A Novel Genomic Approach Identifies Bacterial DNA-Dependent RNA Polymerase as the Target of an Antibacterial Oligodeoxynucleotide, RBL1

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Received November 19, 2004; Revised Manuscript Received February 9, 2005

ABSTRACT: Rapid emergence of antibiotic-resistant bacterial pathogens limits the applicability of existing drugs, which has created an urgent need for novel antibiotics preferably with entirely new mechanisms of action. Oligodeoxynucleotides (ODNs) and their modified forms have been shown to inhibit bacterial gene expression, representing a potential for developing highly specific and efficacious antibacterial agents. In this study, a tetracycline (Tet)-inducible, randomized single-stranded DNA (ssDNA) expression library was constructed and screened for conditional growth-defective or lethal phenotypes in an *Escherichia coli* system. From ∼5000 transformants screened, 12 bacterial colonies were identified with either growth-defective or lethal phenotypes. One clone, CY01, with a lethal phenotype was selected and sequenced, and the ODN sequence that it generates was designated as RBL-1. Because RBL-1 shows no significant homologies to any bacterial gene sequence, a potential RBL-1 targeting protein was isolated by affinity purification. Using mass spectrometry analysis, this protein was identified as bacterial DNA-dependent RNA polymerase (RNAP). RBL-1 was further shown to effectively inhibit RNA polymerase activity *in vitro*. The usage of this randomized ssDNA expression library screening technology to selectively modulate production and/or function of proteins may provide a powerful strategy in both identifying novel gene targets for antibiotic discovery and developing novel antibacterial agents.

In recent years, the emergence of bacterial pathogens with resistance to antibiotics has become a major public health concern (*1*, *2*). This increasingly serious problem, particularly with multidrug-resistant pathogens, has spurred a resurgent interest in the search for new antibacterial agents. The primary approaches in the past in addressing this problem have been to seek incremental improvements in existing drugs. However, their benefits generally are limited. A better approach involves the discovery of novel antibiotics whose action(s) is not affected by the prevailing mechanisms of resistance. This approach will ultimately lead to new chemical classes and mechanisms of action.

The rapid advances in DNA sequencing have made it possible to obtain complete genome sequences at an unprecedented speed. The first complete bacterial genome sequence was published by The Institute for Genome Research (TIGR) for *Haemophilus influenzae* Rd in 1995 (*3*). Since then, over 50 microbial genomes have been sequenced and more than 100 additional genomes are in progress (*4*). Sequencing of bacterial pathogen genomes provides a rational, target-based approach to develop novel antibiotics.

Oligodeoxynucleotides (ODNs)[1] such as aptamer ODNs, antisense ODNs, and triplex-forming ODNs, have been used effectively to regulate gene expression in both eukaryotic and prokaryotic cells (*5*). Despite the presence of a natural antisense mechanism in prokaryotic cells (*6*), application of ODNs for regulating bacterial gene expression has been limited. Modified ODNs such as phosphorothioate-modified ODNs or DNA mimics such as peptide nucleic acids (PNAs) and phosphorodiamidate morpholino oligomers (PMOs) have been used to control gene expression in prokaryotic cells (*7*−*12*). These ODNs or DNA mimics suppress gene expression either by blocking the initiation of translation process or activating endogenous *RNase*H, which in turn cleaves the targeted template mRNA (*13*).

Ji et al. (*14*) and Forsyth et al. (*15*) have recently developed antisense RNA technologies to identify essential genes of *Staphylococcus aureus*. Instead of generating antisense RNA molecules *in vivo*, we have developed a series of single-stranded DNA (ssDNA) expression vectors that can generate ssDNA or ODN molecules intracellularly for regulating gene expression in both eukaryotic and prokaryotic cells (*16*−*21*). In our previous study, we demonstrated that a "10−23" DNA enzyme generated in *Escherichia coli* cells using the ssDNA expression vector can inhibit the expression of an essential bacterial gene, *fts*Z (*21*). The "10−23" DNA enzyme is an ODN that has the potential to cleave any target mRNA containing a purine−pyrimidine junction (*22*, *23*).

We report here the construction of an ssDNA expression library that can generate randomized ODNs in *E. coli* cells under the control of a tetracycline (Tet)-regulated promoter. When we screened for a conditional bacterial growth phenotype, an ODN, designated as RBL-1, was identified to contain antibacterial activity. Using a proteomic approach, the bacterial DNA-dependent RNA polymerase (RNAP) was further identified as the target protein of RBL-1.

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[1] Abbreviations: ssDNA, single-stranded DNA; ODN, oligodeoxyribonucleotides; Tet, tetracycline; aTc, anhydrotetracycline; RNAP, DNA-dependent RNA polymerase; Cm, chloramphenicol; Spec, spectinomycin.

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Published on Web 04/09/2005

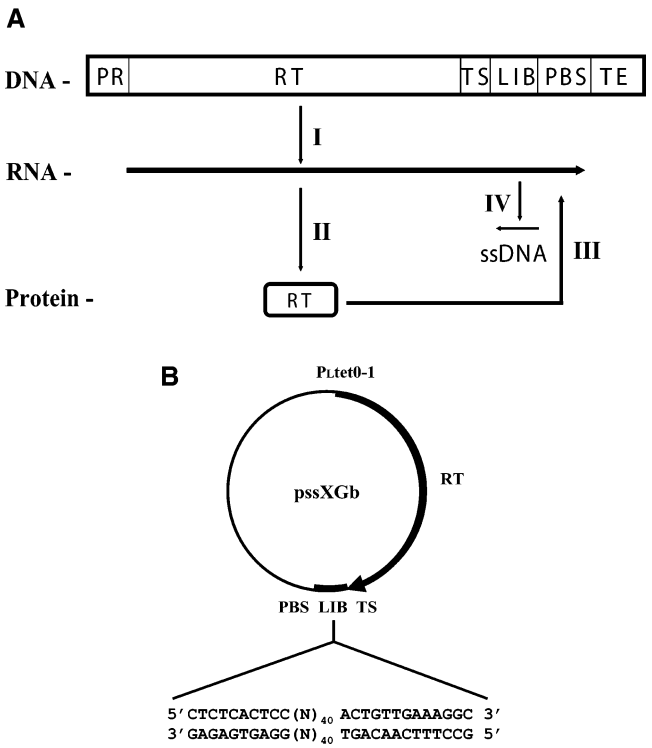

FIGURE 1: Randomized ssDNA expression library. (A) Schematic representation of the ssDNA expression library. PR, inducible promoter, P_{LtetO}-1; RT, moloney murine leukemia virus reverse transcriptase gene; TS, reverse transcription termination signal; LIB, random ODN library; PBS, primer binding site; and TE, terminator sequence. I, transcription; II, translation; and III and IV, reverse transcription. (B) Tet-inducible randomized ssDNA expression library vector. $(N)_{40}$ represents randomized 40-mer ODNs.

MATERIALS AND METHODS

Bacterial Strain and Plasmid. *E. coli* strain, DH5αpro (*deoR*, *endA1*, *yrA96*, *hsdR17*($r_k^- m_k^+$), *recA1*, *relA1*, *supE44*, *thi-1*, Δ(*lacZYA-argF*)*U169*, *ϕ80δlacZM15*, F^-, λ^-, P_{N25}/*tetR*, P_{laci}^q/*laci*, and Sp^r), a derivative of DH5α capable of producing Tet repressor protein, was purchased from Clontech (Palo Alto, CA). To maintain the selection of the Tet-inducible ssDNA expression vector, DH5αpro cells were grown in LB medium containing 34 μg/mL chloramphenicol (Cm) and 50 μg/mL spectinomycin (Spec). To generate the plasmid pssXGb, a double-stranded ODN insert was formed by annealing two ODNs: 5′-TAAGCTAGCTCTAGATGGTGCGTCCGAGTGGACCGGGAGACCCCTGCTCGAGC-3′ and 5′-CTAGGCTCGAGCAGGGGTCTCCCGGTCCACTCGGACGCACCATCTAGAGCTAGCTTAAT-3′ (Integrated DNA Technologies, Coraville, IA). This insert was subcloned into the *Pac*I and *Xba*I sites of pssXGa (*21*). The newly created plasmid pssXGb was used for constructing the randomized ssDNA expression library.

Construction of a Tet-Inducible Randomized ssDNA Expression Library. The structure of the ssDNA expression vector and randomized ssDNA library are shown in Figure 1. To generate the randomized ssDNA library insert, two ODNs, CY(*Sac*II)-40, 5′-CTCTCACTCC-$(N)_{40}$-ACTGTTGAAAGGC-3′ (N_{40} represents 40 random nucleotides) and CY(*Sac*II)-R, 5′-CTTTCAACAGT-3′ (Integrated DNA Technologies, Coraville, IA) were annealed by mixing them at the molar ratio of 1:20 (CY(*Sac*II)-40/CY(*Sac*II)-R). The partial double-stranded DNA thus formed was filled using the Klenow fragment of *E. coli* DNA polymerase (New England Biolabs, Beverly, MA). The reaction was carried out in a buffer containing 0.16 μg/μL partial double-stranded DNA, 0.16 unit/μL Klenow, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 7.5 mM DTT, and 33 μM dNTP. After incubation at 25 °C for 15 min, the mixture containing the double-stranded DNA was ethanol-precipitated, resuspended, and ligated into the prepared pssXGb vector, which was predigested with *Pac*I and *Xba*I and blunt-ended using the Klenow fragment. The ligation products were subsequently transformed into DH5αpro cells by electroporation using the MicroPulser apparatus (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer.

Screening of Antibacterial ODNs. The transformants were recovered on LB plates containing 34 μg/mL Cm and 50 μg/mL Spec after overnight incubation at 37 °C. The LB plates were then replica-plated onto inducing [200 ng/mL anhydrotetracycline (aTc), a modified form of Tet] and noninducing (without aTc) LB plates and incubated overnight at 37 °C. Colonies that grew normally on noninducing LB plates but did not grow or showed growth defect on the inducing plates were selected and confirmed by resuspending the colonies in LB medium and retesting cell growth on both inducing and noninducing plates. DNA sequencing of the expression vector isolated from a selected clone CY01 showed a coding sequence for an ODN designated as RBL-1. CY01c is a control containing an expression vector coding for a complementary sequence of RBL-1, designated RBL-1c.

Measurement of Cell Growth. An overnight culture (200 μL) of CY01 cells was inoculated into 10 mL of LB media with 34 μg/mL Cm and 50 μg/mL Spec. Various concentrations of aTc (0, 10, 50, 100, or 200 ng/mL of aTc) were added. A total of 1 mL of culture was removed after incubation for 1 h at 37 °C for a cell growth assay by measuring viable cells. Viable cell counts were determined by serially diluting the cells and plating them in triplicate on LB plates with the appropriate antibiotics. The plates were incubated overnight at 37 °C, and the number of colonies was enumerated by visual inspection.

Bacterial Infection of HeLa Cells. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 μg/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 34 μg/mL Cm and incubated at 37 °C in a humidified 5% CO_2 incubator. HeLa cells were seeded in 6-well plates at a density of 1×10^5 cells per well the day before infection. A total of 10^5 CFU/mL of freshly grown CY01 cells and various concentrations of aTc (0, 50, 100, or 200 ng/mL) were added to the culture medium. The plates were incubated overnight at 37 °C, and bacterial growth was visualized using light microscopy.

Purification of RBL-1 Binding Protein. 5′-biotinylated RBL-1 was synthesized by Integrated DNA Technologies (Coraville, IA) and used to purify RBL-1 binding protein(s) according to Codosh (*24*) with some modifications. Briefly, 130 μL of a packed volume of streptavidin agarose (Invitrogen, Carlsbad, CA) was collected by centrifugation for 2 min. The collected agarose was incubated with 500 μL of blocking buffer (10 mM Tris-HCl at pH 7.5, 100 mM NaCl,

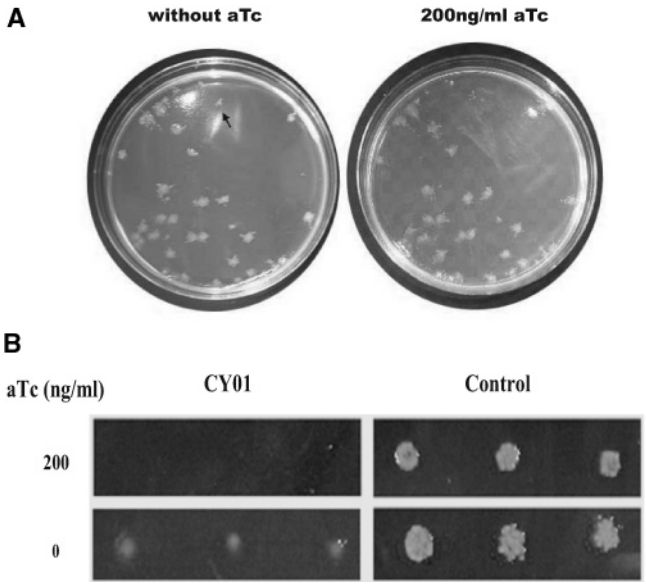

FIGURE 2: Screening of a Tet-inducible randomized ssDNA expression library. (A) Transformants were recovered on LB plates containing 34 μg/mL Cm and 50 μg/mL Spec after overnight incubation at 37 °C. The LB plates were then replica-plated onto the inducing (200 ng/mL aTc) and noninducing (without aTc) LB plates. Clone CY01, marked by an arrow, grew normally on noninducing LB plates but did not grow on the inducing plates, which were selected for further characterization. (B) Confirmation of lethal phenotype of clone CY01. The phenotype of clone CY01 cells was confirmed by resuspending cells in LB medium and retesting growth on both inducing and noninducing plates. Control cell contains pssXGb vector only.

4% glycerol, and 200 μg/mL salmon sperm DNA) on ice for 30 min and then washed 3 times with 500 μL of binding buffer (10 mM Tris-HCl at pH 7.5, 100 mM NaCl, and 4% glycerol). To prepare cell lysates, 5 mL DH5αpro cells of log-phase culture ($\sim OD_{600} = 1.0$) was collected by centrifugation. Cell pellets were resuspended in 200 μL of binding buffer and lysed by ultrasonification using Virsonic 100 (VirTis, Gardiner, NY) on ice for 40 s. Cell lysates were collected by centrifugation and then incubated with 500 ng of biotinylated RBL-1 or biotinylated RBL-1c (Integrated DNA Technologies, Coraville, IA) on ice for 30 min. After precipitated protein was removed by centrifugation, the pretreated streptavidin-agarose beads were added to the reaction. The protein-bound streptavidin-agarose beads were then collected by centrifugation and washed twice with 500 μL of binding buffer. The protein was eluted from the agarose beads by incubation with 50 μL of elution buffer (50 mM Tris at pH 7.5, 100 mM NaCl, 2 M KCl, and 4% glycerol) on ice for 30 min. The supernatant was collected by centrifugation and subjected to SDS−PAGE analysis. The protein was visualized by silver staining using the SilverQuest kit (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer.

Nano-HPLC/Electrospray Mass Spectrometry Analysis. Nano-HPLC/electrospray mass spectrometry analysis was performed by Yinming Zhao of Mass Spectrometry Facility Lab at University of Texas Southwestern Medical Center (*25*, *26*). Briefly, after separation by SDS−PAGE, the RBL-1 binding protein band (~160 kDa) was visualized by silver staining. The protein band was excised and digested *in situ* with porcine trypsin overnight at 37 °C. The peptide mixture was then dissolved and injected into reversed phase HPLC/ion trap mass spectrometry instruments with a nanospray source. The resulting profiles were then searched against the NCBI-nr protein sequence database for protein identification.

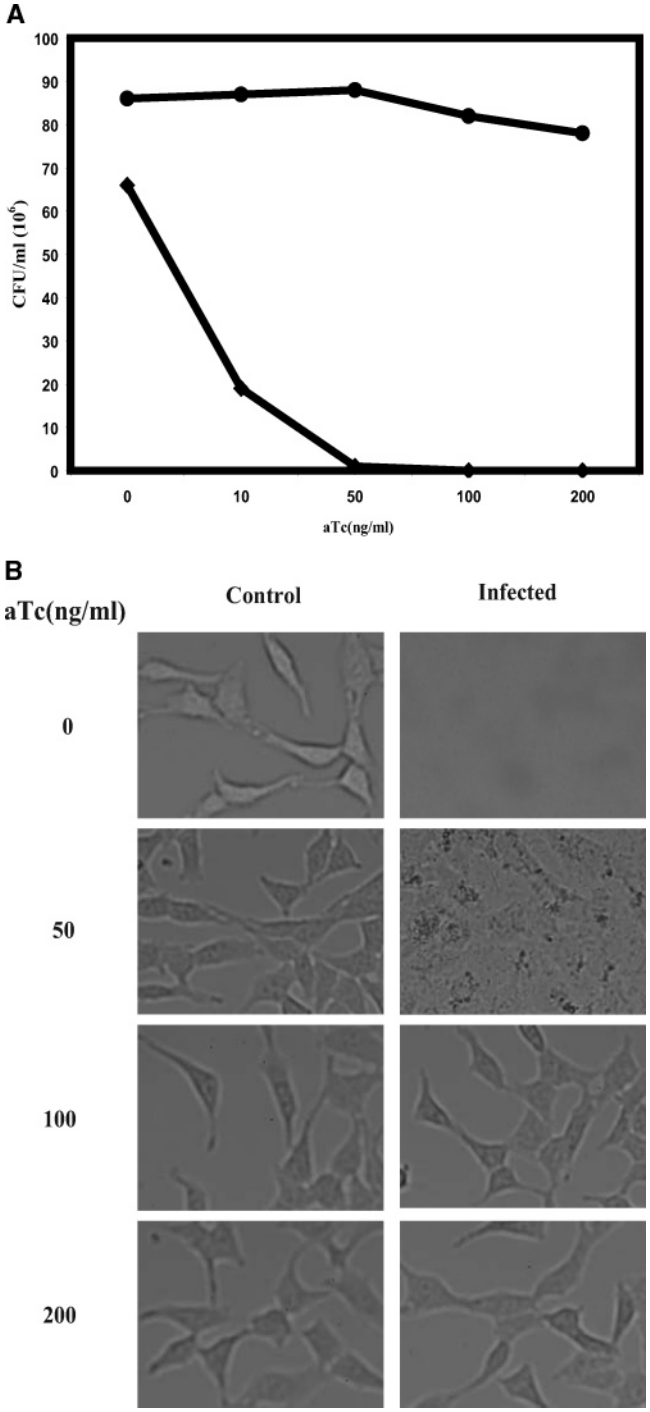

FIGURE 3: Inhibition of bacterial growth by *in vivo* generated RBL-1. (A) Inhibition of bacterial cell growth. CY01 cells were grown in the presence of 0, 10, 50, 100, or 200 ng/mL of aTc for 1 h. Bacterial cell growth was shown in squares (CY01 cells) or circles (control CY01c cells). (B) Inhibition of bacterial infection of HeLa cell cultures by *in vivo* generated RBL-1. (Left column) HeLa cell cultures without *E. coli* infection. (Right column) HeLa cell cultures infected with 10^5 CFU/mL of CY01 cells. CFU: colony-forming unit. HeLa cell cultures were incubated overnight in the presence of 0, 50, 100, or 200 ng/mL of aTc.

In Vitro RNA Polymerase Activity Assay. The ODN inhibitory activity against RNA polymerase was determined using an *in vitro* transcription assay. Briefly, the activity assay was

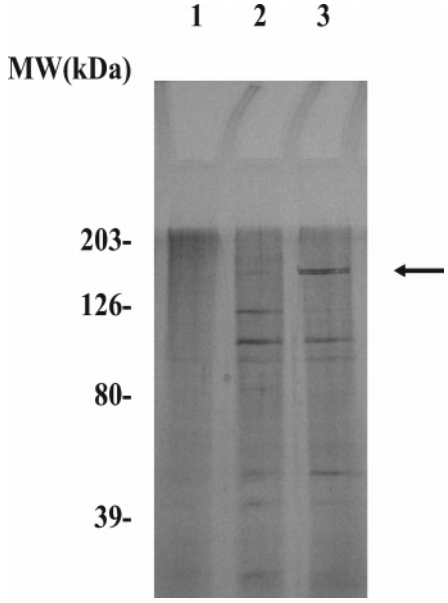

FIGURE 4: Identification of RBL-1 binding protein. RBL-1 binding protein was purified using the avidin-biotin method. DH5αpro cell lysates were incubated with 500 ng of biotinylated RBL-1 (lane 3), RBL-1c (lane 2), or none (lane 1), and then streptavidin-agarose beads were added to the reaction. Proteins that bound to the beads were then eluted (50 mM Tris at pH 7.5, 100 mM NaCl, 2 M KCl, and 4% glycerol) and subjected to SDS−PAGE analysis. The protein was visualized by silver staining using a SilverQuest kit (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. The protein with a molecular weight of ∼160 kDa (lane 3), marked by an arrow, was excised for sequence identification as described in the Material and Methods.

Table 1: Peptide Sequences of RBL-1 Binding Protein, RNAP

sequence	region
KITQGDDLAPGVLK	1046−1059
DLSEELQILEAGLFSR	970−985
AVAVDSGVTAVAK	718−730
VAFMPWNGYNFEDSILVSER	813−832
ITQGDDLAPGVLK	1047−1059
VDLSTFSDEEVMR	1170−1182
LGEPVFDVQECQIR	86−99
ALEIEEMQLK	956−965
SPGVFFDSDK	163−172
ALNYTTEQILDLFFEK	223−238
RIETLFTNDLDHGPYISETLR	343−363
EAAESLFENLFFSEDR	390−405
EFFGSSQLSQFMDQNNPLSEITHK	515−538
WLELGLTDEEK	1008−1018
IETLFTNDLDHGPYISETLR	344−363
EAAESLFENLFFSEDRYDLSAVGR	390−413
DQVDYMDVSTQQVVSVGASLIPFLEHDDANR	659−689
RGGVVQYVDASR	731−742
GMPIATPVFDGAK	1190−1202
SVFPIQSYSGNSELQYVSYR	66−85
EEIEGSGILSKDDIIDVMK	423−441
MNIGQILETHLGMAAK	1118−1133
VPNGVSGTVIDVQVFTR	931−947
LDESGIVYIGAEVTGGDILVGK	876−897
SKGESSLFSR	549−658
INPIEDMPYDENGTPVDIVLNPLGVPSR	1090−1117
LNHLVDDK	1246−1253
VDHPTHYGR	560−568
VNEDEMYPGEAGIDIYNLTK	747−766
GETQLTPEEK	902−911
MMRPGEPPTR	380−389
LPATIILR	215−222

performed in a 20 μL reaction containing 1 unit of *E. coli* holo RNA polymerase (RNAP, Epicentre, Madison, WI), 1 μg of linearized DNA template, pTRI18(ICP47)-1, 250 μM NTP, 40 mM Tris-HCl (pH 7.5), 250 mM KCl, 10 mM $MgCl_2$, 0.01% Triton X-100, and 10 mM DTT. Various concentrations of RBL-1 or truncated RBL-1 ODNs were added to the reaction. After incubation at 37 °C for 40 min, the RNA products were analyzed on a 1% agarose gel and the inhibitory activity was scored by visualization of band intensity.

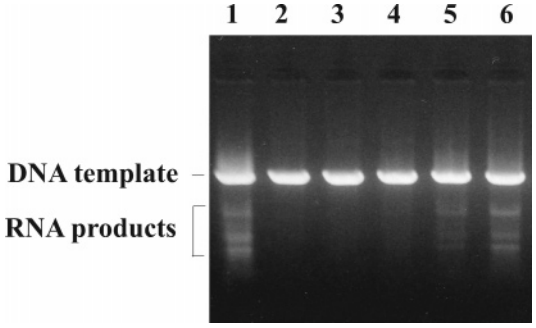

FIGURE 5: Inhibition of RNAP activities by RBL-1. *In vitro* RNA polymerase activity assays were performed as described in the Methods and Materials. RNA products were separated on a 1% agarose gel. Lane 1; control, without RBL-1; lane 2, 0.5 μM RBL-1; lane 3, 0.25 μM RBL-1; lane 4, 0.125 μM RBL-1; lane 5; 0.05 μM RBL-1; and lane 6, 0.5 μM control RBL-1c.

RESULTS

Construction and Screening of a Tet-Inducible Randomized ssDNA Expression Library. In our previous study, we demonstrated that the Tet-inducible ssDNA expression vector, pssXGb, is able to generate sufficient copies of DNA enzyme molecules to suppress *fts*Z gene expression, which leads to the inhibition of bacterial cell proliferation (*21*). Under the control of a Tet-regulated promoter $P_{LtetO-1}$ (*27*), this vector includes coding sequences for (1) the moloney murine leukemia virus reverse transcriptase gene (RT), (2) a reverse transcription termination signal (TS), (3) the random ODN library (LIB), and (4) a primer binding site (PBS). Once the vector is delivered into the cell, RT is synthesized in the presence of aTc and the newly synthesized RT then uses the endogenous $tRNA^{val}$ as a primer, which binds to the PBS on the 3′ end of the RNA transcript, for ssDNA synthesis. The reverse transcription reaction will be terminated by the TS (Figure 1A).

In this study, we constructed an ssDNA expression library by inserting randomized double-stranded ODNs (40-mers) into the pssXGb vector (Figure 1B). The randomized ssDNA expression library was then transformed into DH5αpro cells and screened by replica-plating onto the aTc inducing and noninducing LB plates. Colonies that grew normally on the noninducing plates but did not grow or had a growth-defective phenotype on inducing plates were selected. From ∼5000 transformants screened, a total of 12 colonies were selected. One clone, CY01, shown in Figure 2A, had a lethal phenotype, while the remaining 11 colonies were growth-defective (not shown). The lethal phenotype of the clone CY01 was further confirmed by resuspending cells in LB medium and retesting growth on both inducing and noninducing plates (Figure 2B). DNA sequencing of the plasmid isolated from CY01 cells revealed the coding sequence for an ODN, TTTGATGACCTTTGCTGACCATACAATTGCGATATCGTGG, designated as RBL-1.

Table 2: Identification of the Minimal Functional Region of RBL-1[a]

ODN	activity
TTTGATGACCTTTGCTGACCATACAATTGCGATATCGTGG	
RBL-1	++++
RBL1(01-35)	++++
RBL1(01-30)	++++
RBL1(01-25)	-
RBL1(01-20)	-
RBL1(01-15)	-
RBL1(06-40)	++++
RBL1(11-40)	++++
RBL1(16-40)	++++
RBL1(21-40)	++++
RBL1(26-40)	-
RBL1(21-30)	-
RBL1(21-32)	+
RBL1(21-34)	++
RBL1(21-36)	++++
RBL1(21-38)	++++
RBL-1c	-

[a] ODN inhibitory activities against RNA polymerase were determined by visualization of band intensity in gel. Score: ++++, 100%; +++, ~75%; ++, ~50%, +, ~25%; −, ~0%.

Inhibition of Bacterial Cell Growth by In Vivo Generated RBL-1. To further characterize the antibacterial effect of RBL-1, we then investigated the aTc dose response for cell-growth inhibition by RBL-1 generated *in vivo*. CY01 cells were grown in the presence of various concentrations of aTc (0, 10, 50, 100, or 200 ng/mL) for 1 h, and viable cells were enumerated as described in the Materials and Methods. CY01c cells, containing a plasmid for generating a RBL-1 complementary sequence, RBL-1c, were used as the control. Figure 3A shows that cell-growth inhibition by RBL-1 is aTc concentration-dependent. However, there is no significant inhibition of cell growth when RBL-1c is generated in control CY01c cells, indicating that the inhibition is RBL-1 sequence-specific and not caused by aTc.

To examine the antibacterial potential of RBL-1 in the presence of eukaryotic cells, we tested CY01 cell growth in HeLa cell culture medium. HeLa cell cultures were infected with 10^5 CFU/mL of CY01 cells in the presence of various concentrations of aTc (0, 50, 100, or 200 ng/mL). As shown in Figure 3B, RBL-1 were generated to fully cure the Hela cell culture of the infection when 100 ng/mL of aTc or higher was used.

Identification of RBL-1 Targeting Protein(s). To identify potential gene target(s) of RBL-1 ODNs, we searched the NCBI Genbank database. However, we failed to identify matches that were complementary to RBL-1, although three *E. coli* genes, btuE, CaiB, and dgt, contained sequences that were partially complementary to different portions of RBL-1. We then constructed expression vectors to generate these partial RBL-1 sequences in bacterial cells, but none of them showed any inhibitory activities (data not shown). These results are consistent with some earlier reports that all three genes are not essential (*28*−*30*) and suggest that, instead of mRNA, RBL-1 may directly target critical proteins or other structural molecules.

To identify potential RBL-1 binding protein(s), an affinity purification procedure was developed. DH5α cell lysates were incubated with biotinylated RBL-1 ODNs and then immobilized onto streptavidin-agarose beads. The beads complexes were washed, and bound proteins were eluted and fractionated by SDS−PAGE. Cell lysates incubated with RBL-1c or agarose beads alone were used as controls. As shown in Figure 4, the affinity profile of RBL-1 binding proteins was different from both controls and a least one protein with a molecular weight of ~160 kDa (marked by an arrow) binds specifically to RBL-1. This protein band was excised and analyzed by nano-HPLC/electrospray mass spectrometry. Table 1 shows that 34 of the identified peptides localize to 29 regions of bacterial RNAP in the Genbank database, suggesting the RNAP as a potential RBL-1 targeting protein.

Inhibition of RNA Polymerase Activity by RBL-1. We then assessed the effect of RBL-1 on RNA polymerase activity *in vitro* on the presumption that RBL-1 binds to RNA polymerase (Figure 4 and Table 1). The inhibitory effect of RBL-1 on RNA polymerase was determined using an *in vitro* transcription assay as described in the Materials and Methods. Figure 5 shows that, at the concentrations of 0.5, 0.25, or 0.125 μM, RBL-1 can effectively inhibit RNA polymerase activity.

Identification of the Minimal Functional Region of RBL-1. To identify the minimal functional region of RBL-1 for inhibition of RNA polymerase activity, a series of truncated RBL-1 ODNs were synthesized (Table 2) and their inhibitory activities were assessed using the *in vitro* RNA polymerase activity assays. We first synthesized truncated RBL-1 with

3′ deletions of various lengths. At the concentration of 5 μM, only RBL1(1−35) and RBL1(1−30) showed inhibitory activities, but not RBL1(1−25), RBL1(1−20), and RBL1(1−15) (Table 2). We then investigated the effects of the 5′-end sequence on the activity of RBL-1. Except RBL1(26−40), all 5′-truncated RBL-1, RBL1(6−40), RBL1(11−40), RBL1(16−40), and RBL1(21−40), were shown to be active (Table 2). These results indicate that the sequence between 21 and 30 might be essential for RBL-1 activity. However, the lack of activity of RBL1(21−30) suggests that the region between 21 and 30 is necessary but not sufficient. We then added additional nucleotides to the 3′-end of RBL1(21−30). RBL1(21−36) and RBL1(21−38) but not RBL-1(21−32) or RBL-1(21−34) were shown to have similar inhibitory activities as RBL-1. Control ODN, RBL-1c, did not show any activity.

DISCUSSION

In our previous studies, we and our collaborators have demonstrated that ssDNA expression vector that we constructed can effectively generate (1) "10−23" DNA enzyme for inhibiting gene expression in both eukaryotic and prokaryotic cells (*16*, *18*, *19*, *21*), (2) triplex-forming ODN (TFO) for inducing gene recombination (*17*), and (3) G-quartet containing ODN for inhibiting cell proliferation (*20*). We report here the construction of a Tet-inducible bacterial ssDNA expression library. A total of 12 colonies with either lethal or growth-defective phenotypes from ∼5000 transformants screened. An isolated clone, CY01, with lethal phenotype, generates an antibacterial ODN, designated as RBL-1. In addition, this ODN could cure the bacterial infection of HeLa cell cultures. Using a proteomic approach, we then identified the bacterial RNAP as the RBL-1 binding protein. *In vitro* RNA polymerase activity assay further confirmed RBL-1 as an effective inhibitor of RNAP. These results suggest that antibacterial activity of RBL-1 is mediated through its binding to RNAP. In addition, our study indicated that the region between 21 and 30 of RBL-1 is required for the inhibitory activity but not sufficient. Additional nucleotides at either the 5′ or 3′ end are needed.

RNAP is an essential enzyme for bacterial gene expression, responsible for all cellular RNA biosynthesis (*31*). In bacteria, the catalytically competent core RNAP contains five subunits: αI, αII, β, β', and ω with a total molecular weight of 400 kDa. Additional subunit, σ, which binds the core RNAP to form the holoenzyme, is required for promoter-specific recognition and efficient initiation of RNA transcription (*32*). Owing to its central role in bacterial RNA biosynthesis, RNAP has become an attractive target for antibiotics. However, even with intensive research in the last 50 years, few small-molecule inhibitors that are specific for bacterial RNAP have been described and only one inhibitor, rifampicin (Rif), and its derivatives have reached clinical use (*33*). The inhibitor Rif, which is one of the most potent and broad-spectrum antibiotics against bacterial pathogens, directly blocks the RNA elongation process (*34*). Other RNAP inhibitors, such as MccJ25, streptolydigin, and CBR703, might act by interfering with the nucleotide addition or translocation cycle (*35*). The mechanism of RBL-1 interaction with RNAP is still unknown and requires further investigation.

In conclusion, this study demonstrated the utility of the ssDNA expression library screening technology for the isolation of both RNAP as a target and a novel RNAP inhibitory ODN. However, in comparison to other screening methods (*14*, *15*), the efficiency of this library screening technology still needs to be improved.

ACKNOWLEDGMENT

We thank Harilyn McMicken for technical support. We also thank Gerald Joyce for helpful discussion and Malcolm Skolnick, John Rossi, Sam Kaplan, Cy Stein, Peter Glazer, and Cindy Ewell for critical reading of the manuscript.

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BI0475626