

Revised Abstract

Background. Many clinically useful antibiotics target the translation machinery of bacteria. Despite otherwise broad-spectrum activity, tetracyclines (TETs), among many other translation antibiotics, do not possess clinically useful activity against *Pseudomonas aeruginosa* (PA). To assess the target-based potency of TETs in PA, as compared to *Escherichia coli* (EC), an *in vitro* TNT assay was developed for PA.

Methods. Using modifications of methods from Liu et al. [1] and Kim et al [2], S30 extracts were prepared from a PA strain expressing a T7 RNA polymerase (T7RNAP) gene (Kang et al. 2007. Prot. Expr. Purif. 55:325). The reporter gene, firefly luciferase (*luc*), was expressed from either pBEST-*luc* (Promega, Madison, WI) or a plasmid, pET3a-*luc*, expressing *luc* from a T7 promoter. Compound titrations were tested in 20 µl reactions incubated at 37°C for 1 hour. *luc* expression was detected using the Promega E1500 luciferase substrate and a LumiStar Optima luminometer (BMG Labtech). Inhibition of PA TNT was compared to EC TNT from Promega with a panel of diverse translation antibiotics, in triplicate.

Antibiotic	PA IC ₅₀ (µg/ml)	EC IC ₅₀ (µg/ml)	EC:PA IC ₅₀ Ratio
Doxycycline	1.96 ± 1.37	2.07 ± 0.35	1.05
Tetracycline	1.54 ± 0.70	1.87 ± 0.21	1.21
Tigecycline	0.11 ± 0.03	0.25 ± 0.07	2.3
Spectinomycin	0.10 ± 0.04	0.30 ± 0.15	2.91
Amikacin	0.04 ± 0.01	0.05 ± 0.01	1.21
Erythromycin	0.19 ± 0.09	0.48 ± 0.03	2.58
Linezolid	0.96 ± 0.4	1.53 ± 0.06	1.59
Chloramphenicol	0.44 ± 0.26	1.30 ± 0.10	2.94
Rifampicin	0.05 ± 0.02	0.05 ± 0.01	1.05
Meropenem	>100	>100	N/A
Levofloxacin	>100	>100	N/A
Average uninhibited luminescence units:	PA: 2825	EC: 6622	

Results. Optimal *luc* expression in the PA TNT was from pBEST-*luc*; expression from pET3a-*luc* was negligible, even with T7RNAP enzyme supplementation. The ranking of compounds for each TNT system was similar and 50% inhibitory concentration (IC₅₀) values were within 1 to 3-fold, illustrative of the overall high degree of conservation between the EC and PA transcription/translation machinery.

Conclusion. A PA-specific TNT assay should enable ranking of novel inhibitors targeting translation and the characterization of relative species-specific sensitivities to certain translation inhibitors.

Introduction

Both nature and man have had success exploiting the essentiality and conservation of bacterial transcription and translation machinery for the development of chemically and mechanistically diverse broad spectrum antibacterials. Despite the attractiveness of targeting this highly conserved pathway, the activities of antibacterial compounds can be significantly affected by subtle species-specific variations in antibiotic binding sites, as well as intrinsic bacterial resistance mechanisms limiting cellular accumulation. Successful treatment of infections caused by the gram-negative pathogen *Pseudomonas aeruginosa* (PA) is often impeded by the prevalence of multidrug-resistant phenotypes seen in clinical isolates. Over-expression of intrinsic resistance mechanisms in this organism significantly reduces the potency of a wide variety of antibiotics, including translation inhibitors, and has stimulated interest in developing novel therapies to combat antibiotic resistance in PA.

Here we report the development of a coupled transcription/translation (TNT) assay derived from PA. This assay can be used to assess target-based potency of new compounds targeting the PA ribosome. While *Escherichia coli* (EC) S30 extract systems are readily available commercially, the vast difference in antimicrobial susceptibility profiles between the two species provided the impetus to develop an assay that can be used to differentiate drug-target interactions from permeability/efflux-related effects for the discovery of novel antibacterial compounds targeting PA.

Methods

PA S30 preparation. A modified S30 protocol was developed based upon the methods described by Lui et al. [1] and Kim et al. [2] using two *P. aeruginosa* strains, PAO1 and a PAO1 derivative expressing the T7 RNA polymerase (PAO1-T7pol-FRT; [3]). *P. aeruginosa* isolates were grown shaking at 37°C in 2XYT medium containing additional sodium phosphate (40 mM dibasic, 22 mM monobasic) and glucose (100 mM) to an OD₆₀₀ of approximately 2 to 4 units. For T7 polymerase expression, cells were induced with IPTG for one hour prior to harvesting. Cells were pelleted and washed once with S30 buffer (10 mM Tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, 1 mM DTT). Cells were resuspended in 1 ml S30 buffer per gram of wet cell weight plus 30 U/ml RNase Out (Invitrogen) and disrupted using a bead-beater with 0.1 mm zirconia/silica beads (BioSpec Products). Lysates were then spun down twice at 30,000 x g, followed by incubation in pre-incubation buffer, shaking at 280 rpm, 37° C for 80 minutes. The pre-incubation buffers consisted of 370 mM Tris-acetate pH 8.2, 11.1 mM ATP, 5.5 mM DTT, 50 µM each amino acid, with either 27 µg/ml creatine kinase/200 mM creatine phosphate or 8.4 U/ml pyruvate kinase/105 mM phosphoenol pyruvate. If protease inhibitors were used, they were added at 1% during the run-off reaction (Protease-50 EDTA free, Gibco). Lysates were then spun down for 10 minutes at 4000 x g and dialyzed for 40 minutes at 4° C using a 3500 MWCO Slide-A-Lyzer cassette (Pierce Cat. # #66110) in a 40-fold volume of S30 buffer. Dialyzed lysates were collected and frozen in a dry ice/ethanol bath prior to storage at -80°C.

Transcription-Translation assays. *E. coli* assays were run using commercially available S30 and T7-S30 extract kits for circular DNA (Promega Cat # L1020, L1130 respectively). *P. aeruginosa* extracts were supplemented with S30 buffer from Promega (L512A) and DNA either prepared in house using a Qiagen maxi-prep kit (Plasmid Plus Maxi Cat. # 12963) or with DNA purchased from Promega (Cat. # L492A). For S30 extracts derived from PAO-T7pol-FRT and the commercially available *E. coli* T7-S30 kits, a pET3a vector carrying the luciferase gene cloned by PCR from the pBEST-*luc* (Promega) was used. Reactions were carried out in a total volume of 20 µl in black-walled 96-well flat-bottom assay plates (Costar Cat. # 3915) for one hour at 37° C. Each reaction contained 5 to 5.23 µl of extract, 0.05 to 0.6 µl of plasmid, 8 µl of S30 buffer and 3 µl of compound dilution. Reactions were stopped by placing on ice for 5 minutes followed by the addition of 25 µl of luciferase substrate (Promega Cat. # E1500) per well. Luminescence was read using a LumiStar Optima (BMG Labtech) with gain at 3600, 0.2 second read time, 0 seconds between wells. Percent luminescence was plotted against inhibitor concentration and the compound 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 PA extracts.

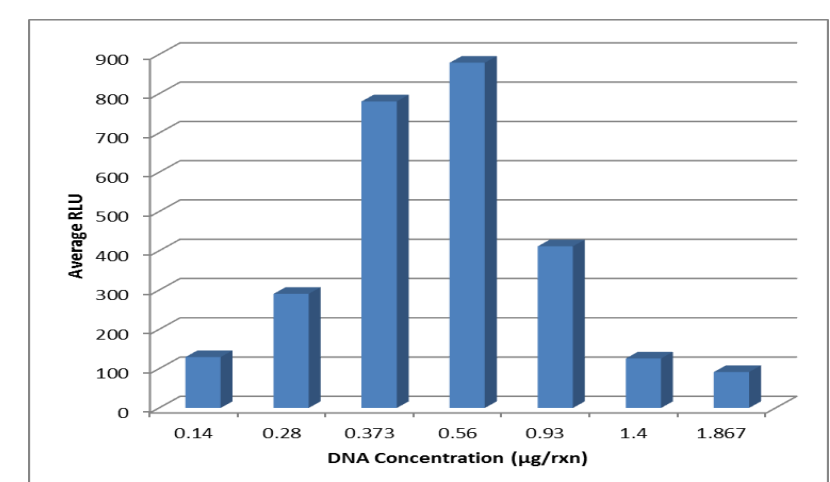
Antibiotic susceptibility assays. Minimum inhibitory concentration (MIC) assays were run in Mueller Hinton Broth (BD #212322) following CLSI guidelines [4] using Costar (Cat. # 3788) round bottom assay plates. Assay plates and viable counts were incubated overnight at 35° C. *E. coli* wild type (MG1655; ATCC 700926), and the isogenic *tolC* mutant (MG1655 *tolC::kan*; FB22092 cured of pKD46 plasmid) were from Fred Blattner at the University of Wisconsin, Madison. *P. aeruginosa* wild type, and the isogenic Mex mutant, are PAO1 and PAO750 Δ(mexAB-oprM) Δ(mexAB-oprM) Δ(mexDC-oprN) Δ(mexEF-oprN) Δ(mexJK) Δ(mexXY) ΔoprM, ΔpscC, obtained from Herbert Schweizer at Colorado State University [5].

Optimization of Activity in *P. aeruginosa* S30 Extracts

Plasmids pBEST-*luc* and pET3a-*luc* have similar activity in EC extracts

Promega EC Extract	Plasmid	Plasmid Concentration (µg)	Av RLU
S30-T7	pET3a- <i>luc</i>	0.1	28373 ± 1724
S30	pBEST- <i>luc</i>	0.1	21182 ± 2519

Plasmid pBEST-*luc** concentration requires optimization in each new PA S30 extract



* Commercially available pBEST-*luc* produced the highest level of activity in PA S30 extracts

The best activity in PA S30 extracts is seen with creatine kinase and pBEST-*luc*

Kinase	S30 Extract	Plasmid	Plasmid Concentration (µg/rxn)	T7 RNAP* (units/rxn)	1% PI**	Av RLU ± SD
Pyruvate	PAO1-T7	pET3a- <i>luc</i>	0.22	6.7	-	96.67 ± 9.50
			0.22	6.7	+	26.67 ± 6.02
			0.22	67	-	57 ± 5.29
	PAO1	pBEST- <i>luc</i>	0.22	67	+	31 ± 7.94
			0.13	N/A	-	528.33 ± 159.6
			0.13	N/A	+	162.33 ± 31.07
Creatine	PAO1-T7	pET3a- <i>luc</i>	0.26	N/A	-	1216.67 ± 482.83
			0.26	N/A	+	465.33 ± 36.00
			0.22	6.7	-	376.67 ± 92.4
	PAO1	pBEST- <i>luc</i>	0.22	6.7	+	168 ± 12.50
			0.22	67	-	146.67 ± 24.27
			0.22	67	+	80 ± 20.22
PAO1	pBEST- <i>luc</i>	0.13	N/A	-	2026 ± 404.60	
		0.13	N/A	+	2082 ± 399.40	
		0.26	N/A	-	2928.67 ± 309.04	
			0.26	N/A	+	2139 ± 34.40

*T7 RNAP, T7-RNA polymerase
**PI, protease inhibitor cocktail

Comparison of the Activities of Diverse Antibiotics in EC vs. PA S30 Extracts and MIC Assays

Class	Antibiotic	Bacterial Ribosome Subunit Targeted	General Mechanism of Action	PA TNT IC ₅₀	EC TNT IC ₅₀	EC:PA TNT ratio	EC wild type MIC	EC ToIC MIC	PA wild type MIC	PA Mex ^x MIC
				µg/ml	µg/ml		µg/ml	µg/ml	µg/ml	µg/ml
Tetracycline	Doxycycline	30S	A-site block/inhibit initiation	1.96 ± 1.37	2.07 ± 0.35	1.05	8	1	32	0.25
	Tetracycline			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	0.063	16	0.13
Aminoglycoside	Paromomycin	30S	inhibit initiation/translation fidelity	0.04 ± 0.03	0.02 ± 0.00	0.46	8	>32*	>32	32
	Amikacin			0.04 ± 0.01	0.05 ± 0.01	1.21	4	4	8	2
	Tobramycin			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
Macrolide	Erythromycin	50S	inhibit translocation	0.10 ± 0.04	0.30 ± 0.15	2.91	32	32	>32	>32
	Linezolid	50S	inhibit translocation	0.19 ± 0.09	0.48 ± 0.03	2.58	>32	4	>32	16
Oxazolidinone	Linezolid	50S	inhibit peptide bond formation	0.96 ± 0.40	1.53 ± 0.06	1.59	>32	16	>32	8
	Lincomamide	50S	inhibit peptide bond formation	2.72 ± 1.75	5.08 ± 1.39	1.86	>32	>32	>32	>32
Aminonucleoside	Purromycin	50S	inhibit peptide bond formation	0.15 ± 0.03	0.17 ± 0.01	1.09	>32	4	>32	8
	Chloramphenicol	50S	inhibit peptide bond formation	0.44 ± 0.26	1.30 ± 0.1	2.94	16	2	>32	2
Thiopeptide	Thiostrepton	50S	inhibit translocation	0.59 ± 0.16	1.67 ± 0.41	2.85	8	8	8	8
	Cycloheximide	N/A*	inhibit mammalian translation	>10	>10	n/a	>32	>32	>32	>32
Ansamycin	Rifampin	N/A	inhibit bacterial RNA polymerase	0.05 ± 0.02	0.05 ± 0.01	1.05	16	8	32	32
	Meropenem	N/A	inhibit bacterial cell wall synthesis	>100	>100	n/a	0.063	0.13	8	1
Fluoroquinolone	Levofloxacin	N/A	inhibit bacterial topoisomerases	>100	>100	n/a	0.063	≤0.016	0.5	≤0.016

N/A, not applicable

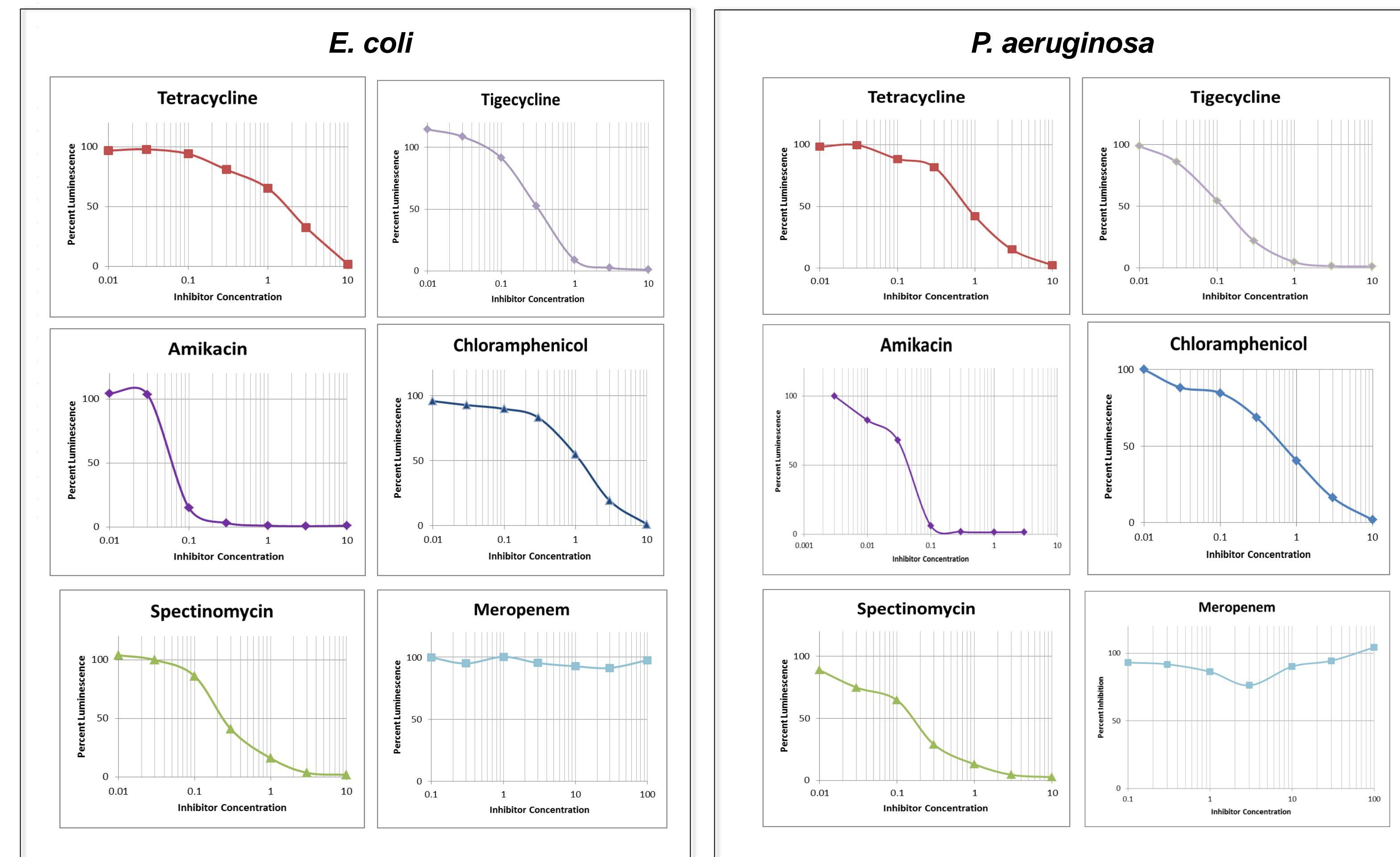
*elevated MIC value is attributed to *aph(3)-II* gene encoded by Tn5 insertion in *tolC*, inactivating paromomycin

References

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Results

Examples of Inhibition Curves from *E. coli* and *P. aeruginosa* TNT Assays



Conclusions

- An optimal set of conditions with a *P. aeruginosa* S30 extract for a coupled transcription/translation assay was identified.
- The best activity for *P. aeruginosa* extracts was attained when utilizing creatine kinase as an ATP source to exhaust 70S ribosomes of “translations-in-progress.”
- Commercially available pBEST-*luc* plasmid from Promega provided the most robust activity in *P. aeruginosa* systems.
- Many antibiotics were similarly active against *E. coli* and *P. aeruginosa* mutants devoid of major efflux pumps.
- P. aeruginosa* and *E. coli* S30 systems gave comparable IC₅₀ values for most antibiotics.