): PT45 (3443474–3443735): PT1116C
rplL	50S ribosomal subunit protein L7/L12	*rplJ, rpoB, rpoC*	(4178602–4178650): PT304
rplM	50S ribosomal subunit protein L13	*rpsI*	(3376226–3376437): PT1306C, PT1386C (3376658–3376776): PT1330C, PT1533C (3376438–3376588): PT1518C, PT1587C
rplN	50S ribosomal subunit protein L14	*rplF, rplX, rplE, rpsE, rpsH, rpsN, rplR, rpmD, rplO, secY*	(3446053–3446165): PT55, PT334

Table 1. Continued

Gene	Product encoded*	Other essential genes within operon†	asRNA clone insert coordinates,‡ clones and clone types
rplO	50S ribosomal subunit protein L30	*rplN, rplX, rplE, rpsN, rpsH, rplF, rplR, rpsE, rplD, secY*	(3442251–3442600): PT189, PT293 (3442191–3442269): PT1228C (3442537–3442667): PT1280C
rplQ	50S ribosomal subunit protein L17	*rpsK, rpsM, rpsD, rpoA*	(3437674–3437796): PT91 (3437801–3438054): PT235, PT241 (3437565–3437690): PT1367C (3437978–3438088): PT1518C, PT1587C (3437691–3437966): PT1610C
rplR	50S ribosomal subunit protein L18	*rplN, rplX, rplE, rpsN, rpsH, rplF, rpsE, rpmD, rplO, secY*	(3443474–3443735): PT1116C (3443279–3443402): PT1385C
rplV	50S ribosomal subunit protein L22	*rpsJ, rplC, rplW, rplB, rpsS, rplD, rpsC, rplP, rpmC, rpsQ*	(3447932–3448050): PT141, PT142 (3448126–3448303): PT1272C, PT1247C
rplW	50S ribosomal subunit protein L23	*rpsJ, rplB, rplC, rplD, rpsS, rplV, rpsC, rplP, rpmC, rpsQ*	(3449247–3449437): PT1316C (3449574–3449772): PT1578C
rplX	50S ribosomal subunit protein L24	*rplN, rplE, rpsN, rpsH, rplF, rplR, rpsE, rpmD, rplO, secY*	(3445539–3445602): PT1602C
rpmD	50S ribosomal subunit protein L30	*rplN, rplX, rplE, rpsN, rpsH, rplF, rplR, rpsE, rplO, secY*	(3442605–3442849): PT80, PT479 (3442251–3442600): PT189, PT293 (3442537–3442667): PT1280C
rpoA	RNA polymerase, alpha subunit	*rpsM, rpsK, rpsD, rplQ*	(3438419–3438661): PT300, PT328 (3438059–3438210): PT326 (3438300–3438414): PT379 (3437978–3438088): PT1518C, PT1587C
rpoC	RNA polymerase, beta prime subunit	*rplJ, rplL, rpoB*	(4186643–4186713): PT11, PT50 (4184366–4184694): PT306 (4185255–4185547): PT1130C (4184355–4184497): PT1509C
rpsA	30S ribosomal subunit protein S1	None	(961514–961694): PT15 (961506–961694): PT239 (962090–962383): PT1408C (962523–962628): PT1566C
rpsB	30S ribosomal subunit protein S2	*tsf*	(190451–190554): PT17, PT172
rpsC	30S ribosomal subunit protein S3	*rpsJ, rplC, rplD, rplW, rplB, rpsS, rplV, rplP, rpmC, rpsQ*	(3447274–3447439): PT1124C (3447272–3447441): PT1259C
rpsE	30S ribosomal subunit protein S5	*rplN, rplX, rplE, rpsN, rpsH, rplF, rplR, rpmD, rplO, secY*	(3443076–3443203): PT36 (3442605–3442849): PT80, PT479
rpsH	30S ribosomal subunit protein S8	*rplN, rplX, rplE, rpsN, rpsE, rplF, rplR, rpmD, rplO, secY*	(3444556–3444785): PT12 (3444089–3444227): PT18, PT35, PT263, PT265, PT266, PT267, PT268, PT269, PT272, PT275, PT276, PT277, PT355, PT362, PT365
rpsI	30S ribosomal subunit protein S9	*rplM*	(3376226–3376437): PT1306C, PT1386C
rpsK	30S ribosomal subunit protein S11	*rpsM, rpsD, rpoA, rplQ*	(3440016–3440145): PT74 (3439846–3439981): PT1308C
rpsL	30S ribosomal subunit protein S12	*rpsG, fusA*	(3472339–3472689): PT93
rpsM	30S ribosomal subunit protein S13	*rpsK, rpsD, rpoA, rplQ*	(3440016–3440145): PT74 (3440150–3440288): PT148
rpsN	30S ribosomal subunit protein S14	*rplN, rplX, rplE, rpsE, rpsH, rplF, rplR, rpmD, rplO, secY*	(3444556–3444785): PT12 (3444903–3444963): PT1297C (3444668–3444857): PT1364C (3444766–3444902): PT1534C, PT1549C
rpsP	30S ribosomal subunit protein S16	*trmD, rplS*	(2743908–2743982): PT84, PT133 (2744137–2744339): PT1293C
rpsR	30S ribosomal subunit protein S18	None	(4423294–4423920): PT113 (4424028–4424446): PT1275C

Table 1. Continued

Gene	Product encoded*	Other essential genes within operon†	asRNA clone insert coordinates,‡ clones and clone types
rpsS	30S ribosomal subunit protein S19	*rpsJ, rplC, rplD, rplW, rplB, rplV, rpsC, rplP, rpmC, rpsQ*	(3448126–3448303): PT1160C, PT1272C, PT1247C (3448304–3448550): PT1411C, PT1502C
mutL§	Methyl-directed mismatch repair protein	*yjeE*	(4396833–4396880): PT1540C, PT1542C
priB§	Primosomal protein N	*rpsR*	(4423724–4423857): PT1303C, PT1505C
rimM§	16S rRNA gene processing protein	*rpsP, trmD, rplS*	(2743908–2743982): PT84, PT133
rplA§	50S ribosomal subunit protein L1	*rplJ, rplL, rpoB, rpoC*	(4176746–4176904): PT1351C
rplI§	50S ribosomal subunit protein L9	*rpsR*	(4424183–4424555): PT70 (4424028–4424446): PT1275C (4424321–4424446): PT1349C (4424306–4424446): PT1546C (4424462–4424572): PT1579C
rplK§	50S ribosomal subunit protein L11	*rplJ, rplL, rpoB, rpoC*	(4176746–4176904): PT1351C (4176522–4176719): PT1520C, PT1551C, PT1580C
rpsO§	30S ribosomal subunit protein S15	*nusA, infB*	(3309412–3309551): PT1499C
rpsU§	30S ribosomal subunit protein S21	*dnaG, rpoD*	(3208846–3209066): PT1156C
tufA§	Protein chain elongation factor EF-Tu (duplicate of *tufB*)	*rpsL, rpsG, fusA*	(3469077–3469201): PT32, PT599 (3468846–3469072): PT323 (1 mismatch at 3469061)
tufB§	Protein chain elongation factor EF-Tu (duplicate of *tufA*)	*thrU, glyT*	(4174117–4174241): PT32, PT599 (4174246–4174472): PT323

*From PEC database.

†From RegulonDB website.

‡Based on GenBank accession number NC_000913.

§Denotes nonessential genes which share operons with other essential gene(s).

Selective cell sensitization

To determine whether an asRNA clone can be sensitized towards inhibitors targeting the essential protein involved, the *fusA* asRNA clone (PT44) was studied in cell-based assays. The strategy is to inhibit the function of an essential protein to a threshold level (but without totally shutting down the cells' growth) whereby the cell is sensitive to any additional assault on the same essential protein. First, a seven-point IPTG dose–response curve for PT44 was generated (Fig. 2a) with the IC_{50} value of 28 μM. Subsequently, the inducer concentrations for sensitizing PT44 clone against fusidic acid (which targets EF-G) were further optimized (Fig. 2b). Our results indicated that at 45 μM IPTG, the asRNA clone exhibits 12-fold increase (IC_{50} at 0 μM divided by IC_{50} at 45 μM) in sensitivity to the specific inhibitor (Fig. 2b). The optimized cell-based assay was performed against serial dilutions of nine other antibiotics (Fig. 2c). Results showed that the *fusA* asRNA clone was the most sensitive to fusidic acid (12-fold), followed by erythromycin (fivefold) and tetracycline (fourfold), both are well-known antibiotics targeting protein synthesis (Fig. 2c).

Discussion

It was recognized that conditional silencing by introduced asRNAs in Gram-negative bacteria is less efficient than in Gram-positive bacteria (Wagner & Flardh, 2002). Specifically, while global essential genes in *S. aureus* (Ji *et al.*, 2001; Forsyth *et al.*, 2002) and *S. mutans* (Wang & Kuramitsu, 2005) have been identified by regulated asRNAs, the adoption of such approach in Gram-negative bacteria has not been reported (Good & Stach, 2011). Although the reasons for such discrepancy are not well defined, one possible explanation lies in the reduced stability of plasmid-borne artificial asRNAs in *E. coli* probably due to the presence of RNase E in this bacterium (Xu *et al.*, 2010), but not in *S. aureus*. For this reason, Nakashima and colleagues (Nakashima *et al.*, 2006) designed a series of *E. coli* plasmid vectors which produce RNA molecules with paired-termini to increase the asRNA stability and conditional gene silencing. Targeted antisense fragment cloning using such paired-termini vectors has produced asRNA constructs which have shown to knockdown or silence the expression of a number of essential genes in *E. coli* (Nakashima *et al.*, 2006). In this communication, we report a genome-wide application of regulated asRNA expression in *E. coli* using the vector pHN678. Here, we demonstrated that employing this paired-termini vector indeed identified a large number of asRNA constructs targeting *E. coli* essential genes and, to a lesser extent, some nonessential genes which share operons with essential genes.

While asRNA constructs targeting essential genes of a number of cellular processes in *E. coli* were identified

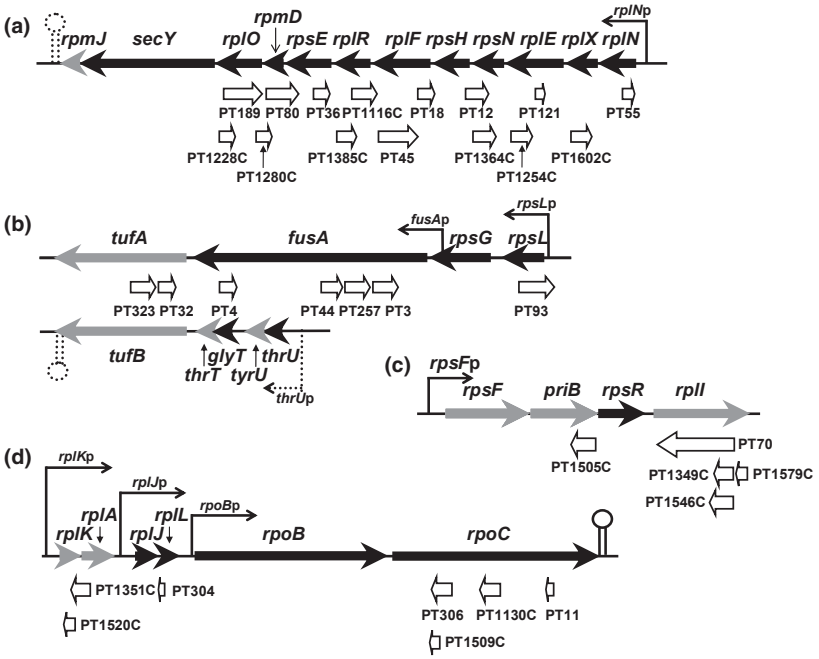

Fig. 1. Representative *Escherichia coli* operons targeted and silenced by asRNAs. Solid dark arrows are essential genes, while gray arrows are nonessential genes based on PEC database. All open arrows are asRNAs, each capable of inhibiting the growth of *E. coli* cells upon induction by 1 mM IPTG. (a) Operon *rplN* contains 11 essential genes and one nonessential gene. Every essential gene except *secY* is targeted by at least one asRNA construct. Two additional asRNAs are not shown because of space limit. (b) Operon *rpsL* contains three essential genes plus one nonessential gene (*tufA*). Four and one asRNA constructs were discovered to silence *fusA* and *rpsL* genes, respectively. Immediately below the *rpsL* operon, an unlinked operon containing three nonessential genes (*tufB*, *thrT,* and *tyrU*) and two essential tRNA genes (*glyT* and *thrU*) was shown to illustrate common nucleotide regions of *tufA* and *tufB* genes potentially targeted by two independent asRNA constructs (PT32 and PT323). PT323 was apparently derived from *tufB* gene because there is one nucleotide mismatch located at genome position 3469061 of *tufA* gene. (c) Operon *rpsF* showing that five independent asRNA constructs targeting nonessential genes, illustrating their capacity to independently silence the essential gene (*rpsR*) within the same operon. (d) In operon *rplK*, five asRNA constructs target essential genes, while two independent asRNA constructs target nonessential genes. Operon structures were based on information from RegulonDB website.

(Table 1 and Table S1), particularly striking was the observation that the asRNAs predominately silence the expression of essential genes (77% of total genes) involved in protein synthesis processes (tRNAs, tRNA synthetases, transcription, ribosomal proteins, and translation factors) (Table S1). We speculate that this bias may have been caused by high basal level (leaky) promoter (P_{trc}) activity from the vector in the absence of IPTG (Nakashima & Tamura, 2009) during the library transformation process. It was possible that for those asRNA constructs derived from essential genes, which normally are expressed at low levels, even the background level of asRNAs could render the *E. coli* clones unable to grow into colonies after transformation. In contrast, asRNA clones in which highly expressed genes are being targeted would be able to grow into colonies and selected during the subsequent phenotypic (+IPTG) screens. This hypothesis is supported by data from DNA array-based *E. coli* gene expression profiling (Tao *et al.*, 1999). For example, 53 of the 79 essential genes (67%) targeted by asRNA constructs (Table S1) are within the top 10% highly expressed genes among the 4290 ORFs examined when *E. coli* cells were grown exponentially in LB broth plus glucose (Tao *et al.*, 1999). To increase the diversity of asRNA clones identified, possible technical improvements include replacing *Ptrc* with a more stringent promoter element on the cloning vector or employing a number of plasmid vectors each containing a promoter with different range of activities (Nakashima *et al.*, 2006; Xu *et al.*, 2010).

The recovery of 18 asRNA constructs derived from 10 nonessential genes which share operons with essential genes provides strong support for a hypothesis that expressed asRNAs silence gene function in *E. coli* at the operon level. The mechanism of asRNA inhibition in *S. aureus* was examined previously by Young and coworkers (Young *et al.*, 2006) who demonstrated that asRNAs exert their inhibition by eliciting degradation of mRNAs upstream (5′) of the regions where the asRNAs bind, which lends support to our hypothesis. If the hypothesis is confirmed, an asRNA construct or synthetic oligonucleotide could

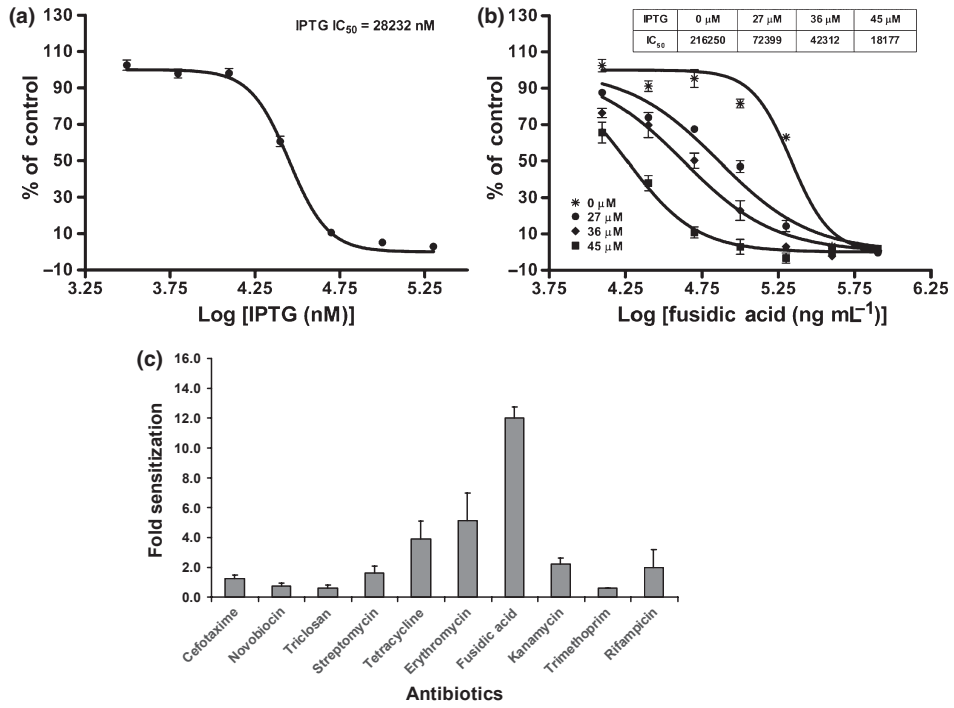

Fig. 2. Inducer optimization and selective sensitization of *fusA* targeted PT44 clone. (a) Inducer dose–response curve for PT44 clone. *X*-axis shows the log values of inducer concentrations, while *Y*-axis shows the percent growth (as compared to no-induction cell control) of cells treated under various inducer concentrations shown. (b) PT44 cells become more sensitive to fusidic acid as the amount of IPTG increases. *X*-axis shows the log values of inhibitor concentrations. *Y*-axis is the same as in (a). (c) Induction of asRNA to *fusA* mRNA (in PT44 clone) selectively sensitizes the cells to fusidic acid, which specifically targets EF-G encoded by *fusA* gene. Also included in the assay were nine other antibiotics targeting essential processes such as cell wall synthesis (cefotaxime), DNA replication (novobiocin), RNA transcription (rifampicin), fatty acid synthesis (triclosan), protein synthesis (streptomycin, tetracycline, kanamycin, and erythromycin) and folate synthesis (trimethoprim). For each condition four replicate data points were obtained per experiment and each experiment was performed three times (n = 3) with SD error bars shown.

inhibit as many as 11 essential genes simultaneously on the *rplN* operon (Fig. 2a), rendering it difficult for multiple resistant mutations to occur in multiple genes. If such multigene mechanism of gene silencing turns out to be prevalent among bacteria, it will facilitate design and development of antisense-based antimicrobial therapeutics which are 'polypharmaceutical' (Good & Stach, 2011) or 'multitargeting' (Silver, 2007): antibiotics (e.g. most of the successful antibiotics in clinical use) target or interact with two or more bacterial target proteins.

In this study, two genomic libraries were constructed successfully and screened for inducible growth inhibitory asRNA clones. The asRNA constructs discovered could knock-down or silence the expression of 79 *E. coli* essential genes. While the genes being targeted are not yet comprehensive, likely due to a leaky P_{trc} promoter of pHN678, this communication represents a first published report to successfully apply regulated asRNA technology to discover *E. coli* asRNA clones at the genome level. Such conditional asRNA clones will not only stimulate studies of global functions of genes and operons in *E. coli* but also facilitate discovery and development of novel antimicrobial agents to combat multidrug-resistant pathogens.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Construction and phenotypic screening of *E. coli* shot-gun genomic libraries and confirmation of inducer sensitive clones.

Table S1. *E. coli* essential genes targeted by asRNA clones directly or indirectly (via operon effects).

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