

A liquid handling platform for standardized quantification of cell-free enzymatic activity encoded by antimicrobial resistance genes

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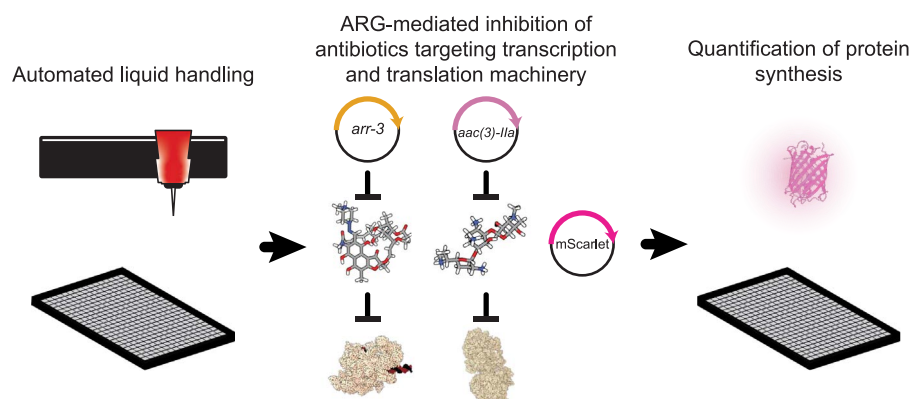
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Abstract

Antimicrobial resistance (AMR) is a growing global threat to human health, and rapid methods for characterizing emerging antimicrobial resistance genes (ARGs) are needed. Here, we develop a semi-automated workflow using cell-free gene expression systems to measure the activity of two ARGs encoded on plasmid DNA that produce rifampicin-inactivating and gentamicin-inactivating enzymes. We validated the use of a small benchtop Myra liquid handling system compared to manual pipetting, with no statistical differences observed. After optimizing the pre-incubation time of ARGs and dispensing protocol, expression of *aac(3)-IIa* increased the half-maximal inhibition concentration (IC₅₀) of gentamicin by over 150-fold, whilst *arr-3* increased the IC₅₀ of rifampicin by ~20-fold compared to controls. This methodology for rapid, semi-automated ARG characterization offers a strategy to combat AMR by assessing novel ARGs identified through genomic surveillance or profiling activity of new or derivative antibiotics.

Graphical abstract



Keywords cell-free gene expression, antimicrobial resistance, liquid handling, automation, aminoglycoside resistance, rifampicin resistance

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Introduction

Antimicrobial resistance (AMR) is a global concern to human health, contributing to millions of deaths each year [1]. Bacteria can acquire novel AMR through intrinsic genetic changes, including chromosomal mutation and recombination, as well as horizontal gene transfer (HGT), with the latter enabling rapid dissemination of antimicrobial resistance genes (ARGs) between strains and species [2–4]. Genomic surveillance enables the identification of emerging ARGs and resistance-associated mutations; however, distinguishing between genes that confer clinically relevant resistance and sequence-similar homologues with negligible impact remains a bottleneck, often requiring labour-intensive genotype-to-phenotype characterization [3, 5]. It is also difficult to assess the relative contributions of different AMR mechanisms, for example, drug target mutations, efflux, and enzymatic modifications, that contribute to the overall resistance to a specific antimicrobial agent in bacterial cells [6].

Here, we apply a liquid handling workflow to characterize two ARGs from distinct families as a proof-of-concept for profiling antibiotic-inactivating enzymes in *Escherichia coli* cell-free expression (CFE) systems. Enzymatic modification is one of the most important forms of resistance to aminoglycosides and is poorly understood given the large, diverse family of aminoglycoside-modifying enzymes able to act on these substrates [2, 7]. The gene *aac(3)-IIa* provides an example of this critical mechanism, encoding an aminoglycoside *N*-acetyltransferase that deactivates gentamicin, which otherwise inhibits protein synthesis by binding with the 30S ribosomal subunit [8–10]. Whilst a less common mechanism for rifampicin resistance, *arr-3* encodes an enzyme that catalyzes ADP-ribosylation of rifampicin, impairing its ability to inhibit transcription via interaction with the RNA polymerase (RNAP) β -subunit [11]. Both *aac(3)-IIa* and a close homologue of *arr-3* have been demonstrated to be active in cell-free lysates [12–14], and previous work has demonstrated the potential utility of CFE for screening ribosome-targeting inhibitors [15] and investigating enzymatic substrate-specificity on β -carbapenem antibiotics [16].

Integrating CFE systems with automated workflows and biofoundries has enabled increased experimental throughput and reproducibility, with recent applications in biological engineering and natural product discovery; however, accessibility to biofoundries and large-scale automation remains limiting [17, 18]. In this study, we design a semi-automated workflow to characterize ARG activity in *E. coli* cell-free lysates with a compact benchtop Bio Molecular Systems Myra liquid handling robot that offers a low-cost alternative to large-scale automated systems. The ARGs *aac(3)-IIa* and *arr-3* are well-suited for developing this CFE-based methodology due to their direct, intracellular enzymatic activity on antibiotics and the activity of their cognate antibiotics being directed towards native transcription and translation machinery present in lysate-based CFE systems [17]. This study adds to recent work developing automated and semi-automated CFE workflows using Opentrons OT-2 robotics [19, 20] and Echo Acoustic Liquid Handlers [21–23], with the focus of making CFE experimentation standardized, reproducible, and accessible to a broader range of users. Our work serves as a proof-of-concept for pairing automated liquid handling with CFE systems to rapidly characterize the antibiotic-inactivating activities of ARGs, addressing a key aspect of the global AMR crisis.

Results

Automated liquid handling is equivalent to manual pipetting for CFE-based ARG activity assays

To establish the reliability of automated liquid handling for characterizing ARG activity, we first compared results between automated and manual workflows. For initial characterization, a plasmid harbouring *aac(3)-IIa* or a water control was added to a 384-well plate followed by the immediate addition of cell-free reaction mix, which included *E. coli* BL21 Star™ (DE3)pLysS lysate, supplemental substrates and co-factors known to maximize protein synthesis, and a plasmid encoding mScarlet, which was used as a quantitative readout of lysate protein synthesis (Fig. 1a). The cell-free reactions were incubated for 2 h to enable expression of *aac(3)-IIa* before the addition of gentamicin or water. Liquid transfers were designed for a total reaction volume of 15 μ L with plasmid concentrations of 15 nM, and the complete reactions were then incubated for 16 h. Expression of *aac(3)-IIa* protected cell-free reactions from gentamicin-dependent inhibition of protein synthesis, and no statistically significant differences ($n=9$) were observed between manual and automated liquid dispensing across all conditions tested (Fig. 1b).

Pre-incubation is essential for reproducible ARG activity in CFE assays

We rationalized that ARG activity within cell-free lysates would depend on a pre-incubation phase, allowing ARG synthesis prior to antibiotic-mediated inhibition of the RNAP or ribosomes by rifampicin and gentamicin, respectively. We tested different ARG pre-incubation durations in which mScarlet was either co-expressed during pre-incubation (Fig. 1c) or added after pre-incubation (Fig. 1d). We found that dependency on pre-incubation time differed between ARGs, with *aac(3)-IIa* requiring 1–2 h for optimal activity, whilst *arr-3* was at least partially active immediately. Co-expression of mScarlet during pre-incubation beyond 1 h resulted in detectable fluorescence under antibiotic treatments in the absence of ARGs, attributable to maturation of a small mScarlet pool synthesized before antibiotic-mediated inhibition (Fig. 1c). Adding the mScarlet plasmid after pre-incubation eliminated this background signal (Fig. 1d).

Automated CFE quantifies aminoglycoside and rifampicin resistance gene activity

ARG-expressing cell-free reactions were challenged with increasing antibiotic concentrations to determine if the optimized semi-automated protocol could be used for robust quantification of enzymatic activity (Fig. 1e). Expression of *aac(3)-IIa* and *arr-3* notably increased the half-maximal inhibitory concentration (IC_{50}) of gentamicin and rifampicin, respectively (Fig. 2a and c). Across three independent replicates, expression of *aac(3)-IIa* conferred a gentamicin IC_{50} value of $10.04 \pm 0.84 \mu$ M, which was over 150-fold greater and statistically distinct (adjusted P -values $< 5 \times 10^{-5}$) from negative controls expressing either no ARG (55 ± 3.7 nM) or *arr-3* (64 ± 05.4 nM; Fig. 2b). Likewise, expression of *arr-3* conferred a rifampicin IC_{50} value ($1.00 \pm 0.13 \mu$ M) that was ~20-fold greater and statistically distinct (adjusted P -values $< 5 \times 10^{-4}$) from empty (63 ± 10 nM) and *aac(3)-IIa*-expressing controls (49 ± 14 nM; Fig. 2d). As predicted, IC_{50} values when *aac(3)-IIa* expression was challenged with rifampicin

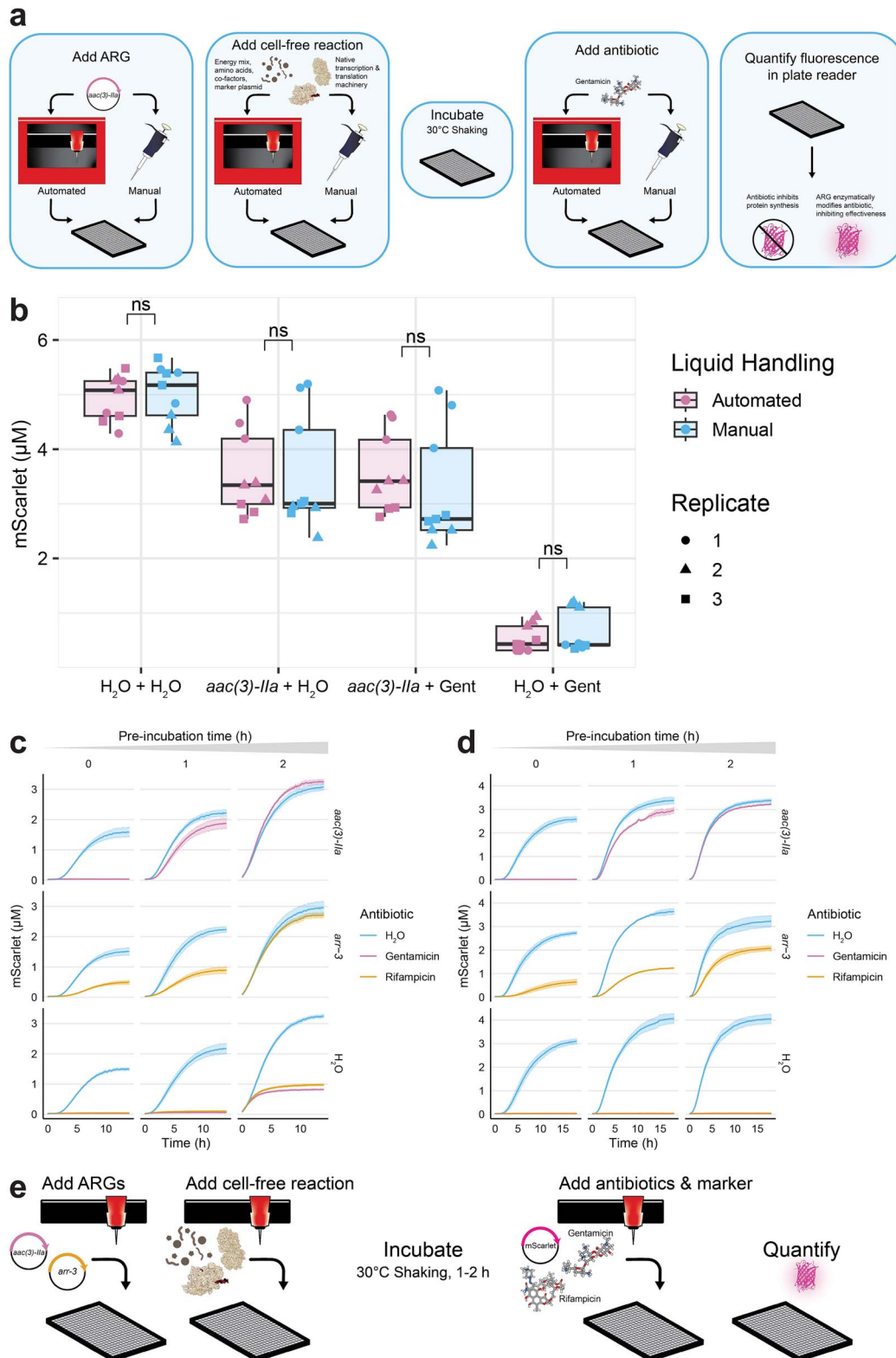


Figure 1 Automated liquid handling workflow for cell-free ARG expression functions equivalently to manual pipetting. (a) Schematic for cell-free workflow comparing automated liquid handling to manual pipetting. Ribosome (PDB 4YBB), RNAP complex (PDB 6X43), and gentamicin (PubChem CID 3467) are shown for illustrative purposes, not to scale. (b) mScarlet (μM) produced after 16 h across three independent replicates. Welch's t-tests followed with Benjamini & Hochberg correction indicate no significant differences between manual and automated pipetting for each combination. (c and d) ARG pre-incubation time determines protection efficacy in CFE antibiotic inhibition assays. mScarlet production over 16 h after ARG plasmids *aac(3)-IIa* and *arr-3* were either incubated with the cell-free reaction and mScarlet marker plasmid for 0–2 h prior to adding antibiotic (c) or incubated with the cell-free reaction for 0–2 h prior to adding both the antibiotic and mScarlet marker plasmid (d). Error bars indicate standard deviation across three technical replicates. Similar results were observed for three independent replicates. (e) Optimized automated liquid handling workflow. Plasmids, proteins, and antibiotics (rifampicin: PubChem CID 135398735) not shown to scale.

and *arr-3* was challenged with gentamicin did not statistically differ from null controls. Therefore, enzymes encoded by *aac(3)-IIa* and *arr-3* behave as expected in cell-free reactions, enzymatically modifying, and inhibiting gentamicin or rifampicin [12–14], and provide a proof-of-concept for semi-automated characterization of intracellular AMR activity in cell-free (Fig. 2e).

Discussion

In this study, we established a semi-automated CFE workflow with the Myra liquid handling system for the characterization of ARGs. We validated the use of this automated system compared to skilled manual pipetting and optimized a 1–2 h pre-incubation period for robust ARG expression prior to antibiotic treatment. The requirement for a pre-incubation phase is supported by previous studies that indicate proteins in CFE systems undergo an initial phase of transcription that lasts up to 1 h for enhanced GFP (eGFP), a quasilinear protein synthesis phase for up to 8 h for eGFP, and a plateau upon completion [24]. Maturation times are protein-dependent, causing these times to vary [24, 25].

Whilst we cannot exclude the possibility that the disparity in pre-incubation times for *aac(3)-IIa* and *arr-3* is attributed to protein maturation kinetics, differing expression levels, or enzyme activity, we theorize it reflects the distinct targets of the respective antibiotics within the gene expression pathway. Rifampicin targets RNAP-mediated transcription but does not prevent the translation of *arr-3* mRNA transcripts synthesized during a brief pre-incubation. Conversely, gentamicin inhibits ribosomal translation, meaning that *aac(3)-IIa* mRNA transcripts must be translated prior to antibiotic addition, necessitating a longer pre-incubation period. Additionally, the activity of a close homologue of Arr-3, Arr-2, has been shown to require NAD⁺ [12]. Whilst all cell-free reactions were supplemented with NAD⁺, we did not investigate the relationship between NAD⁺ concentration and enzyme activity. For our purpose of developing a semi-automated workflow, a 1–2 h pre-incubation time sufficiently enabled detection of activity; however, future work characterizing protein expression and enzymatic activity could further elucidate the discrepancy observed.

Scripts utilized for liquid handling are open-access and enable 384 unique 15- μ L cell-free reactions on a single plate. Liquid transfer functioned with Myra default settings without concerns of bubbles causing variation in dispensed amounts before optimisation, which has been addressed in previous workflows [19, 21]. However, whilst an Echo Acoustic Liquid Handler is capable of transferring nL volumes to screen cell-free reactions with a total volume of 1 μ L, a limitation of the Myra and OT-2 robots is the minimum dispensing volume of 1 μ L.

Although this proof-of-concept study is limited to two ARGs from distinct families, future work can utilize this accessible, semi-automated workflow to perform high-throughput characterization of ARGs as a tool for global AMR assessment and mitigation. Whilst cell-free ARG characterization is constrained to intracellular mechanisms, this limitation enables the determination of activity and substrate specificity for each ARG, presenting an alternative to whole-cell assays in which resistance could be achieved through any combination of efflux pump expression, import mechanisms, target modifications, or multiple ARGs [6]. Additional intracellular mechanisms, such as target modifications of the RNAP and ribosome complex, may also be explored in with cell-free, as was previously shown with *Klebsiella pneumoniae* [26].

Recent advancements with one-pot mutagenesis and linear DNA template expression in CFE systems could be employed for rapid phenotypic quantification of novel ARG activity with critical applications in healthcare [16, 27–29]. Understanding substrate specificity of ARGs is also important for new antibiotic discovery, especially where drugs are being designed to evade known enzymatic modification, for example in resistance-breaker approaches, or for inhibitors targeting ARGs [30–33]. A recent study found that sudden gain of tobramycin resistance in pathogens causing lung infections in human patients was largely due to the plasmid-mediated transfer of an aminoglycoside *N*-acetyltransferase (*aac(3)-IIIId*) that had not been previously described as a cause of clinical resistance [34]. Our cell-free approach offers a method to efficiently assess substrate specificity of genes, such as *aac(3)-IIIId*, as they emerge to improve AMR surveillance and patient treatment options.

Materials and methods

Cell-free lysate preparation

Cell-free extracts of *E. coli* BL21 Star™ (DE3)pLysS were prepared according to a previously published workflow [35]. Briefly, cells were streaked and grown on an LB agar plate overnight. A loopful of colonies was collected with a sterile loop and used to inoculate a 1-L culture of CFAI media (86 mM NaCl, 44 mM KH₂PO₄, 80 mM K₂HPO₄, 2.8 mM D-glucose, 11 mM D-lactose, 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.6% (v/v) glycerol, pH adjusted to 7.2 with KOH) in a 2-L glass baffled flask. The culture was incubated overnight at 30°C whilst shaking at 200 rpm. Cells were cooled on ice, pelleted (5000 $\times g$, 10 min, 4°C), and resuspended in S30 buffer (10 mM Tris acetate pH 8.2, 14 mM Magnesium acetate tetrahydrate, 60 mM Potassium acetate, 2 mM dithiothreitol). Cells were pelleted again, and the supernatant was removed before resuspending the pellet in S30 buffer at a 1:1 (w/v) ratio. Extracts were lysed under the following sonication settings: 10 s pulse on, 10 s pulse off at 55%–65% amplitude for an approximate active sonication time of 4 min. Lysed cells were centrifuged (15 000 $\times g$, 10 min, 4°C), and the supernatant was collected, aliquoted, and flash frozen in dry ice. A Bradford assay was used to estimate total protein concentration with Pierce™ Bradford Protein Assay Kit (Thermo Scientific) using bovine serum albumin as a standard, and extracts were diluted in S30 buffer to ~25–35 mg/mL for CFE.

CFE

E. coli lysate-based cell-free reactions were comprised of the following: 248 μ M CoA, 320 μ M NAD, 731 μ M cAMP, 65 μ M folinic acid, 926 μ M spermidine, 30 mM 3-PGA, 1.36 mM ATP, 1.36 mM GTP, 0.68 mM CTP, 0.68 mM UTP, 955 μ M amino acids (except for leucine at 796 μ M), 6 mM Mg-glutamate, 175 mM K-glutamate, 15 nM SP44-mScarlet-1 plasmid (Addgene #163756), 3% (w/v) PEG-8000, 48 mM Hepes buffer (pH 8.0), and ~10 mg/mL lysate [36]. Plasmids encoding ARGs were added at final concentrations of 10–15 nM. Gentamicin was suspended in nuclease-free H₂O (10 mg/mL stock), and rifampicin was suspended in DMSO (50 mg/mL stock). For serial dilutions of rifampicin, the maximum final concentration of DMSO in the cell-free reaction was 0.16% (range: 0.16–1.6 $\times 10^{-5}$ %), which was shown to not affect cell-free activity (Supplementary Fig. S1). Antibiotics were added at the specified concentrations after the specified pre-incubation period. A FLUOstar Omega (BMG Labtech) plate reader was used to measure

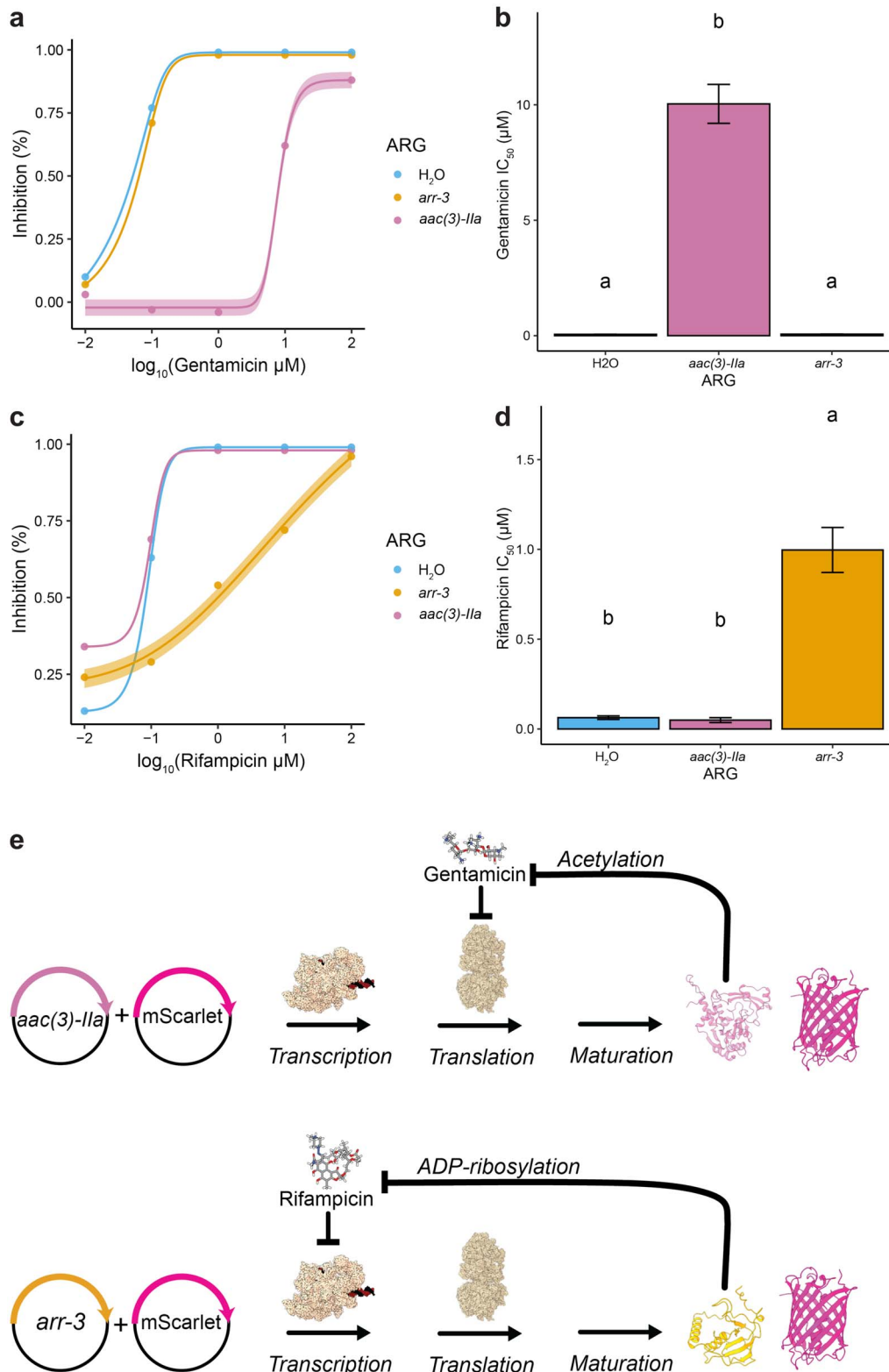


Figure 2 Cell-free quantification of *aac(3)-IIa* and *arr-3* activity. Cell-free expression of *aac(3)-IIa* and *arr-3* confers resistance to gentamicin and rifampicin, respectively. (a and c) Percent inhibition of mScarlet cell-free protein synthesis for gentamicin (a) and rifampicin (c) treatments with points representing the median values of three technical replicates. (b and d) The mean and standard error of the mean are shown across three independent replicates. A one-way ANOVA followed by TukeyHSD test indicates *aac(3)-IIa* mediates a statistically higher gentamicin IC_{50} value compared to *arr-3* and H_2O controls (b) and that *arr-3* mediates a statistically higher rifampicin IC_{50} value compared to *aac(3)-IIa* and H_2O controls (d). (e) Schematic showing hypothesized inhibitory mechanisms in cell-free. Drawings not to scale.

relative mScarlet fluorescence over 16 h at 30°C, with double orbital shaking at 200 rpm between readings, and 584 nm/620–10 nm excitation/emission settings.

Automated liquid handling

Unless otherwise noted, pipetting was performed with the Bio Molecular Systems (BMS) Myra model using the scripting interface (BMS Workbench v1.2.0). For 15 μ L reactions, ARG plasmids were first added (120–180 nM 1.25 μ L), followed by the cell-free reaction mixture with or without the mScarlet marker plasmid as specified (10–11.25 μ L). After incubation at 30°C with shaking at 200 rpm for 0–120 min, antibiotics, which were pre-mixed with mScarlet at a 1:1 ratio if specified, were added to each well (1.25–2.5 μ L). R scripts for generating the indexed sample input file and Myra-readable scripts to assign specified ARGs and antibiotics to each well are available on Github (https://github.com/Mo42-mb/Myra_automation.git).

mScarlet standardization

Purified mScarlet standards were used to convert relative fluorescence to μ M concentration. For purification, *E. coli* BL21 Star™ (DE3)pLysS carrying pET-15b-6xHis-mScarlet-I (Addgene # 255536) was grown in 500 mL LB with 100 μ g/mL carbenicillin at 37°C, 200 rpm to an OD₆₀₀ of 0.6, induced with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG), and incubated at 16°C, 200 rpm for 16–18 h. Cells were harvested (5000 $\times$ g, 5 min, 4°C) and resuspended in binding buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 10 mM imidazole, EDTA-free Pierce™ Protease Inhibitor (Thermo Scientific) per manufacturer's instructions) in a 1:10 (w/v) ratio. Cells were lysed by sonication and clarified (15 000 $\times$ g, 10 min, 4°C). The clarified lysate was purified by gravity-flow Ni²⁺-Immobilized Metal Affinity Chromatography (IMAC) by loading on a 20-mL equilibrated column with Ni²⁺-charged Chelating Sepharose™ Fast Flow resin (Cytiva). Buffers with consistent Tris–HCl and NaCl compositions but increasing imidazole concentrations were used to wash (30 mM then 70 mM imidazole) and elute (400 mM imidazole) proteins. mScarlet was quantified by absorbance at 280 nm using an extinction coefficient predicted by ProtParam (ExPASy). Relative fluorescence units (RFU) values were recorded for a standard series (0–8 μ M) with three technical replicates and three independent replicates, and a linear regression (Adjusted R² = 0.9999) was used to convert all experimental RFU values to μ M concentration (Supplementary Fig. S2).

Statistical analyses

All analysis was performed in R. For each independent replicate, the median of three technical replicates was calculated to reduce the effect of outliers. Data was evaluated for outliers, normality, and homogeneity of variances prior to performing Welch's t-tests with Benjamini & Hochberg correction (rstatix; [10.32614/CRAN.package.rstatix](https://doi.org/10.32614/CRAN.package.rstatix)) or a one-sided ANOVA followed by a TukeyHSD test (α = 0.05).

IC₅₀ characterization

Percent inhibition was calculated as the difference between the mScarlet concentration for the dH₂O control and the mScarlet concentration under the specified antibiotic treatment, divided by the mScarlet concentration of the dH₂O control. N-parameter regressions were calculated and visualized with the nplr package in R, with the

goodness of fit for each prediction being at least 0.992 ([10.32614/CRAN.package.nplr](https://doi.org/10.32614/CRAN.package.nplr)).

Plasmids

ARG plasmids were developed with EcoFlex Golden Gate Assembly using the J23100 promoter, pET RBS, BB0012 terminator, and pTU1-B backbone [37]. Coding sequences of *aac(3)-IIa* (Addgene #255535) and *arr-3* (Addgene #255534) expression vectors were derived from NCBI $\times$ 13543 and NCBI AJ277027, respectively, and *Klebsiella pneumoniae* codon-optimized. All plasmids used for CFE were purified using a QIAGEN Plasmid Midi Kit. DNA was resuspended in 1:1 (v/v) nuclease-free H₂O: isopropanol with 149 mM sodium acetate and incubated at –20°C overnight. DNA was pelleted (10 000 $\times$ g, 2 min), washed three times with 70% ethanol, vacuum-dried (~10 min, 60°C), and resuspended in nuclease-free H₂O. Concentration was measured using a Qubit Fluorometer.

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Author contributions

Molly Bergum (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing—original draft, Writing—review & editing), Sahana Suthakaran (Methodology), Bethany K. Martin (Investigation, Writing—review & editing), J Mark Sutton (Conceptualization, Funding acquisition, Project administration, Writing—review & editing), and Simon J Moore (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing—review & editing)

Supplementary data

Supplementary data is available at SYN BIO online.

Conflict of interest disclosure

No potential conflict of interest was reported by the authors.

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Data availability

All data generated or analyzed during this study is provided on Figshare (doi:[10.6084/m9.figshare.32055030](https://doi.org/10.6084/m9.figshare.32055030)).

Supporting information

DMSO concentrations used in cell-free reactions do not statistically impact *E. coli* extract productivity (Supplementary Fig. S1).

Quantification of mScarlet with purified standards (Supplementary Fig. S2).

Material availability statement

All plasmids used in this work have been deposited on Addgene (#163756, #255534-6).

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