Published in final edited form as:

J Microbiol Methods. 2012 September ; 90(3): 256–261. doi:10.1016/j.mimet.2012.05.018.

Development and characterization of a *Pseudomonas aeruginosa in vitro* coupled transcription-translation assay system for evaluation of translation inhibitors

Corey Fyfe, Joyce A. Sutcliffe, and Trudy H. Grossman*

Tetraphase Pharmaceuticals, Inc., Watertown, MA

Abstract

Bacterial transcription and translation have proven to be effective targets for broad-spectrum antimicrobial therapies owing to the critical role they play in bacterial propagation and the overall conservation of the associated machinery involved. *Escherichia coli* is the most common source of S30 extract used in bacterial *in vitro* coupled transcription-translation assays, however, transcription-translation assays in other important pathogens including *Staphylococcus aureus* and *Streptococcus pneumoniae* have been described (Murray *et al.*, 2001; Dandliker *et al.*, 2003). *Pseudomonas aeruginosa* is an important and difficult-to-treat Gram-negative pathogen. In a drug discovery program, to de-risk any potential species specificity of novel inhibitors, we developed and optimized a robust method for the preparation of S30 extract from *P. aeruginosa* strain PAO1. Further, a *P. aeruginosa* transcription-translation assay using a firefly luciferase reporter plasmid was validated and compared to an *E. coli* S30-based system using a wide range of antibiotics encompassing multiple classes of translation inhibitors. Results showed a similar ranking of the activities of known inhibitors, illustrative of the high degree of conservation between the transcription-translation pathways in both organisms.

Keywords

coupled transcription-translation assay; translation antibiotics; S30 extract; *Pseudomonas aeruginosa*

1. Introduction

Protein biosynthesis is a fundamental process of living cells, and the target of several clinically important classes of antibiotics (Sohmen *et al.*, 2009; Wilson *et al.*, 2009). Owing to the high degree of conservation in the transcription and translation machinery of bacteria, antibiotics which target this pathway have the potential for broad-spectrum activity against a wide variety of Gram-positive and Gram-negative species. This is reflected in the current antibiotic pipeline containing a substantial number of new bacterial translation inhibitors targeting many different clinical indications (Butler and Cooper, 2011; Sutcliffe, 2011). Despite the attractiveness of targeting this highly conserved pathway, the antibacterial activities of translation inhibitors can be significantly affected by target-modifying enzymes, target-based resistance mutations, species-specific variations in antibiotic binding sites, as well as intrinsic bacterial resistance mechanisms that limit intracellular accumulation (Li and Nikaido, 2004). Thus, for early stage drug discovery it is critical to have informative *in vitro* and cell-based assays to guide compound development.

*Corresponding author. Mailing address: Tetraphase Pharmaceuticals, Inc. 480 Arsenal Street, Suite 110, Watertown, MA 02472. Phone: (617) 715-3559. Fax: (617) 926-3557. tgrossman@tphase.com.

Successful treatment of infections caused by the Gram-negative pathogen *P. aeruginosa* is often impeded by the prevalence of multidrug-resistant phenotypes seen in clinical isolates and has stimulated interest in developing novel therapies to combat antibiotic resistance in *P. aeruginosa* and other drug-resistant pathogens (Infectious Diseases Society of America, 2010). Bacterial translation is an anti-pseudomonal target validated by aminoglycoside antibiotics used clinically such as gentamicin, amikacin and tobramycin. Other classes of translation inhibitors are ineffective against *P. aeruginosa* due to the high degree of intrinsic efflux and reduced permeability in this organism (Li and Nikaido, 2004; Nikaido, 2003). While numerous methods for the preparation of *E. coli* S30 extracts for studying coupled *in vitro* transcription-translation have been described in the literature (Ma *et al.*, 2010; Kim *et al.*, 2008; Kim *et al.*, 2006; Liu *et al.*, 2005), and commercially-prepared extracts are readily available, there are no reports of similar assay systems for *P. aeruginosa*. Reports of S30 extract preparations from species including *Staphylococcus aureus* (Murray *et al.*, 2001), *Streptococcus pneumoniae* (Dandliker *et al.*, 2003) and *Bacillus subtilis* (Eustice *et al.*, 1988) demonstrate that *in vitro* transcription-translation assays can be developed for other bacterial species. Further, a study using S30 extracts prepared from *S. aureus* versus *E. coli* suggests that species-specific differences in the sensitivity to certain antibiotics could occur (Murray *et al.*, 2001).

Using a novel, fully synthetic chemistry platform, Tetrphase Pharmaceuticals is optimizing tetracycline-class compounds for antibacterial activity against *P. aeruginosa*. The vast difference in antimicrobial susceptibility profiles between *E. coli* and *P. aeruginosa* provided the impetus to develop a *P. aeruginosa* coupled transcription-translation assay. This assay allows for the assessment of *P. aeruginosa*-specific target-based potency of novel translation inhibitors, in the absence of barriers to intracellular accumulation. Further, this assay enables the side-by-side comparison of the activities of inhibitors tested in *P. aeruginosa* and any other organism's S30 extract, allowing the detection of species-specific differences in inhibition.

2. Materials and Methods

2.1 Reagents

Cation-adjusted Mueller Hinton Broth (MHB) powder, YT broth powder, and trypticase soy agar plates were purchased from BD (BBL/BD, Franklin Lakes, NJ). T7 RNA polymerase, restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA). The FailSafe PCR System (Epicentre, Madison, WI) was used for DNA amplification for cloning. RNase Out was purchased from Invitrogen (Carlsbad, CA). Phosphoenol pyruvate was purchased from MP Biomedicals (Solon, OH). A protease inhibitor cocktail (Protease-50 EDTA free) was from G-biosciences (Maryland Heights, MO). Amino acid mixture and S30 pre-mix were obtained from Promega (Madison, WI). Creatine phosphokinase, tRNA, 3',5'-cyclic adenosine monophosphate, adenosine 5'-triphosphate, guanosine 5'-triphosphate, uridine 5'-triphosphate, cytidine 5'-triphosphate, DL-dithiothreitol, sodium creatine phosphate, isopropyl β -D-1-thiogalactopyranoside, magnesium acetate, potassium hydroxide, polyethylene glycol, ammonium acetate, trizma acetate, L-glutamic acid, sodium phosphate monobasic, sodium phosphate dibasic, D-(+)-glucose, dimethyl sulfoxide, ethanol, doxycycline, minocycline, tetracycline, paromomycin, amikacin, tobramycin, streptomycin, hygromycin B, spectinomycin, erythromycin, puromycin, chloramphenicol, thiostrepton, cycloheximide, rifampicin, meropenem, levofloxacin and water were procured from Sigma-Aldrich (St. Louis, MO). Linezolid was purchased from AK Scientific (Union City, CA) and tigecycline was purchased from Wyeth (Pfizer) Pharmaceuticals.

2.2 Bacterial strains

E. coli wild type strain MG1655, and the isogenic *tolC* insertion mutant (MG1655 *tolC::kan*; FB22092 cured of pKD46 plasmid) were from Fred Blattner at the University of Wisconsin, Madison (strains available at: <http://www.genome.wisc.edu/sequencing.htm>). *P. aeruginosa* wild type strain PAO1, and the isogenic multiple Mex pump mutant, PAO750 ($\Delta(\text{mexAB-oprM})$, $\Delta(\text{mexCD-oprJ})$, $\Delta(\text{mexEF-oprN})$, $\Delta(\text{mexJK})$, $\Delta(\text{mexXY})$, ΔopmH , ΔpscC), were obtained from Herbert Schweizer at Colorado State University (Kumar *et al.*, 2006). A PAO1 derivative expressing a chromosomally-encoded T7 polymerase gene, PAO1-T7pol-FRT, was obtained from Tung Hoang at the University of Hawaii, Manoa (Kang *et al.*, 2007).

2.3 Plasmids

Plasmid pBEST*luc* (1 $\mu\text{g/ml}$; Promega, Madison, WI) was used for the expression of firefly luciferase from a *tac* promoter. Plasmid pT7-*luc* was created for expression of firefly luciferase from a T7 promoter. The firefly luciferase gene was amplified from pBEST*luc* by PCR using the following primers (Forward primer: GCCGCGCGGGCGCCGCATATGGAAGACGCCAAAAACATAAAG; reverse primer: GCCGCGCGGGCGCCGGGATCCTTACAATTTGGACTTTCCGCCCTT), which introduced 5' NdeI and 3' BamHI restriction sites. The fragment was digested with NdeI and BamHI and ligated into pET3a vector (EMD Millipore, Billerica, MA) digested with NdeI and BamHI using T4 DNA ligase. Ligation mixtures were transformed into electrocompetent DH10B (Cat# 18290-015, Invitrogen, Grand Island, NY) and the plasmid DNA from ampicillin-resistant transformants was prepared by Qiagen spin prep (Qiagen, Germantown, MD). Plasmids were screened by restriction digest to confirm the construction of pET3a-*luc*. Large scale plasmid DNA preparations were made from 100 ml DH10B(pET3a-*luc*) or DH10B(pBEST*luc*) cultures grown in 2XYT (twice strength YT broth) using the Qiagen Plasmid Plus Maxi kit (Qiagen, Germantown, MD) or purchased from Promega (pBEST*luc*).

2.4 MIC assays

Minimum inhibitory concentration (MIC) assays were run in cation-adjusted Mueller Hinton Broth (MHB) following Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2009) using Costar round bottom 96-well microtiter assay plates (Corning, Lowell, MA). MIC assays routinely included quality control strains *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 to confirm antibiotic activity was within CLSI ranges, where available, and the reproducibility of the assay conditions. Microtiter plates and agar plates delineating the viable counts of each inoculum were incubated overnight at 35° C. Compounds were suspended in sterile deionized water or dimethyl sulfoxide (DMSO, final DMSO concentration in MIC assays was less than 0.5%).

2.5 S30 Extract Procedure

The protocol for producing *P. aeruginosa* S30 extracts was developed based upon modification of the methods for *E. coli* described by Lui *et al.* (2004) and Kim *et al.* (2008) using two *P. aeruginosa* strains, PAO1 and a PAO1 derivative expressing an T7 RNA polymerase gene under the control of an inducible promoter (PAO1-T7pol-FRT; (Kang *et al.*, 2007). *P. aeruginosa* cells were grown in either well-aerated shake flasks at 37°C (150 – 200 mL cultures grown in-house to an OD₆₀₀ ~ 2 to 4 units) or 5L fermentations at 37°C (grown to OD₆₀₀ ~ 10 - 12 units at the Biomolecule Core Facility, New England Regional Center of Excellence (NERCE) for Biodefense and Emerging Infectious Disease Research, Harvard Medical School). All cultures were grown in 2× YT medium (BD Biosciences, Franklin Lakes, NJ) supplemented with sodium phosphate (40 mM dibasic, 22 mM monobasic) and glucose (100 mM). For T7 RNA polymerase expression, cells were induced

with 1 mM IPTG for one hour prior to harvesting. Cells were pelleted and washed once with S30 wash buffer (10 mM Trisacetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, 1 mM dithiothreitol). Cells were resuspended in 1 ml S30 wash buffer per gram of wet cell weight plus 30 units/ml RNase Out and disrupted using a bead-beater with 0.1 mm zirconia/silica beads prepared according to the manufacturer's instructions (BioSpec Products, Bartlesville, OK). Optimal lysis was typically achieved after 15 minutes total beating in an ice-filled jacket, using a cycle of 1 minute on, 30 seconds off, in a 20 ml chamber. Lysates were then centrifuged twice at 14,500 rpm for 58 minutes at 4°C in an F15S-8 × 50cy rotor (Thermo Scientific, Asheville, NC) which was equivalent to 30,000 × g for 30 minutes in a Sorvall SS-34 rotor. The supernatants were pooled and retained. Pre-incubation buffer (200-300 µl per milliliter of total extract) was added to the pooled S30 supernatants to allow translation of any nascent mRNAs ("run-off reaction"); reaction mixes were shaken vertically in 15 ml conical tubes (BD Biosciences, Franklin Lakes, NJ) at 280 rpm, 37° C for 80 minutes to complete translations in progress. Pre-incubation buffer contained 370 mM Tris-acetate pH 8.2, 11.1 mM magnesium acetate, 16.5 mM ATP, 5.5 mM dithiothreitol, and 50 µM of each amino acid, supplemented with either 27 µg/ml creatine kinase, 200 mM creatine phosphate or 8.4 units/ml pyruvate kinase, 105 mM phosphoenol pyruvate, as noted. If protease inhibitors were used, the cocktail was added to lysate at a final concentration of 1% during the run-off reaction. Following run-off pre-incubation, lysates were centrifuged for 10 minutes at 4000 × g and supernatants were dialyzed for 40 minutes at 4° C using a 3500 molecular weight cut-off Slide-A-Lyzer cassette (Pierce, Rockford, IL) in a 40-fold volume of S30 wash buffer. Dialyzed lysates were collected, aliquoted into 500 - 600 µl portions, and rapidly frozen in a dry ice/ethanol bath prior to storage at -80°C.

2.6 Transcription-translation assay

E. coli transcription-translation assays used commercially available S30 and T7-S30 extract kits for circular DNA (Promega, Madison, WI). *P. aeruginosa* extracts were supplemented with S30 pre-mix from Promega or S30 pre-mix prepared according to Murray *et al.* (2001). Plasmid pBEST*luc* DNA was prepared using a Qiagen Plasmid Plus kit or purchased from Promega. Plasmid pET3a-*luc* was used for luciferase expression from S30 extracts derived from *P. aeruginosa* PAO-T7pol-FRT and the commercially available *E. coli* T7-S30 kit. Reactions were carried out in a total volume of 20 µl in black-walled 96-well flat-bottom assay plates (Corning, Lowell, MA) for one hour at 37°C. Each reaction contained 5 to 5.23 µl of extract, 0.05 to 0.6 µl of plasmid stock, 8 µl of S30 pre-mix, 3 µl of compound dilution and sterile deionized water. Compounds were diluted in sterile deionized water and, where necessary, with DMSO (final concentration less than 2%). T7 RNA polymerase was added at a concentration of 0.7 -70 units per reaction (Kohrer *et al.*, 1996) when trying to boost expression from pET3a-*luc* in S30 extracts derived from PAO-T7pol-FRT. Reactions were stopped by placing on ice for 5 minutes, followed by the addition of 25 µl of luciferase substrate (Promega, Madison, WI) per well. Luminescence was read using a LUMISStar Optima (BMG Labtech, Cary, NC) with gain at 3600, 0.2 second read time, 0 seconds between wells. Percent of uninhibited luminescence (average of 12 wells without compound per plate) was plotted against inhibitor concentration, and the concentration producing 50% inhibition (IC₅₀) was determined. Inhibitor IC₅₀ values were calculated as an average of a minimum of three independent IC₅₀ determinations, with each inhibitor tested against a minimum of two independently prepared S30 extracts. Background luminescence measured in extracts without plasmid addition was typically 25 – 45 relative light units (RLUs).

3. Results and Discussion

3.1 S30 extract preparation protocol and assay development

In an effort to attain the most robust and reproducible level of activity in a *P. aeruginosa* transcription-translation assay system, as measured by firefly luciferase production, multiple parameters were explored including conditions for cell lysis, *P. aeruginosa* strain, luciferase reporter gene promoter construct, addition of protease inhibitors, conditions for the run-off reaction, reporter plasmid concentration, time of assay duration and temperature of assay incubation.

While the efficiency of lysis appeared to impact overall activity of extracts, quantification of the degree of lysis by measuring the release of soluble protein and nucleic acid proved unreliable; extracts that appeared to have the same or similar soluble protein and nucleic acid content as commercially available *E. coli* S30 preparations (as quantified by absorbance at 260 nm for nucleic acid, and absorbance at 280 nm or by colorimetric protein assay for protein; data not shown) had vastly different levels of overall transcription-translation activity. Therefore, in order to confirm adequate cell lysis and activity was achieved, completion of the full S30 extract preparation procedure followed by a transcription-translation assay detecting firefly luciferase production was necessary to quantify the total activity of extracts prior to additional optimization. The use of protease inhibitors did not produce a significant net increase in the activity of extracts, and were omitted from later extract preparations (Table 1). While the S30 assay pre-mix purchased from Promega (contents described in Murray *et al.*, 2001) contained phosphoenol pyruvate and pyruvate kinase, run-off reactions performed with creatine kinase and creatine phosphate as the ATP-generation system in the pre-incubation buffer produced the most active *P. aeruginosa* extracts as compared to run-off reactions containing pyruvate kinase and phosphoenol pyruvate in the pre-incubation buffer (Table 1); therefore later extract preparations used creatine kinase and creatine phosphate in the run-off reaction.

Extracts from strain PAO1-T7pol-FRT using pET3a-*luc*, even with the addition of exogenous T7 RNA polymerase, produced relatively lower levels of firefly luciferase activity as compared to extracts from PAO1 using pBEST*luc* (Table 1). Interestingly, extracts from PAO1-T7pol-FRT were capable of producing 2600-3200 RLU's using 0.13 – 0.26 µg per reaction of pBEST*luc* plasmid, showing that these lysates were competent for translation of firefly luciferase mRNA transcribed from a *tac* promoter using the host RNA polymerase. Confirmation that plasmid pET3a-*luc* was properly constructed and could produce high levels of active firefly luciferase activity was demonstrated in an *E. coli* extract supplemented with T7 RNA polymerase, where RLU production was found to be similar to expression from pBEST*luc* (Table 2). These results suggested that while pET3a-*luc* and pBEST*luc* worked similarly in *E. coli* extracts, pBEST*luc* was the better reporter plasmid for *P. aeruginosa* extracts. Thus, further assay optimization was done with PAO1 extracts using pBEST*luc*.

A comparison of pBEST*luc* plasmid prepared by Qiagen Plasmid Plus Maxi kit versus plasmid purchased from Promega suggested that the commercially available plasmid more reliably produced the greatest activity per microgram of DNA, and was therefore the preferred source of pBEST*luc* (not shown). Further, it was found that each new *P. aeruginosa* S30 extract preparation and each new batch of plasmid DNA required re-optimization by testing a range of plasmid DNA concentrations (~0.2 to 2 µg per reaction) to identify a concentration in the linear range which produced adequate luminescence (~2000 – 3000 RLU's) in the transcription-translation assay (Figure 1). However, once optimized, assay results using *P. aeruginosa* S30 extracts were highly reproducible. Since *E. coli* extracts generally produced ~5 – 10-fold greater maximal luminescence than *P.*

aeruginosa extracts, for compound-inhibition comparisons, total luminescence from *E. coli* extracts was brought down to the range of *P. aeruginosa* extracts by adjusting plasmid concentration. Typically a higher concentration of pBEST*luc* was required in the *P. aeruginosa* assay to achieve similar relative luminescence as the *E. coli* assay; in the case of one specific plasmid preparation from Promega, to produce approximately 2000-3000 RLU, the amount of pBEST*luc* per reaction from one plasmid preparation was 0.4 µg in *P. aeruginosa* extracts and 0.08 µg in *E. coli* extracts (not shown). The amount of extract added to reactions was also examined. Investigation of varying the volume of S30 *P. aeruginosa* extract per reaction showed that approximately 5 - 6.5 µl typically produced the highest luminescence for most extracts used in a 20 µl total reaction volume (not shown).

Temperature and duration of assay incubation were also examined. Assays incubated at either 25°C or 30°C produced ~1.5 to 2.5-fold greater luminescence than assays incubated at 37°C (Figure 1). Since compound-inhibition curves and IC₅₀ determinations performed at 25°C versus 37°C for a test set of compounds were similar (not shown), experiments were standardized to the physiologically-relevant temperature, 37°C, despite the lower overall total luminescence. Examination of the time of assay incubation from 15 to 120 minutes showed that, similar to the *E. coli* S30 assay, luminescence production in the *P. aeruginosa* assay appeared to plateau after 60 minutes (Figure 2). To be consistent with the manufacturer's recommendation for incubation of *E. coli* S30 extracts, experiments with *P. aeruginosa* extracts were standardized to 60 minute incubations.

3.2 Transcription-translation assay results

The activities of a wide variety of mechanistically distinct antibiotics were compared in *P. aeruginosa* and *E. coli* transcription-translation assays (Table 3). Compounds were serially diluted over a range of concentrations and inhibition of total luminescence was plotted as a percent of averaged uninhibited values, and an IC₅₀ was determined. The ratio between *E. coli* and *P. aeruginosa* IC₅₀ values was also calculated as an indicator of differences in assay inhibition. Most translation antibiotics inhibited both assays similarly, with transcription-translation IC₅₀ ratios of ~1-2, reflecting the conservation of antibiotic targets in the bacterial translation pathway. In the case of streptomycin, spectinomycin, chloramphenicol and thiostrepton, the transcription-translation ratio was closer to 3, suggesting that the *P. aeruginosa* transcription-translation assay may be more sensitive to these antibiotics than *E. coli*, possibly reflecting differences in the translation machinery in both organisms. Representative dose-response curves from at least two independent experiments per antibiotic are shown in Figure 3; differences in curve shape are presumably due to inhibitor mechanistic differences.

Many translation inhibitors, such as paromomycin, hygromycin B, spectinomycin, erythromycin, linezolid, lincomycin and puromycin were potent in both transcription-translation assays but had limited, or no, whole cell antibacterial activity (Table 3 and see below). Rifampin, a bacterial RNA polymerase inhibitor, showed similarly potent inhibition in both assays, as expected. Cycloheximide (a mammalian-specific translation inhibitor), meropenem (a bacterial cell wall inhibitor), and levofloxacin (a bacterial topoisomerase inhibitor), served as negative controls, and as anticipated were not active in either transcription-translation assay at any concentration tested, supporting the specificity of these assays.

3.3 Antibiotic susceptibility of wild type and efflux-defective *E. coli* and *P. aeruginosa* strains

The same set of antibiotics was tested for microbiological activity against wild type and isogenic efflux-deficient mutants of *P. aeruginosa* and *E. coli*. The majority of translation

inhibitors were 2 to >100-fold more potent against efflux-knockout strains versus the corresponding wild-type parental strains, consistent with Gram-negative intrinsic efflux being a major barrier to the antibacterial potency of many of the translation inhibitors tested. Interestingly, many antibiotics were similarly active against *E. coli* and *P. aeruginosa* mutants devoid of major efflux pumps, further exemplifying the high degree of conservation of antibiotic targets in the translation pathways in both organisms (Table 3). Notably, several antibiotics such as hygromycin B, spectinomycin, and lincomycin exhibited no antibacterial activity against any of the strains, presumably due to poor cellular uptake into Gram-negative bacteria, disconnecting their potent transcription-translation assay IC₅₀ values from cell-based activity. The *E. coli* *tolC* knockout mutant showed resistance to paromomycin due to the presence of an *aph(3')-II* gene encoded by the *Tn5* transposon insertion into the chromosomal *tolC* gene (Alvarez and Mendoza, 1992). The eukaryotic translation inhibitor cycloheximide also failed to inhibit bacteria, consistent with its mammalian-specific activity.

4. Conclusions

We describe a robust method for preparing *P. aeruginosa* S30 extracts and validate the use of these extracts for measuring the potency of bacterial transcription and translation inhibitors. Since the antibacterial potency of most antibiotics is subject to permeability and intrinsic efflux barriers, especially in Gram-negative bacteria, the availability of species-specific S30 extracts allows for cell-free determination of inhibitor potency against the translation pathway of the targeted bacterial species. While each new preparation of *P. aeruginosa* S30 extract required optimization of luciferase reporter plasmid concentration, once optimized, assay results were consistent between batches of *P. aeruginosa* S30 extracts, and comparable to the robustness of a commercially available *E. coli* assay. Similar to the *E. coli* transcription-translation assay, the *P. aeruginosa* assay allows for ranking of compounds with regard to inhibition of transcription or translation and, in combination with cell-based assays, should prove useful in guiding the development of novel antibacterials targeting *P. aeruginosa*.

Acknowledgments

The authors thank Theresa Farrell for excellent technical assistance and Dr. Robin Ross at the Biomolecule Core Facility, New England Regional Center of Excellence (NERCE) for Biodefense and Emerging Infectious Disease Research, Harvard Medical School, for providing PAO1 fermentations funded by NIH/NIAID Grant No. U54 A1057159.

References

1. Alvarez M, Mendoza MC. Epidemiological survey of genes encoding aminoglycoside phosphotransferase APH (3') I and APH (3') II using DNA probes. *J Chemother.* 1992; 4:203–210. [PubMed: 1328557]
2. Butler MS, Cooper MA. Antibiotics in the clinical pipeline in 2011. *J Antibiotic.* 2011; 64:413–425.
3. Clinical and Laboratory Standards Institute (CLSI). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Eighth Edition. CLSI document M07-A8 [1-56238-689-1]. Clinical and Laboratory Standards Institute; 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA: 2009.
4. Dandliker PJ, Pratt SD, Nilius AM, Black-Schaefer C, Ruan X, Towne DL, Clark RF, Englund EE, Wagner R, Weitzberg M, Chovan LE, Hickman RK, Daly MM, Kakavas S, Zhong P, Cao Z, David CA, Xuei X, Lerner CG, Soni NB, Bui M, Shen LL, Cai Y, Merta PJ, Saiki AYC, Beutel BA. Novel antibacterial class. *Antimicrob Agents Chemother.* 2003; 47:3831–3839. [PubMed: 14638491]

5. Eustice DC, Feldman PA, Slee AM. The mechanism of action of DuP 721, a new antibacterial agent: effects on macromolecular synthesis. *Biochem Biophys Res Comm.* 1988; 150:965–971. [PubMed: 2449210]
6. Infectious Diseases Society of America. The 10 × '20 initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. *Clin Infect Dis.* 2010; 50:1081–1083. [PubMed: 20214473]
7. Kang Y, Son MS, Hoang TT. One step engineering of T7-expression strains for protein production. *Protein Express Purif.* 2007; 55(2):325–333.
8. Kim TW, Kim HC, Oh IS, Kim DM. A highly efficient and economical cell-free protein synthesis system using the S12 extract of *Escherichia coli*. *Biotechnol Bioprocess Eng.* 2008; 13:464–469.
9. Kim TW, Keum JW, Oh IS, Choi CY, Park CG, Kim DM. Simple procedures for the construction of a robust and cost-effective cell-free protein synthesis system. *J Biotechnol.* 2006; 126:554–561. [PubMed: 16797767]
10. Kohrer C, Mayer C, Grobner P, Piendl W. Use of T7 RNA polymerase in an optimized *Escherichia coli* coupled *in vitro* transcription-translation system: application in regulatory studies and expression of long transcription units. *Eur J Biochem.* 1996; 236:234–239. [PubMed: 8617270]
11. Kumar A, Chua K, Schweizer HP. Method for regulated expression of single-copy efflux pump genes in a surrogate *Pseudomonas aeruginosa* strain: identification of the BpeEF-OprC chloramphenicol and trimethoprim efflux pump of *Burkholderia pseudomallei* 1026b. *Antimicrob Agents and Chemother.* 2006; 50:3460–3463. [PubMed: 17005832]
12. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria. *Drugs.* 2004; 64:159–204. [PubMed: 14717618]
13. Liu DV, Zawada JF, Swartz JR. Streamlining *Escherichia coli* S30 extract preparation for economical cell-free protein synthesis. *Biotechnol Prog.* 2005; 21:460–465. [PubMed: 15801786]
14. Ma R, Yang Z, Huang L, Zhu X, Kai L, Cai J, Wang X, Xu Z. Construction of an efficient *Escherichia coli* cell-free system for *in vitro* expression of several kinds of protein. *Eng Life Sci.* 2010; 10:333–338.
15. Murray RW, Melchior EP, Hagadorn JC, Marotti KR. *Staphylococcus aureus* cell extract transcription-translation assay: firefly luciferase reporter system for evaluating protein translation inhibitors. *Antimicrob Agents Chemother.* 2001; 45:1900–1904. [PubMed: 11353649]
16. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev.* 2003; 67:593–656. [PubMed: 14665678]
17. Sohmen D, Harms JM, Schlünzen F, Wilson DN. Enhanced snapshot: antibiotic inhibition of protein synthesis II. *Cell.* 2009; 139(1):212–212. e1. [PubMed: 19804764]
18. Sutcliffe JA. Antibiotics in development targeting protein synthesis. *Ann NY Acad Sci.* 2011; 1241:122–152. [PubMed: 22191530]
19. Wilson DN. The A – Z of bacterial translation inhibitors. *Crit Rev Biochem Mol Biol.* 2009; 44:393–433. [PubMed: 19929179]

Figure 1. Effect of plasmid concentration and temperature of assay incubation on luminescence in a representative S30 extract from *P. aeruginosa* PAO1

A titration of plasmid pBEST/luc concentrations was tested in a representative S30 extract at 25°C, 30°C and 37°C. Extracts were incubated for 1 hour. Total luminescence after incubation was measured as described in Materials and Methods. The plasmid preparation used in this experiment was from Promega. Each bar represents the average of 8 replicates.

Figure 2. Effect of time of assay incubation on luminescence in a representative S30 extract from *P. aeruginosa* PAO1 versus a commercial *E. coli* S30 extract
P. aeruginosa and *E. coli* assays contained 0.4 μ g/reaction and 0.08 μ g/reaction of plasmid pBEST $_{luc}$, respectively. The plasmid preparation used in this experiment was from Promega. Assays were incubated at 37°C for 15 min, 30 min, 60 min and 120 min. Each bar represents the average of 7 replicates. Luminescence was quantified as described in Materials and Methods.

Figure 3. Inhibitory activity profiles for various antibiotics in the *P. aeruginosa* and *E. coli* transcription-translation assays

Compound titrations were tested for inhibition of total luminescence in *P. aeruginosa* and *E. coli* extracts as described in Materials and Methods. The plasmid preparation used in these experiments was from Promega. Sample titration curves from assays run in duplicate are shown. Average IC_{50} values from a minimum of 2 replicate assays per compound are presented in Table 3.

Optimization of *P. aeruginosa* S30 extracts.

Table 1

Run-off Reaction Kinase	<i>P. aeruginosa</i> S30 Extract	Plasmid	Plasmid Concentration (μg/rxn)	T7 RNAP ¹ (units/rxn)	1% PI ²	Average RL U ³ ± SD
Pyruvate	PAO1-T7pol-FRT	pET3a- <i>luc</i>	0.22	6.7	-	97 ± 10
			0.22	6.7	+	27 ± 6
			0.22	67	-	57 ± 5
			0.22	67	+	31 ± 8
Creatine	PAO1	pBEST- <i>luc</i> ⁴	0.26	N/A	-	1217 ± 483
			0.26	N/A	+	465 ± 36
			0.22	6.7	-	377 ± 92
			0.22	6.7	+	168 ± 13
Creatine	PAO1-T7pol-FRT	pET3a- <i>luc</i>	0.22	67	-	147 ± 24
			0.22	67	+	80 ± 20
Creatine	PAO1	pBEST- <i>luc</i> ⁴	0.26	N/A	-	2929 ± 309
			0.26	N/A	+	2139 ± 34

¹ T7 RNAP, T7-RNA polymerase
² PI, protease inhibitor cocktail
³ RL U, relative light units± standard deviation, average of 3 replicates
⁴ pBEST*luc* was purified in-house by Qiagen Plasmid Plus Maxi Kit

Table 2Activity of pET3a-luc and pBEST*luc* plasmids in *E. coli* S30 extracts.

<i>E. coli</i> Extract	Plasmid	Plasmid Concentration (μg)	Av RLU ^I ± SD
S30-T7	pET3a- <i>luc</i>	0.1	28373 ± 1724
S30	pBEST <i>luc</i>	0.1	21182 ± 2519

^IRLU, relative light units ± standard deviation; average of 4 replicates

Table 3
Activities of a diverse set of antibiotics in *E. coli* and *P. aeruginosa* transcription-translation and MIC assays

Class	Antibiotic	Bacterial Ribosome Subunit Targeted	Transcription/Translation Assay			MIC Assay			
			<i>P. aeruginosa</i> IC ₅₀ µg/ml	<i>E. coli</i> IC ₅₀ µg/ml	IC ₅₀ ratio ¹	<i>E. coli</i> wild type MIC µg/ml	<i>E. coli tolC</i> MIC µg/ml	<i>P. aeruginosa</i> wild type MIC µg/ml	<i>P. aeruginosa</i> Mex ⁻ MIC µg/ml
Tetracycline	Doxycycline		1.96 ± 1.37	2.07 ± 0.35	1.05	8	1	32	0.5
	Tetracycline	30S	1.54 ± 0.70	1.87 ± 0.21	1.21	4	2	32	0.25
	Minocycline		0.43 ± 0.17	1.13 ± 0.25	2.65	4	0.5	32	0.25
	Tigecycline		0.11 ± 0.03	0.25 ± 0.07	2.30	0.13 8	0.063	16	0.13
Aminoglycoside	Paromomycin		0.04 ± 0.03	0.02 ± 0.00	0.46		>32 ²	>32	32
	Amikacin		0.04 ± 0.01	0.05 ± 0.01	1.21	4	4	8	2
	Tobramycin	30S	0.01 ± 0.00	0.03 ± 0.01	2.13	1	1	1	0.5
	Streptomycin		0.04 ± 0.01	0.10 ± 0.03	2.78	8	4	32	16
	Hygromycin B		0.01 ± 0.00	0.02 ± 0.00	1.39	>32	32	>32	>32
	Spectinomycin		0.10 ± 0.04	0.30 ± 0.15	2.91	32	32	>32	>32
Macrolide	Erythromycin	50S	0.19 ± 0.09	0.48 ± 0.03	2.58	>32	4	>32	16
Oxazolidinone	Linezolid	50S	0.96 ± 0.40	1.53 ± 0.06	1.59	>32	16	>32	8
Lincosamide	Lincomycin	50S	2.72 ± 1.75	5.08 ± 1.39	1.86	>32	>32	>32	>32
Aminonucleoside	Puromycin	50S	0.15 ± 0.03	0.17 ± 0.01	1.09	>32	4	>32	8
Phenicol	Chloramphenicol	50S	0.44 ± 0.26	1.30 ± 0.1	2.94	16	2	>32	2
Thiopeptide	Thiostrepton	50S	0.59 ± 0.16	1.67 ± 0.41	2.85	8	8	8	8
Glutarimide	Cycloheximide	N/A ³	>10	>10	N/A	>32	>32	>32	>32
Ansamycin	Rifampin	N/A	0.05 ± 0.02	0.05 ± 0.01	1.05	16	8	32	32
Carbapenem	Meropenem	N/A	>100	>100	N/A	0.063	0.13	8	1
Fluoroquinolone	Levofloxacin	N/A	>100	>100	N/A	0.063	0.016	0.5	0.016

¹Ratio of *E. coli* *P. aeruginosa* IC₅₀s

²Elevated MIC value is attributed to *aph(3')-II* gene encoded by *Tn5* insertion in *tolC*, inactivating paromomycin

³N/A, not applicable