

Resistance against aminoglycoside antibiotics via drug or target modification enables community-wide antiphage defense

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Abstract

The ongoing arms race between bacteria and phages has forced bacteria to evolve a sophisticated set of antiphage defense mechanisms that constitute the bacterial immune system. In our previous study, we highlighted the antiphage properties of aminoglycoside antibiotics, which are naturally secreted by *Streptomyces*. Successful inhibition of phage infection was achieved by addition of pure compounds and supernatants from a natural producer strain emphasizing the potential for community-wide antiphage defense. However, given the dual functionality of these compounds, neighboring bacterial cells require resistance to the antibacterial activity of aminoglycosides to benefit from the protection they confer against phages. In this study, we tested a variety of different aminoglycoside resistance mechanisms acting via drug or target (16S rRNA) modification and demonstrated that they do not interfere with the antiphage properties of the molecules. Furthermore, we confirmed the antiphage impact of aminoglycosides in a community context by coculturing phage-susceptible, apramycin-resistant *Streptomyces venezuelae* with the apramycin-producing strain *Streptoalloteichus tenebrarius*. Given the prevalence of aminoglycoside resistance among natural bacterial isolates, this study highlights the ecological relevance of chemical defense via aminoglycosides at the community level.

Keywords: phage; infections; aminoglycoside; resistance; strains; aminoglycosides

Introduction

Owing to the constant threat of viral predation exerted by bacteriophages (or phages), bacteria have evolved an extensive repertoire of antiphage defense mechanisms. In recent years, advancements in high-throughput bioinformatics and experimental approaches have expanded our understanding of bacterial antiphage defense to over a hundred different systems, unveiling an unexpectedly rich portfolio of systems and mechanisms (Georjon and Bernheim 2023). While previous knowledge of antiphage mechanisms was largely confined to restriction-modification and CRISPR-Cas systems targeting invading nucleic acids, recent discoveries have exposed remarkably intricate bacterial immune strategies. These include systems that rely on intracellular signal transduction facilitated by cyclic (oligo-)nucleotides and systems recognizing conserved structural patterns in viral proteins or RNAs to initiate immune responses (Cohen et al. 2019, Ofir et al. 2021, Tal et al. 2021, Gao et al. 2022, Tal and Sorek 2022). Recent studies also revealed several mechanisms that are shared among cells within a microbial community. These communal defenses encompass the extrusion of membrane vesicles serving as a phage decoy, quorum sensing-based activation of defense systems, biofilm formation, and secretion of antiphage small molecules (Luthe et al. 2023).

Soil-dwelling bacteria of the genus *Streptomyces* are sophisticated producers of bioactive compounds endowed with antibacterial, antifungal, or even anticancer properties (Barka et al. 2016). The natural producers usually possess self-resistance mechanisms to prevent autotoxicity (Hopwood 2007), thereby conferring them a fitness advantage over other microorganisms (Tyc et al. 2017). Shortly after their discovery, bacterial secondary metabolites emerged as a pivotal breakthrough to combat bacterial and fungal infections or applications in chemotherapy (Al-Shaibani et al. 2021). Their production is usually closely linked to the multicellular life cycle of *Streptomyces*. When conditions are favorable, spores germinate and develop into a vegetative mycelium in response to adverse conditions, such as nutrient deprivation, *Streptomyces* sacrifices parts of this vegetative mycelium via programmed cell death, supplying nutrients for the erection of aerial hyphae, which further differentiate into chains of spores. Typically, this developmental switch to reproductive growth aligns with the onset of secondary metabolite production, presumably to protect released nutrients from competing soil organisms (Flärdh and Buttner 2009, McCormick and Flärdh 2012, Schlimpert and Elliot 2023). Interestingly, recent studies indicate that bacterial small molecules also exhibit antiphage activity (Hardy et al. 2023). The *Streptomyces*-derived, naturally secreted anthracyclines doxo- and daunorubicin, which are primarily recognized as an-

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ticancer drugs, have been shown to efficiently inhibit infection of dsDNA phages, presumably by specifically intercalating into phage DNA (Kronheim et al. 2018). In our previous report, we expanded the repertoire of known antiphage molecule classes by demonstrating the antiphage properties of aminoglycoside antibiotics in widely divergent bacterial hosts (Kever et al. 2022, Hardy et al. 2023).

Aminoglycosides are bactericidal antibiotics that inhibit protein translation by binding to the 16S rRNA of the 30S ribosomal subunit (Krause et al. 2016). While different aminoglycosides exhibit varying specificity for distinct regions on the ribosomal A-site, they all induce a conformational change leading to the promotion of mistranslation by inducing codon misreading upon the delivery of aminoacyl transfer RNA. Despite the initial recognition of their antiphage properties as early as the 1960s (Brock et al. 1963, Schindler 1964, Bowman 1967), the exploration of these features in the context of bacterial antiphage immunity was not further pursued. In-depth analysis of this observation using aminoglycoside-resistant strains revealed a strong inhibition of phage infection by addition of pure apramycin as well culture supernatant (=spent medium) of the natural apramycin producer *Streptoalloteichus tenebrarius* (suborder *Pseudonocardineae*, order *Actinomycetales*) (Tamura et al. 2008), hinting toward an ecological relevance of such chemically mediated antiphage defense via aminoglycosides. Their secretion into the environment could create an antiviral microenvironment, thereby providing a chemical defense against phages at the community level (Hardy et al. 2023, Luthe et al. 2023). However, in order to benefit from this, neighboring cells or mycelial structures must possess resistance mechanisms to counteract the antibacterial impact of the compound without abolishing the antiphage effect. For aminoglycosides, three main resistance mechanisms are currently known including aminoglycoside-modifying enzymes (AME) for drug modification, 16S rRNA methyltransferases for target site modification, and increased export or decreased uptake (Garneau-Tsodikova and Labby 2016). Recent investigations reveal the widespread distribution of AMEs across different continents and terrestrial biomes, wherein ~25% of sequenced bacteria carrying at least one AME (Pradier and Bedhomme 2023). This distribution is expected to be closely correlated with the high mobility of AMEs. Remarkably, ~40% of the detected AMEs are located on mobile genetic elements, facilitating their acquisition and broadening of the resistance spectrum via horizontal gene transfer (Pradier and Bedhomme 2023). Additionally, they are often carried by phage plasmids, allowing them to be easily transferred across bacteria upon infection without direct cell–cell contact (Pfeifer et al. 2022).

In this study, we systematically examined the influence of various resistance mechanisms, encompassing a set of AMEs and a 16S rRNA methyltransferase, on the antiphage properties of aminoglycosides. Remarkably, our results demonstrated that diverse aminoglycoside resistance mechanisms acting through drug modification as well as target site modification did in fact abolish the antibacterial properties, while the antiphage activity was retained. This evidence provides the prerequisite for making a community-wide antiphage defense, even on an interspecies level, conceivable.

Material and methods

Bacterial strains and growth conditions

All resistance cassettes, bacterial strains and phages used in this study are listed in Tables S1 and S2. For *Streptomyces venezuelae*, precultures for growth and infection assays were in-

oculated from spore stocks in glucose–yeast extract–malt extract (GYM) medium containing 50% tap water, pH 7.3 and cultivated at 30°C and 120 rpm for 18 h. Precultures were subsequently used to inoculate main cultures in the same medium to the indicated OD₄₅₀. For *Escherichia coli*, cultures were inoculated from a single clone grown on LB agar plates and cultivated at 170 rpm and 37°C O/N in LB medium for being subsequently used to inoculate main cultures to the indicated OD₆₀₀. In all cases, antibiotics were added to cultures as indicated.

For standard cloning procedures and protein overproduction *E. coli* DH5 α and *E. coli* BL21 (DE3) were used, respectively. Infection assays with phage λ were conducted in *E. coli* LE392 and conjugation between *Streptomyces* spp. and *E. coli* was done via the conjugative *E. coli* ET12567/pUZ8002.

In order to determine the titer of plaque-forming units during infection assays, spot assays were conducted. To this end, 2 μ l of decimal dilution series of culture supernatant in sodium chloride/magnesium sulfate buffer (10 mM Tris–HCl pH 7.3, 100 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂) were spotted on a bacterial lawn propagated on a double-agar overlay with the top layer inoculated to an optical density of OD₄₅₀ = 0.3 and OD₆₀₀ = 0.2 for *S. venezuelae* and *E. coli*, respectively. Plaque assays with *S. venezuelae* were done in a comparable manner by adding 10³ PFU/ml of phage Alderaan to GYM soft-agar inoculated with mycelium to an OD₄₅₀ of 0.3. Plaque areas were analysed using ImageJ and the color thresholding tool (Schneider et al. 2012). To this end, images were cropped and the color threshold settings were adjusted to select plaques as accurately as possible. Subsequently, plaque areas were measured using the “analyse particle” option with the following parameters: size (pixel²): 40 to infinity, circularity: 0.1–1.0.

Recombinant DNA work and cloning

All plasmids and oligonucleotides used in this study are listed in Tables S3 and S4. Standard cloning techniques such as polymerase chain reaction (PCR), restriction digestion with indicated restrictions enzymes and Gibson assembly were performed according to standard protocols (Sambrook and Russell 2001, Gibson 2011). DNA sequencing as well as synthesis of oligonucleotides and (codon-optimized) genes was performed by Eurofins Genomics (Ebersberg, Germany).

Phage infection curves

Growth assays and phage infection curves were performed in the BioLector microcultivation system [Beckman Coulter Life Sciences (formerly m2p-labs), Krefeld, Germany] using a shaking frequency of 1200 rpm and cultivation temperature of 30°C. Biomass was measured as a function of backscattered light intensity with an excitation wavelength of 620 nm (filter module: $\lambda_{Ex}/\lambda_{Em}$: 620 nm/620 nm, gain: 25) from three independent biological replicates.

Cultivation of *S. venezuelae* was performed in 1 ml GYM medium (pH 7.3, 50% tap water) inoculated with an overnight culture to a starting OD₄₅₀ of 0.15. Infection was conducted by supplementing the indicated initial phage titers to the respective wells. Culture supernatants were collected in specific time intervals to monitor phage amplification over time via double-agar overlay assays.

Cocultivation in submerged cultures

Streptoalloteichus tenebrarius strains were inoculated from mycelial stocks in 5 ml GYM medium (pH 7.3, 50% tap water) and cultivated at 170 rpm and 30°C for 24 h. Subsequently, these precultures were used to inoculate 20 ml GYM medium (pH 7.3, 50% tap water) to an initial OD₄₅₀ of 0.2. Cultures were incubated for 2

days at 120 rpm and 30°C to allow production and secretion of apramycin or its biosynthesis intermediates. One day prior to the cocultivation assay, *S. venezuelae* NRRL B-65442- pIJ10257-*kamB* was inoculated from spores in 5 ml GYM medium (pH 7.3, 50% tap water) and cultivated at 170 rpm and 30°C for 18 h. For cocultivation, 1 OD unit of *S. tenebrarius* mycelium was mixed with 1 OD unit of *S. venezuelae* mycelium in 1 ml GYM medium (pH 7.3, 50% tap water). Cultivation was performed in the BioLector microcultivation system as described in “Phage infection curves” in biological triplicates. Phage infection was performed by addition of 10⁸ PFU/ml Alderaan particles. As comparison, mycelium of *S. tenebrarius* was either directly transferred to cocultivations or washed twice beforehand to remove produced metabolites.

Preparation of culture supernatants (referred to as spent media)

In order to collect spent medium of the natural apramycin producer *S. tenebrarius* and respective biosynthesis mutants, all strains were cultivated as described in “Cocultivation in submerged cultures” using a final cultivation volume of 70 ml GYM medium (pH 7.3, 50% tap water). Culture supernatants were harvested by centrifugation at 5000 × *g* at 4°C for 20 min and subsequently sterile filtered before storage at 4°C for subsequent usage in infection assays.

Infection assays in spent medium

Infection assays of apramycin-resistant *S. venezuelae* NRRL B-65442-pIJ10257-*kamB* in spent medium were conducted in the BioLector microcultivation system (Beckman Coulter Life Sciences) as described in “Phage infection curves” with the following modifications: 1.25x GYM medium (pH 7.3, 50% tap water) was supplemented with spent medium to a final concentration of 20% (v/v).

Purification of biosynthesis intermediates

Paromamine and oxyapramycin were generated from Δ *aprD4* mutant WDY288 (Lv et al. 2016). Acetylated apramycin and acetylated pseudotetrasaccharide were generated from Δ *aprP* mutant WDY321 (Zhang et al. 2021). The two mutant strains were cultivated on SPA medium (2% soluble starch, 0.1% beef extract, 0.05% MgSO₄, 0.1% KNO₃, 0.05% NaCl, 0.05% K₂HPO₄, 2% agar) at 37°C for spore production. Their seed culture was prepared in 5 ml TSBY medium (3% tryptone soya broth and 0.5% yeast extract) at 37°C with shaking at 220 rpm for 2 days. The seed culture of Δ *aprD4* and Δ *aprP* were subcultured into 50 ml SPC fermentation medium (4% glucose, 1% peptone, 0.4% soybean meal, 1% corn meal, 0.4% MgSO₄·7H₂O, 0.5% NH₄Cl, 0.05% FeSO₄, 0.03% MnCl₂, 0.003% ZnSO₄, 0.5% CaCO₃) at 37°C with shaking at 220 rpm for 7 days. The culture supernatant was collected by centrifugation at 5000 rpm for 30 min. Afterwards, the supernatant was adjusted to a pH of 2–3 with saturated oxalate and the insoluble fraction was removed by centrifugation at 5000 rpm for 30 min. Next, the supernatant was loaded onto the 732 cation exchange resin (Hebi Juxing Resinco., Ltd, Hebi, China), before being eluted by 3% ammonia hydroxide solution. Finally, the ammonia hydroxide eluate was purged with a nitrogen stream.

The filtered eluate was concentrated and subjected to a semipreparative HPLC System equipped with Evaporative Light Scattering Detector (ELSD, Alltech 2000ES) and an Agilent ZORBAX SB-C18 column (5 μm, 250 mm × 9.4 mm). Gradient elution was performed at a flow rate of 3.0 ml/min. The gas flow and temperature of ELSD was set to 2.9 l/min and 109°C. Gradient elution was performed at a flow rate of 3 ml/min with solvent A (water con-

taining 10 mM heptafluorobutyric acid) and solvent B (CH₃CN): 0–3 min, constant 80% A/20% B; 3–5 min, a linear gradient to 75% A/25% B; 5–9 min, a linear gradient to 70% A/30% B; 9–17 min, a linear gradient to 59% A/41% B; 17–18 min, a linear gradient to 80% A/20% B; and 18–25 min, constant with 80% A/20% B. The eluent containing the target components was collected and subsequently dried through lyophilization.

Protein purification via affinity chromatography

For heterologous protein overproduction, *E. coli* BL21 (DE3) cells containing C-terminal Strep-tagged versions of the respective resistance genes on the pAN6 plasmid were precultivated in LB medium supplemented with 50 μg/ml kanamycin (LB Kan₅₀) at 37°C and 120 rpm. The preculture was used to inoculate the main culture in the same medium to an OD₆₀₀ of 0.1 and cultivation was continued at 37°C and 120 rpm until gene expression was induced with 100 μM IPTG at an OD₆₀₀ of 0.6. Cells were harvested after further cultivation at 120 rpm and 18°C for 16 h.

Cell harvesting and disruption were performed with the multi shot cell disruptor at 20000 psi using buffer A (100 mM Tris-HCl, pH 8.0) with cOmplete™ Protease inhibitor (Roche, Basel, Switzerland) for cell resuspension and disruption. After centrifugation at 20000 × *g* and 4°C for 30 min, buffer B (100 mM Tris-HCl, 500 mM NaCl, pH 8.0) was used for purification.

To this end, supernatant containing either the C-terminal Strep-tagged ApmA or the AAC(3)-IVa apramycin acetyltransferase was applied to an equilibrated 2 ml Strep-Tactin-Sepharose column (IBA, Göttingen, Germany). After washing with 30 ml buffer B, the protein was eluted with 5 ml buffer B containing 15 mM D-desthiobiotin (Sigma Aldrich, St. Louis, USA). After purification, elution fractions with the highest protein concentrations were pooled and checked by SDS-PAGE (Laemmli 1970) using a 4%–20% Mini-PROTEAN gradient gel (BioRad, Munich, Germany). Final protein concentration of the pooled elution fraction was determined with the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) before being used for in vitro acetylation.

In vitro acetylation of apramycin

Purification of the two acetyltransferases AAC(3)-IVa and ApmA was performed as described above. Acetylation of apramycin was done as described in Kevery et al. (2022) with the following modifications: assay mixtures were composed of 350 μl 100 mM Tris-HCl (pH 8.0), 50 μl purified enzyme (end concentration: 30 μg/ml), 50 μl apramycin (end concentration: 10 mM) and 50 μl acetyl-CoA trisodium salt (Sigma Aldrich; end concentration: 10 mM). The assay mixtures were incubated at 37°C for 30 min. Reactions lacking acetyl-CoA trisodium salt as a cofactor served as controls.

Direct injection mass spectrometry

For checking acetylation efficiency, direct injection mass spectrometry was performed. To this end, samples were diluted 1:100 (v/v) with deionized water and further 1:10 (v/v) with 50% methanol containing 0.1% acetic acid. The individual samples were directly injected into the HESI-source of a QExactive Plus mass spectrometer (Thermo Fisher) using a 500 μl syringe with a constant flow of 5 μl/min. The source parameters were set as follows: spray voltage (+): 3200 V, capillary temperature: 320.00°C, sheath gas: 8.0, aux gas: 2.0, S-Lens RF Level: 50.00. Once the spray stabilized data were acquired for 2 min in positive mode with a full scan resolution of 70000 and an AGC target of 3e6. Representative spectra data were retrieved from mid run scans for each sample.

Results

Impact of drug and target site modification of aminoglycosides on their antibacterial and antiphage activity

Natural producers of secondary metabolites typically exhibit resistance to the antimicrobial molecules they synthesize (Hopwood 2007, Tenconi and Rigali 2018). This characteristic gains significance, particularly in the screening of small molecules for antiviral properties, as potential toxic effects on bacterial growth could mask any observed inhibition of phage infection. In a previous study, we showed the inhibition of phage infection for a broad range of aminoglycoside antibiotics by using strains resistant to the respective molecules (Kever et al. 2022). In case of the used model aminoglycoside apramycin belonging to the subclass of aminoglycosides with mono-substituted 2-desoxystreptamin (2-DOS) ring (Krause et al. 2016), this resistance mechanism relied on the acetylation of the 3' amino group of the 2-desoxystreptamin ring via aminoglycoside N(3)-acetyltransferase AAC(3)-IVa (Magalhaes and Blanchard 2005) (Fig. 1A). In this study, we aimed to investigate the impact of diverse bacterial (self-) resistance mechanisms on the antiphage properties of different aminoglycoside antibiotics. Our goal was to discern whether the observed decoupling of antibacterial and antiphage properties through resistance mechanisms is a pervasive trait among different mechanisms found in bacterial genomes.

As already shown in Kever et al. (2022), infection in absence of apramycin results in a complete culture collapse and a progressive phage amplification over time, whereas no more growth defect and increase in extracellular phage titer was detectable upon apramycin treatment for the virulent phage Alderaan infecting *S. venezuelae* (Fig. 1B and C).

To investigate the antiphage properties of the unmodified compound, the 16S rRNA methyltransferase KamB encoded in the apramycin biosynthesis cluster of the natural producer *S. tenebrarius* was harnessed as an alternative resistance mechanism (Holmes et al. 1991). This methyltransferase catalyses the N1-methylation of the 16S rRNA at position A1408 conferring high-level resistance to the structurally divergent aminoglycosides apramycin, kanamycin, and tobramycin (Koscinski et al. 2007) (Fig. 1D). As observed for drug modification, target site modification abolished the antibacterial mode of apramycin, but simultaneously allowed a complete inhibition of phage infection to an almost identical extent as AAC(3)-IVa (Fig. 1E and F). The same applied to the other tested aminoglycosides tobramycin and gentamicin or kanamycin and tobramycin, which are also covered by the resistance spectrum of AAC(3)-IVa and KamB, respectively (Fig. 1G, Figure S1). Although these compounds share the 2-desoxystreptamin (2-DOS) core structure with apramycin, they differ structurally in their substitutions and belong to the class of 4,6-disubstituted 2-DOS (Krause et al. 2016).

For resistance via acetyltransferase AAC(3)-IVa or methyltransferase KamB, analogous results concerning the influence of aminoglycosides were also obtained for infection of *E. coli* with the temperate phage λ , which has previously been demonstrated to be sensitive toward apramycin and kanamycin treatment (Figure S2) (Kever et al. 2022). Overall, this led us to the conclusion that acetylation neither positively nor negatively affects the antiphage activity of apramycin or tobramycin. Based on these findings, we infer that the inhibition of phage infection is most likely not due to a residual blockage of bacterial translation.

Different aminoglycoside modifications do not interfere with the antiphage properties of the molecules

To test whether this result is in fact of broad relevance, we tested different AMEs targeting different positions on the aminoglycoside scaffold. In case of apramycin, just two different AMEs are described in literature including the already used acetyltransferase AAC(3)-IVa and ApmA (Fig. 2A). ApmA reveals a unique regiospecificity by acetylating apramycin at the N2' position of the octadiose element (Bordeleau et al. 2021, Bordeleau et al. 2023).

Since the apramycin resistance level mediated by the acetyltransferase ApmA was reported to be more than 8-fold lower than for AAC(3)-IVa (Bordeleau et al. 2021), plaque assays offering higher resolution of the antiphage effect were used as a direct comparison of both modification positions. A gradual decrease in the number of plaques was detected upon increasing apramycin pressure independent of the underlying resistance gene (Fig. 2B and C). This was also in line with results gained during infection of *E. coli* with phage λ . As with apramycin, different modifications of kanamycin via phosphotransferases APH(3')-Ia and APH(2'')-IIa or the acetyltransferase AAC(6')-Ih did not interfere with the antiphage properties of this compound and caused a dose-dependent reduction in λ plaque formation. The same applied for modification of neomycin, an aminoglycoside with a 4,5-disubstituted 2-DOS, via APH(3')-Ia (Figure S2).

To further verify the observed effects with a focus on apramycin effects on phage Alderaan infecting *S. venezuelae*, both acetyltransferases (AAC(3)-IVa and ApmA) were purified by affinity chromatography and used for *in vitro* modification of apramycin. Successful acetylation was confirmed by mass spectroscopy (Figure S3A). When supplementing acetylated apramycin to infection assays with the *S. venezuelae* wild type strain not resistant to the antibacterial effect of apramycin, no or just a slight effect on bacterial growth could be detected for AAC(3)-IVa- and ApmA-mediated acetylation, respectively. Under infection conditions, both acetylated versions severely impacted phage infection as indicated by an omitted cell lysis and a drop in titer after 24 h of infection (Fig. 2D and E). However, time-resolved quantification of phage titers revealed differences between the influence of intracellularly and extracellularly acetylated apramycin, which is likely caused by differences in uptake compared to the unmodified molecules. In case of AAC(3)-IVa-acetylated apramycin, phage titers rose in the early stages of infection before falling below the starting level, whereas ApmA-acetylated apramycin allowed no initial increase and a more pronounced decrease in titer of ~1000-fold after 24 h. (Figure S3B). The higher sensitivity of phage infection to ApmA-acetylated apramycin was also observed when the modified apramycin versions were added to spot assays with phage Alderaan and the *S. venezuelae* wildtype strain (Figure S3C). To conclude, together with the results presented in the previous sections, drug and target site modification were shown to efficiently abolish the antibacterial properties while retaining the antiphage properties.

Dissecting the apramycin biosynthesis pathway reveals different intermediates with antiviral properties

Our previous study about the antiphage properties of aminoglycosides revealed that the effect of the pure compound apramycin could be reproduced with spent medium of the natural apramycin producer *S. tenebrarius* (Kever et al. 2022). To ascertain the role of apramycin as main antiphage molecule in the supernatant inter-

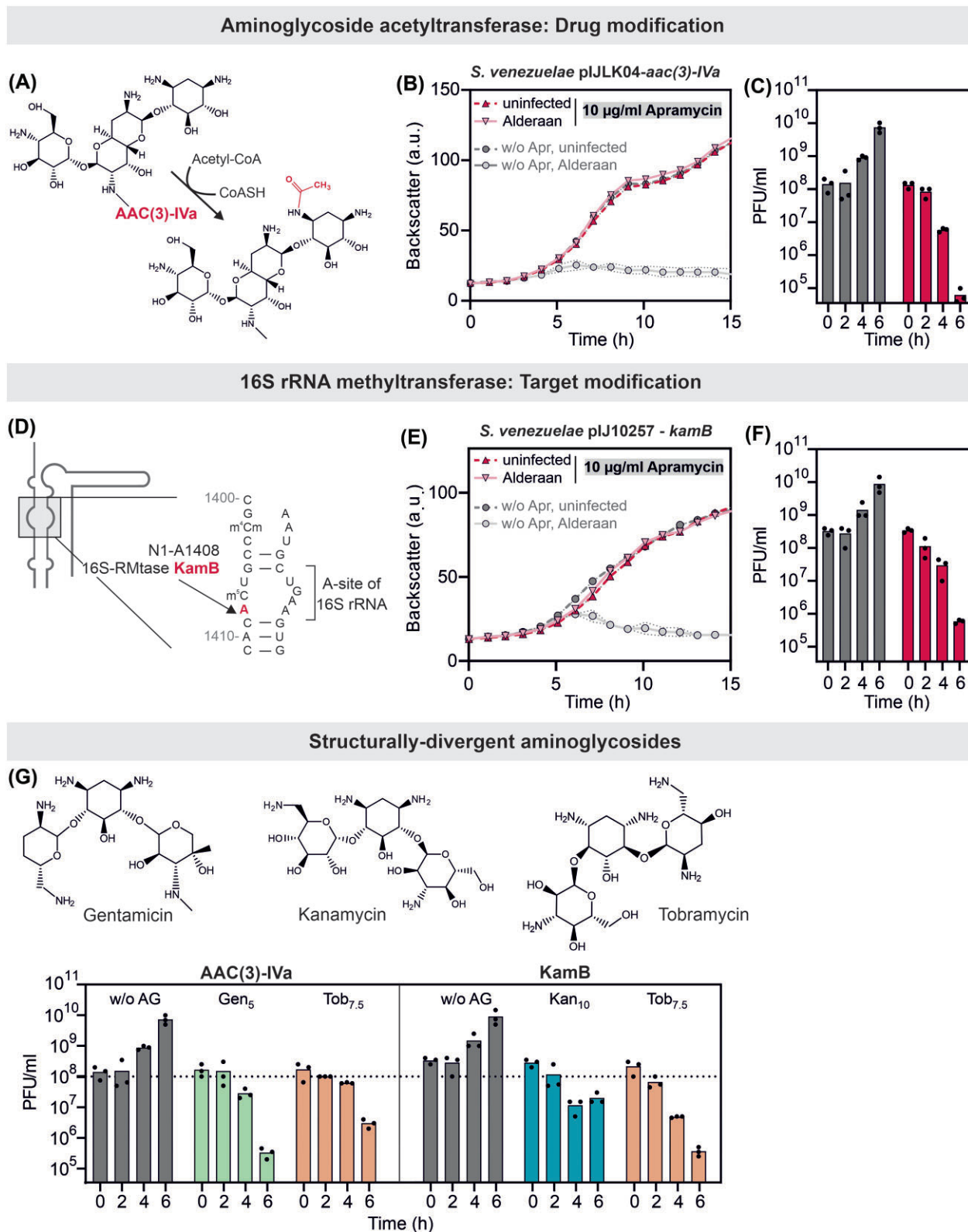


Figure 1. Aminoglycoside resistance mechanisms via drug and target site modification do not interfere with the antiphage properties of structurally divergent aminoglycosides. (A) Acetylation reaction of apramycin catalysed by AAC(3)-IVa. (B) Growth of *S. venezuelae* ATCC 10712-pJLK04-*aac(3)-IVa* upon infection with phage Alderaan in presence and absence of 10 µg/ml apramycin. (C) Time course of phage titers during Alderaan infection shown in (B). (D) A-site of 16S rRNA showing the methylation position (A1408) used by the methyltransferase KamB. Schematic illustration was designed according to Wachino and Arakawa (2012). (E) Growth of *S. venezuelae* NRRL B-65442-pJ10257-*kamB* upon infection with phage Alderaan in presence and absence of 10 µg/ml apramycin. (F) Time course of phage titers during Alderaan infection shown in (E). (G) Phage amplification during drug and target site modification via AAC(3)-IVa and KamB, respectively, in presence of gentamicin, kanamycin, and tobramycin. All infection assays were conducted with an initial phage titer of 10^8 PFU/ml in biological triplicates.

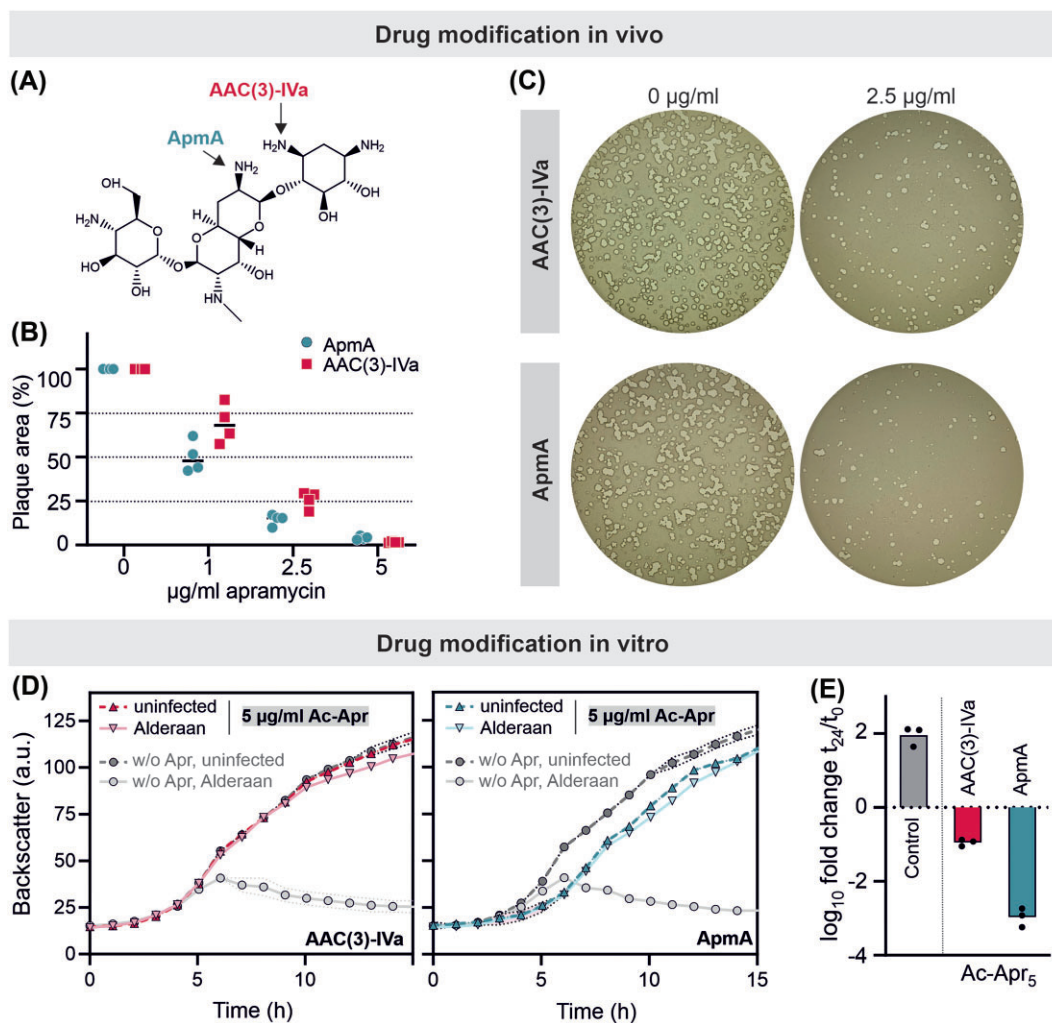


Figure 2. Influence of different acetylation positions on the antiphage properties of apramycin. (A) Acetylation position of the two apramycin acetyltransferases AAC(3)-IVa and ApmA. (B) Correlation between normalized percentage plaque area (Apr_x/Apr_0) and apramycin concentration upon infection of the two apramycin-resistant strains *S. venezuelae* NRRL B-65442 encoding either AAC(3)-IVa or ApmA. (C) Representative plaque assays to the data shown in (B). (D) Growth of *S. venezuelae* NRRLB-65442 wild type upon infection with phage Alderaan in presence and absence of 5 µg/ml AAC(3)-IVa- or ApmA-acetylated apramycin. (E) \log_{10} fold change in PFU/ml during infection shown in (D). Assays shown in (D) and (E) were performed in biological triplicates.

fering with phage infection and to determine the position in the biosynthetic pathway at which antiphage properties appear, we tested spent media obtained from different *S. tenebrarius* mutant strains lacking different enzymes in the biosynthetic pathway (Lv et al. 2016, Zhang et al. 2021) (Fig. 3A). To analyse the impact of these spent media on phage amplification, we harnessed the apramycin-resistant *S. venezuelae* strain encoding the 16S rRNA methyltransferase KamB to prevent further modification of the apramycin biosynthesis intermediates. Resistance via target site modification allowed complete inhibition of phage amplification by addition of *S. tenebrarius* wild type spent medium (Fig. 3B).

Deletion of *aprD4* encoding a putative Fe-S oxidoreductase interferes with apramycin biosynthesis by preventing C3 deoxygenation, thereby increasing production of the apramycin analogue oxyapramycin and paromamine (Lv et al. 2016). Supplementing of *S. tenebrarius* $\Delta aprD4$ spent medium to infection assays of the apramycin-resistant *S. venezuelae* strain carrying the methyltransferase KamB showed a similar extent of phage inhibition than the wild type spent medium. This could be traced back to the antiphage properties of oxyapramycin, as demonstrated

by the addition of the purified intermediates to infection assays (Fig. 3B and C, Figure S4A). Contrary to this, deletion of *aprU* encoding an aminoglycoside phosphotransferase and deletion of *aprP* encoding a putative creatinine amidohydrolase showed comparable amplification kinetics than the control infection without any supplementation, although slightly lower maximal phage titers were detected. This was in line with the successful phage infection upon addition of the acetylated demethylaprosamin and 7'-N-acetylated apramycin shown to accumulate in the *S. tenebrarius* $\Delta aprU$ and $\Delta aprP$ mutant, respectively (Fig. 3B and C) (Zhang et al. 2021). However, comparing phage titers after 24 h of infection exposed a significant reduction in plaque-forming units upon addition of acetylated apramycin reaching almost the starting level, which could not be observed for the acetylated pseudotrisaccharide or paromamine (Figure S4A). Interestingly, a similar trend could be observed for the spent medium of the $\Delta aprZ$ mutant lacking the extracellular alkaline phosphatase as final enzyme of the apramycin biosynthesis pathway (Zhang et al. 2021). Within the first 8 h, a similar extent of phage amplification was measured, but the decrease in titer after 24 h was substantially more pro-

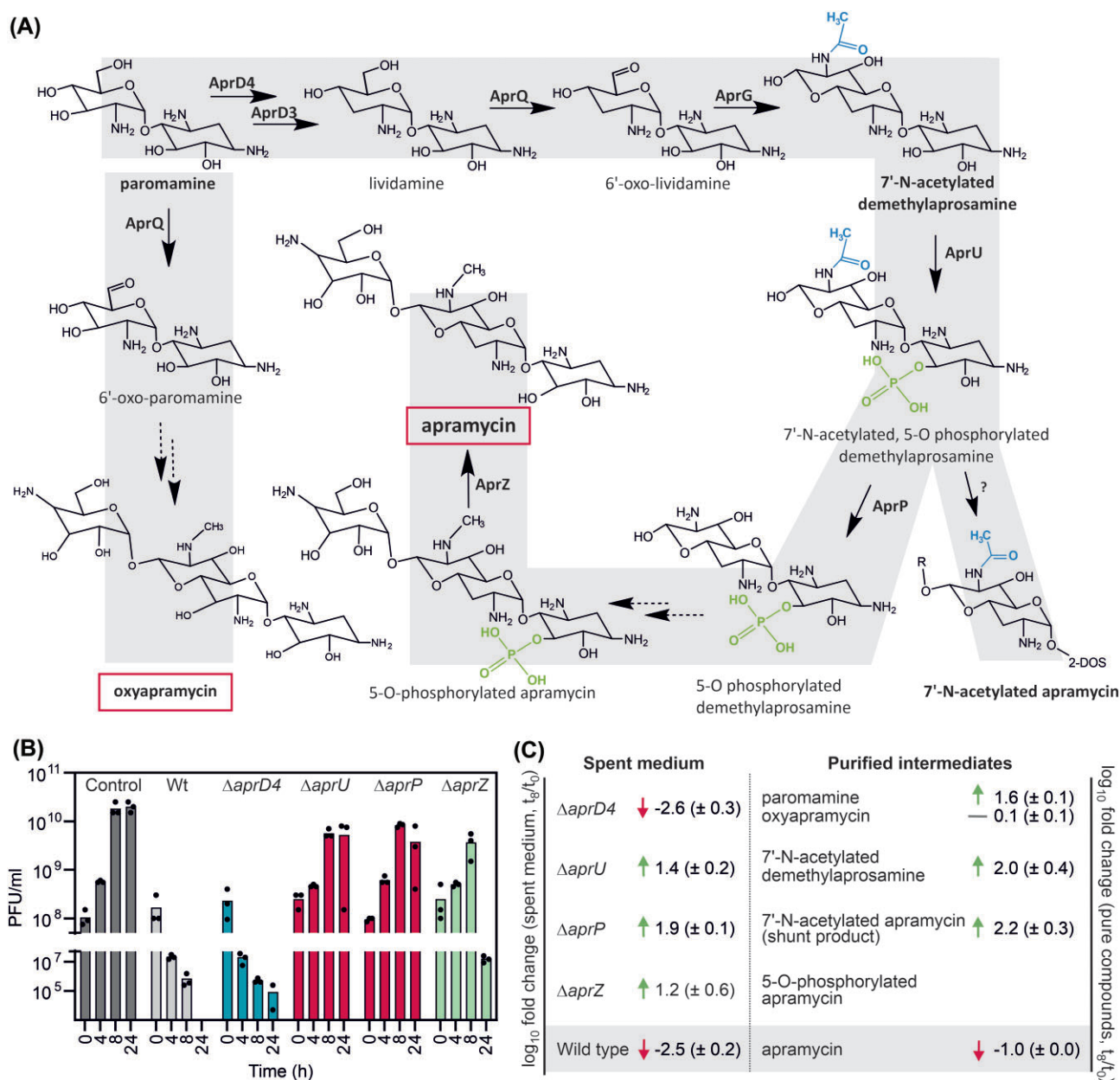


Figure 3. Influence of spent medium of different *S. tenebrarius* apramycin biosynthesis mutants on infection dynamics of phage Alderaan infecting *S. venezuelae* NRRL B-65442-pIJ10257-*kamB*. (A) Biosynthetic pathway of apramycin biosynthesis in *S. tenebrarius* (2-DOS: 2-deoxystreptamin ring) (Lv et al. 2016, Zhang et al. 2021, Fan et al. 2023). Compounds harboring antibacterial activity are marked with boxes. (B) Phage titers during phage infection in presence and absence of 20% of the indicated spent media. (C) Log₁₀ fold change (t_8/t_0) in PFU/ml calculated for infection in presence of 20% spent medium and 10 μ g/ml of purified biosynthesis intermediates assumed to accumulate in the different mutant strains. All assays were performed in biological triplicates.

nounced dropping from $\sim 4 \times 10^9$ PFU/ml at 8 h to $\sim 2 \times 10^7$ PFU/ml at 24 h. This was accompanied by a less pronounced growth defect under infection conditions (Fig. 3B and C, Figure S4B). Accordingly, intracellular formation of phosphorylated apramycin appears to prevent autotoxicity by deactivating the antibacterial properties of the compound (Zhang et al. 2021), but at the same time provides some degree of protection against phage infection for the producer already during apramycin biosynthesis. Subsequent extracellular dephosphorylation activates the dual functionality of apramycin, which can be hypothesized to confer community-wide protection for resistant cells in the same ecological niche. Based on the results obtained for the different mutant strains defective in apramycin biosynthesis, we conclude that the antiphage prop-

erties of apramycin and its intermediates emerge after the 7'-N'-acetylated demethylaprosamine step, since no antiphage properties were observed for mutants lacking AprU or AprP.

Cocultivation of *S. venezuelae* and *S. tenebrarius* confirms the antiphage impact of apramycin in the context of microbial communities

To mimic community-wide antiphage defense more properly, phage-susceptible *S. venezuelae* NRRL B-65442-pIJ10257-*kamB* was cocultured with apramycin-producing *S. tenebrarius*. In presence of the producer mycelium, which was precultivated for 2 days to allow apramycin biosynthesis, Alderaan was no longer able to propagate on *S. venezuelae* (Fig. 4A and B).

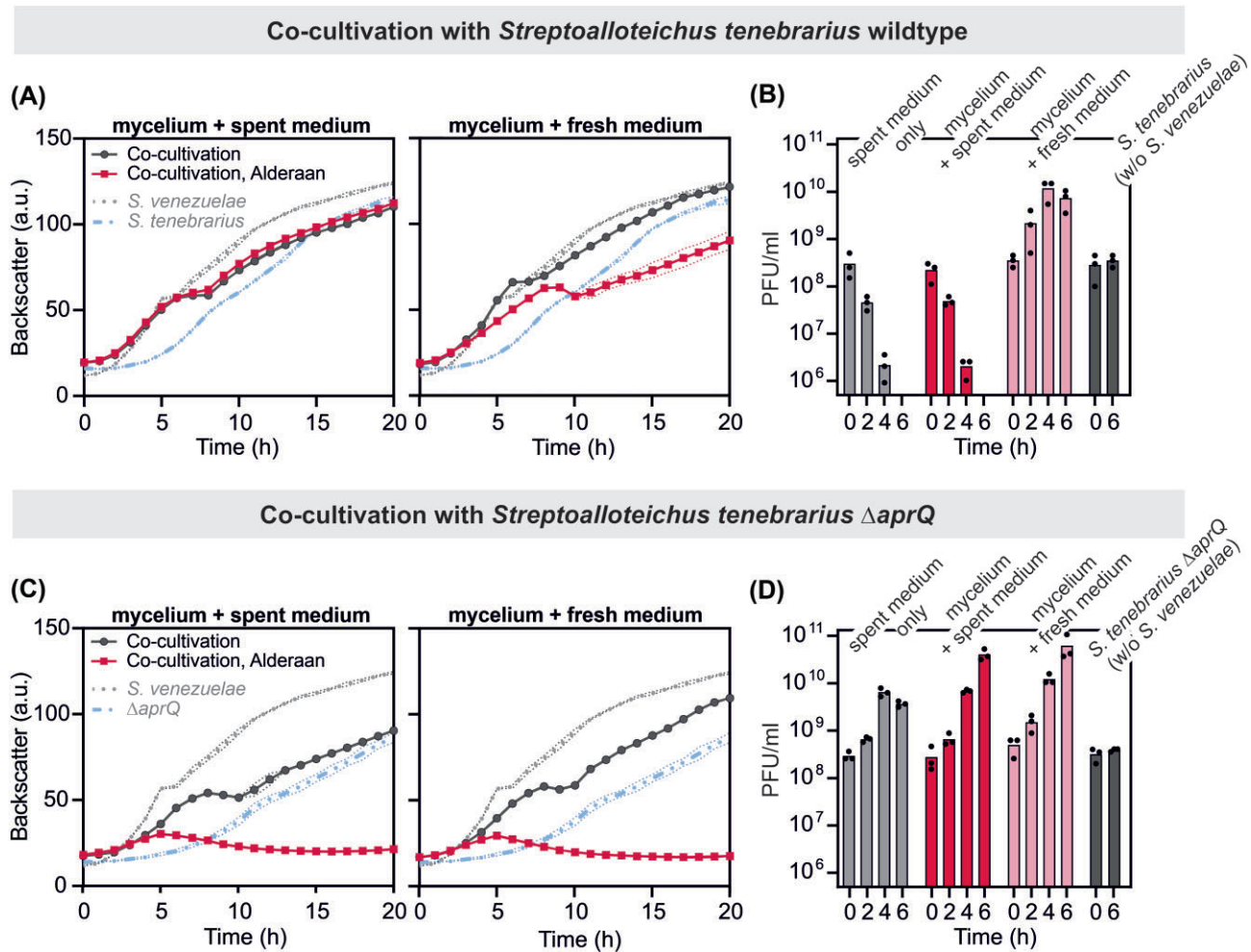


Figure 4. Cocultivation of *S. venezuelae* NRRL B-65442–pIJ10257–*kamB* with two different *S. tenebrarius* strains during Alderaan infection. (A) Infection curves during cocultivation of *S. venezuelae* with *S. tenebrarius* wild type directly transferred from preculture (+ spent medium) or after intensive washing to remove spent medium (+ fresh medium). Growth of *S. venezuelae* and *S. tenebrarius* separately from each other is shown for comparison. (B) Corresponding time course of phage titers during cocultivation and infection. Influence of *S. tenebrarius* spent medium on infection dynamics and *S. tenebrarius* itself on phage infectivity is added as control. (C) Analogous data set to (A) showing infection curves during cocultivation of *S. venezuelae* with *S. tenebrarius* $\Delta aprQ$. (D) Corresponding time course of phage titers during cocultivation and infection. All assays were performed in biological triplicates.

Interestingly, phage infection could be restored by cocultivating *S. venezuelae* with *S. tenebrarius* mycelium, which has previously been intensively washed to remove produced apramycin, while the simple addition of spent medium separated from mycelium was sufficient for phage inhibition. Furthermore, no negative effect of *S. tenebrarius* mycelium and apramycin production on extracellular Alderaan phage particles was detected, further supporting the previously published hypothesis of interference with phage infection at an intracellular level (Fig. 4A and B) (Kever et al. 2022). In contrast, continuous phage amplification and a complete culture collapse was observed in the presence of the *S. tenebrarius* $\Delta aprQ$ mutant, altogether ruling out general issues in phage amplification due to the presence of other cocultured bacteria. (Fig. 4C and D). However, it is striking that no more cell growth was observed upon phage infection, even though Alderaan is not able to infect *S. tenebrarius*. This might be due to the release of as yet unidentified growth-inhibiting molecules or proteins, potentially accounting for the suppression of *S. tenebrarius* growth. In addition to showing the protective effect of *S. tenebrarius* against viral predation of *S. venezuelae*, these results highlight the need for

a background level of apramycin already produced prior to phage attack.

Discussion

Aminoglycosides antibiotics were previously shown to be efficient antiphage agents in widely divergent bacterial hosts (Kever et al. 2022). However, a pivotal question emerged from this discovery: how does antibiotic resistance impact the antiviral capabilities of aminoglycosides? Resistance within this versatile antibiotic class predominantly arises through modifications either to the drug itself or to the bacterial target (16S rRNA). In this study, we screened a broad portfolio of aminoglycosides and resistance genes to evaluate the potential uncoupling of the dual functionality via antibacterial resistance. Regardless of the use of target site or drug modification, all tested resistance mechanisms ensure bacterial growth under infection conditions by retaining the antiphage mode and abolishing the antibacterial mode of action, as exemplified by the virulent phage Alderaan and the temperate phage λ .

As recently demonstrated by Pradier and Bedhomme (2023), aminoglycoside-resistance genes are widely distributed among all biomes, with the soil playing a pivotal role in AME gene transfer events. Natural ecosystems are considered to harbor a reservoir of antibiotic resistance genes, stemming from both, anthropogenic transfer of antibiotics and resistance genes as well as the presence of natural aminoglycoside producers such as *Streptomyces* and *Micromonospora* spp. residing in soil (Tripathi and Cytryn 2017, Durand et al. 2019). Gene transfer events are facilitated by the frequent localization of such resistance genes on mobile genetic elements, allowing broadening of the resistance spectrum (Pradier and Bedhomme 2023). Given the molecular versatility of aminoglycosides confirmed in this study, the acquisition of resistance becomes not only advantageous as defense against the chemical warfare exerted by the secretion of aminoglycosides, but could ensure concurrent protection from phages as already discussed in our previous review article (Hardy et al. 2023). The prevalence of aminoglycoside-resistant bacteria in natural habitats puts further emphasis on the ecological significance of such chemical antiphage defense. However, taken into account the predominance of subinhibitory antibiotic concentrations and the existence of local extremes in the soil environment (Fajardo et al. 2009), one can infer that such community-wide antiphage defense is constrained to mutualistic interactions within the genetic kin or between different species. The latter is thought to be facilitated by increased horizontal gene transfer events of aminoglycoside resistance genes in the presence of low antibiotic concentrations (Cairns et al. 2018). Overall, this would add to the notion of the pan-immune system describing antiphage defense as a shared community resource (Bernheim and Sorek 2020).

An additional aspect, which reinforces the role of aminoglycosides as “public goods” in antiphage defense, is the natural growth phenotype as well as the temporal onset and organization of antibiotic production in *Streptomyces* spp. These multicellular growing microorganisms are characterized by a complex developmental program, starting with the germination of a spore and the establishment of a vegetative mycelium, which further develops into aerial hyphae and spores upon nutrient depletion (Bush et al. 2015, Schlimpert and Elliot 2023). Antibiotic production is typically linked to the morphological differentiation to aerial mycelium in order to protect released nutrients of sacrificed mycelial parts from other terrestrial competitors (Bibb 2005, van der Meij et al. 2017). To enhance colony-wide fitness, this antibiotic production can be coordinated by a division of labor, involving the differentiation into an altruistic subpopulation specialized in antibiotic production (Zhang et al. 2020, Zhang et al. 2022), which in turn is facilitated by the genomic instability of *Streptomyces* at the chromosomal arms (Bentley et al. 2002, Thibessard and Leblond 2014). Given the clonal nature of the *Streptomyces* colonies, secretion of aminoglycosides might play a pivotal role in shielding susceptible segments of the colony against both bacterial competitors and phage predation.

In addition to the ecological relevance, this study provides structural insights into the antiphage effect of these compounds. According to assays with the different *S. tenebrarius* mutant strains and purified apramycin biosynthetic intermediates, the antiphage effect occurred at a late step of the biosynthetic pathway (Fig. 3). This could be due to either differences in the intracellular interaction with phage amplification or in the uptake of the respective molecules. Modification via acetylation or phosphorylation reduces the amount of positively charged amino groups at neutral pH or even adds a negative charge to the cationic aminoglycosides, which could affect their interaction with the nega-

tively charged components of the bacterial surface for uptake (Taber et al. 1987). In the case of the *in vitro* acetylated apramycin, the interference with phage infection was less pronounced in the early stages of infection, but comparable to the *in vivo* modification in the long-term, supporting the hypothesis of a reduced uptake of the molecules when modified *in vitro*. The same could apply to the phosphorylated apramycin accumulating in the $\Delta aprZ$ mutant, possibly explaining the delayed phage-inhibiting effect. These considerations highlight the importance of discriminating between differences in molecule uptake and a potential antiphage property exerted at the intracellular level (Kever et al. 2022). As discussed previously, investigations into the mechanism of action suggest that the inhibition of phage infection occurs early, between the injection and replication stages. While the precise molecular target remains unclear, it may involve direct targeting of the injected, yet unreplicated, phage DNA and/or interference with the phage DNA injection process (Kever et al. 2022). Aminoglycosides are known to bind to the bacterial cell membrane and subsequently accumulate within the cell via electron-transport mediated processes, EDPI and EDPII (Krause et al. 2016). Using fluorescently labeled antibiotics, the membrane-bound and EDPII elements were shown to constitute a substantial proportion of the overall fluorescence levels tightly bound to *E. coli* cells (Sabeti Azad et al. 2020). In cells expressing resistance genes, one could reasonably expect to predominantly encounter the membrane-bound fraction of aminoglycosides. This anticipation stems from the premise that the disruption of membrane integrity and the subsequent diffusive entry of the drug, typically induced by mis-translated proteins during EDPII, might not occur in the presence of resistance mechanisms.

While the acquisition of aminoglycoside resistance holds several advantages for bacteria, it poses a drawback for antibiotic-phage combinatory treatments (Jiang et al. 2020, Zuo et al. 2021). Although the clinical use of aminoglycosides declined with the introduction of other classes of antibiotics such as cephalosporins or fluoroquinolones, the global spread of multidrug-resistant pathogens has led to a renewed demand for this class of antibiotics, frequently for combinatory treatments (Krause et al. 2016). Moreover, increasing knowledge about modifications overcoming aminoglycoside-resistance allows design of semisynthetic antibiotics, thereby improving the therapeutic window of aminoglycosides to combat this global health issue (Krause et al. 2016, Serio et al. 2018, Zárata et al. 2018). However, attention should be paid to the preselection of phages for antibiotic-phage combinatory treatments to avoid antagonistic effects. Antiphage activities of aminoglycosides were observed in the background of diverse aminoglycoside resistance genes tested in this study.

In conclusion, this study emphasizes the ecological relevance of natural aminoglycoside secretion for a community-wide defense against phages. Moreover, it sheds light on crucial considerations for the integration of phage-antibiotic combinatory treatments in the context of clinical infections.

Author contributions

Conceptualization: LK, AH, JF; Data Curation: LK; Formal Analysis: LK, QZ, PW; Funding acquisition: YY, JF; Investigation: LK, QZ, AH, PW; Methodology: All; Project administration: JF; Resources: YY, JF; Supervision: YY, JF; Validation: All; Visualization: LK; Writing—original draft: LK; Writing—review and editing: All; All authors have read and agreed to the published version of the manuscript.

Supplementary data

Supplementary data is available at [FEMSML Journal](#) online.

Conflict of interest: The authors declare no conflict of interest.

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