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Knockdown of *recA* gene expression by artificial small RNAs in *Escherichia coli*

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ABSTRACT

Bacterial RecA plays a central role in DNA repair and regulation of the SOS response to DNA damage, and has been suggested as a new antibiotic drug target. To develop a new tool to study RecA function, we engineered artificial small RNAs (sRNAs) that can posttranscriptionally repress RecA expression in *Escherichia coli*. The artificial sRNAs mimic the bacterial noncoding sRNAs which possess an antisense domain that is partially complementary to the targeted mRNA. We screened a library of artificial sRNAs with a randomized antisense domain and isolated several anti-*recA* sRNAs that can knockdown the endogenous RecA level in *E. coli*. The cells expressing the anti-*recA* sRNAs were found to exhibit phenotypes consistent with RecA repression such as reduced swarming motility and increased susceptibility to ciprofloxacin, a fluoroquinone antibiotic.

1. Introduction

Bacterial RecA is a highly conserved multifunctional protein that plays a central role in DNA repair and regulation of the SOS response to DNA damage [1,2]. RecA has also been implicated in pathogenic processes such as swarming motility [3], activation of toxin biosynthesis [4,5], competence [6,7], virulence factor production [8] and genetic diversification of biofilms [9]. Thus, RecA has multiple cellular functions that make it vital for an organism's survival and pathogenicity. More recently, the Collins group showed that all the major bactericidal antibiotics lead to the production of toxic hydroxyl radicals which trigger the bacteria to counteract by activating the SOS response [10]. Their work provides an explanation for the increased antibiotic sensitivity of the *recA* mutants that are not capable of activating the SOS response [11,12], and suggests RecA or other genes involved in the SOS response as potential novel drug targets to combat the ever-increasing problem of antibiotic resistance.

The goal of the present work is to develop "artificial small RNAs (sRNAs)" that can posttranscriptionally repress RecA expression. In contrast to the permanent *recA* knockout strains that have been used to study the gene functions, our *trans*-acting artificial sRNAs can dynamically and finely modulate the RecA expression level. Such artificial sRNAs may enable a more accurate emulation of RecA-targeting drugs whose effects are often transient and imperfect, and could be used to study temporal and dosage effects of RecA-targeting drugs.

Our artificial sRNAs are inspired by the natural noncoding bacterial sRNAs that posttranscriptionally regulate their target mRNAs *in trans* [13]. This regulation occurs via imperfect Watson–Crick base pairing between the sRNA and its target mRNA and is facilitated by the chaperone protein Hfq [14]. Pairing occurs over a seed region of 6–8 base-pairs although longer interactions have been discovered [15]. Several recent studies have highlighted the modular nature of these sRNAs to possess three discrete domains: (1) mRNA target interacting (antisense) domain, (2) Hfq binding domain, and (3) transcriptional terminator domain [16–19]. Taking advantage of this modular nature, our group constructed a library in which a randomized antisense domain is fused to an Hfq-binding/transcription terminator domain (sRNA scaffold) derived from natural sRNAs. Using a reporter plasmid that contains the 5′ leader sequence from the targeted mRNA, we isolated a number of artificial sRNAs that efficiently repressed expression of an outer-membrane porin (OmpF) and flagellin (FliC) in *Escherichia coli* [20].

In the present study, we performed a high-throughput screen to identify artificial anti-*recA* sRNAs and demonstrate that the wild-type *E. coli* expressing the sRNAs exhibit phenotypes consistent with RecA knockdown. The artificial sRNAs targeting RecA can be useful for studying the diverse functions of RecA as well as for developing new antibiotic strategies.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli TOP10 cells (Invitrogen, F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K

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*rps*L (Str^R) *end*A1 *nup*G λ^-) were used in all experiments, unless specified otherwise. Western blot analysis, swarming motility assay and ciprofloxacin sensitivity assay were performed using *E. coli* K-12 MG1655 cells (F$^-$ λ^- *ilv*G$^-$ *rfb*-50 *rph*-1) obtained from Coli Genetic Stock Center (CGSC). The cells were grown at 37 °C (unless specified otherwise) from single-colony isolates or diluted from overnight cultures in LB medium. For plasmid maintenance, ampicillin (Fisher Scientific) and kanamycin (Sigma) were used at concentrations of 100 and 50 μg/mL, respectively.

2.2. Construction of artificial sRNA library plasmid and pKP33-recAGFPuv reporter plasmid

Plasmid pPROTetAS contains a ColE1 origin of replication and an ampicillin resistance marker (*bla*) and is used to clone sRNAs downstream of the constitutive promoter $P_L$tetO1 [20]. Plasmid pPROTetAS-Spot42 (Supplementary Fig. S1A) was obtained by cloning the *spot*42 sRNA [17] gene into pPROTetAS. The library was constructed as described recently [20,21] by performing polymerase chain reaction (PCR) to amplify the whole pPROTetAS-Spot42 plasmid while randomizing the antisense domain of Spot42 using partially degenerate primers. The PCR was performed with a common reverse primer Artsrna-rev (5′ NNNNN NNNNN NNNNN NNNNN GTCGA CATGT GCTCA GTATC TCTAT C 3′) and either Spot-Fw (5′ ATTTG GCTGA ATATT TTAGC CGC 3′) or Spotart-Fw (5′ NNNNN NNNNN ATTTG GCTGA ATATT TTAGC CGC 3′) as a forward primer and pProtetAS-Spot42 as a template. Phusion DNA polymerase (New England Biolabs) was used according to the manufacturer's protocol. The PCR product was treated with Dpn I (New England Biolabs) to digest the template plasmid, column purified (DNA Clean & Concentrator-5, Zymo Research), and phosphorylated by T4 polynucleotide kinase (New England Biolabs). After column purification, the linear DNA was self-ligated using Quick Ligation Kit (New England Biolabs) and immediately transformed into competent TOP10 cells (Invitrogen). The library complexity was estimated to be 3×10^6. The reporter plasmid pKP33-RecA-GFPuv (Supplementary Fig. S1B) was constructed by cloning the 5′ UTR + 12 codons of *E. coli recA* mRNA in the pKP33-GFPuv plasmid [21].

2.3. Library screening

Screening of artificial sRNAs was performed as described previously [20,21]. Briefly, TOP10 competent cells harboring pKP33-RecA-GFPuv reporter plasmid were transformed with the artificial sRNA library and plated on LB agar plates supplemented with ampicillin and kanamycin. The plates were incubated at 37 °C overnight and visually examined over a UV transilluminator (UVP) at 365 nm for colonies with diminished fluorescence.

2.4. GFP assay as a measure for artificial sRNA activity

Fluorescent reporter assay to measure artificial small RNA activity was performed as described previously [20,22]. Briefly, TOP10 cells transformed with both artificial sRNA library plasmid and pKP33-RecA-GFPuv reporter plasmid were grown as 1 mL cultures to late-log phase (OD600 0.8–0.9) at 37 °C. Two hundred microliters of this culture was harvested by centrifugation, washed once with PBS, and transferred to a 96-well plate in PBS (200 μL). Optical density at 600 nm (OD600) and GFPuv fluorescence (excitation wavelength 395 nm; emission wavelength 509 nm) of the cells were measured using Safire^2 microplate reader (Tecan). Fluorescence data from TOP10 cells transformed with pPROTetAS were used to subtract the background cellular fluorescence. The background-corrected fluorescence data was further normalized by OD600. Finally, fluorescence of the reporter cells carrying the empty plasmid (pPROTetAS) was compared to the cells carrying the artificial small RNA plasmid in order to evaluate the artificial small RNA activity.

2.5. Western blot

MG1655 cells transformed with an appropriate sRNA expression plasmid were diluted 1000-fold from an overnight culture and grown for five hours in25 mL LB medium supplemented with ampicillin. The cells were pelleted and washed twice with 0.1 M Tris buffer (pH 7.4), and suspended in 1.5 mL 0.1 M Tris buffer (pH 7.4). After addition of phenylmethanesulfonyl fluoride (Sigma) to a final concentration of 0.1 mM, the cell suspensions were sonicated five times on ice with 15 s bursts with 1 min intervals using Branson Sonifier 250. The samples were centrifuged at 10,000*g* for 10 min to remove cell debris. The extracted total proteins (1 mg) were separated on 12% SDS–PAGE and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked using blotto (5% [w/v] nonfat dry milk in pH 7.6 Tris-buffered saline with 0.1% Tween-20 [TBST]) at room temperature. Blots were incubated with monoclonal anti-*E. coli* RecA antibody (MBL) (1:2000) diluted in blotto, followed by incubation with HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology) (1:10,000) diluted in TBST. Blot was developed using ECL plus western blotting detection reagents (GE Healthcare) and imaged with Storm 860 scanner (Molecular Dynamics).

2.6. Swarming motility assay

The semi-solid medium used for swarming assays consisted of 1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% glucose, and 0.45% agar. Glucose was added after autoclaving the medium. Swarm agar plates were allowed to dry overnight at room temperature and preheated to 30 °C before use. The surface at the center of the swarm plate was inoculated with $\sim 10^5$ cells, incubated at 30 °C overnight and photographed.

2.7. Ciprofloxacin sensitivity assay

MG1655 cells harboring the artificial sRNA plasmids were grown in LB medium supplemented with ampicillin overnight. On the following day, 2 μL cells (10^4–10^5) were spot inoculated on the surface of an LB ampicillin plate supplemented with an appropriate concentration of ciprofloxacin (TCI). The plates were incubated at 37 °C overnight and photographed.

3. Results and discussion

In this work, a plasmid library was designed to constitutively express artificial sRNAs with a randomized antisense domain (20–30 nt) fused to the Spot42 sRNA scaffold as previously described (Supplementary Fig. S1A) [20]. This library was subsequently transformed into TOP10 *E. coli* cells containing the reporter plasmid pKP33-RecA-GFPuv which transcribes the 5′ UTR and the first 12 codons derived from the *recA* mRNA fused to *gfpuv* coding region from a synthetic constitutive promoter (Supplementary Fig. S1B). The resulting 1.7×10^4 colonies on agar plates were visually screened over a UV transilluminator for diminished fluorescence. Further confirmation and sequencing yielded seven artificial sRNAs (Table 1) that exhibit varying degrees of target gene repression. As seen in Fig. 1A, 4–10-fold repression of *recA*::*gfpuv* was observed in the presence of anti-*recA* sRNAs in liquid culture. For further characterization, aRecA-12 and aRecA-46 were chosen which repressed *recA*::*gfpuv* by 86% and 78%, respectively.

We first examined if the artificial sRNAs are capable of regulating the intended endogenous gene (i.e. *recA*). Therefore, we transformed

Table 1
Antisense domain sequences of the anti-*recA* artificial sRNAs.

sRNA (aRecA-#)	Antisense domain sequence[a,b]
3	GCCCAUGUACCAACUUUGUCAUGCGGG
4	CUUUUUUACCUCCUGUUAAAUAG
9	UGCUAAUGGCAGCGCCAACCUGUUAUGCAC
10	UGGCGGCUACACUCCGUCAUGGUUA
12	GCAAGCUGCCACCACUCCUGUCGUACCUUA
16	GCCAGCAGUACCCCGUCAUG
46	CACUCCCUGUCACUUGACGC

[a] Note that all sRNAs start with 5′-AUGUCGAC derived from the vector (not shown above).
[b] Region of the sRNA predicted to either completely or partially hybridize with the SD region of *recA* mRNA is underlined.

the plasmid expressing aRecA-12 or aRecA-46 into MG1655 (*recA*$^+$) cells and analyzed the RecA levels by Western blot. As expected, the cells expressing the anti-*recA* sRNAs showed reduced RecA levels compared to the cells transformed with the empty vector (Fig. 1B). In agreement with the reporter gene assay (Fig. 1A), RecA repression by aRecA-12 appears to be stronger compared to aRecA-46.

Swarming is a coordinated multicellular movement of bacteria on solid surfaces driven by flagella which may be important for bacterial pathogenesis [23]. Gómez-Gómez et al. recently reported that *recA* knockout results in the loss of swarming motility [3]. Consistent with this observation, MG1655 cells expressing the artificial sRNAs showed markedly reduced swarming activity (Fig. 2A).

Recent revelations regarding the critical role of DNA damage (SOS) response in antibiotic resistance have triggered interest in RecA as a potential drug target to combat the emergence of antibiotic resistance [10,24]. RecA has also been identified in genome-wide screen of genes that are involved in sensitivity to the existing antibiotics [25]. In this study, we examined the sensitivity of MG1655 cells expressing the artificial sRNAs to a fluoroquinolone antibiotic ciprofloxacin. As shown in Fig. 2B, MG1655 cells transformed with an empty vector (no sRNA) grew robustly up to 5 ng/mL ciprofloxacin, as did the cells expressing artificial sRNAs targeting OmpF [20]. However, cells expressing aRecA-12 did not grow at the same concentration of the antibiotic, consistent with the expected downregulation of RecA. Notably, TOP10 cells which lack *recA* showed even higher sensitivity to the antibiotic, with no growth even at 2 ng/mL. Cells expressing aRec-46, however, did not exhibit sensitivity to ciprofloxacin which may be due to insufficient RecA repression and/or off-target effects that my partially compensate for RecA repression.

Next, we used IntaRNA [26] to predict the interaction between the artificial sRNAs and *recA* mRNA. Both aRec-12 and aRec-46 were predicted to engage with *recA* mRNA near its Shine-Dalgarno (SD) region (Supplementary Fig. S2). This interaction seems to be facilitated by the presence of the sequence 5′-CUCCU-3′ in the antisense domain of the sRNAs which is complementary to the SD sequence of *recA* mRNA, 5′-AGGAG-3′. In addition, the superior repression displayed by aRecA-12 in our fluorescent reporter assay (Fig. 1A), may be attributed to the potential of this artificial sRNA

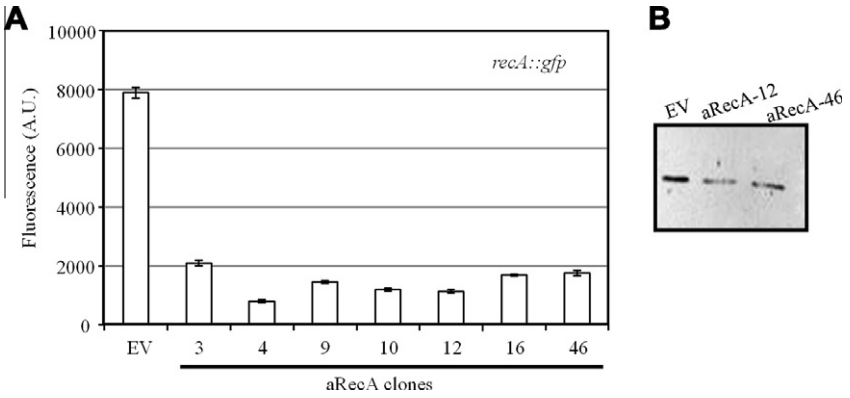

Fig. 1. Artificial sRNAs targeting *recA*. (A) Fluorescence reporter assay (*recA*::*gfpuv*) of the anti-*recA* artificial sRNAs. Cell fluorescence was measured during the late-log phase (OD_{600} 0.8–0.9) of growth as described in Section 2. The data are averages of triplicate cultures and the error bars indicate SD. EV: empty vector (pPROTetAS), and (B) endogenous RecA levels in MG1655 expressing anti-*recA* sRNAs (aRecA-12 and aRecA-46) detected by Western blot. EV: empty vector (pPROTetAS).

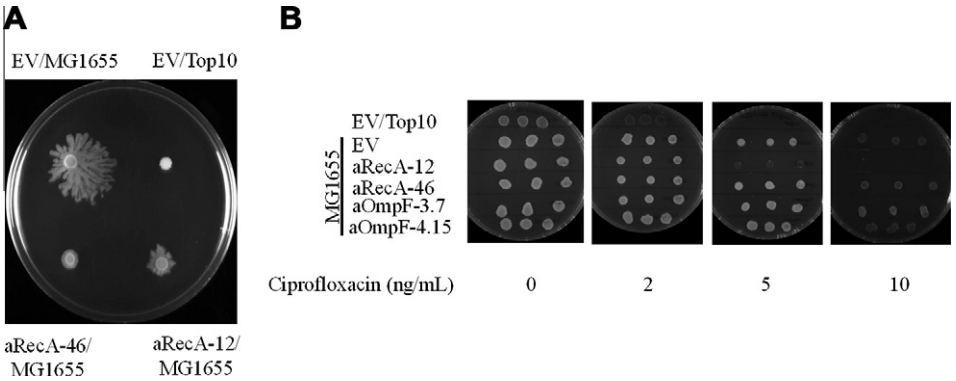

Fig. 2. Swarming motility and antibiotic sensitivity assays of the anti-*recA* sRNAs. (A) Swarming motility of *E. coli* cells expressing the artificial sRNAs. Approximately 10^5 cells were inoculated on the surface of a 0.45% soft tryptone agar plate and incubated at 30 °C overnight. EV/TOP10: TOP10 (*recA*$^-$) cells with an empty vector (pPROTetAS), and (B) ciprofloxacin sensitivity of *E. coli* cells expressing artificial sRNAs. Cells were spotted on LB agar plates containing the indicated concentrations of ciprofloxacin and incubated for 24 h at 37 °C. EV: empty vector (pPROTetAS). TOP10 cells were spotted as *recA*$^-$ control.

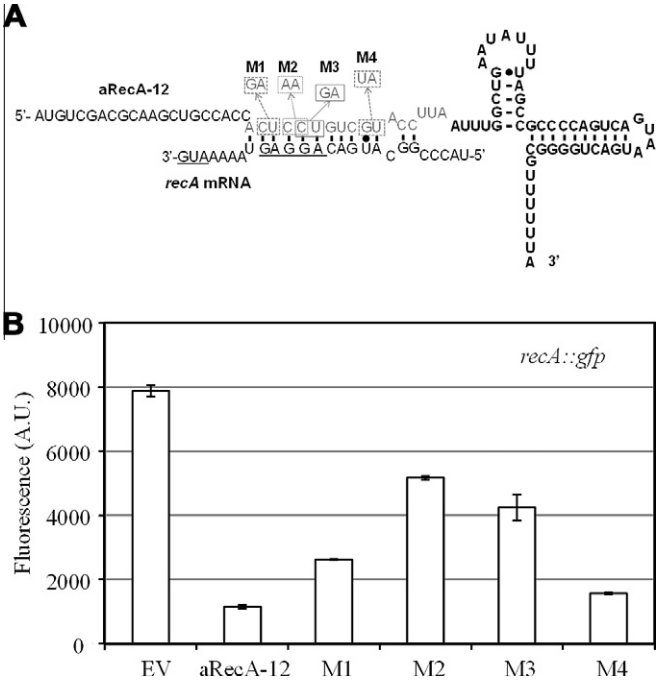

Fig. 3. Mutational analysis of aRecA-12. (A) Model of aRecA-12 interacting with the 5′ UTR of *recA* mRNA as predicted by IntaRNA [26], and (B) fluorescence reporter assay (*recA*::*gfpuv*) of the anti-*recA* artificial sRNA mutants. Cells were monitored for fluorescence in the late-log phase (OD_{600} 0.8–0.9) of growth. The data are averages of triplicate cultures and the error bars indicate SD. EV: empty vector (pPROTetAS).

to form eleven consecutive Watson–Crick base pairs near the SD region in *recA* mRNA (Fig. 3A). To support the model depicted in Fig. 3A, we designed several two-base mutants of aRec-12 and evaluated their activities by reporter gene assay. Introduction of a mismatch at the center of the 11-bp duplex (M2 and M3) resulted in the most severe loss of artificial sRNA activity, with 35% (M2) and 46% (M3) repression compared to 86% repression by aRecA-12 (Fig. 3B). A mutation closer to the 3′ end of the duplex (M1) resulted in a less pronounced but significant reduction in activity (67%) but the mutation at the 5′ end of the duplex (M4) had no noticeable effect (80%). The difference might be attributed to the fact that M1 affects the part of the duplex that is near the SD region which contributes more directly to the observed gene regulation.

Despite its numerous important functions in bacteria, researchers have almost entirely relied on the conventional genetic knockout techniques to investigate RecA. The ability of the artificial sRNAs to dynamically regulate RecA to desired levels may allow us to study how temporal modulation of RecA (i.e. by a drug) affects bacterial phenotypes. Moreover, the diversity of the *recA* phenotypes (swarming motility, antibiotic sensitivity, etc.) provides opportunities to use the anti-*recA* sRNAs as an output in synthetic gene circuits to control the cellular behavior [27].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.141.

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