

Lytic phage potentiates aztreonam–avibactam in *Escherichia coli* with layered resistance by disrupting adaptive stress tolerance

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Abstract

Phage–antibiotic synergy (PAS) can enhance bacterial killing; however, the physiological states that make resistant bacteria vulnerable during co-exposure remain poorly defined. We paired the lytic phage EC10P1 with aztreonam–avibactam (ATM–AVI) against a clinical NDM-5-producing *Escherichia coli* isolate carrying a PBP3/FtsI insertion and a frameshifted *ompF*. Time-kill assays showed that EC10P1 plus sub-MIC ATM–AVI produced a discrete 4-6h window of maximal killing, reaching approximately 4- $\log_{10}$ reduction relative to ATM–AVI alone, while isothermal microcalorimetry was consistent with sustained metabolic suppression in the combination condition that was not observed with either monotherapy. Time-resolved mRNA sequencing at 1h, 4h and 24h with an interaction-based model localized non-additive responses to the 4h killing phase. Interaction-defined responses were concentrated at the 4h killing phase and showed attenuated induction of alternative respiratory, proteostatic and redox-buffering programs during co-exposure. In contrast, the major porin and *mar/acr* responses were predominantly phage-driven or additive, providing no evidence that potentiation resulted from transcriptional restoration of canonical drug influx or suppression of the central efflux pathway. Together, the data support transient functional resensitization through reduced stress-buffering capacity rather than reversal of a single resistance determinant.

Introduction

Multidrug-resistant Gram-negative infections remain difficult to treat because resistance is rarely explained by a single determinant¹. In *Escherichia coli*, metallo- β -lactamases such as New-Delhi metallo- β -lactamase (NDM) enzymes withdraw carbapenems and many other β -lactams from the therapeutic repertoire², while additional changes in penicillin-binding proteins, outer-membrane permeability, efflux, and β -lactamase expression can further reduce the activity of last-line beta-lactam/beta-lactamase inhibitor combinations^{3,4}. This layered resistance architecture allows isolates to retain partial protection even when one resistance mechanism is pharmacologically bypassed.

Aztreonam–avibactam (ATM–AVI) was developed to address one part of this problem. Aztreonam is stable to metallo- β -lactamase hydrolysis, and avibactam inhibits many co-produced serine β -lactamases that would otherwise inactivate aztreonam^{5,6}. However, ATM–AVI activity still depends on drug access to the periplasm and productive engagement of PBP3/FtsI. Clinical isolates with penicillin binding protein 3 (PBP3) insertions, reduced porin function, altered envelope regulation or efflux-associated changes are therefore less susceptible^{4,6}. Strategies that weaken the physiological capacity of such isolates to withstand β -lactam exposure could help restore the practical value of the antibiotic even when conventional susceptibility is compromised.

Bacteriophages offer one such strategy as they attack bacteria through mechanisms that are distinct from those of conventional antibiotics. In phage–antibiotic synergy (PAS), improved killing may arise in several ways. In some cases, sub-lethal antibiotic exposure enhances phage replication or promotes filamentation, increasing phage-mediated killing^{7,8}. In other cases, bacteria that evolve phage resistance

pay a collateral cost, such as altered surface structures, reduced fitness, or increased antibiotic susceptibility^{9,10}. PAS can also occur without stable genetic resensitization: active phage infection and lysis may transiently disturb the bacterial envelope, metabolism, and stress-response capacity, making cells less able to tolerate antibiotic exposure. This distinction is particularly important for β -lactam-based combinations such as ATM–AVI, because β -lactam killing depends not only on drug–target interaction but also on outer-membrane access, periplasmic stress and the ability of the cell to repair envelope damage.

Despite increasing evidence for PAS, the cellular programs that distinguish effective co-exposure from the sum of phage-only and antibiotic-only stress remain incompletely defined. Most phenotypic synergy assays identify whether the combination kills better, but not when the relevant bacterial state emerges or which adaptive responses fail during that interval. Transcriptomics can help resolve this temporal physiology, particularly when early, peak-killing and late-adaptive time points are analyzed separately. However, conventional pairwise comparisons can overinterpret shared stress responses as synergy. A factorial interaction framework is therefore useful for separating additive co-exposure from non-additive responses that occur specifically when phage and antibiotic are present together.

Here, we used a clinical NDM-5-producing *E. coli* isolate (EC45622, see Methods), carrying both a PBP3/FtsI YRIK insertion and a frameshifted *ompF*, to determine whether EC10P1^{11,12} could potentiate sub-MIC ATM–AVI and to define the bacterial state associated with enhanced killing. Time-kill assays and isothermal microcalorimetry (IMC) established the temporal phenotype, while transcriptomes collected at 1, 4 and 24h captured early exposure, maximal combination benefit and late adaptation. A factorial interaction model was then used to distinguish monotherapy-driven and additive responses from combination-specific departures from additivity, allowing us to test whether potentiation was associated with restoration of canonical permeability or with impaired adaptive stress buffering.

Results

3.1. Time-kill analysis demonstrates phage–antibiotic synergy

To characterize the bactericidal activity of phage EC10P1 and ATM–AVI individually and in combination, time-kill assays were conducted using the *E. coli* isolate EC45622 (Supplementary File 1). ATM-AVI alone produced limited killing at sub-MIC concentrations (0.5–4 mg/L), whereas at 8 mg/L bacterial counts declined markedly by 4h and reached the detection limit (10^2 CFU/mL) in all replicates by 6h, consistent with bactericidal activity at MIC (Fig. 1A). Phage alone produced little reduction initially at 2h, however at 4h it exhibited an approximately 4-log₁₀ (10,000-fold) reduction compared with the untreated control. Bacterial counts subsequently increased from 6h onward, with pronounced regrowth at 10 and 24h, a pattern consistent with expansion of phage-resistant or less susceptible subpopulations (Fig. 1B). In contrast, the combination of phage EC10P1 with sub-MIC ATM–AVI concentrations produced markedly

greater bacterial killing than either agent alone (Fig. 1B). By 4h, bacterial counts in the combination conditions had fallen substantially below those observed with antibiotic alone, and this suppression was sustained through 6 hours.

The pooled $\Delta\log_{10}$ combination benefit ($[phage + antibiotic] - antibiotic\ alone$; technical plating replicates were averaged to yield one value for each antibiotic concentration) was small at 2h (0.24) and 10h (0.83), largest at 4h (4.00) and 6h (4.07), and reversed by 24h (-0.34) (Fig. 1C, D). A Friedman test confirmed that this trajectory varied non-randomly across timepoints ($\chi^2 = 13.4$, $df = 4$, $p = 0.0095$); at $n = 4$ concentrations per timepoint, pairwise comparisons between individual timepoints lack power to reach significance regardless of effect size, so no single-timepoint difference is claimed. The rise was consistent across all four sub-MIC concentrations (Fig. 1C), and we describe 4–6h as the window of maximal combination benefit, with 4h as the earliest point at which it was reached.

3.2. Isothermal microcalorimetry

Isothermal microcalorimetry provided a continuous metabolic readout that supported the time-kill findings (Supplementary File 2). The untreated bacterial control showed a rapid increase in heat flow, with an early shoulder at approximately 4h, followed by a maximum at approximately 7h (Figure S1A). Sub-MIC ATM–AVI concentrations of 0.5–2 mg/L produced smaller peaks at approximately 3.9–4.0 h, followed by persistent residual metabolic activity throughout the approximately 23-hour recording. In contrast, ATM–AVI at 8 mg/L produced an almost flat trace and did not reach sustained detectable metabolic activity, consistent with inhibition at the isolate's known MIC.

Phage EC10P1 alone initially maintained heat flow close to baseline, followed by sustained metabolic recovery at 7.2 ± 0.3 h and a maximum heat-flow peak at approximately 8.5h. The phage–antibiotic combinations showed a distinctly prolonged suppression profile. At 4h, heat flow remained near baseline across all available combination concentrations, while the corresponding sub-MIC antibiotic-only cultures showed substantial metabolic activity. By 8h, phage-only cultures had recovered considerable heat production, whereas the combination cultures remained close to baseline (Figure S1B).

Using an operational recovery threshold of heat flow exceeding 10 μ W for at least 30 consecutive minutes, sustained metabolic recovery occurred at 15.4 ± 1.3 h, 13.9 ± 1.2 h, and 16.8 ± 1.5 h for phage combined with 0.5, 1, and 2 mg/L ATM–AVI, respectively. The separation between the combination and monotherapy conditions was maintained across alternative recovery thresholds, although the precise recovery times were threshold-dependent. These findings indicate that phage plus ATM–AVI co-exposure delayed population-level metabolic recovery substantially longer than either sub-MIC ATM–AVI or phage alone.

Together, the time-kill and IMC data identify 4h as the key time point for transcriptomic profiling: it is the earliest point at which maximal phage-antibiotic killing was observed, and it coincides with near-complete suppression of bacterial metabolic activity in the combination condition. The 1h and 24h

samples provide flanking early and late comparisons, while the 4h samples form the main basis for interpreting the transcriptional response associated with synergistic killing.

3.3. Global transcriptional landscape

Time was the dominant source of transcriptomic variation, followed by phage exposure, whose effect was strongest during the 4h killing window (Fig. 2A, Supplementary File 3). All transcriptomic experiments involving ATM–AVI were performed at 4 mg/L. After accounting for this dominant temporal structure, phage exposure represented the next major source of variation and produced a clear separation between phage-exposed and unexposed samples. PERMANOVA confirmed phage-associated structure at 1h ($R^2 = 0.33$, $F = 4.08$, $p_{\text{adj}} = 0.012$), with the effect most pronounced at 4h ($R^2 = 0.52$, $F = 10.86$, $p_{\text{adj}} = 0.0093$), coinciding with the peak bactericidal phase in the time-kill assays. By 24 h, phage-exposed and unexposed samples were no longer significantly separated ($R^2 = 0.098$, $F = 0.86$, $p_{\text{adj}} = 0.425$), consistent with transcriptional convergence among surviving populations. Consistent with this divergence, phage treatment produced a markedly broader transcriptional perturbation at 4h (1445 exclusive differentially expressed genes), whereas antibiotic treatment alone yielded 74 differentially expressed genes at the same time point (Fig. 2B, Supplementary File 4).

Pathway-level enrichment further distinguished the physiological programs engaged by each stress (Fig. 2C, Supplementary File 5). Under antibiotic treatment, early (1h) and mid (4h) time points were dominated by negative enrichment of motility-associated pathways (flagellar assembly and bacterial chemotaxis), with additional suppression of biofilm formation and two-component signaling at 4h, indicating reduced motility, surface-associated behaviors, and signaling during antibiotic exposure. In contrast, phage treatment elicited a broader, time-dependent response: at 1h, pathways linked to nitrogen utilization, transport systems (including ABC transporters and phosphotransferase systems), and glycerophospholipid metabolism were negatively enriched, while ribosome, amino acid biosynthesis, and cofactor metabolism were positively enriched. By 4h, phage-treated cells showed negative enrichment of the TCA cycle and alternative carbon fixation pathways alongside positive enrichment of the pentose phosphate pathway, purine metabolism, and continued ribosomal and biosynthetic activity, consistent with extensive metabolic reprogramming during sustained phage challenge.

The combination condition displayed the largest breadth of significant pathway shifts, particularly at 1h, where translation/biosynthesis (ribosome, amino-acid and cofactor pathways) and redox-supporting metabolism (e.g., pentose phosphate and glutathione pathways) were positively enriched while motility programs were concurrently suppressed; at 4h, the combination retained strong negative enrichment of chemotaxis/flagellar, biofilm, and two-component systems together with positive enrichment of nucleotide and central metabolic pathways.

Together, these analyses define the dominant temporal structure of the dataset and establish that phage, antibiotic, and combination treatment are associated with distinct pathway-level responses during the 4h killing window (Fig. 2C), motivating an explicit interaction analysis to separate additive co-exposure from combination-specific effects.

3.4. Interaction-defined responses reveal stress-tolerance collapse

The distribution of transcriptional response classes changed markedly over time (Fig. 3, Supplementary File 4). At 1h, the response was dominated by phage-driven genes, whereas at 4h both phage-driven and shared additive responses increased, and the interaction-defined class reached its maximum. These genes were identified by a factorial model in which the interaction coefficient represents departure of the observed combination response from the modeled sum of the two monotherapy effects. By 24 h, the phage-driven and interaction-defined components had largely diminished. Thus, combination-specific transcriptional responses were temporally concentrated in the same 4h window in which the phenotypic interaction was strongest.

Figure 4. **Interaction-defined transcriptional responses at the 4h killing timepoint.** (A) Scatter plot showing the relationship between the antibiotic $\log_2\text{FC}$ and the interaction coefficient ($\Delta\log_2\text{FC}$) for genes at 4h. Points are colored by response class. (B) Heatmap of $\log_2\text{FC}$ for representative genes grouped by functional category, showing phage, antibiotic, observed combination, and interaction effects. (C) Branching slope plots for major functional clusters. For each gene, phage and antibiotic mono-treatment $\log_2\text{FC}$ are connected to (i) "Sum" the arithmetic sum of the two independently shrunk mono-treatment estimates, shown as a descriptive reference point, and (ii) "Combination" the observed, independently shrunk combination-treatment estimate.

To contextualize these interaction signals against the multilayered resistance architecture of this isolate, which carries *bla*_{NDM-5}, a YRIK insertion in PBP3/FtsI, and a frameshifted *ompF*, we examined the $\log_2\text{FC}$ profiles across all four conditions for functionally annotated gene clusters (Fig. 4B). The heatmap shows that the strongest and most coherent negative interactions are concentrated in anaerobic respiration, proteostasis, and oxidative stress, whereas the outer membrane, zinc homeostasis, acid stress, and *mar/acr* regulon clusters show phage-driven remodeling and additive responses with conspicuously absent interaction terms.

To resolve the expected versus observed combination response at the individual gene level within the four most mechanistically critical clusters, we traced gene trajectories from monotherapy $\log_2$ fold changes through the expected additive value, to the observed combination $\log_2\text{FC}$ (Fig. 4C), making the direction and magnitude of non-additivity directly visible for each gene.

3.4.1. Anaerobic respiratory escape routes are blocked during co-exposure

The strongest and most coherent negative interaction cluster involved anaerobic electron acceptor pathways (Fig. 4B-C). The *tor* operon, which encodes the trimethylamine N-oxide (TMAO) reductase system, was strongly induced by aztreonam-avibactam alone, with *torC* reaching an antibiotic $\log_2\text{FC}$ of

+ 7.2, consistent with published observations that bactericidal antibiotics perturb central metabolism and stimulate alternative respiratory flux^{13,14}. However, this induction was markedly attenuated in the combination, with negative interaction coefficients for *torA* (Δ -2.8), *torD* (Δ -2.6), and *torC* (Δ -1.9). A similar pattern was observed in the formate hydrogenlyase (FDH) complex¹⁵, *hycC-hychI*, and *fdhF* genes, where antibiotic-driven induction was consistently reduced under phage co-exposure. The DMSO reductase-associated chaperone *dmsD* (Δ -0.89) extended this pattern to a third anaerobic respiratory pathway.

Together, these operons represent a coordinated metabolic escape program: under cell wall stress, bacteria divert electron flow toward alternative anaerobic acceptors to manage fermentative overflow and reduce the energetic costs of envelope repair. Phage co-exposure consistently attenuated all three systems below the additive expectation, indicating that the lytic program specifically disrupts this adaptation. It should be noted that transcriptional attenuation does not confirm the functional abolition of these pathways; however, the magnitude and coherence of the interaction signal across all three electron acceptor systems support the interpretation that bacteria are unable to fully deploy anaerobic respiratory compensation during the peak killing window.

3.4.2. Proteostatic buffering is attenuated by the combination

A second coherent negative interaction cluster involved molecular chaperones and protein quality control proteases (Fig. 4B-C). These genes were generally induced by both phage and antibiotic individually, indicating that each treatment imposed a proteotoxic burden. However, this combination did not produce the stronger induction expected from adding the two mono-treatment effects. Instead, the expression in the combination remained close to or only modestly above the level observed with either treatment alone.

This pattern was evident across the major chaperone systems, where *dnaK/dnaJ*, *groL/groS*, and *hspG* were all induced by phage and antibiotic individually, as expected^{16,17}. However, they showed negative interaction coefficients in the combination (*dnaK* Δ -1.4; *dnaJ* Δ -1.0; *groL* Δ -0.8; *groS* Δ -1.0; *hspG* Δ -0.7). This interaction means that the proteostasis response failed to increase proportionally when both stresses were present together.

The same non-additive attenuation was observed for the ATP-dependent protease system *hslU/hslV* (*hslU* Δ -0.9; *hslV* Δ -1.0), suggesting that both protein folding and protein degradation arms of the quality-control network failed to scale during co-exposure^{18,19}. Attenuation of *pspA* (Δ -0.88), an inner membrane stress marker, further supports impaired envelope stress buffering during the combination treatment.

Together, these data suggest that the combination imposes overlapping proteostatic demands, but the bacterial response reaches a ceiling rather than increasing additively. In this sense, the proteostasis cluster reflects a negative interaction: phage and antibiotic each activate the chaperone network alone,

but co-exposure prevents the cell from mounting the larger compensatory response that would be expected if the two stresses were independently buffered. This failure to scale proteostatic protection likely contributes to the broader collapse of stress tolerance during the 4h killing window.

3.4.3. Oxidative stress defenses are blunted despite predicted oxidative burden

The oxidative stress cluster showed a similar pattern of attenuated protection during co-exposure. The alkyl hydroperoxide reductase system *ahpC/ahpF*, the primary scavenger of low-level organic peroxides at physiological concentrations²⁰, was induced by each treatment alone but showed negative interaction in the combination (*ahpC* Δ -0.86; *ahpF* Δ -1.09). This suggests that the observed combination response was weaker than expected despite the simultaneous presence of two stressors. Additional redox-buffering systems followed the same pattern: chaperedoxin *cnoX* (Δ -0.66), which protects oxidatively damaged proteins²¹, and periplasmic disulfide isomerase *dsbC* (Δ -0.54), which maintains correct disulfide bond formation in envelope proteins²², suggested impaired management of protein oxidation in the periplasm. The NADPH-dependent aldehyde reductase *yqhD* (Δ -0.74), which detoxifies reactive aldehydes generated during membrane lipid peroxidation²³, extended this mismatch from peroxide stress to lipid-derived oxidative damage.

Although the contribution of β -lactam antibiotics to intracellular ROS generation has been debated^{24,25}, phage lytic replication is an established source of membrane oxidative stress through physical envelope disruption^{26,27}. The combination is therefore predicted to impose a greater cumulative oxidative burden than either agent alone, yet the antioxidant and redox-buffering response is paradoxically attenuated across multiple independent systems. This failure leaves bacteria vulnerable to oxidative damage at precisely the 4h killing window and is mechanistically consistent with the simultaneous proteostatic attenuation described in section 3.4.2, as both reflect overloading of the overlapping σ^{32} -mediated stress response network.

3.4.4. Motility–biofilm tolerance transition is disrupted by co-exposure

A separate interaction-defined cluster involved motility, chemotaxis, and surface-adaptation genes (Fig. 4B-C). Aztreonam-avibactam alone strongly repressed the flagellar regulator *fliA* (σ^{28}), together with several flagellar assembly genes, including *flgB*, *flgD*, and *flgG*. In the *E. coli* flagellar hierarchy, *fliA* is part of the FlhDC-controlled flagellar programme and σ^{28} contributes to late flagellar and chemotaxis gene expression, including the *tar-tap-cheRBYZ* operon²⁸. In the combination, this repression was partially relieved, producing positive interaction coefficients for *fliA* (Δ + 0.92), *flgB* (Δ + 1.31), *flgD* (Δ + 1.05), and *flgG* (Δ + 1.26). Chemotaxis genes such as *cheR* (Δ + 0.87) and *tap* (Δ + 1.79) showed the same pattern. Thus, phage co-exposure partly counteracted the antibiotic-driven shutdown of the motility programme.

Genes linked to surface adaptation and biofilm regulation also showed interaction, but in a mixed direction. *rdcB* and *mlrA*, which are connected to c-di-GMP and curli/biofilm regulation, showed positive interaction, whereas type 1 fimbrial genes such as *fimB* and *fimI* showed negative interaction. This suggests that the combination does not simply restore motility or promote biofilm formation. Instead, it disrupts the normal transition between motile and surface-associated states. Such disruption may prevent bacteria from entering a coordinated sessile, antibiotic-tolerant physiology during the 4h killing window.

3.4.5. Outer membrane remodeling does not explain resensitization

The outer membrane response indicates that functional porin-mediated resensitization is unlikely to account for synergy in this isolate (Fig. 4B, C). *ompW* was strongly phage-repressed ($\log_2FC$ -5.6), consistent with phage receptor downregulation rather than enhanced antibiotic influx. *ompC*, the only intact general diffusion porin in this strain given the *ompF* frameshift, was also phage-repressed ($\log_2FC$ -1.7) with no significant interaction term. This means the combination did not restore expression of the sole functional porin, and canonical outer membrane permeability remained reduced in co-exposure ($\log_2FC$ -1.8).

ompF showed a positive interaction coefficient ($\Delta + 1.26$), indicating partial transcriptional derepression in the combination relative to the additive expectation. In isolates with intact *ompF*, a positive interaction coefficient of this kind would suggest that co-exposure partially counteracts the antibiotic-driven repression of the primary aztreonam entry channel, a regulatory outcome that, if translated to functional protein, could contribute to sustained drug permeability during combination treatment. Here, however, *ompF* carries a frameshift and cannot encode a functional β -barrel porin, so this signal reflects a latent regulatory response with no functional permeability consequence. The positive interaction is best understood through the MarA/Rob–MicF axis²⁹, which represses *ompF* transcription downstream of membrane stress signals. These signals are generated under both phage and antibiotic conditions, producing a non-additive regulatory outcome at the *ompF* locus without any change in outer membrane permeability. The observation that *ompR*, the regulator of both *ompF* and *ompC* transcription³⁰, was additively induced, yet *ompC* remained phage-repressed reinforces this decoupling between porin regulatory signals and functional porin output. Taken together, these data indicate that any enhanced aztreonam access during co-exposure arises from phage-mediated physical envelope disruption during lytic replication³¹ rather than from transcriptional restoration of functional porins.

3.4.6. Classical efflux-mediated resistance is not differentially modulated by the combination

In direct contrast to the interaction-defined stress-response clusters, the *mar/acr* regulon showed no evidence of non-additive modulation by the combination (Fig. 4B). *marA* was strongly phage-induced, while the antibiotic effect was minimal and non-significant. Several other *mar/acr*-associated genes,

including *acrA*, *acrR*, *acrZ*, *acrD*, *acrE*, *acrF*, and *tolC*, were also primarily phage-driven rather than interaction-defined.

Importantly, *acrB*, encoding the central RND permease of the AcrAB-TolC efflux pump, showed no significant transcriptional change. Thus, phage-induced activation of the *mar* regulon does not translate into measurable induction of the principal efflux transporter. The strong phage-associated repression of *acrZ*, which modulates *AcrB* substrate specificity, may alter efflux function at the complex level, but this effect is not specific to the combination. The absence of interaction terms across the broader *mar/acr* network indicates that efflux is not a major battleground of phage-antibiotic synergy in this isolate. The combination neither suppresses efflux beyond the effect of phage alone nor induces a compensatory efflux response to aztreonam-avibactam.

Discussion

In this study, we investigated whether a lytic phage could enhance aztreonam–avibactam activity against a multi-layered resistant isolate. After selecting the EC45622–EC10P1 pair, we combined time-kill assays, isothermal microcalorimetry, and time-resolved transcriptomics to characterize both the phenotypic and physiological consequences of co-exposure. The combination produced substantially greater killing at sub-MIC ATM–AVI levels and caused sustained metabolic suppression compared with either agent alone. These findings indicate that EC10P1 enhances ATM–AVI activity in a resistant background. Rather than indicating restored drug access or stable resensitization, the data point to a transient failure of bacterial stress tolerance during phage–antibiotic co-exposure.

A straightforward way to interpret the mechanism is adaptive overloading. ATM–AVI imposes cell-wall and periplasmic stress; lytic phage replication adds a second envelope-centred pressure through adsorption, replication, and lysis³¹. Either stress alone can trigger responses that help bacteria maintain envelope integrity, manage damaged proteins, adjust metabolism, and buffer redox imbalance. Under co-exposure, these responses appear insufficient. The attenuation of anaerobic respiratory compensation (*tor*, *hyc*), proteostatic buffering (*dnaKJ*, *groLS*, *hslUV*), and oxidative stress defense (*ahpCF*, *cnoX*, *dsbC*) should not be read as a list of independent pathway effects, but as evidence that the bacterial population could not fully sustain the adaptive state required to tolerate β -lactam injury while simultaneously managing phage infection. The combination acts less like a specific reversal of one resistance mechanism and more like a second pressure that pushes an already-stressed cell beyond its buffering capacity.

An important aspect of the data is what they do not support. Because ATM–AVI must cross the outer membrane to reach its periplasmic target, restoration of permeability would be an obvious explanation for enhanced killing. The transcriptional pattern argues against this. The only intact major porin, *ompC*, was not restored during combination treatment, and *ompF* carries a frameshift whose transcriptional signal is unlikely to translate into functional drug influx^{4,29}. The central AcrAB–TolC efflux axis showed no combination-specific suppression³⁰. If phage improves antibiotic access, it is therefore more likely

through physical envelope disruption during lytic replication than through regulated restoration of permeability^{26,27}.

These findings support a form of phage-mediated functional resensitization, but not stable genetic resensitization. In other systems, phage resistance can generate heritable collateral antibiotic susceptibility through receptor or envelope modification^{10,32,33}. Here, EC10P1 did not restore porin-mediated ATM–AVI entry or suppress the major efflux pathway. Instead, phage co-exposure weakened the physiological buffering capacity needed to survive β -lactam stress, making the bacterium transiently more vulnerable to ATM–AVI during treatment. Whether this acute functional resensitization is accompanied by stable susceptibility changes in EC10P1-resistant survivors remains to be tested.

These findings have direct relevance for the development of phage adjunct strategies against MDR Enterobacterales. The ability of EC10P1 to drive substantial killing at one-quarter of the ATM–AVI MIC indicates that phage co-exposure can convert a poorly active antibiotic concentration into a bactericidal condition in this resistant isolate. This does not imply that lower antibiotic doses would be sufficient *in vivo*, because clinical efficacy depends on tissue exposure, inoculum size, host immunity, phage pharmacokinetics, and the emergence of phage resistance. However, it supports a clinically testable concept: in isolates where ATM–AVI activity is reduced by PBP3 alteration, and porin loss coupled with other resistance mechanisms, matched lytic phages may create a transient physiological vulnerability that restores effective β -lactam killing. Validation across diverse clinical isolates and infection-relevant models will be essential to define when this strategy is likely to work.

This study examined one clinical isolate and one phage–antibiotic pairing; therefore, the generalizability of the findings across broader NDM-producing *E. coli* backgrounds remains to be tested. The transcriptomic data provide population-level evidence for altered stress physiology, but functional assays are needed to confirm the proposed roles of envelope damage, proteostatic stress, redox imbalance and respiratory adaptation. Future studies should therefore extend this analysis to additional isolates and phages, directly measure envelope integrity and antibiotic uptake, and determine whether phage-resistant survivors acquire altered ATM–AVI susceptibility.

Conclusion

EC10P1 potentiated ATM–AVI against an NDM-5-producing, PBP3-modified *E. coli* by creating a transient state in which adaptive stress responses failed to scale with combined phage and β -lactam pressure. The effect was not explained by restoration of functional porin-mediated influx or combination-specific suppression of major efflux, supporting functional resensitization through stress-capacity overload rather than reversal of a canonical resistance determinant. This framework provides a testable basis for identifying additional phage– β -lactam pairs capable of recovering activity against antibiotic-compromised Enterobacterales.

Methods

6.1. Bacterial isolate panel and selection of the phage–host pair

The *E. coli* isolate panel used in this study was derived from the clinical collection described previously by Tellapragada *et al.*⁴. Resistant isolates (MIC > 4 mg/L) in this panel were screened against a panel of lytic bacteriophages to identify phage–host pairs with reproducible infectivity (Figure S2). Phage susceptibility was assessed by spot assay and recorded as clear lysis, dim lysis or no lysis. EC45622 (SRA: SRR27641205) was selected for downstream experiments because it had an elevated ATM–AVI MIC of 8 mg/L and showed reproducible clear lysis with phage EC10P1. This isolate therefore provided a clinically relevant model in which to test whether phage exposure could enhance ATM–AVI activity in a multi-mechanism β -lactam-compromised background.

6.2. Phage propagation and infectivity testing

Phage EC10P1 was isolated from Finnish environmental samples and has been described previously (Mattila *et al.*, 2015)¹¹. The bacterial receptor used by EC10P1 has not been experimentally identified. Phage propagation was performed as described previously (Patpatia *et al.*, 2022)¹², with minor modifications. Briefly, 200 μ l of an overnight bacterial culture and 20 μ l of phage suspension were added to 10 ml LB broth and incubated for 4h at 37°C. The culture was then centrifuged at 12,000 $\times g$ for 10 min, and the clarified lysate was filtered through a 0.22 μ m filter and stored at 4°C until use.

Phage infectivity was tested against the full isolate panel by spot assay. Briefly, 5 μ l of phage stock or serial phage dilutions in LB broth was spotted onto bacterial overlay lawns prepared from 200 μ l overnight bacterial culture and 2.7 ml soft agar (0.4% w/v) on 1.5% LB agar plates. Plates were incubated overnight at 37°C. Lysis phenotypes were classified as clear lysis, dim lysis or no lysis. The phage–host screening results are summarized in Figure S2.

6.3. MIC determination

MIC values for aztreonam-avibactam (ATM-AVI) were determined as described in a previous study (Tellapragada *et al.* 2024)⁴. All isolates were tested in triplicates and isolates with MIC values $\geq$ 4 mg/L for ATM-AVI were used in the present study. *E. coli* strain ATCC 25922 was used for quality control of the broth microdilution method for MIC determination.

6.4. Inoculum preparation and testing conditions

Bacterial suspensions were prepared from fresh overnight culture plates and adjusted to a turbidity equivalent to a 0.5 McFarland standard. The suspensions were subsequently diluted 1:100 in cation-adjusted Mueller-Hinton Broth II (CAMHB; Sigma-Aldrich) and used for all experiments. Antibiotic powders were obtained from MedChemExpress (Stockholm, Sweden).

Aztreonam (ATM) was tested at concentrations ranging from 0.5 to 4 mg/L in combination with a fixed concentration of avibactam (AVI) of 4 mg/L. The bacteriophage EC10P1 was evaluated at a multiplicity of infection (MOI) of 1.

Experimental conditions included: (i) untreated bacterial control, (ii) bacteria exposed to EC10P1 alone, (iii) bacteria exposed to ATM-AVI at varying concentrations (0.5/4 to 4/4 mg/L), and (iv) bacteria exposed to the combination of ATM-AVI and EC10P1.

For each condition, bacterial suspensions were prepared in a master tube containing a total volume of 1 mL. From each suspension, 100 μ L per well was transferred to 96-well microtiter plates for time-kill assays, while 120 μ L was used for isothermal microcalorimetry (IMC) experiments. All treated and untreated samples were prepared from the same initial bacterial suspension to ensure experimental consistency.

6.5. Time-kill assays

Bacterial cultures were exposed to ATM–AVI alone, EC10P1 alone or EC10P1 plus ATM–AVI in microtiter plates and incubated at 37°C. Briefly, 100 μ L of the each treated or untreated bacterial suspensions were dispensed per well in a microtiter plate and incubated at 37°C. Wells containing only MHB served as the negative control. Samples were taken after 2, 4, 6, 10, and 24h. Samples were diluted appropriately and 10 μ L of dilutions were spotted in triplicates on blood agar plates which were incubated overnight at 37°C. The following day, colonies were counted and averaged to CFU/mL.

For each condition, log₁₀ CFU/mL reduction from the initial inoculum was averaged across the three technical (plating) replicates of each biological culture before statistical testing, yielding one value per antibiotic concentration (0.5, 1, 2, 4 mg/L) at each timepoint. At each timepoint, the phage + antibiotic and antibiotic-alone conditions were compared using a two-sample Wilcoxon rank-sum test ($n = 4$ concentrations per arm), with Benjamini–Hochberg correction across the five timepoints. The combination-benefit magnitude (Δ log₁₀ CFU/mL, phage + antibiotic minus antibiotic alone) was compared across the five timepoints using a Friedman test, blocked by antibiotic concentration to account for the paired, repeated-measures structure of the design; this test evaluates whether the overall trajectory varies non-randomly across time and does not itself identify which individual timepoints differ. All statistical analyses were performed in R using the rstatix package.

6.6. Isothermal microcalorimeter (IMC)

An isothermal microcalorimetry device, CalScreener™ (Symcel, Sweden) was used for testing in this study. One plate can accommodate up to 48 screw-capped titanium wells of which the top and bottom row wells are used as thermodynamic references. From the master tube containing exposed and unexposed bacterial suspensions, 120 μ L was dispensed in each well fitted with a sterile plastic insert. The plates were prepared for loading in the device as per the manufacturer's instructions. Mueller-Hinton broth was included as a negative control. Heat flow was recorded continuously at $37 \pm 0.001^\circ\text{C}$ for at least 24h using the CalScreener™ system.

Raw heat-flow signals were baseline-corrected for each well using the baseline values stored by the Symcel calView® software and were subsequently analyzed in R using technical replicate wells. Time to sustained metabolic recovery was defined operationally as the first-time baseline-corrected heat flow exceeded 10 μ W for at least 30 consecutive minutes. The 10 μ W threshold was informed by the stable-signal criterion used in the IMC framework described by Tellapragada et al.³⁴, but was applied here as an operational measure of delayed metabolic recovery rather than as a validated growth or MIC cutoff. Robustness was assessed by repeating the analysis using alternative absolute and control-relative thresholds. Conditions that did not reach the threshold during the recording period were classified as not having reached sustained metabolic recovery. Heat-flow curves and derived metrics were generated in R using ggplot2 (v4.0.2). All replicate wells originate from a single experiment (technical, not biological, replicates), so no inferential statistics were computed on these data.

6.7. RNA extraction, library preparation, and sequencing

Samples from bacterial cultures (5ml) grown in the presence and absence of treatment (phage, EC10P1 alone, phage plus ATM-AVI at 4mg/L) were collected at 1h, 4h and 24h. We tested each test condition in triplicate, and samples were collected for RNA extraction. Total RNA was extracted using the Quick-RNA Mini Prep kit (ZymoResearch, USA) as per the manufacturer's instructions. The concentration and purity of the extracted RNA were assessed using a Thermo Nano Drop One. Ribosomal RNA was depleted using the RiboCop rRNA depletion kit (Lexogen GmbH, Austria), and libraries for RNA-Seq were prepared using the ALFA-SEQ Directional RNA Lib Prep kit (FINDROP, China), and the quality of the libraries was assessed on an Agilent Bioanalyzer 2100. Sequencing was carried out on NovaSeq platform (Illumina, San Diego CA) with PE150 mode.

6.8. RNA-seq data processing and differential expression analysis

Raw FASTQ files were assessed for quality using FastQC (v0.12.1), and adapter sequences and low-quality bases were trimmed using Trim Galore (v0.6.10, wrapping Cutadapt v4.8) with a Phred quality threshold of 20, a minimum post-trimming read length of 35 bp, and removal of Illumina TruSeq adapters and poly-G tails. Trimmed reads were aligned to the *E. coli* EC45622 reference genome using STAR (v2.7.11b), and gene-level counts were quantified from the resulting BAM files using featureCounts (Subread v2.0.6) (Supplementary Files 7 and 8).

Of the 36 samples collected (three biological replicates per condition and time point), four did not pass sequencing quality control and were excluded before count generation (Supplementary File 6); a further sample (A38, 24 h) was excluded from the primary analysis as a quality control outlier, based on its VST-PCA first principal component (> 4-fold the next most extreme24hsample), its DESeq2-estimated library size (approximately half the average of other24hsamples), and a single locus accounting for 11.7% of its library versus a 3.6% average in other24hsamples; its raw mapping rate was normal (86.5%), consistent with a compositional/expression outlier rather than a sequencing failure. A38 is retained in the full

sample metadata (Supplementary File 6) but excluded from the primary count matrix and all DESeq2 models.

All downstream analyses were performed in R (v4.4.2). Due to the dominant effect of time on transcriptional variation observed in principal coordinate analysis (PCoA; Fig. 2A), samples were analysed separately for each time point (1h, 4h, 24h) using DESeq2 (v1.44.0).

To formally test the significance of transcriptional separation between phage-exposed and unexposed samples at each time point, permutational multivariate analysis of variance (PERMANOVA) was performed using the *adonis2* function from the *vegan* package (v2.7.3), applied to the same VST Euclidean distance matrix used for PCoA. Separate models were fitted for each time point with phage presence as the sole predictor, and resulting p-values were adjusted for multiple comparisons across time points using the Benjamini–Hochberg method. Homogeneity of within-group dispersions was assessed using *betadisper* with permutation testing to verify that significant PERMANOVA results reflected centroid separation rather than differences in group spread.

At each time point, surrogate variable analysis (SVA; sva package v3.54.0) was applied to estimate latent sources of variation. Counts were first library-size-normalized using DESeq2-estimated size factors and log-transformed prior to SVA input. SVA estimated two surrogate variables at 1h and one surrogate variable at both 4h and 24h; all estimated surrogate variables were included as covariates in the corresponding time-specific DESeq2 design formula, Eq. (1):

$$\sim SV1 [+SV2] + phage + antibiotic + phage : antibiotic$$

1

with the bracketed SV2 term included only in the 1h model.

The interaction term in this model estimates the departure of the observed combination response from the sum of the individual mono-therapy effects (expected additive response), expressed as Eq. (2).

$$interaction = \mu_{PA} - \mu_P - \mu_A + \mu_C$$

2

Where μ_{PA} , μ_P , μ_A , and μ_C denote the fitted mean $\log_2$ expression for the combination, phage monotherapy, antibiotic monotherapy, and untreated control conditions, respectively, at the same time point. A non-zero interaction coefficient therefore indicates that the antibiotic effect depends on phage presence, operationally defining genes whose combination response is non-additive.

$\log_2$ fold changes were shrunk to stabilize estimates for genes with low counts or high variance. For pairwise comparisons (phage vs. untreated; antibiotic vs. untreated), shrinkage was performed using the *apeglm* method. For interaction coefficients and combination contrasts, shrinkage was performed using the *ashr* method, which is more appropriate for interaction terms that may not conform to the unimodal

prior assumed by *apeglm*. These shrunken $\log_2$ FCs were used for all downstream visualization and gene ranking; statistical significance was determined from the corresponding Benjamini–Hochberg-adjusted p-values ($p_{adj} < 0.05$) from the DESeq2 model.

Genes were classified into five mutually exclusive response categories based on the significance of each model coefficient: (i) Interaction-defined (non-additive): significant interaction term; (ii) Shared (additive, no interaction): significant phage and antibiotic effects without a significant interaction term; (iii) Antibiotic-driven: significant antibiotic effect only; (iv) Phage-driven: significant phage effect only; and (v) No significant change.

To visualize global transcriptional relationships, PCoA was performed on variance-stabilized count data. Raw counts were filtered to retain genes with at least 10 reads in a minimum of three samples, size factors were estimated using the DESeq2 model, and counts were transformed using the variance-stabilizing transformation (VST) with *blind = TRUE*. Euclidean distances were computed between all sample pairs from the VST matrix, and principal coordinates were obtained by classical multidimensional scaling (*cmdscale*, R stats package v4.4.2).

Gene set enrichment analysis (GSEA) was performed on pre-ranked gene lists derived from DESeq2 shrunken $\log_2$ FCs for each condition and time point. Genes were ranked by signed $\log_2$ FC and tested for enrichment against KEGG pathway gene sets for *Escherichia coli* K-12 MG1655, downloaded using the clusterProfiler *download_KEGG* function (clusterProfiler v4.14.6). KEGG pathways were downloaded once and shared across all conditions within an analysis run to ensure consistency. Gene set size was restricted to 10–500 members, and enrichment p-values were adjusted using the Benjamini–Hochberg method. KEGG-based pathway mapping was performed using K-12 MG1655 orthologues as reference given the limited functional annotation available for EC45622-specific genes. All figures were generated in R (v4.4.2) using ggplot2 (v4.0.2). Sankey diagrams were produced with ggsankey (v4.4), Venn diagrams with ggVennDiagram (v1.5.7) and GSEA heatmaps with ComplexHeatmap (v2.22.0).

Declarations

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Data availability

Data supporting the findings of this study are available in the article and Supplementary Information. Short Illumina reads data are available in NCBI Sequence Read Archive under accession code

Code availability

The code used to process raw sequencing read are available at <https://github.com/xrazmo/bactrnaflow>. The downstream analysis of count data including statistical tests and visualization is available at <https://github.com/xrazmo/PAS-analysis>.

Authors contributions

S.P., H.L., A.J., A.Ö., and C.T. performed the experimental work and data generation, including phage selection and characterization, time-kill experiments, and isothermal microcalorimetry. C.T. and C.G.G. conceived and designed the study. C.T. supervised the experimental works. M.R. performed the bioinformatic and statistical analyses, prepared the visualizations, and drafted the manuscript. All authors contributed to interpretation of the results, reviewed and edited the manuscript, and approved the final version.

Competing interests

The authors declare no competing interests.

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Figures

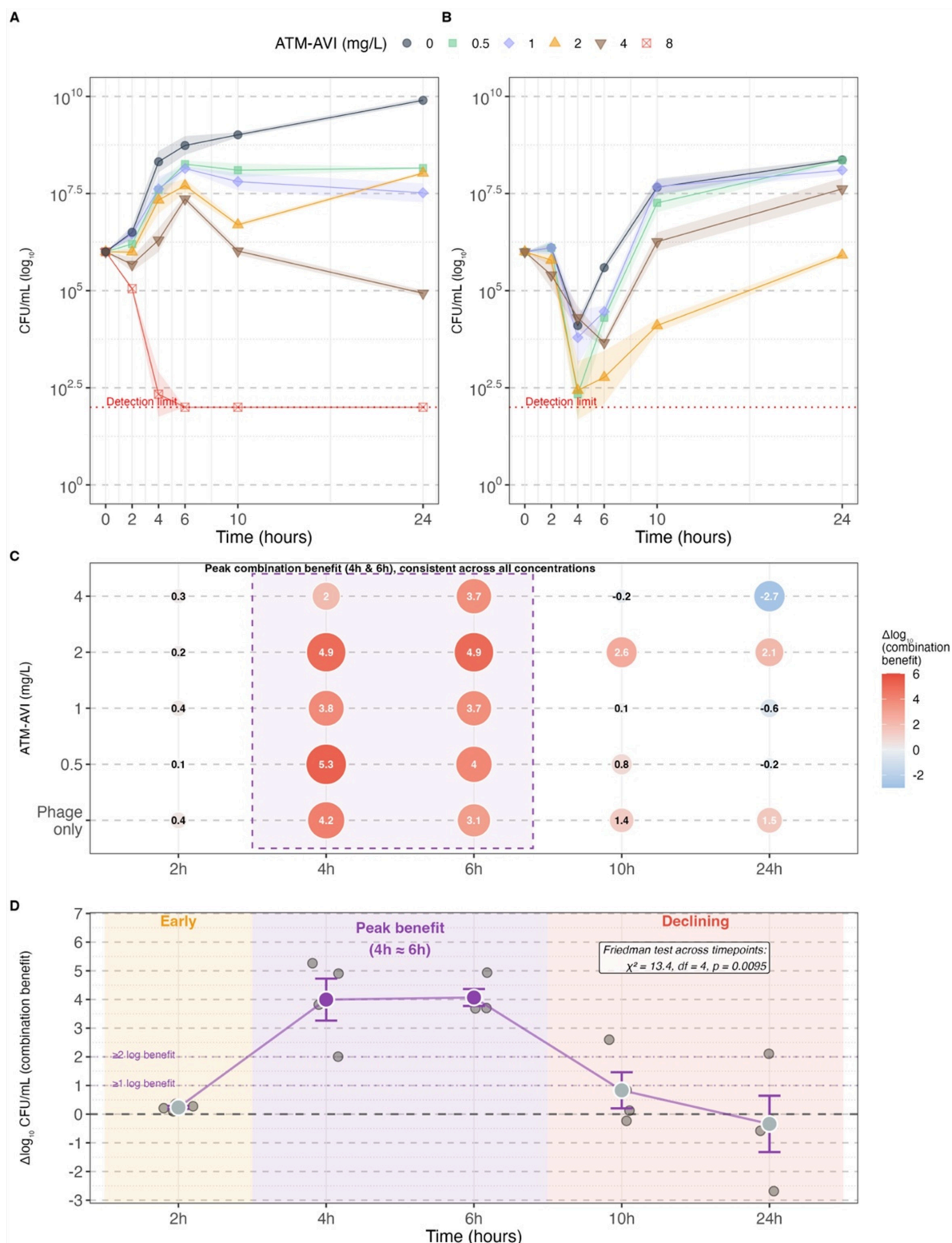


Figure 1

Time-kill analysis of the EC10P1-ATM-AVI interaction in *E. coli* EC45622. (A) Bacterial survival following ATM-AVI alone at 0, 0.5, 1, 2, 4, and 8 mg/L over 24 hours. (B) Survival following EC10P1 plus the same ATM-AVI concentrations. Data are shown as $\log_{10}$ CFU/mL (mean $\pm$ SD, $n = 3$ technical replicates); the dotted line indicates the approximate detection limit (10^2 CFU/mL). (C) Bubble plot showing $\Delta \log_{10}$ combination-benefit magnitude at each time point and ATM-AVI concentration. (D)

$\Delta\log_{10}$ values pooled across sub-MIC ATM-AVI concentrations. Dunn post-hoc groups are shown by letters; groups sharing the same letter do not differ significantly ($p > 0.05$).

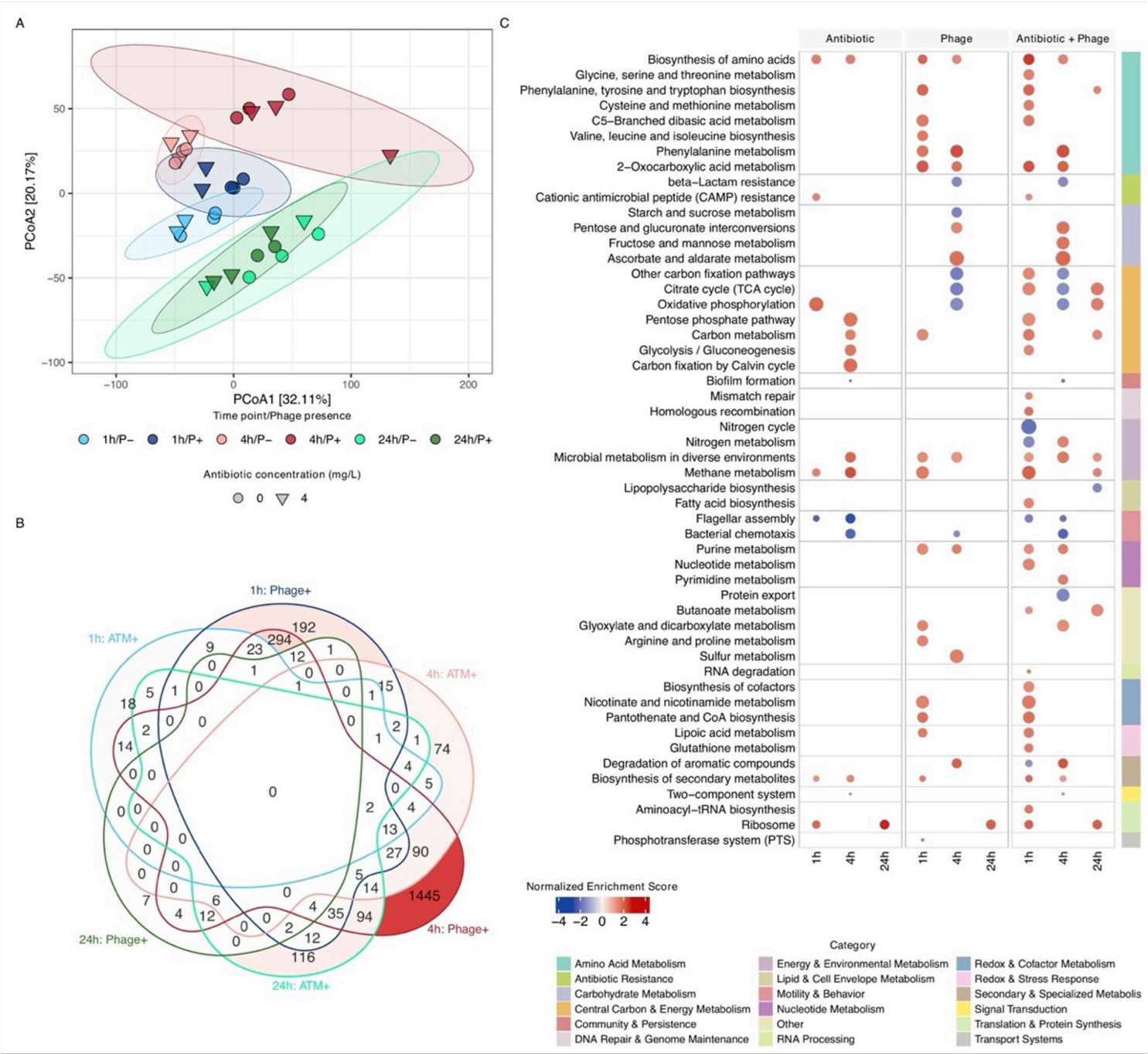


Figure 2

Time-structured transcriptional profiles during antibiotic, phage and combination exposure. **(A)** PCoA of normalized transcriptomes shows strong separation by time point (1h, 4h, 24h), with treatment-specific divergence most evident at 4h. **(B)** Venn diagram summarizing overlap of differentially expressed genes (adjusted P value (p_{adj}) < 0.05 and $|\log_2$ fold change| ≥ 1) across treatments and time points, highlighting early-only, sustained, and late-emerging gene sets. **(C)** Dot plot summarizes KEGG gene set enrichment analysis (GSEA) across phage, antibiotic, and combination treatments (p_{adj} < 0.05). Dot

color indicates the normalized enrichment score (NES; positive or negative enrichment), and dot size reflects the leading-edge signal (a larger dot indicates that a greater fraction of genes in the pathway contributed to the enrichment).

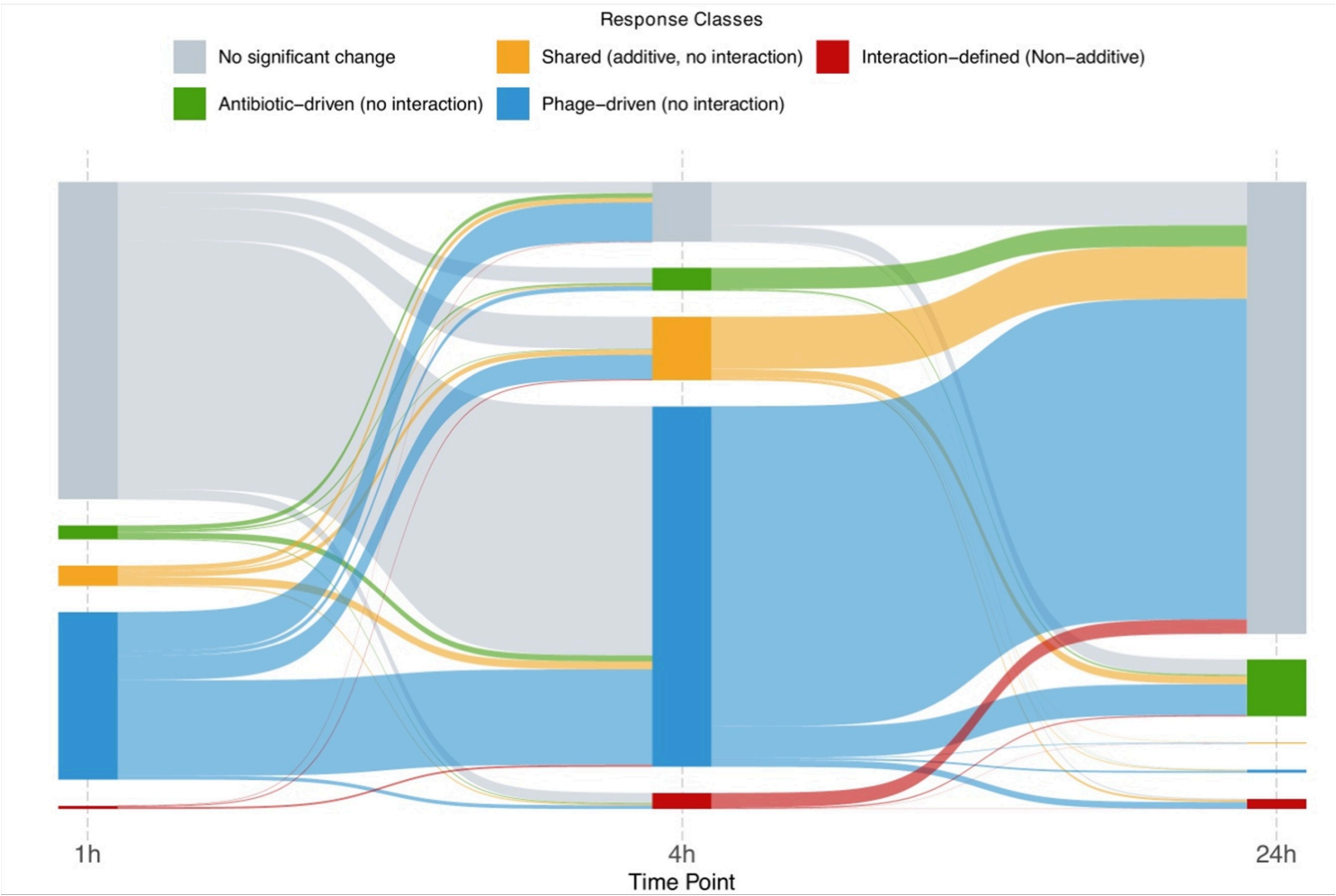


Figure 3

Temporal redistribution of transcriptional responses. Sankey diagram summarizing the temporal redistribution of genes across 1h, 4h, and 24h based on interaction model outputs. Nodes are proportional to gene counts at each time point, and the flows track how genes shift between categories over time. Only genes significant ($p_{adj} < 0.05$) in at least one model coefficient across the experiment are included.

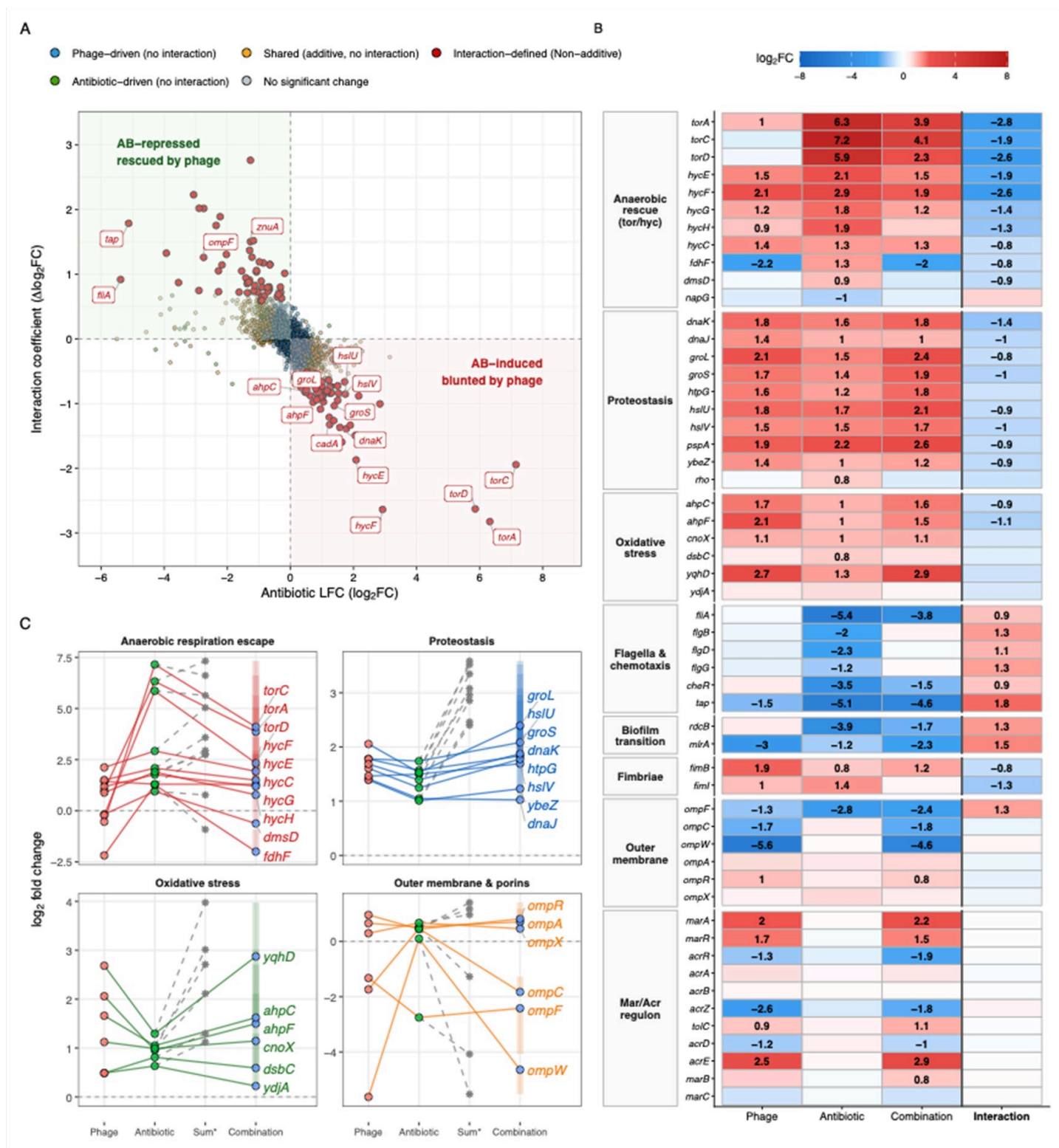


Figure 4

Interaction-defined transcriptional responses at the 4h killing timepoint. (A) Scatter plot showing the relationship between the antibiotic $\log_2FC$ and the interaction coefficient ($\Delta\log_2FC$) for genes at 4h. Points are colored by response class. (B) Heatmap of $\log_2FC$ for representative genes grouped by functional category, showing phage, antibiotic, observed combination, and interaction effects. (C) Branching slope plots for major functional clusters. For each gene, phage and antibiotic mono-treatment

$\log_2FC$ are connected to (i) "Sum" the arithmetic sum of the two independently shrunken mono-treatment estimates, shown as a descriptive reference point, and (ii) "Combination" the observed, independently shrunken combination-treatment estimate.

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