Defence systems provide synergistic anti-phage activity in *E. coli*

Yi Wu[1], Anne van den Hurk[1], Cristian Aparicio-Maldonado[1], Simran Krishnakant Kushwaha[1,2], Claire M. King[1], Yaqing Ou[3], Thomas C. Todeschini[1], Martha R.J. Clokie[4], Andrew D. Millard[4], Yilmaz Emre Gençay[5], Franklin L. Nobrega[1]*

[1] School of Biological Sciences, University of Southampton, SO17 1BJ Southampton, United Kingdom

[2] Department of Biological Sciences, Birla Institute of Technology & Science (BITS), Pilani, Rajasthan, India

[3] Wellcome Centre for Cell-Matrix Research, Faculty of Biology, Medicine and Health, University of Manchester, United Kingdom

[4] Department of Genetics and Genome Biology, University of Leicester, Leicester, UK

[5] SNIPRBiome, Copenhagen, Denmark

* Correspondence: F.Nobrega@soton.ac.uk

Abstract

Anti-phage defence systems in bacterial genomes have a vast intra- and inter-species diversity. Here we investigated whether combinations of defence systems are selected for or against due to the effects of anti-phage protection. Analysis of defence system co-occurrence in *Escherichia coli* genomes revealed that positive and negative co-occurrences are common, and surprisingly both can provide synergistic anti-phage activities. Furthermore, we did not find conserved patterns of defence system co-occurrence across bacterial taxa. This suggests that the selection of strains carrying defence system combinations is more likely a consequence of environmental conditions than mechanistic incompatibilities between defence systems. Additionally, we showed that Gabija and Zorya type II can sense the anti-restriction protein Ocr to activate defence, and that phages also make use of these shared sensing mechanisms to evade multiple defence systems with a single escape strategy. Together, our results highlight the complex co-evolutionary dynamics of phage defence and anti-defence.

Keywords: defence system co-occurrence, synergy, Gabija, tmn, Druantia, Zorya, Kiwa, ietAS, escape mutants, Ocr

Introduction

Bacteria employ multiple lines of active defence to withstand phage predation. In recent years, dozens of new anti-phage defence systems were discovered based on the observation that defences are commonly located in defence islands in the bacterial genome (Doron et al., 2018; Gao et al., 2020; Makarova et al., 2013; Millman et al., 2022; Rousset et al., 2022; Vassallo et al., 2022). These genomic islands have been suggested to facilitate mobilization of the defence systems between bacterial strains and are usually of variable composition (Hochhauser et al., 2022; Piel et al., 2022; Tesson et al., 2022). Evidence suggests that certain sets of defence systems seem to be more conserved than others (Costa et al., 2022), suggesting that specific combinations of defence systems may co-occur in bacterial genomes for enhanced anti-phage defence. For example, different CRISPR-Cas subtypes co-occur and their CRISPR arrays appear to interact with other *cas* genes (Bernheim et al., 2019). Toxin-antitoxin RNA pairs safeguard CRISPR immunity by making cells dependent on CRISPR-Cas for survival (Li et al., 2021), while CRISPR-Cas and restriction-modification (RM) systems (Dupuis et al., 2013), as well as BREX and the restriction enzyme BrxU (Picton et al., 2021) co-occur to expand phage protection. However, the co-occurrence of defence systems has not been explored on a larger scale.

Here we looked at the co-occurrence of defence systems in thousands of *Escherichia coli* genomes and investigated their relevance to phage defence. We observed that multiple pairs of defence systems co-occur more frequently than expected and we analysed three of these pairs to reveal strong synergistic anti-phage activities. Defence systems that co-occur less frequently than expected were also numerous in *E. coli* but testing of two pairs also revealed synergistic anti-phage effects. These results show that negative co-occurring defence systems are not necessarily mechanistically incompatible, and instead may be selected against due to functional redundancy or the existence of more favourable defence system combinations in specific environments. This aligns with our observation that defence system co-occurrence is not conserved across bacterial taxonomic groups. We additionally found that the anti-defence gene Ocr is detected by the Gabija and Zorya type II defence systems, as previously seen for PARIS (Rousset et al., 2022). However, sensing mechanisms that are shared among defence systems are explored by phages to evolve broad-range escape mutations that allow simultaneous escape from multiple defence systems.

Results

Defence system distribution in *E. coli* genomes

We analysed 26,384 *E. coli* genomes of the NCBI Reference Sequence (RefSeq) database for the presence and nature of defence systems (Payne et al., 2021; Tesson et al., 2022), and found that restriction-modification (RM) (99.8%) and CRISPR-Cas (68.8%) are the most prevalent (**Figure 1A**, **Figure S1A**), followed by AbiE (20.0%), CBASS (19.6%), Retrons (16.0%), Druantia (14.0%), DRT (13.1%), and Zorya (11.2%). We next investigated whether these defence systems are located in the chromosome, prophages, plasmids, or prophages in plasmids (**Figure 1A**). While most of the defence systems occur in the chromosome, some systems primarily appear on prophages and plasmids. BstA, Old, tin, DUF4238, 2TM-1TM-TIR, Nixl, and Rst_3HP are mostly found in prophage regions. These systems, with the exception of DUF4238, have been previously reported to be mainly encoded by prophages (Tesson et al., 2022). Curiously, the defence systems Gabija, PifA, and especially tmn, ppl, AbiQ and viperins are mostly found in plasmids, suggesting that some defence systems are specific to certain mobile genetic elements. However, RM systems, while more often encoded in the bacterial chromosome, are also the defence systems most frequently found in prophages and plasmids (**Figure S1A**,**B**), showcasing their wide distribution.

E. coli genomes carry on average six defence systems (**Figure 1B**), in accordance with previous analysis (Tesson et al., 2022), with some strains carrying as few as one and as many as 20 defence systems. The distribution and the average number of defence systems found per strain do not significantly change when considering only systems found in the chromosome (**Figure 1C**). Mobile genetic elements provide the bacteria with an average of one additional defence system, and up to seven (**Figure 1D**, **Figure S1C,D**), most likely due to restrictions of genome size in comparison to that of the bacterial chromosome.

In summary, we show that defence systems are abundant and diverse in *E. coli* genomes and that some of the defence systems are preferentially carried in specific mobile genetic elements.

Positive and negative defence system co-occurrence is common in *E. coli*

As *E. coli* strains encode multiple diverse defence systems, we questioned if certain combinations of defence systems are favoured due to anti-phage synergism. To address this, we identified pairs of defence systems that co-occur more frequently (positive co-occurrence) or less frequently (negative co-occurrence) than expected based on their individual prevalence in *E. coli* genomes (**Table S1**). Our results show that multiple defence systems positively co-occur (**Figure 1E**), including RM type IIG and CRISPR-Cas type I-E,

agreeing with previous experimental work showing that RM and CRISPR-Cas systems work together in *Streptococcus thermophilus*, increasing the defence against phage predation (Dupuis et al., 2013). On the other hand, we also found that several pairs of defence systems negatively co-occur, including CRISPR-Cas type I-E and type I-F1, as previously observed in Proteobacteria (Bernheim et al., 2019).

We observe that all defence systems have positive and/or negative co-occurring systems, with the exception of RM type II, DprA_PRTase and Helicase_DUF2290 (**Figure 1F**). Some defence systems have many positive co-occurring systems (>10), including AbiE, CRISPR-Cas type I-E, Gabija, PsyrTA, RM IV, Thoeris I and tmn, suggesting that their mechanisms can synergise with multiple other systems for anti-phage activity, or that their combined activities are favourable in the environments inhabited by *E. coli* strains. In contrast, other defence systems have multiple negative co-occurring systems (>15), including Dpd, Druantia III, retron I-C, RM II-G, Zorya II, AbiE and PsyrTA. The negative co-occurrences can indicate negative epistasis, in which there is a counter-selection of cells with both defence systems, or functional redundancy of the defence systems that leads to the loss of one by genetic drift (Bernheim et al., 2019). To understand which of these hypotheses supports the observed negative co-occurrences, we looked at the pairs of negative co-occurring defence systems to see if they still co-occur in genomes, or if no co-occurrence can be found (**Table S1**). For most defence system combinations, these can still be found in the same genomes, suggesting that their negative co-occurrence is likely the result of functional redundancy. No co-occurrence was only observed for a few pairs of systems, including Zorya type II and CRISPR-Cas I-F1, retron I and Lit, dpd and Lit, retron II-A and PsyrTA, and CRISPR-Cas I-F1 and PsyrTA, suggesting negative epistasis, but due to the low expected number of co-occurrences (16 to 66, **Table S1**) no conclusions can be drawn from these observations.

Interestingly, subtypes of the same defence system have distinct patterns of co-occurrence with other systems (**Figure 1E, F**). For example, Druantia type III has 27 negative co-occurring defence systems and positively co-occurs with Zorya type II, which is the only system that negatively co-occurs with Druantia type I. Druantia type III contains *druH* (unknown function), which is absent in Druantia type I (Doron et al., 2018), and thus may be associated with the numerous negative co-occurrences observed here. Similarly, Zorya type II has 32 negative co-occurring defence systems, while Zorya type I has only two. Genes *zorC* (unknown function) and *zorD* (helicase and sometimes containing Mrr-like nuclease domains) present in Zorya type I are absent in Zorya type II, which instead contains *zorE*, an HNH endonuclease (Doron et al., 2018) that may be responsible for the negative co-occurrences with multiple defence systems.

In addition to looking at defence system co-occurrence in the main genome, we also screened *E. coli* prophages and plasmids (**Figure S1E,F**), which revealed a vast majority of negative co-occurrences, in contrast to our general observation in *E. coli* where we found a majority of positive co-occurrences (**Figure 1E**). This striking difference is likely explained by a limitation in the number of defence systems that prophages and plasmids can carry in their smaller genomes.

Overall, our results reveal that both positive and negative defence system co-occurrences are common in *E. coli*, and suggest that negative co-occurring systems are the result of functional redundancy rather than negative epistasis due to conflicting molecular mechanisms.

Defence system co-occurrence varies across bacterial taxonomic groups

To assess if co-occurring defence systems are conserved across bacterial taxa, we performed our co-occurrence analysis for Enterobacterales (9,124 strains, **Figure 2A**, **Table S1**), Pseudomonadales (1,288 strains, **Figure 2B**), Bacillales (3,952 strains, **Figure 2C**) and Burkholderiales (2,199 strains, **Figure 2D**). Data show that positive and negative defence system co-occurrences are more frequent in Enterobacterales than in other orders. Importantly, positive and negative co-occurring defence systems are distinct across bacterial taxa. Among all the co-occurring pairs of systems, only four negative co-occurring pairs (Gabija and Mokosh, Zorya and Mokosh, CBASS and Mokosh, and AbiE and Mokosh) are shared by two taxa, Enterobacterales and Burkholderiales. This is not unexpected, since variation in defence system prevalence among taxa was previously reported (Tesson et al., 2022). Interestingly, pairs of defence systems that co-occur positively in one taxon can co-occur negatively in a second taxon, and the opposite is also true. For example, CRISPR and Zorya co-occur positively in Enterobacterales and negatively in Burkholderiales, and CBASS and Gabija co-occur positively in Bacillales and negatively in Enterobacterales. These observations suggest that the negative co-occurring systems are not necessarily functionally incompatible, and the negative co-occurrence is more likely a consequence of selection determined by environmental factors (e.g. phages encountered).

Additionally, we observed that negative co-occurrences of defence systems are more frequent than positive co-occurrences in all taxonomic orders analysed, except for Pseudomonadales (**Figure 2B**) in which 7 positive and 2 negative co-occurrences are observed.

In summary, our results indicate that defence system co-occurrence diverges among bacterial taxa, and seems to be more frequent in Enterobacterales than in Pseudomonadales, Bacillales or Burkholderiales. Negative co-occurring systems in one bacterial taxon were found to positively co-occur in different taxa, suggesting that defence systems are in their majority mechanistically compatible.

Positive co-occurring systems provide synergistic anti-phage protection

To test whether defence system co-occurrence is indicative of added benefits in terms of strength or broadness of anti-phage activity, we selected the co-occurring pairs Gabija and tmn, Druantia type III and Zorya type II, and ietAS and Kiwa. The defence systems were cloned individually and together in *E. coli* (**Figure 3A**) and confirmed to have no deleterious effect on bacterial growth (**Figure S2A**). The protective effect of the systems against a panel of 13 phages was tested using efficiency of plating (EOP) and liquid culture assays.

For the Gabija and tmn pair, the EOP assays show that Gabija alone protects against all 11 phages tested with varied strengths (**Figure 3B**, left) while tmn protects against seven of the 13 phages. When together, these systems provide synergistic protection (i.e., stronger than the combined effects of the individual systems) for phages T1, T3, T4 and JK16, with lysis from without (LO, i.e. non-productive infection) occurring for all four phages. For example, the infectivity of phage T1 is reduced $<10^1$-fold by Gabija, $<10^2$-fold by tmn, and $>10^6$-fold by the combination. For T7, a synergistic activity is observed in terms of reduction of plaque size (**Figure S2B**). Curiously, synergism is not restricted to phages that are targeted by both individual systems, since T4 is not affected by tmn individually, but the combination of Gabija and tmn results in increased defence. Moreover, synergism was not observed with all phages targeted by the individual systems, with some (e.g. SLUR435 and SLUR432) showing the same reduction provided by the strongest individual system (tmn). This shows that synergism in defence occurs only against specific phages. Infection dynamics in liquid cultures confirm synergism of the defence systems against phage T3 (**Figure 3B**, right). This is not obvious for T1 since tmn individually provides almost full protection to the cell population at a low multiplicity of infection (MOI).

For the Druantia type III and Zorya type II pair, EOP assays showed that the individual systems provide low levels of protection ($<10^2$-fold reduction in EOP) for the phages tested (**Figure 3C**, left). However, when in combination, these systems offer synergistic protection for phages T1, T3 (LO), phi113 ($\approx 10^1$-fold additional reduction compared to individual systems), and especially 670 and 672 ($>10^4$-fold reduction compared to individual systems, LO). For phage T3, the synergism is also visible in liquid cultures (**Figure 3C**, right) where

the cell population grows as the uninfected population at a low phage MOI. Synergism was observed for phage T7 in EOP assays only as a reduction of plaque size (**Figure S2**), and the liquid cultures show a clear benefit to the population of having the two systems when targeted by phage T7 at low MOIs.

For the ietAS and Kiwa pair (**Figure 3D**), the individual defence systems provide no significant protection against our panel of phages, and their combination does not result in synergistic effects, although protection is slightly increased against phage T3 in EOP assays (10^1-fold reduction). Considering the low levels of activity of the defence systems against our panel of phages, it is possible that stronger effects of the individual systems and their combinations can be observed against other phage types (e.g. ssDNA or RNA) or mobile genetic elements.

Overall, we show that combinations of systems that positively co-occur can act synergistically against specific phages, providing an increased anti-phage defence to the cell.

Negative co-occurring systems can provide synergistic anti-phage protection

Next, we investigated the anti-phage activity of defence systems pairs that were found to co-occur less than expected, including Druantia type III and Gabija, and Zorya type II and ietAS (**Figure 4A**). For Druantia type III and Gabija, we surprisingly observed synergistic protection of the combination for multiple phages in the EOP assays (**Figure 4B**, left), including phages T1, T3 (LO), T4 (LO), SLUR435, SLUR432, and especially JK16 (10^2-fold reduction by Gabija, no reduction by Zorya type II, and $>10^6$-fold reduction by the combination of both systems). This is confirmed in liquid cultures with phages T1 and T3 (**Figure 4B**, right). For Zorya type II and ietAS, a synergistic effect is observed for phage T3 (LO) in both EOP and liquid culture assays (**Figure 4C**). For phage T7, synergistic protection is observed in plaque size (**Figure** S2B) and in the liquid cultures (**Figure 4C**). Importantly, no negative effect was seen in the protection against any of the phages tested when the negatively co-occurring systems were combined.

Overall, these results indicate that negative co-occurrence of defence systems does not imply mechanistic incompatibility, and synergistic effects can still be observed against specific phages.

Phage escape mechanisms can provide broad evasion from defence systems

To investigate the mechanisms underlying the synergistic anti-phage effects described above, we isolated evolved phage escape mutants for the combinations of Gabija and tmn, and Druantia type III and Zorya type II. To isolate the phage mutants, we selected phage plaques obtained in EOP assays with each defence system combination and tested these phages for their ability to evade the defence systems (i.e., culture collapse) in liquid culture assays (**Figure 5A**).

The phage T1 escape mutants of Gabija and tmn (GT#) were able to overcome the defence provided by tmn completely and by the combination of Gabija and tmn almost completely in both EOP and liquid cultures (**Figure 5B**, **Figure 4B**, **Figure S3A**). This suggests that the combination of systems is more difficult to overcome by the escape mutants than the individual systems, and that this effect is mostly achieved by escaping targeting by tmn. We sequenced the T1 escape mutants and identified a common mutation in a gene coding for a protein of unknown function (*46*, locus tag T1p46, **Table S2**), with a dedicated promoter and rho-independent terminator (Roberts and Kropinski, 2011). No further information is known about the role of this gene in T1 infection, and therefore in the detection by tmn.

For phage T3 escape mutants of Druantia type III and Zorya type II (DZ#), we observed partial escape from the combination of systems (EOP and liquid cultures in **Figure 5C**, **Figure 4C**, **Figure S3B**), and escape from Zorya type II visible only in the liquid cultures. Sequencing of the escape phages revealed mutations in the tail fibre gene (*g17*, locus tag T4p48), suggesting that the tail fibre is being sensed by Zorya type II to activate the immune response.

We checked also for cross-escape mechanisms by testing how the escape phages respond to other defence systems individually and combined. While no additional effect was detected for T1 GT# escape mutants (**Figure 5B**), the T3 DZ# escape mutants show cross-escape with several defence systems. DZ# escape mutants have a restored ability to infect Gabija and tmn individually, but not the combination of both (**Figure 5C**). On the other hand, the DZ# mutants overcome the strong protection provided by the combinations of Druantia type III and Gabija (10^4-fold), and Zorya type II and ietAS (10^4-fold), indicating that Zorya type II, Gabija, and tmn all use the tail fibre *g17* (T3p48) to sense infection by phage T3. Interestingly, Gabija and tmn together seem to be able to overcome this evasion mechanism and sense infection by phage T3.

In summary, we demonstrate that phage escape mutants can be generated for pairs of defence systems that act synergistically against the wild type phage and that some escape mutations provide the phages with the ability to escape from multiple individual and combined defence systems.

Anti-restriction protein Ocr is sensed by Gabija and Zorya type II

During our experiments with the defence systems, we tested phage T7Select, a version of phage T7 typically used for phage display experiments (Sharma et al., 2009). The genome of T7Select is identical to that of phage T7 except for the deletion of a set of genes known to be involved in host take over, among which *0.3*, which codes for the anti-restriction protein Ocr that inhibits RM (Zavilgelsky and Kotova, 2014) and BREX (Isaev et al., 2020) defence systems.

To investigate if Ocr is involved in anti-defence activities against the systems tested here, we expressed Ocr in the strains containing the individual and combined defence systems and assessed the impact on T7Select infectivity (**Figure 6A**). Interestingly, protein Ocr elicits a stronger protection against T7Select by Gabija (10^2-fold), Zorya type II (10^1-fold) and the combination of Druantia type III and Gabija (10^2-fold, same as Gabija alone) (**Figure 6B**, **Figure S4**). Furthermore, the phage plaques formed in these systems were turbid and had a significant decrease in size (**Figure 6C**). This suggests that the Ocr protein is sensed by Gabija and Zorya type II, as previously described for the PARIS defence system (Rousset et al., 2022). On the other hand, when Zorya type II is combined with Druantia type III, Ocr improves the infectivity of phage T7Select by 10^1-fold (**Figure 6B,C**, $p < 0.05$), suggesting that this combination of systems interferes with sensing of Ocr by Zorya type II, and reduces the anti-phage protection. Curiously, when combined with Gabija, Druantia type III does not interfere with sensing of Ocr, suggesting that the antagonistic effect is specific to the combination with Zorya type II.

In summary, we show that Gabija and Zorya type II sense the anti-restriction protein Ocr to activate their defence mechanisms against T7Select. However, sensing of Ocr by Zorya type II seems to be blocked by Druantia type III, suggesting a mechanistic disadvantage of combined defence systems.

Discussion

To assess whether the clustering of defence systems in genomic defence islands is indicative of cooperation between defences, we investigated the co-occurrence of defence systems in *E. coli*. We found several pairs of defence systems to co-occur more frequently than theoretically expected by chance alone, suggesting the establishment of beneficial anti-phage effects that are favourably selected for in bacterial populations. When testing three of the co-occurring pairs of defence systems (Gabija and tmn, Druantia type III and Zorya type

II, ietAS and Kiwa), we detected synergistic defence against some of the phages from our panel. Phage escape mutants that were generated in the combinations of defence systems could escape especially well from one of the defences, and at least partially from the combination of defence systems, suggesting that only one of the defence systems is sensing phage infection, while both defences act in concert for enhanced anti-phage protection via unknown mechanisms.

Notably, we also detected several negative co-occurring defence systems in *E. coli*, by testing two of these pairs (Druantia type III and Gabija, Zorya type II and ietAS) revealing synergistic defence against specific phages. Since most of the negative co-occurring defence systems can be found together in a few genomes, negative co-occurrences do not seem to predict incompatibility between defence systems. Instead, they likely suggest functional redundancy of the defence systems (as previously reported for CRISPR-Cas systems (Bernheim et al., 2019)) or selection for defence system combinations that are more favourable in certain environments, e.g. type of phages encountered. This hypothesis is consistent with the uneven distribution of defence systems among microbial species previously reported (Tesson et al., 2022), the lack of conservation in co-occurring defences across bacterial taxa that we report here, and the fact that negative co-occurring systems in one taxon are found to positively co-occur in other taxa.

Additionally, we show that pairs of synergistic defence systems are not sufficient to prevent phages from evolving to escape defence. More importantly, these evasion mechanisms can provide broad escape from multiple individual and combined defence systems that share sensing strategies. Recently, the evolution of phage T4 against the toxin-antitoxin *toxIN* was shown to constrain its ability to escape from other defence systems due to compensatory losses in the genome to accommodate for the genome size increase caused by the segmental amplification of the gene responsible for the escape mechanism (Srikant et al., 2022). In contrast, the T3 escape mutants generated in our work acquire an expanded host range. This is most likely because the point mutation in the tail fibre gene did not cause rearrangements elsewhere in the phage genome (i.e. no loss or mutation of genes required for infection of other strains), and allows escape from three defence systems that sense the same phage protein (Gabija, tmn, Zorya type II, and combinations thereof). This indicates that phage escape mechanisms do not necessarily come with a fitness cost, and indeed they may provide a fitness benefit.

We show further examples of defence systems sharing sensing strategies, with Gabija and Zorya type II detecting the anti-RM and anti-BREX protein Ocr (Isaev et al., 2020; Zavilgelsky and Kotova, 2014), as previously shown for the PARIS defence system (Rousset

et al., 2022). Therefore, these systems may act as a second line of defence when RM or BREX systems fail to control phage infection. In this context, we revealed a disadvantage of specific combinations of defence systems, with Druantia type III negatively interfering with the Zorya type II sensing of Ocr, highlighting the complex co-evolutionary dynamics of phage defence and anti-defence (**Figure 7**).

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Author contributions

Conceptualization, F.L.N.; Methodology, Y.W. and F.L.N.; Formal Analysis, Y.W., A.v.d.H., and Y.O; Investigation, Y.W., A.v.d.H., C.A.M., S.K., Y.O., C.M.K., and T.T.; Resources, M.C., A.M., E.G., and F.L.N.; Data Curation, Y.W.,and F.L.N.; Writing – Original Draft, Y.W. and F.L.N.; Writing – Review & Editing, all authors; Visualization, Y.W. and F.L.N.; Supervision, E.G. and F.L.N.; Funding Acquisition, F.L.N.

Declaration of interests

Y.E.G. is a full-time employee of SNIPR Biome.

Methods

Bacteria

E. coli strain Dh5α was used to clone plasmids pACYCDuet-1 or 8A with individual defence systems. *E. coli* BL21-AI cells containing plasmid(s) with the defence systems were used for phage assays. All bacterial strains were grown at 37 °C in Lysogeny Broth (LB) with 180 rpm shaking for liquid cultures, or in LB agar (LBA) plates for solid cultures. Strains containing plasmid pACYCDuet-1 or 8A were grown in media supplemented with 25 µg/ml of chloramphenicol or 100 µg/ml of ampicillin, respectively.

Phages

Phages used in this study and their origins are described in **Table S3**. All phages were produced in LB with their host strain, centrifuged, filter-sterilized, and stored as phage lysates at 4 °C.

Defence system detection

The FASTA amino acid (FAA), FAST Nucleic Acid (FNA), and Generic Feature Format (GFF) files of 26,384 *Escherichia coli*, 9,124 Enterobacterales, 1,288 Pseudomonadales, 3,952 Bacillales, and 2,199 Burkholderiales genomes were downloaded from the NCBI Reference Sequence (RefSeq) Database in October 2021. Defence systems were detected in these genomes using PADLOC version 1.1.0 with database version 1.4.0 (Payne et al., 2021) and DefenseFinder version 1.0.8 (Tesson et al., 2022). Defence systems outputted by PADLOC in categories *other* and *adaptation* were removed from the analysis as they would require manual validation. Some of the genomes had extensive fragmentation, which negatively influenced the number of defence systems detected (two-sided Spearman, $p < 0.001$), but the effect was small enough ($r_s = -0.04$) that we opted to keep the fragmented genomes in our analysis. Defence systems found in all strains can be seen on **Table S4**.

Contig characterization and prophage detection

Platon version 1.6 (Schwengers et al., 2020) on accuracy mode was used to categorize bacterial contigs as plasmid or chromosome. Phigaro version 2.2.6 (Starikova et al., 2020) was used with default settings to detect prophages in the bacterial genomes. Contigs shorter than 20 kbp were excluded from this analysis. Defence systems were considered to be located in a prophage region when at least one defence gene was fully within the prophage limits.

Co-occurrence analysis

The observed co-occurrence of defence systems was calculated as the number of genomes in which they both occur simultaneously. The expected co-occurrence of two defence systems was calculated as the product of the observed occurrence of each defence system, divided by the total number of genomes analysed. The fold change between the observed and the expected co-occurrence was determined as the difference between observed and expected co-occurrence divided by the expected co-occurrence. For visual clarity, the fold change was capped at 1, and all values are available at **Table S1**.

Defence system cloning

The plasmids constructed in this work are listed in **Table S5**, and the primers used can be found in **Table S6**. Plasmid pACYCDuet-1 was modified to contain the pBAD promoter from plasmid 8A (MacroLab) by Gibson assembly. YFP, Druantia type III (from *E. coli* ECOR19), ietAS (ECOR52), and tmn (ECOR25) were cloned into the modified pACYCDuet-1 by Gibson assembly. YFP, Kiwa (ECOR8), Gabija (ECOR49), and Zorya type II (ECOR19) were cloned into plasmid 8A by Gibson assembly. The Ocr gene was amplified from phage T7 and cloned into plasmid pCOLA by Gibson assembly. The plasmids were recovered in Dh5α cells, extracted using NucleoSpin Plasmid QuickPure Kit (Thermo Fisher Scientific) and confirmed by sequencing at Plasmidsaurus (USA). Plasmids containing defence systems or the YFP control were transformed individually or in combinations into competent BL21-AI cells prepared using the Mix&Go! *E. coli* Transformation Kit (Zymo).

Efficiency of platting

Overnight cultures of the bacteria were diluted 1:50 in LB containing antibiotics, induced with 0.2% arabinose, and incubated for 5 hours before being used in double agar overlay assays. For this, bacterial cultures were mixed with 0.6% top agar and overlaid on LBA plates. Ten-fold serial dilutions of the phage stocks were spotted onto the bacterial lawn and the plates incubated overnight at 37 °C.

Liquid assay

Overnight bacterial cultures were diluted to an optical density at 584 nm of 0.25 in LB containing antibiotics and 0.2% arabinose. The bacterial suspension was distributed into wells of a 96-well plate to which phage dilutions or LB were added. The plates were incubated in a Fluostar Optima plate reader at 37 °C with shaking at 200 rpm, with optical density at 584 nm measured every 5 min for 24 hours.

Isolation of phage escape mutants

Phage plaques obtained in double layer agar plates with combinations of defence systems were picked and dissolved in 30 µl of LB. The phage solution was tested for the ability to infect bacteria containing the defence systems using liquid assays as described above. Phages with the ability to kill the bacteria in the liquid assay were produced in liquid using the strain with the defence system combination as the host. Phage titre was determined using the double layer overlay assay.

Phage DNA extraction and sequencing

Phage DNA was extracted using the phenol-chloroform method. Briefly, DNase I and RNase were added to the phage stock at 1 µg/ml each, and the solution was incubated for 30 min. EDTA, proteinase K and SDS were added to the solution at 20 mM, 50 µg/ml and 0.5%, respectively, and incubated for 1 hour at 56 °C. And equal volume of phenol was added to the mixture, vortexed, and centrifuged at 3,000 × *g* for 10 min, at room temperature. The aqueous phase was recovered, mixed with an equal volume of 1:1 phenol:chloroform, and centrifuged. The procedure was repeated with chloroform. After centrifugation, the aqueous phase was recovered and mixed with 0.1 volumes of 3 M sodium acetate, pH 5.0 and 2.5 volumes of ice-cold absolute ethanol. The mixture was incubated at -20 °C for 1 hour and centrifuged at 14,000 × *g* for 15 min. The DNA pellet was resuspended in ice-cold 70% ethanol and centrifuged for 5 min. The pellet was let dry at room temperature and resuspended in nuclease-free water. The quality and quantity of extracted phage DNA were estimated using a Nanodrop and a Qubit fluorometer, respectively. Phage samples were sequenced at SeqCenter (Pittsburgh, USA). Sample libraries were prepared using the Illumina DNA Prep kit and IDT 10 bp UDI indices, and sequenced on an Illumina NextSeq 2000, producing 2x151 bp reads.

Sequencing data analysis

Demultiplexing, quality control and adapter trimming were performed with bcl-convert v3.9.3. Reads were mapped to the reference sequences NC_005833 (phage T1) and NC_003298 (phage T3) using BWA v0.7.17 (Li, 2013), and SNP calling was performed using gatk v4.2.6.1 (van der Auwera and O'Connor, 2020).

Quantification and statistical analysis

A two-sided binomial test was performed to determine if the observed co-occurrence of defence systems differed significantly from the expected co-occurrence, using R (R Core Team, 2018). A Benjamin-Hochberg procedure was used to correct for the multiple testing, with a p value < 0.001 considered significant. Unless stated otherwise, experimental data are presented as the mean of biological triplicates ± standard deviation. Statistical tests were

performed using GraphPad Prism 9.2.0 and one sample t test with a hypothetical value of 1 for the efficiency of plating data.

Data and code availability

Raw data of the phage escape mutants have been deposited at Figshare https://doi.org/10.6084/m9.figshare.20503002. All original code has been deposited at Github https://github.com/FNobregaLab/Defence_cooperation.

Figure legends

Figure 1. Prevalence and co-occurrence of defence systems in *E. coli*. (A) Prevalence of defence systems in 26,384 *E. coli* genomes from the RefSeq database. Frequency of occurrence of the defence systems is shown above, and the percentage of defences found in the chromosome, prophage, plasmid, and prophage in plasmid are shown below. **(B)** Number of defence systems per *E. coli* strain, considering chromosome, prophage and plasmid. The average number of defence systems is indicated as an orange line. **(C)** Number of defence systems per *E. coli* genome, excluding prophages and plasmids. The average number of defence systems is indicated as an orange line. **(D)** Number of defence systems per prophage and plasmid found in *E. coli*. The average number of defence systems is indicated as an orange line. **(E)** Co-occurrence matrix of defence systems in *E. coli*. Positive co-occurrences are shown in orange and negative co-occurrences are shown in green. Significant ($p < 0.001$) co-occurrences are indicated with an asterisk (*). **(F)** Frequency (in number) of positive and negative co-occurrences per defence system subtype. Prevalence of each system is shown on top of the bars.

Figure 2. Co-occurrence of defence systems in multiple bacterial taxa. (A) Co-occurrence of defence systems in 9,124 strains of Enterobacterales. Co-occurrences are shown in orange and mutual exclusions are shown in green. Significant ($p < 0.001$) co-occurrences are indicated with an asterisk (*). **(B)** Co-occurrence of defence systems in 1,288 strains of Pseudomonadales. **(C)** Co-occurrence of defence systems in 3,952 strains of Bacillales. **(D)** Co-occurrence of defence systems in 2,199 strains of Burkholderiales.

Figure 3. Effect of defence systems that positively co-occur on phage infectivity. (A) Experimental set-up for the assessment of the anti-phage activity of individual defence systems and their combinations. YFP, yellow fluorescent protein. **(B)** Efficiency of plating (EOP, left) and liquid cultures (right) of Gabija, tmn, or Gabija and tmn, against a set of *E. coli* phages.. For liquid assays, only the curve of the YFP-YFP control is shown because all other uninfected cultures have similar growth profiles (**Figure S2A**). **(C)** Efficiency of plating (EOP, left) and liquid culture assay (right) of Druantia type III, Zorya type II, or Druantia type III and Zorya type II, against a set of *E. coli* phages. **(D)** Efficiency of plating (EOP, left) and liquid culture assay (right) of ietAS, Kiwa, or ietAS and Kiwa, against a set of *E. coli* phages. * $p < 0.001$, ** $p < 0.0001$. S, small phage plaque morphology compared to control; b.d.,

below limit of detection. Data are shown as the average of three independent biological replicates.

Figure 4. Effect of defence systems that negatively co-occur on phage infectivity. (A) Experimental set-up for the assessment of the anti-phage activity of individual defence systems and their combinations. YFP, yellow fluorescent protein. **(B)** Efficiency of plating (EOP, left) and liquid culture assay (right) of Druantia type III, Gabija, or Druantia type III and Gabija, against a set of *E. coli* phages. **(C)** Efficiency of plating (EOP, left) and liquid culture assay (right) of Zorya type II, ietAS, or Zorya type II and ietAS, against a set of *E. coli* phages. * $p < 0.001$, ** $p < 0.0001$. S, small phage plaque morphology compared to control; b.d., below limit of detection. Data are shown as the average of three independent biological replicates.

Figure 5. Phage escape mutants for defence system combinations. (A) Experimental set-up for obtaining and testing phage escape mutants on combinations of defence systems. **(B)** Phage T1 escape mutants of Gabija and tmn. Top, plate infectivity of wild type phage T1 and escape mutants (GT#) generated in Gabija and tmn, and mutations found in the GT# phages compared to wild type T1. Bottom, fold change infectivity of the escape phages relative to the wild type phage T1, against individual and combined defence systems. LO, lysis from without. Full circles represent productive infection of the cells, while hollow circles indicate lysis from without. Data points with a hollow left half circle indicate LO by the wild type phage, and those with a hollow right half circle indicate LO with the mutant phage. **(C)** Phage T3 escape mutants of Druantia type III and Zorya type II. Top, plate infectivity of wild type phage T1 and escape mutants (DZ#) generated in Druantia type III and Zorya type II, and mutations found in the DZ# phages compared to wild type T3. Bottom, fold change infectivity of the escape phages relative to the wild type phage T3, against individual and combined defence systems. Data points with a hollow left half circle indicate LO by the wild type phage, and those with a hollow right half circle indicate LO with the mutant phage. Data are shown as the average of three independent biological replicates.

Figure 6. Effect of the Ocr protein on the infectivity of phage T7Select. (A) Experimental set up to assess the effect of protein Ocr on the infectivity of phage T7Select. **(B)** EOP assay of T7Select against cells with individual and combined defence systems in the presence and absence of Ocr. EOP of each defence system (individual or combined) with Ocr was determined in relation to its counterpart lacking Ocr. ** $p < 0.0001$. **(C)** Images of EOP

assays to assess effect of Ocr on the infectivity of T7Select in cells containing Druantia type III and Zorya type II, Gabija, and Druantia type III and Gabija. Data are shown as the average of three independent biological replicates.

Figure 7. A model for defence system synergy and immune evasion by phages. Left, combinations of defence systems with positive (orange) and negative (blue) co-occurrence show synergistic anti-phage effects. The exact mechanisms by which these systems exert protection are mostly unknown and are here depicted as DNA cleavage for representation purposes only. Middle, phages escape targeting by combinations of defence systems via mutations in genes such as tail fibre. Right, the anti-restriction protein Ocr inhibits the activity of the combination of Zorya type II and Druantia type III, but is sensed by Gabija individually or combined with Druantia type III or by Zorya type II, which subsequently impair phage infection. The cream colour indicates blocking of phage infection, and the blue-grey colour indicates successful phage infection.

Supplementary information titles and legends

Figure S1 Defence system prevalence and co-occurrence in prophages and plasmids in *Escherichia coli*.

Figure S2 Effect of defence systems on bacterial growth and phage plaque size.

Figure S3 Efficiency of plating (EOP) of phage escape mutants evolved against combined defence systems.

Table S1 Co-occurrence of defence systems in *E. coli*, Enterobacterales, Pseudomonadales, Bacillales, and Burkholderiales

Table S2 List of mutations found in phage T1 and T3 escape mutants

Table S3 List of phages used in this work.

Table S4 Defence systems present in *E. coli*, Enterobacterales, Pseudomonadales, Bacillales, and Burkholderiales genomes as analysed by Padloc and DefenseFinder tools.

Table S5 List of plasmids used and generated in this work.

Table S6 List of primers used in this work.

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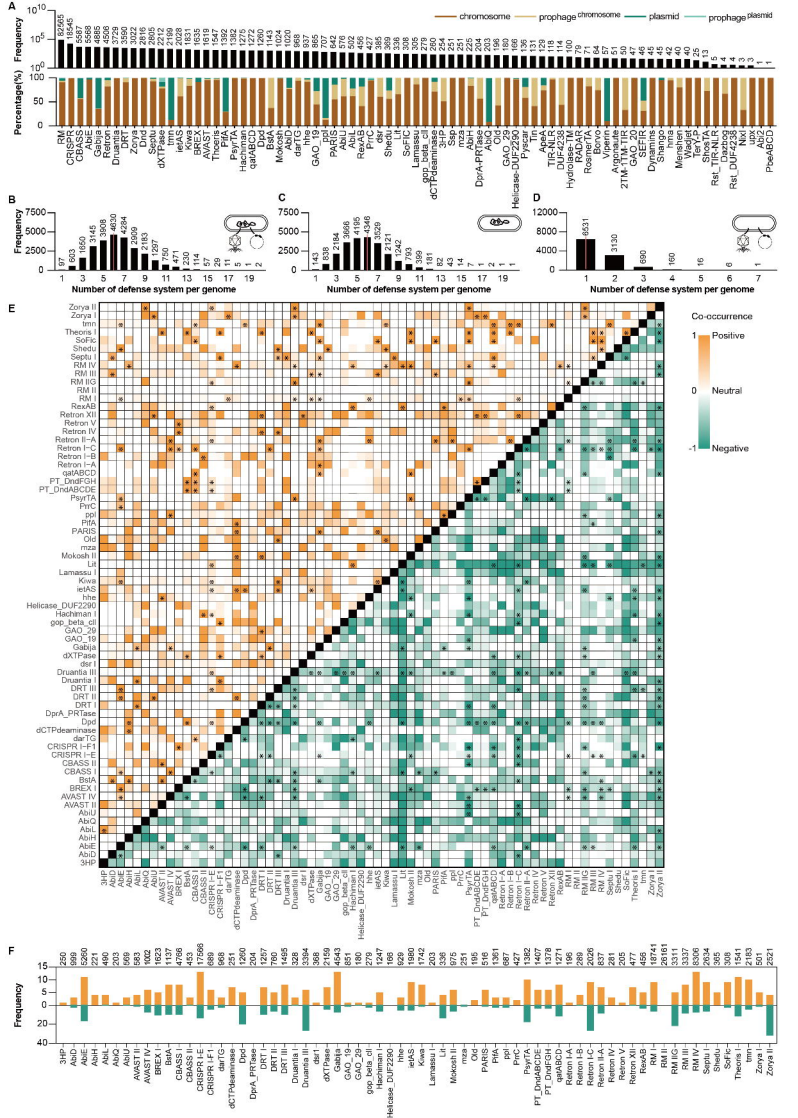
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Frequency
Percentage(%)
chromosome
prophage
plasmid
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C
D
Frequency
Number of defense system per genome
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Co-occurrence
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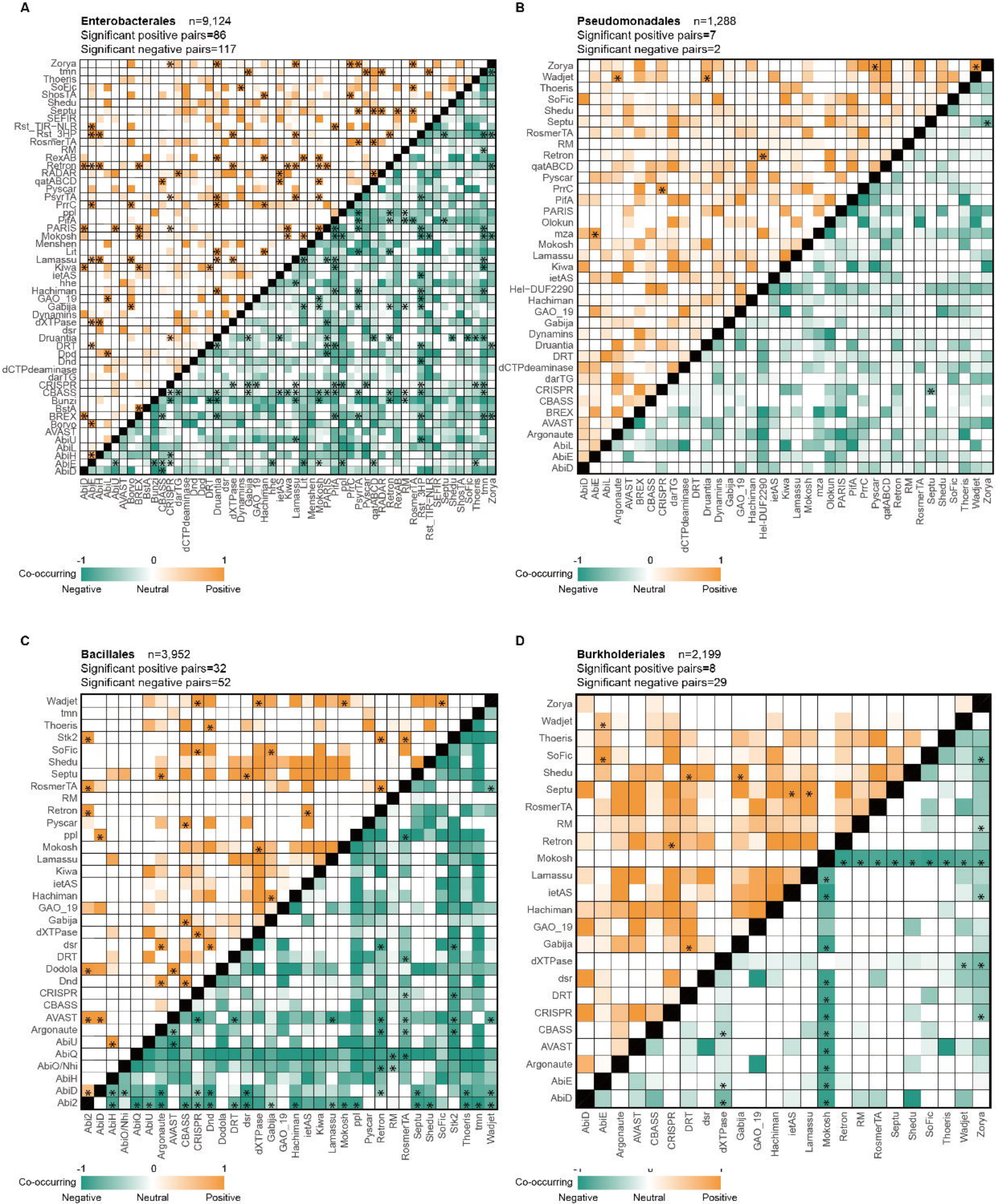
Negative
F
Frequency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Bacillales n=3,952
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Co-occurring
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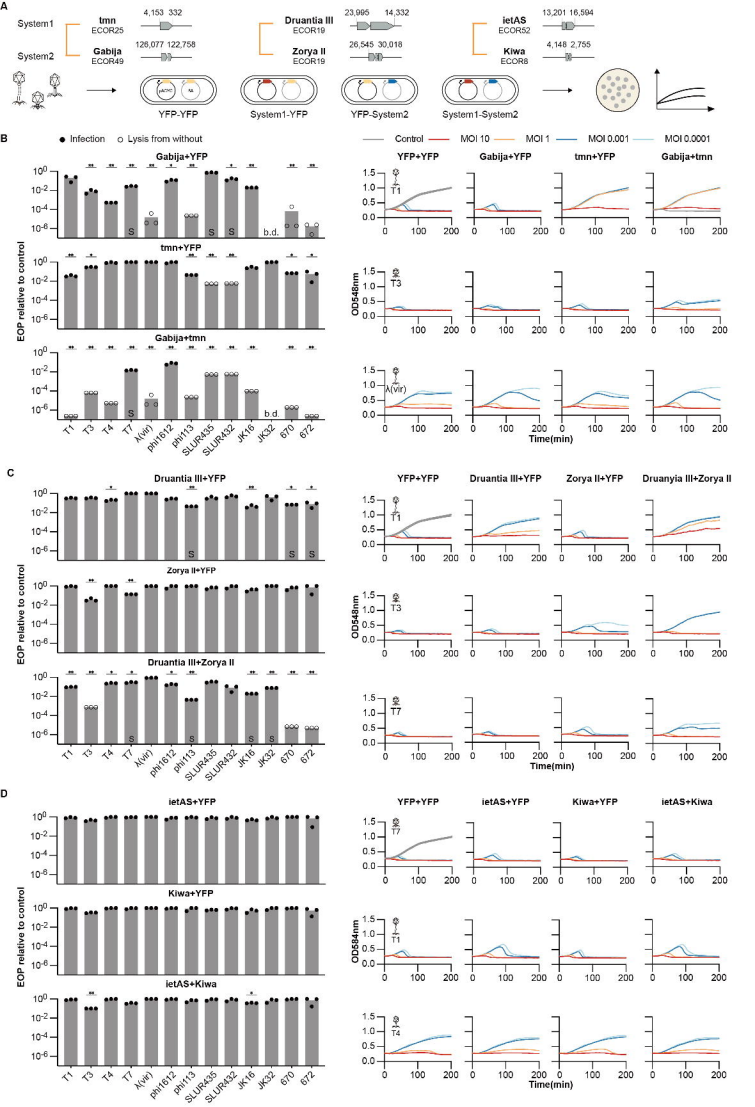
0
1
Negative
Neutral
Positive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System2
tmn
ECOR25
Gabija
ECOR49
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126,077 122,758
Druantia III
ECOR19
Zorya II
ECOR19
23,995 14,332
26,545 30,018
ietAS
ECOR52
Kiwa
ECOR8
13,201 16,594
4,148 2,755
YFP-YFP
System1-YFP
YFP-System2
System1-System2
B
Infection
Lysis from without
Gabija+YFP
tmn+YFP
Gabija+tmn
EOP relative to control
T1
T3
T4
T7
λ(vir)
pH1612
pH113
SLUR435
SLUR432
JK16
JK32
67D
67Z
b.d.
Control
MOI 10
MOI 1
MOI 0.001
MOI 0.0001
YFP+YFP
Gabija+YFP
tmn+YFP
Gabija+tmn
OD548nm
Time(min)
C
Druantia III+YFP
Zorya II+YFP
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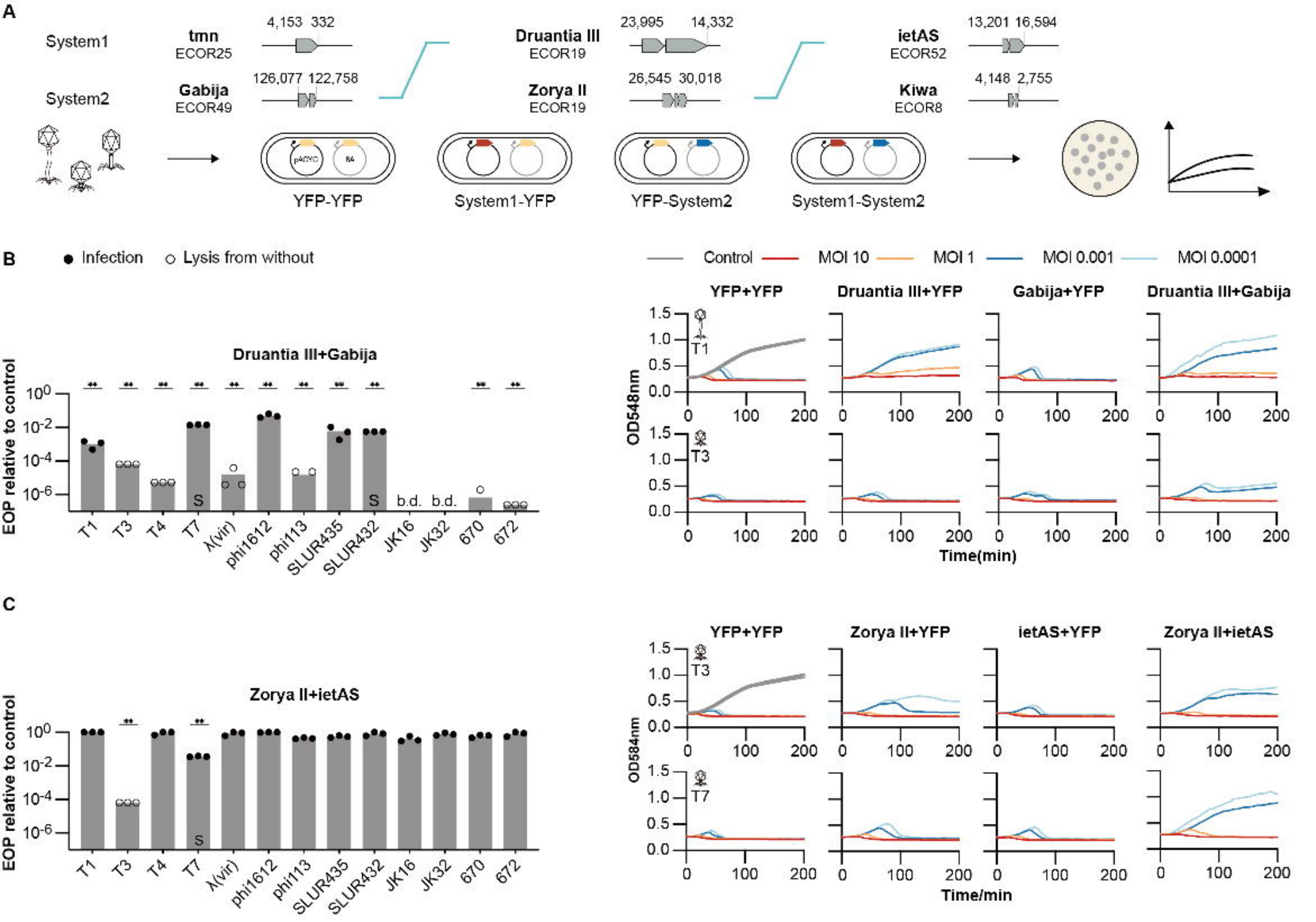
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Kiwa
ECOR8
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YFP-YFP
System1-YFP
YFP-System2
System1-System2
B
Infection
Lysis from without
Druantia III+Gabija
EOP relative to control
T1
T3
T4
T7
λ(vir)
phi1612
phi113
SLUR435
SLUR432
JK16
JK32
670
672
b.d.
Control
MOI 10
MOI 1
MOI 0.001
MOI 0.0001
YFP+YFP
Druantia III+YFP
Gabija+YFP
Druantia III+Gabija
OD548nm
Time(min)
C
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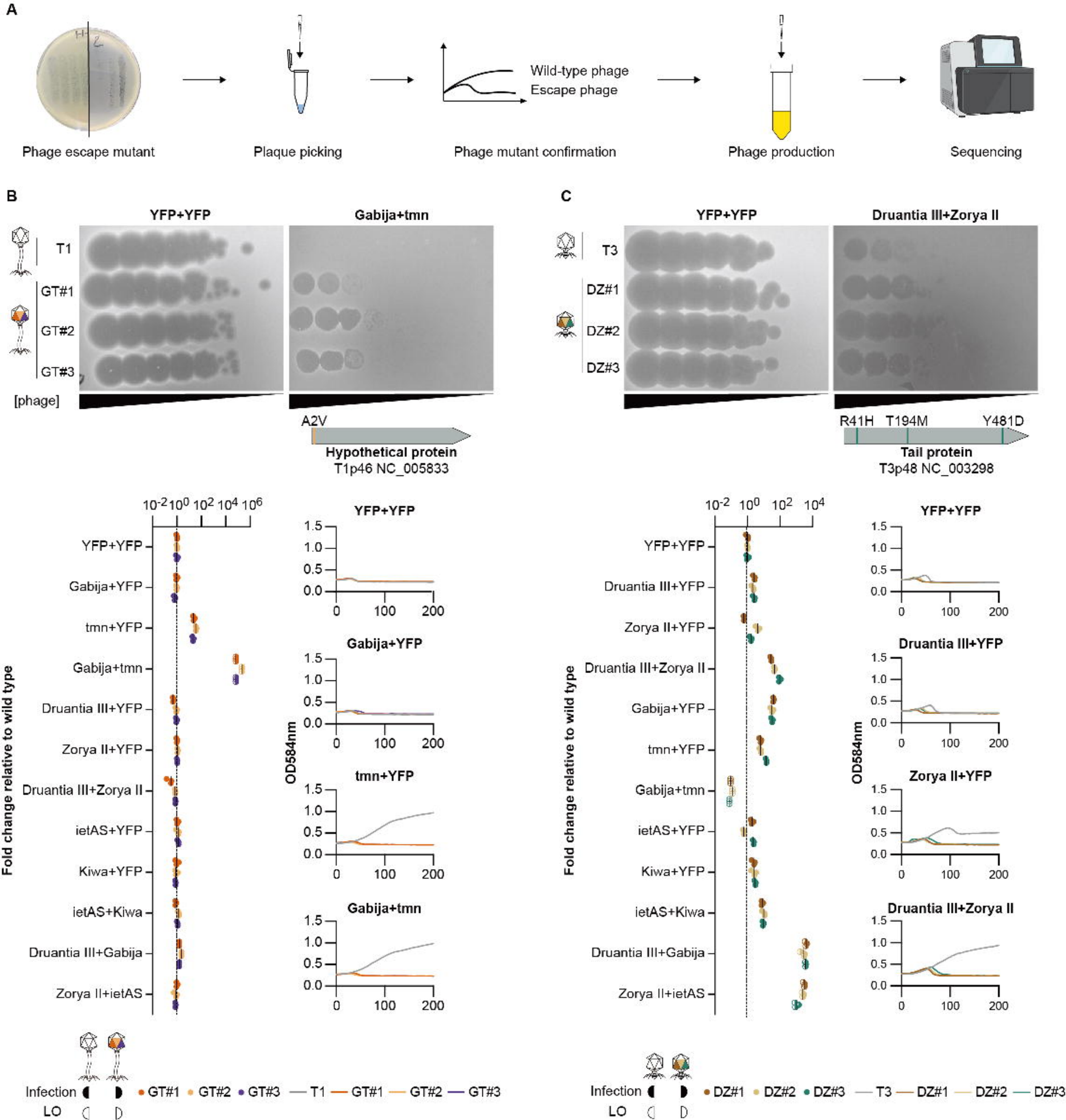
Time/min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Plaque picking
Wild-type phage
Escape phage
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GT#1
GT#2
GT#3
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T3
DZ#1
DZ#2
DZ#3
R41H
T194M
Y481D
Tail protein
T3p48 NC_003298
Fold change relative to wild type
YFP+YFP
Gabija+YFP
tmn+YFP
Gabija+tmn
Druantia III+YFP
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Druantia III+Gabija
Zorya II+ietAS
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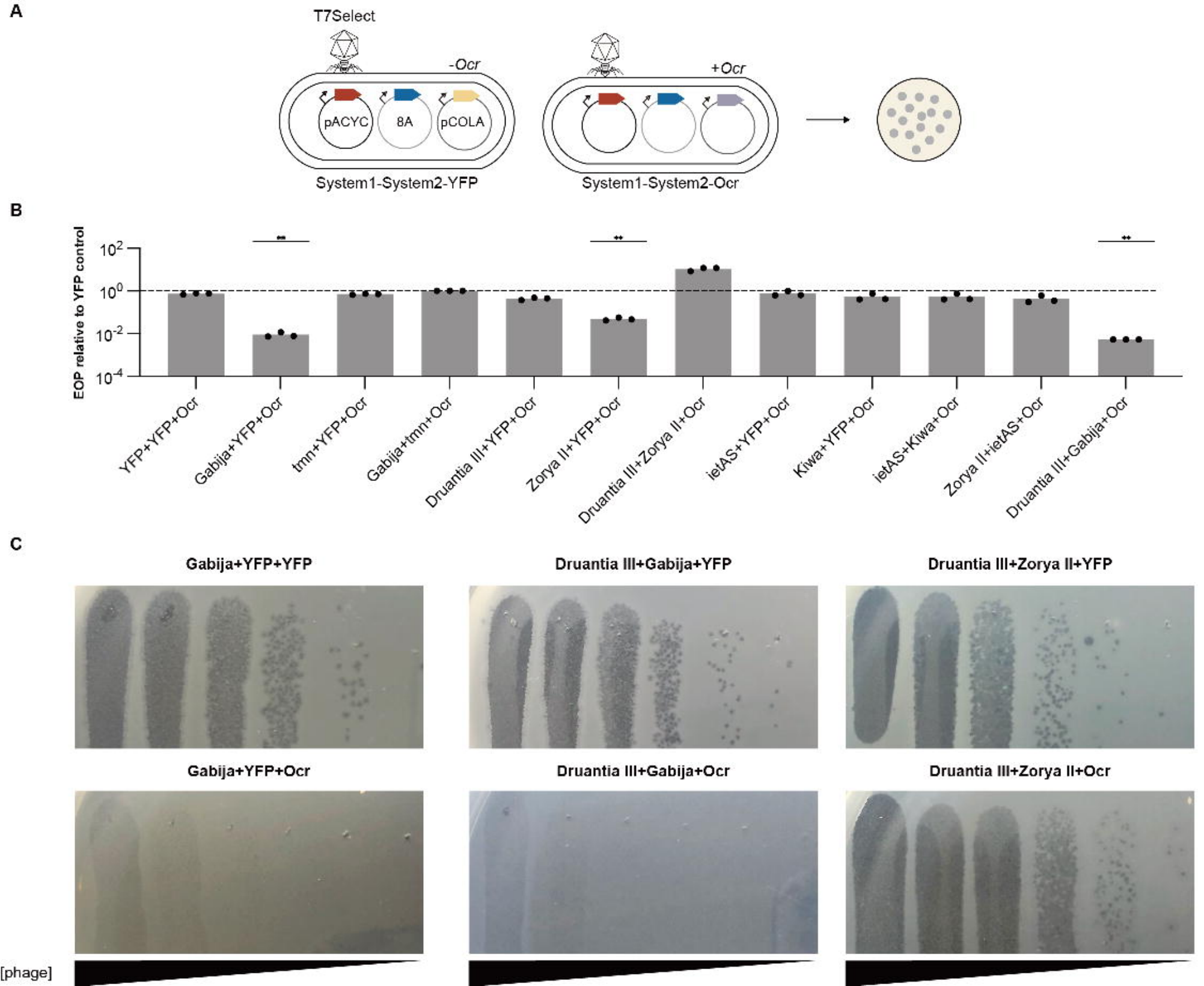
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10^2
10^0
10^{-2}
10^{-4}
YFP+YFP+Ocr
Gabija+YFP+Ocr
tmn+YFP+Ocr
Gabija+tmn+Ocr
Druantia III+YFP+Ocr
Zorya II+YFP+Ocr
Druantia III+Zorya II+Ocr
ietAS+YFP+Ocr
Kiwa+YFP+Ocr
ietAS+Kiwa+Ocr
Zorya II+ietAS+Ocr
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C
Gabija+YFP+YFP
Druantia III+Gabija+YFP
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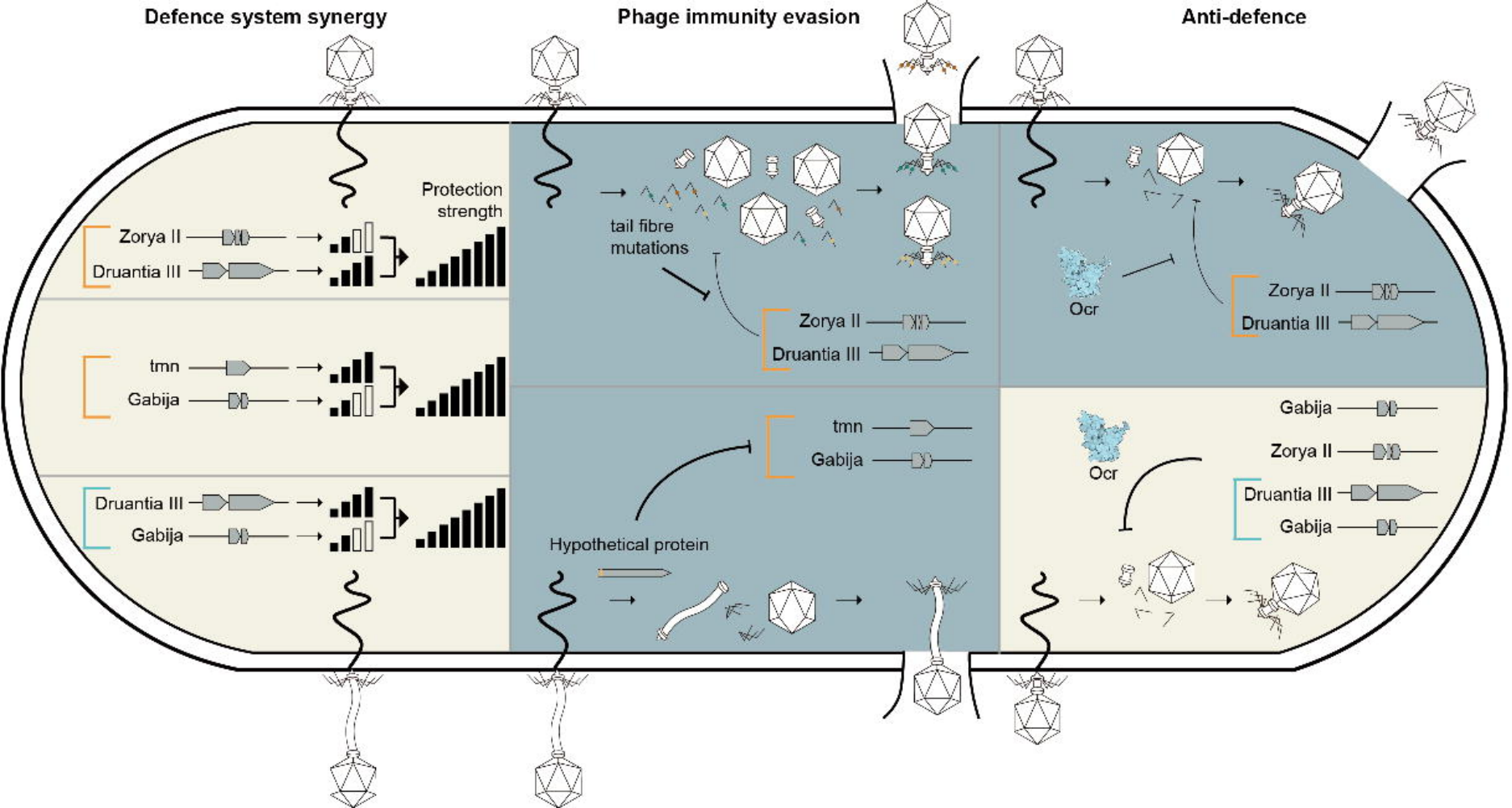
Defence system synergy
Phage immunity evasion
Anti-defence
Protection strength
Zorya II
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tmn
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Gabija
tail fibre mutations
Zorya II
Druantia III
tmn
Gabija
Hypothetical protein
Ocr
Zorya II
Druantia III
Ocr
Gabija
Zorya II
Druantia III
Gabija