Phage-based microbiome manipulation elucidates interactions within community context

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Abstract

Testing microbial interaction hypotheses is a major challenge in microbiome research. Bacteriophages may be used to probe organism-organism interactions within complex microbiomes, yet are rarely used for this purpose. Here, we isolated nine narrow host range phages that replicate in several *Variovorax* species. As *Variovorax* CL14 degrades the plant root stunting hormone auxin, we used phages to eliminate *V.* CL14 from a rhizosphere consortium and reestablished the stunted root phenotype. We used three of the phages to deplete another *Variovorax*, SCN45, from complex communities to test correlation network-based hypotheses of thiamine interdependencies. Genome-resolved metagenomics revealed that three taxa decreased in relative abundance following *Variovorax* depletion and could be rescued by thiamine supplementation. Thus, we confirmed thiamine production as the mechanistic basis for interdependence. These experiments lay the foundation for research that employs wildtype and genetically modified phages to test interaction hypotheses and for targeted microbiome manipulation.

Introduction

Microorganisms form the foundation of food webs, and profoundly influence the health of plants, humans, and other animals on a global scale. Yet microorganisms rarely function in isolation, and instead form complex communities in which a multitude of interactions shape its structure (Pacheco et al., 2019, Ma et al., 2020, Kost et al., 2023, Clegg & Gross, 2025).

Complex microbial communities have long been studied using metagenomics (Tyson et al., 2004), metatranscriptomics (Gilbert et al., 2008, Frias-Lopez et al., 2008), metaproteomics (Ram et al., 2005), and metabolomics (Segata et al., 2013), sometimes informed by stable isotope probing (Starr et al., 2018). Although these methods may generate hypotheses about organism interdependencies, our ability to test predicted interdependencies in a community context is extremely limited and largely restricted to highly specialized techniques (Pierce and Dutton 2022).

Microbial community models can infer the sum of all interactions (Skwara et al., 2023, Quinn-Bohmann et al., 2024), but lack of species-resolved mechanistic understanding limits our ability to engineer these systems. Metabolic models can predict interactions by incorporating microbial metabolic capacities (Dukovski et al., 2021), but this approach is currently limited to co-cultures and simple synthetic communities. This motivates the search for approaches that can elucidate organism-organism interactions in complex microbial ecosystems.

Bacteriophages offer a highly targeted way to probe microbial interactions due to their inherent bacterial specificity and potent host-killing efficiency. Bacteriophages have been predominantly used as therapeutics to remove or deplete pathogenic and antibiotic resistant microorganisms (Buttimer et al., 2022; Thanki et al., 2022; Das et al., 2015, Strathdee et al., 2023; Liao et al., 2025). Bacteriophage can also be further engineered for improved host killing and broadened host range (Selle et al., 2020; Gencay et al., 2023).

Prior work has established that phage hold enormous potential for microbiome manipulation. Carlson et al. (2023) demonstrated that the ammonium-generating capacity of fresh-water enrichments could be altered through the use of applied bacteriophage. Li et al. (2022) demonstrated the depletion of a specific *E. coli* using T7 phage can modulate host phenotypes, specifically animal behaviour. While several studies have engineered phage for increased killing efficiency (Selle et al., 2020), depletion of antibiotic resistance encoding plasmids (Bikard et al., 2014) and modification of host range (Gencay et al., 2023).

Here, we isolated multiple lytic bacteriophages specific to a collection of *Variovorax* isolates, and used them to modulate microbiomes to test several predicted microbial interactions. Our findings demonstrate that phage can be used to probe microbial interactions in the context of complex microbial communities.

Results

Variovorax phage isolation

To isolate *Variovorax* phages capable of selectively killing *Variovorax SCN45* we extracted phage viromes from six rhizosphere and compost samples (Oakland, CA) and tested for infectivity against a panel of *Variovorax* strains (Methods). We isolated nine phages that can replicate in one or more *Variovorax* species. These double-stranded DNA phages ranged in genome size from 43 to 159 Kb and with GC contents of 53% to 66%. Based on protein homology, seven of these phages (all with ~50 kbp genomes) were grouped with other RefSeq phages (Fig. 1a) classified predominantly with *Siphoviridae* (now *Siphovirus*) and share a high degree of average nucleotide diversity (ANI) with each other (Fig. 1b). The remaining two phages clustered with *Myoviridae* and *Autographiviridae* families. Two *Siphoviridae* phages (bapic4 and V.45_iii) and the large *Myoviridae* phage (V.45_v66) showed strong lytic activity against *V.* SCN45 (Fig. 1e). V.45_v66, bapic4 and V.45_iii were shown by TEM to have *Siphovirus*-like morphology, consisting of an icosahedral head and long filamentous tails, similar to the well-known lambda phage (Fig. 1d). All nine of the isolated phage are predicted to be restricted to a lytic lifestyle based on the absence of integrase genes. There was very limited cross-reactivity of the phages between the different *Variovorax* strains (Fig. 1c) based on spot assays. Only one phage, V.45_66, was able to form plaques on multiple hosts - *V.SCN45* and *V. paradoxus DSM 645* (Fig. S1).

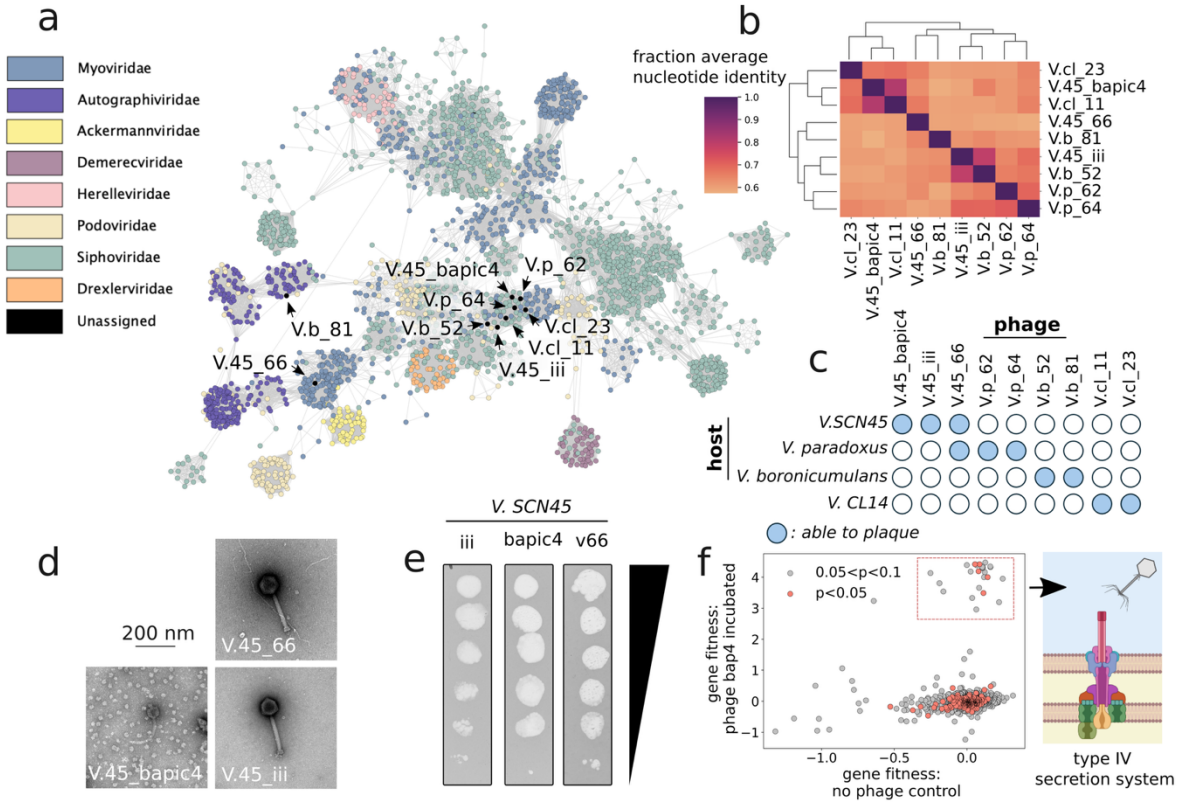

Figure 1. Characterization of nine isolated *Variovorax*-infecting bacteriophages. (a) Protein similarity network of phages of phages deposited in RefSeq and those isolated in this study (black), with edges

indicating a significant shared protein content. (b) Pairwise average nucleotide identity (ANI) between the nine isolated *Variovorax* phages. (c) Plaque assays demonstrate limited cross-reactivity of the nine phages across four different *Variovorax* strains, with filled circles representing the ability to plaque on a given host. (d) Transmission electron microscopy (TEM) of the SCN45-infecting phage, showing icosahedral heads and filamentous tails characteristic of Siphoviridae. (e) High titre phage stocks visualized by 10-fold dilution 1uL spot assays from 10^{-6} to 10^{0}. (f) Genome-wide fitness profiling (RB-TnSeq) for *V. SCN45* genes, following exposure to phage bap4, identifies the Type IV pilus as the likely receptor used by the phage for infection. Phages V.45_66 and V.45_iii generated similar gene fitness results.

Variovorax SCN45 phages infect via chromosome-encoded type IV pilus

To identify loss-of-function gene disruptions that could confer *Variovorax* with increased fitness under phage infection we constructed an Rb-TnSeq mutant library of *Variovorax* SCN45 and employed methods similar to Mutalik et al. (2020). The disruption of genes of the type IV secretion system (T4SS) were found to substantially enhance the fitness of *Variovorax* when incubated with each of its three phages (Fig. 1f). None of these T4SS gene knockouts were enriched in the no-phage controls, indicating that the addition of phage selected for these gene mutants through the elimination of susceptible mutants.

Evaluating Phage-Mediated Depletion in the *Arabidopsis*-*Variovorax*-*Arthrobacter* System

Finkel et al. (2020) demonstrated that *Variovorax CL14* mitigates a root stunting phenotype in *Arabidopsis* by consuming excess auxin secreted by *Arthrobacter* that leads to root stunting. To test whether such well-characterized interactions could be tested through phage-based manipulation, rather than conventional strategies, we hypothesized that a co-culture of the two organisms would result in a normal root phenotype but phage-mediated depletion of *V.* CL14 would result in the accumulation of auxin and cause a stunted root phenotype. We employed a phage cocktail of the Siphoviridae phage V.cl_11 and V.cl_23 to deplete *V.* CL14 from both pure cultures and co-cultures with *Arthrobacter CL28* inoculated onto *Arabidopsis thaliana*. Phage treatment led to the reintroduction of root stunting phenotype (Fig. 2).

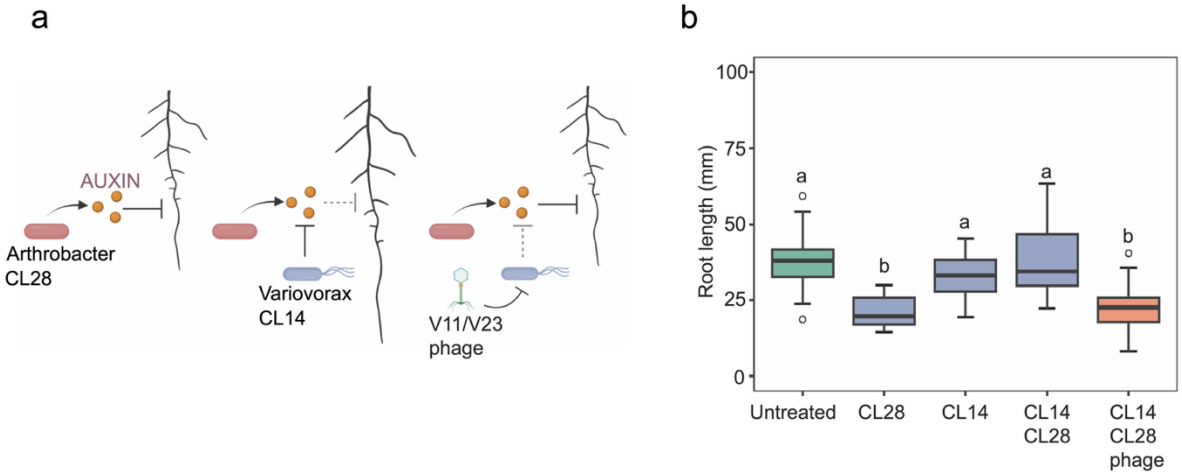

Figure 2. Phage-mediated depletion of Variovorax from an *Arabidopsis*-inoculated synthetic community induces root stunting. (a) Model of root inhibition by the auxin-secreting *Arthrobacter* CL28, which reverts to a normal root phenotype when the auxin-degrading *Variovorax* CL14 is present. If the *Variovorax*-targeting phage cocktail (V_11, V_23) is effective in the rhizosphere, this would disrupt the interaction and the root would revert to stunted growth. (b) *Arabidopsis* primary root elongation with *Arthrobacter* CL28 causes a stunted root phenotype, which recovers with and *Variovorax* CL14 co-culture. The addition of the *Variovorax*-targeting phage cocktail (V_11, V_23) to the co-culture of *Arthrobacter* CL28 and *Variovorax* CL14 recapitulates the root stunting phenotype observed in the presence of *Arthrobacter* alone. Letters above bars indicate the results of a Tukey post hoc test (α=0.05).

Development of stable enrichment communities

To perform community manipulation experiments it is essential to generate stable, reproducible consortia with reasonable complexity, ideally containing organisms that are predicted to be interdependent on other community members. This study leveraged inoculum from lab-scale bioreactors from which the *Variovorax SCN45* pure culture was previously isolated (Hessler et al., 2023). *Variovorax SCN45* overproduces thiamine in pure culture and was predicted to supply this essential vitamin to highly dependent thiamine auxotrophs in bioreactor communities based on ecological network analyses (Hessler et al., 2023). The thiamine auxotrophs that were correlated with *Variovorax* had very few correlations with other thiamine-producing organisms, suggesting a strong dependence.

We supplemented additional *Variovorax* SCN45 cells to increase its abundance in inoculum consortia based on previous findings that initial ratios of organisms can effect the final steady state community composition (Gao et al., 2021). The inoculum was then grown in minimal media, supplemented with various carbon sources, and passaged every 48 hours for a total of seven passages (Fig. 3a) and community compositions assessed. Four carbon sources were chosen for ongoing experiments because the communities were reproducible (Fig. 3, S2). The communities included *Leifsonia*, multiple Xanthomondales and several *Microbacterium*, taxa that were predicted to depend on *Variovorax* for thiamine (Hessler et al., 2023).

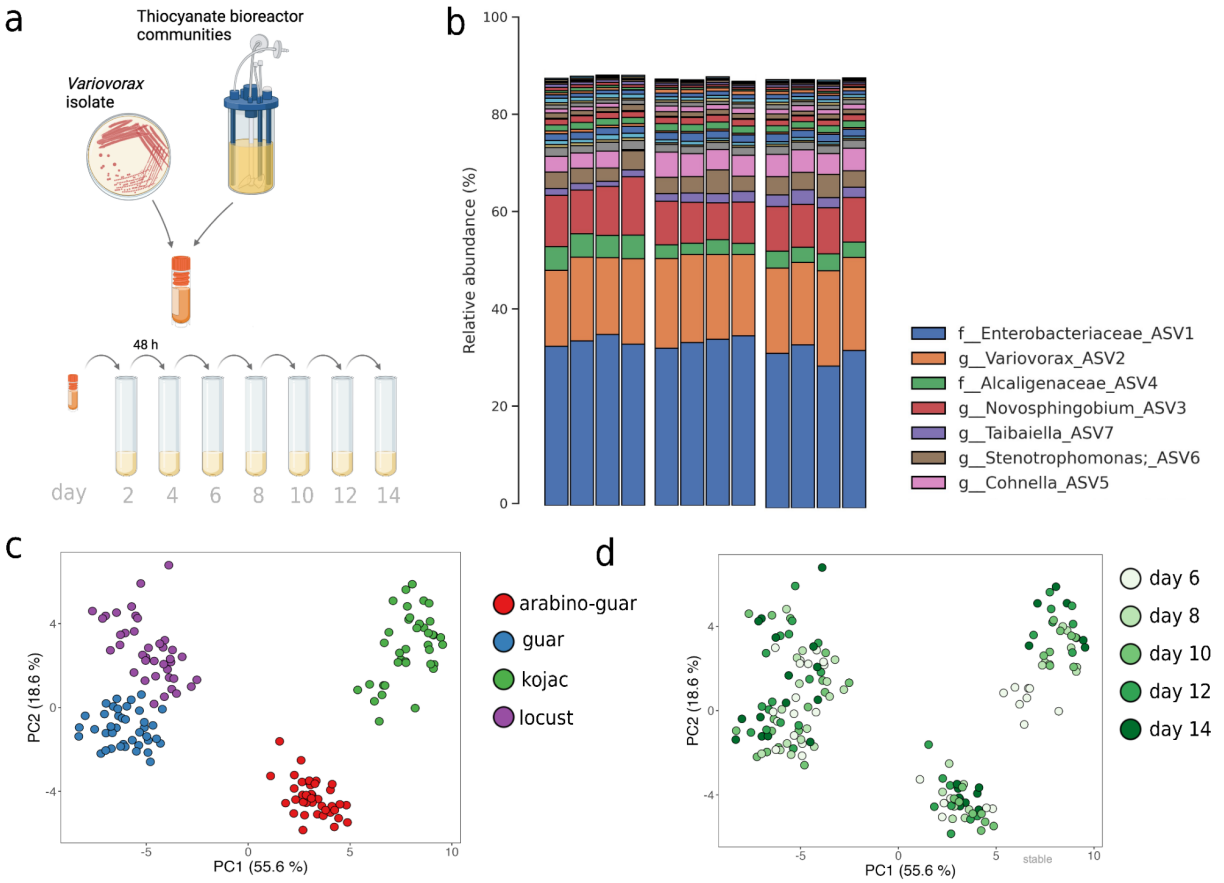

Figure 3. Development of stable enrichment communities. (a) Schematic of Bioreactor-derived inoculum preparation and enrichment passaging. (b) Communities supplemented with guar gum showed reproducible compositions across technical replicates and demonstrated stability across multiple passaging cycles using 16S rRNA gene amplicon sequencing. PCA was performed on robust centered log-ratio (rclr) transformed ASV relative abundances, demonstrating the compositional (c) reproducibility and (d) longitudinal stability were consistent across the cultures supplemented with arabinogalactan with guar gum, guar gum alone, kojac gum, and locust bean gum.

Phage-mediated *Variovorax* depletion leads to broad community impacts

Following the passage of the day 8 communities, either the phage V.45_v66 or a cocktail of three *Variovorax* phages (V.45_v66, V.45_iii, and V.45_bapic4) was added to the communities (Fig. 4 a). The relative abundance of *Variovorax* in the guar gum enrichment was reduced from approximately 22% in the day 8 communities to 2.4% in the day 10 community but further recovered to 11% by day 12 (Fig. 3b). This pattern was consistent across all four communities (Fig. S2). There was minimal difference in the abundance of *Variovorax* between the single phage V.45_v66 and three-phage cocktail, except in the kojac gum- community, where the three-phage cocktail resulted in a lower *Variovorax* abundance (mean 4%) compared to the single V.45_v66 phage (mean 10%) following passage six (day 12).

The overall community structure was impacted by the addition of phages, as evidenced by a notable shift in the principal component two of the PCA (Fig. 4c-e) performed on log-normalized abundance data from amplicon sequencing data. Despite phage addition, community stability was re-established, both in terms of species composition (Fig. 3c-f) and optical density over subsequent passages (Fig. S3). Bacteria other than *Variovorax* that can produce thiamine did not proliferate following *Variovorax* depletion (Fig. 4f). There was no evidence that *Variovorax* phage interacted with non-target organisms that encode a Type IV secretion system (Fig. 4g).

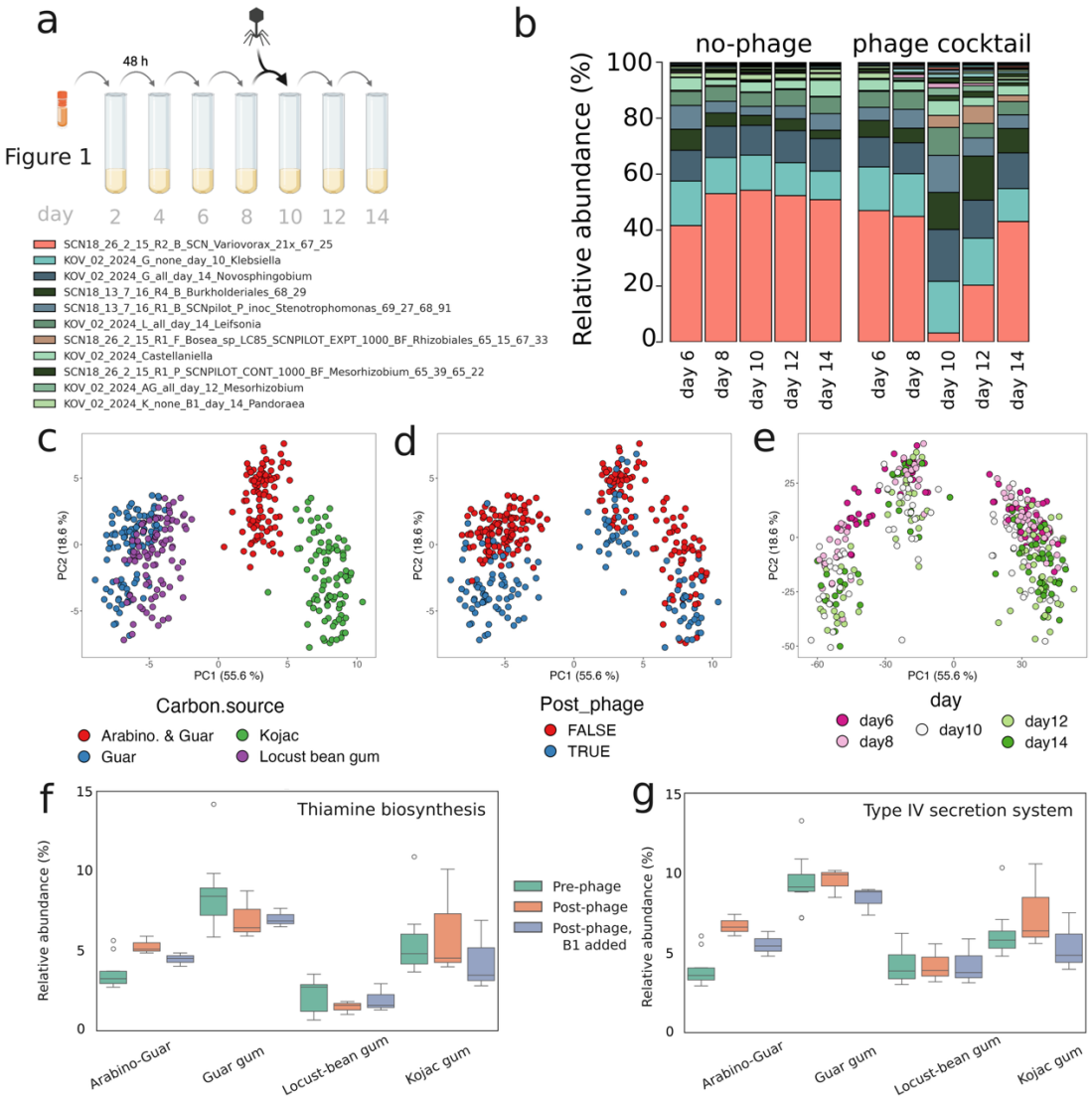

Figure 4. Phage-mediated *Variovorax* depletion across enrichment cultures. (a) Schematic adapted from Figure 3, illustrating the introduction of phage to 2 x10^4 PFU/ml on day 8 following passaging. (b) Genome resolved metagenomics revealed a marked reduction of *Variovorax* (blue) on day 10 post-phage. Principal component analysis (PCA) of log-normalized abundance data from amplicon sequencing data (c) reveals a separation along PC1 driven by carbon source, while (d) PC2 captures the divergence from pre- and post-phage conditions, and (e) day of the experiment. The summed relative abundance of all organisms encoding (f) a complete thiamine biosynthesis pathway and (g) a type IV secretion system (excluding *Variovorax* from 100% relative abundance). Shown across three stages: pre-phage addition (day 6 and day

8 communities), post-phage addition (day 10, 12, and 14 communities) and post-phage communities supplemented with 10 mg/L thiamine.

Variovorax depletion confirms dependence of specific thiamine auxotrophs

We assessed which organisms exhibited statistically significant abundance changes between the 'pre-phage communities' (day six and day eight communities) and the communities following the addition of phage (day 10, 12, 14 communities). After phage administration, we added thiamine to the control enrichment communities to identify the organisms that were specifically reliant on *Variovorax* for thiamine. Amplicon sequencing of the four enrichment cultures revealed that some organisms that decreased in abundance following *Variovorax* depletion, and then increased significantly in abundance following thiamine supplementation (Fig. 5a). These included a *Lysobacter* (Xanthomonadales*)*, a *Leifsonia,* and microorganisms belonging to the family of Microbacteriaceae (Fig. 5b). Genome-resolved metagenomics of the samples revealed that the *Leifsonia* (Fig. S5), a *Microbacterium* and a *Stenotrophomonas* (Xanthomonadales) all lack a complete thiamine biosynthesis pathway (Supplementary data).

To determine if the association between *Leifsonia* species and thiamine-producing bacteria may be more general, we assessed 32 *Leifsonia* genomes present in GTDB for the ability to synthesize thiamine. We found only a single *Leifsonia* genome to have a complete pathway (Supplementary data).

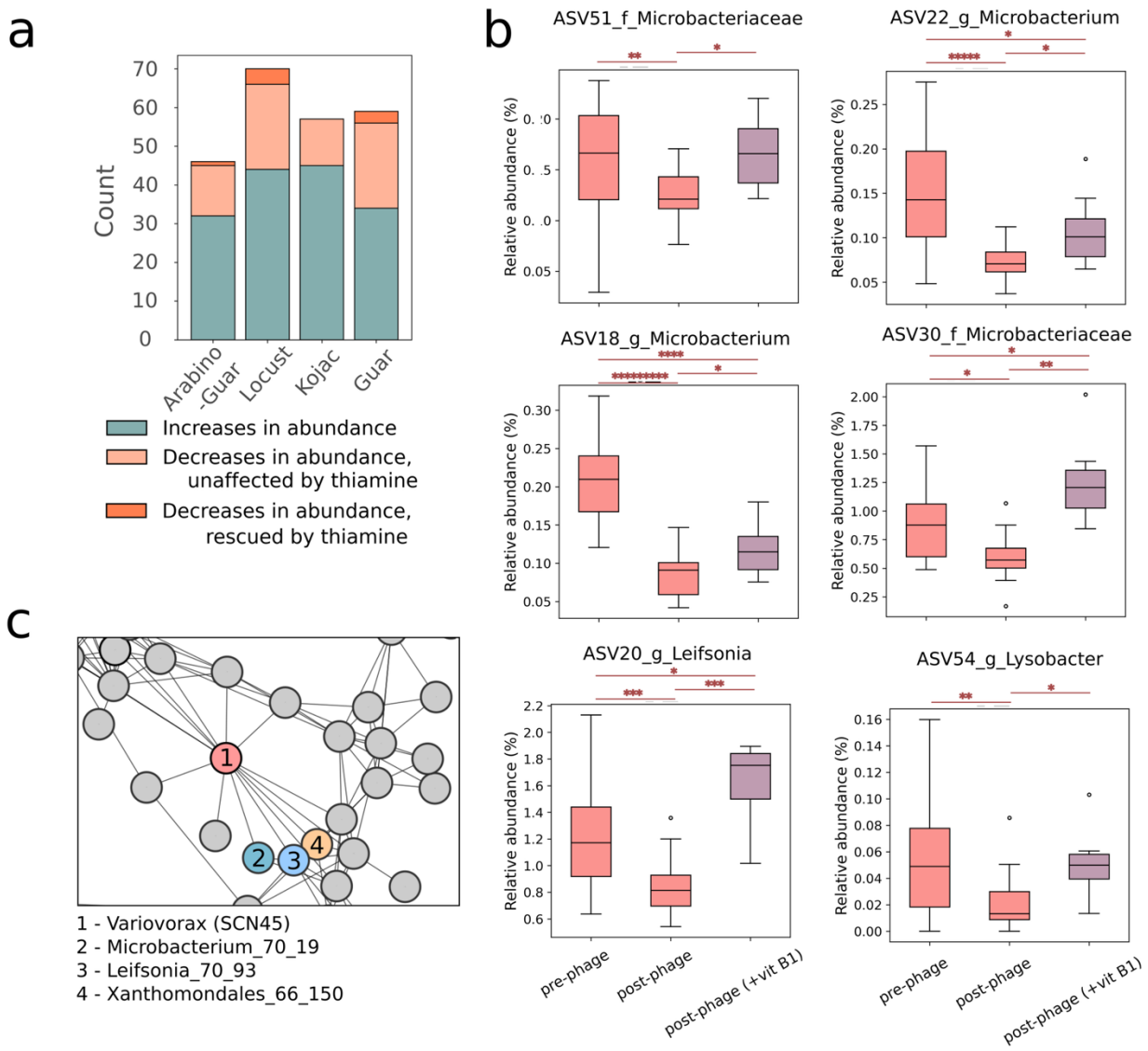

Figure 5. Previously identified organisms experimentally confirmed to rely on *Variovorax* for thiamine. (a) Amplicon sequence variants (ASVs) exhibiting significant changes in relative abundance between the pre- and post phage communities. Many ASVs increased in abundance following *Variovorax* depletion while others showed reduced abundance, and with a subset of these demonstrate recovery following the addition of thiamine. (b) Six ASVs corresponding to multiple *Microbacteria*, a *Leifsonia* and a *Lysobacter* show a reduced abundance following the depletion of *Variovorax* but returned to near original levels with the supplementation of thiamine. (c) Closely related strains of these organisms appear in the correlation network that was initially used to predict *Variovorax* as a thiamine-producer and show correlations with *Variovorax*.

SNPs enabled post-infection recovery of *Variovorax* The *Variovorax* SCN45 genome is comprised of a 6.8 Mb chromosome and 1.2 Mb circular genetic element that is related to a *Variovorax paradoxus S110* sequence described as a “second chromosome” by Han et al. (2010). *Variovorax* SCN45 lacks a CRISPR-Cas systems but encodes 24 PADLOC(v4.3.0)-predicted phage defence systems. We were unable to detect any deletions of these genes, or of any genes (including those

232 of the T4SS), in post-phage *Variovorax* populations based on read mapping and gene
233 abundances.
234
235 To seek evidence for mutations that could confer phage immunity in surviving *Variovorax*
236 populations, we monitored *Variovorax* single-nucleotide polymorphisms (SNPs) across the four
237 experiments.Before the introduction of phage, the majority of reads map to the reference genome
238 without SNPs. However, following phage addition, there is a marked increase in the
239 representation of SNPs and an increase in the number of SNP sites. In prephage communities
240 the majority of wildtype haplotypes are present at approximately 90% (indicating that at a given
241 SNP site, 90% of mapped reads support the wildtype base). However, following phage infection
242 the proportion of SNP sites with the wildtype haplotype reduces, demonstrating selection of new
243 or previously less abundant SNPs (Fig. 6a, S6).
244
245 Although most SNPs post-phage infection were synonymous, a subset were not. To identify new
246 gene variants under strong selection, we identified regions of the genome with SNPs that were
247 closely spaced enough that linkage could be established. Some regions with high densities of
248 linked SNPs occurred in both the no-phage controls and post-phage populations (Fig. 6b) and
249 thus were likely beneficial under all conditions (Fig. 6c). These included genes related to aerobic
250 benzoyl-CoA catabolism, a type V secretion system, enoyl-CoA hydratase and cell envelope
251 structural proteins. However, there was a single region of the *Variovorax* genome that showed
252 high SNP linkage density only in the post-phage populations. This locus encoded multiple
253 conjugal transfer proteins (Fig. 6c). Most importantly, we identified two non-synonymous SNPs in
254 the *trbE* conjugal transfer protein (homologous to *virB4*) within the P-loop domain which alters the
255 charge and hydrophobicity of this region of the protein.
256
257 To identify SNPs that were not close enough to other mutations to provide linkage, we recovered
258 all non-synonymous SNPs that increased in abundance by at least 30% in the post-phage
259 populations relative to the pre-phage and no-phage control populations. We identified 275 gene
260 variants found exclusively in post-phage populations (Supplementary data). Several were
261 associated with known phage defence systems including the retron XIII, Lamassu family and
262 putative PDC-S02 defence systems. Most interesting were three SNPs detected in three or more
263 of the four communities. This group of genes included a filamentous haemagglutinin family outer
264 membrane protein, a redoxin/thioredoxin-like sensor protein associated with a fatty acid
265 biosynthesis operon, and a protein with domain of unknown function (DUF932). This DUF932
266 gene occurs within an integrated plasmid that encodes *parA*, *parB*, an integrase, a plasmid
267 transfer protein (*tral*) and a replication initiation protein (*repA*). Notably, multiple non-synonymous
268 SNPs occurred in the DUF932 protein, and these variants were exceptionally abundant,
269 exceeding 70% in the post-phage populations. In contrast, the trbE gene displayed non-
270 synonymous SNPs in 21-33% of reads from post-phage populations. Interestingly, the start codon
271 of this DUF932 gene was mutated to AAG (lysine) in 50-60% of each of the four populations. This
272 likely impacts translation initiation and results in truncation of the protein as the next in-frame AUG
273 codon occurs in position 66.
274

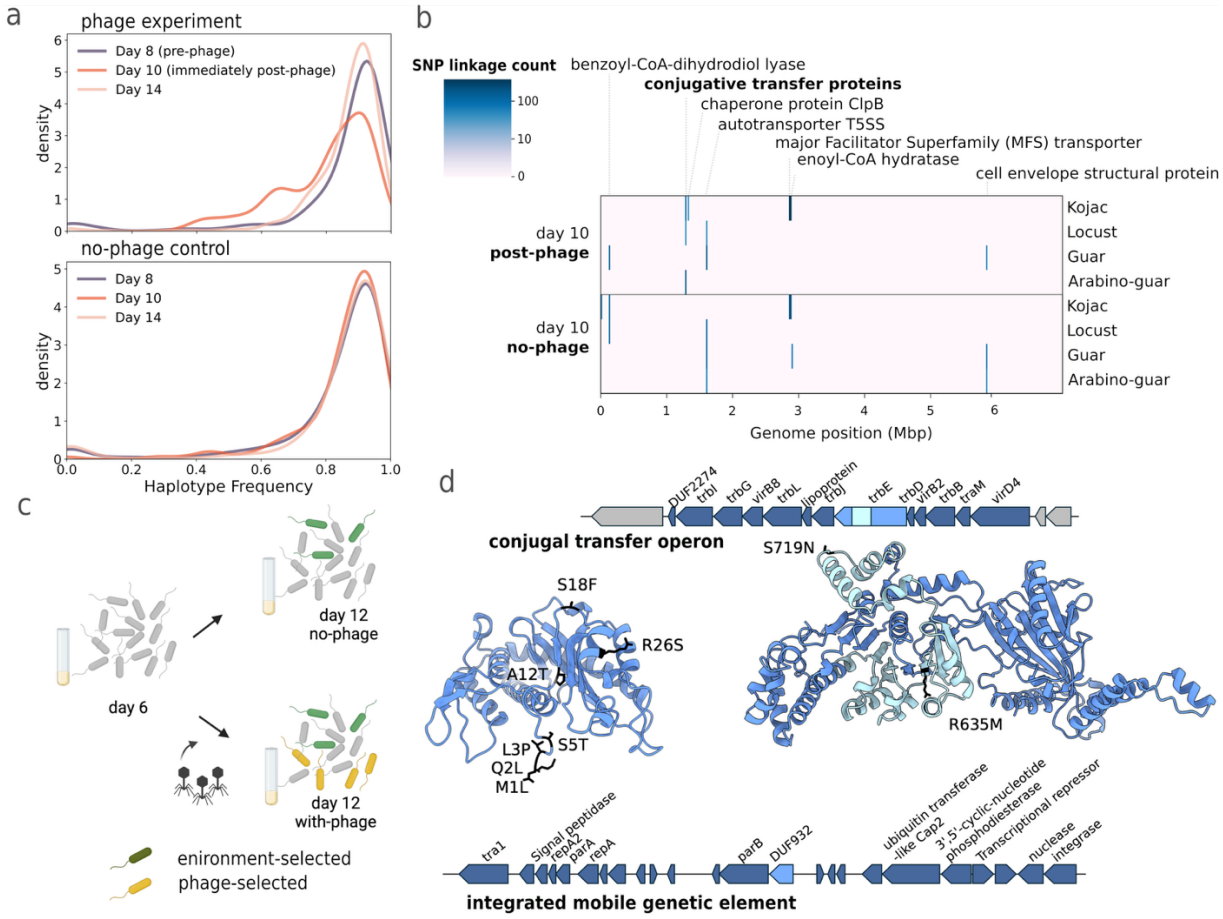

Figure 6. Phage infection induces shifts in *Variovorax* populations and selects for consistent gene variants. (a) Haplotype frequency distributions of *Variovorax* populations in the locust bean gum communities shows the impact by phage application, with expansion of low abundance SNPs, followed by return to pre-phage distributions. (b) Post-phage *Variovorax* populations show increased linked-SNP density in a conjugative transfer gene operon, visualised by a heatmap. Linked-SNPs associated with three 16S rRNA genes have been removed for clarity. (c) A schematic illustrating different categories for selection in day 12 communities. (d) Gene operons and AlphaFold-predicted protein structures showing consistent mutation sites within the conjugal transfer protein TrbE and domain of unknown function within an integrated genetic element.

Variovorax relies on it's own thiamine biosynthesis in all enrichment cultures

To investigate genes important for *Variovorax* fitness in enrichment cultures (in the absence of phage), we introduced *Variovorax* SCN45 mutant libraries into each of the four enrichment cultures (Fig. 7a). In all four of the communities, knockouts of genes involved in the biosynthesis thiamine (*thiC*, *thiG*, *thiE, purE* and *purK)* had a strong negative fitness impact on *Variovorax* (See Fig. S6 for an overview of the pathway). These results indicate that in the four enrichments, *Variovorax* was reliant on its own biosynthesis for thiamine and little extracellular thiamine was likely available.

Rate of recovery of *Variovorax* populations correlates with reduced dependence on Type IV secretion system

Phage treatment reduced the abundance of *Variovorax* to approximately 3% in all day 10 post-phage communities, but *Variovorax* showed the fastest recovery in the guar and locust-bean gum communities over the following days (Fig. 7a). The *Variovorax* populations in these two communities (Fig. 7b, S5) had SNP concentrations close to pre-phage levels by day 14.

We amended enrichments with the Rb-TnSeq mutant library to investigate the consequences of specific gene knockouts for *Variovorax* fitness in the four communities (in the absence of phages; Fig. 7c). In the guar and locust-bean gum communities the knockout of the T4SS was advantageous, based on selection for these genotypes. In contrast, these knockouts were deleterious in the arabino-guar and kojac gum substrate-amended communities.

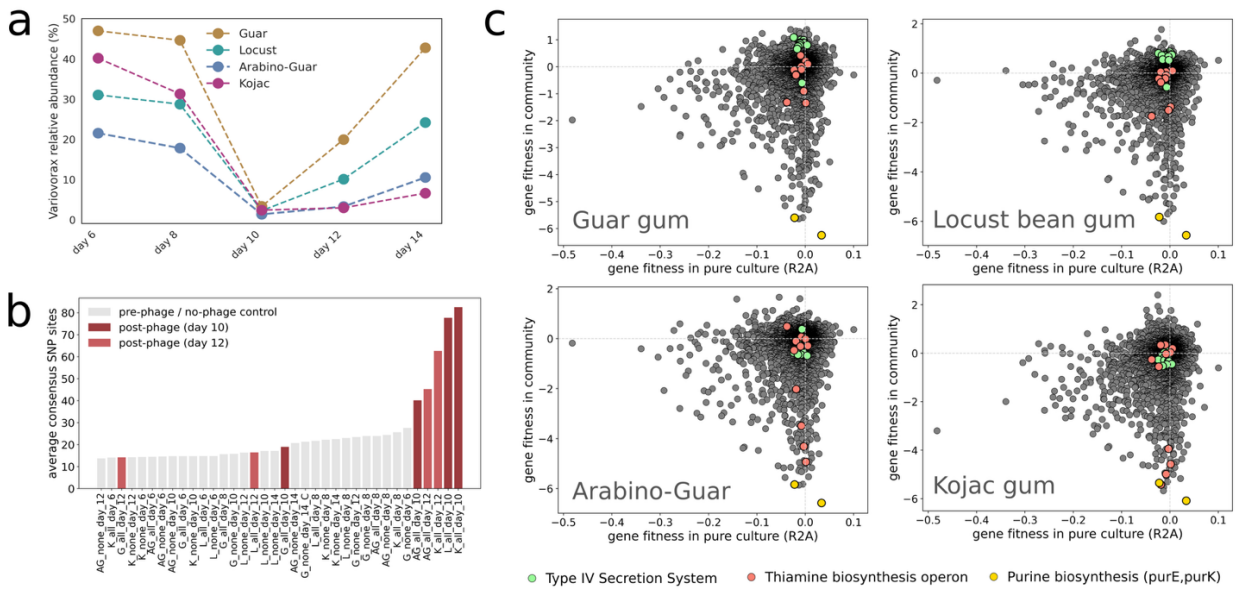

Figure 7. The rate recovery of *Variovorax* populations from phage infection corresponds to the fitness conferred by the type IV secretion system (T4SS). (a) Relative abundance of *Variovorax* is reduced to ~3% in all post-phage communities, with a gradual recovery over the following passages, again fastest in the guar and locust bean gum communities. (b) Genome-wide SNP sites in Variovorax populations increase immediately following phage infection (day 10) but decline to pre-phage levels, with the guar and locust bean gum populations recovering the fastest. Read mapping was normalized to 20x coverage for all *Variovorax* populations. (c) Genome-wide fitness profiling (Rb-TnSeq) for *V. SCN45* showing the fitness of gene knockouts in each of the four communities (y-axis) versus in pure culture in R2A, rich media (x-axis), highlighting the type IV secretion system and thiamine biosynthesis genes.(b)

Discussion

Previously, networks constructed using organism abundance data allowed us to identify thiamine auxotrophs that were strongly correlated with *Variovorax* (SCN45) in the bioreactor communities studied here (Hessler et al., 2023). Subsequently, we showed that *Variovorax* produces high

concentrations of extracellular thiamine in pure culture, however we did not verify that this thiamine production was sustained within natural communities. To better explore possible interdependence related to vitamin provision required experiments conducted in communities from which *Variovorax* could be selectively removed, without directly impacting the rest of the community. We hypothesized that highly targeted community manipulation could be achieved via use of *Variovorax*-specific phages.

To demonstrate the feasibility of testing organism-organism interactions using phage-mediated *Variovorax* depletion, we leveraged the *Arabidopsis*-*Variovorax* CL14-*Arthrobacter* system first characterized by Finkel et al. (2020) in which *V. CL14* mitigates root stunting in *Arabidopsis* by degrading auxin produced by *Arthrobacter*. Following the addition of isolated phages V.cl_11 and V.cl_23 to this co-culture, we observed a pronounced root-stunting phenotype, indicating that the phages successfully depleted *Variovorax* CL14 (Fig. 2). These findings highlight the potential of extending phage-mediated testing of microbe-microbe and microorganism-plant interactions to rhizosphere microbiome manipulations for agricultural applications.

Next, we used three isolated *Variovorax* SCN45 phages to selectively deplete this *Variovorax* from four enrichment communities and monitored the effects on the remaining community (Fig. 4, 5). The subsequent addition of thiamine facilitated the recovery of only a *Leifsonia*, a Xanthomondales and a *Microbacterium*, each lacking complete thiamine biosynthesis pathway. This provides clear evidence that *Variovorax*-produced thiamine was supporting the growth of these specific organisms in the communities and demonstrates the effectiveness of phage-mediated knock-down experiments for capturing these metabolic interactions. Related, but non-identical, strains of *Leifsonia*, *Microbacterium,* and the Xanthomondales were among a larger group of organisms associated with *Variovorax* in the correlation network from which the thiamine-exchange hypothesis was developed (Hessler et al., 2023).

Using a *Variovorax* SCN45 RB-TnSeq mutant library we found that disruptions in the *Variovorax* type IV secretion system genes conferred phage immunity, indicating that the three phages tested all infect via this complex. The T4SS is a common target for phage attachment and genome injection (Russell 1995; Berry et al., 2011). One potential concern is that depletion of *Variovorax* might occur following phage infection, yet decreased abundance of correlated organisms could be due to toxicity following uptake of viral DNA via T4SSs. Countering this, we find that encoding of a type IV secretion system was not a predictor of whether an organism decreased in abundance following phage addition. Thus, we concluded that these phages could be used to directly identify the organisms that are dependent on *Variovorax* SCN45 in the bioreactor consortia and the basis of the dependence.

Variovorax populations demonstrated partial to complete recovery from phage infection by the end of the experiment. We were unable to identify any gene deletions, frameshift mutations or non-synonymous SNPs in T4SS genes. However, by investigating genes with enriched non-synonymous SNPs, we identified gene variants of the *TrbE* conjugal transfer protein in *Variovorax* that arose following phage treatment. *TrbE* physically interacts with the T4SS and is a member of the *VirB4* family of proteins responsible for substrate recruitment and transfer to the secretion

364 channel of T4SS (Li et al., 2018; Redzej et al., 2022). We observed two non-synonymous SNPs
365 within the P-loop domain of this protein which is responsible for ATP binding and hydrolysis. We
366 speculate that the *TrbE* mutations interfere with the functioning of the T4SS required for phage
367 infection.
368
369 In addition to *trbE* variants, we observed non-synonymous SNPs in a small-protein-coding
370 (DUF932) gene within a putative integrated plasmid in all phage-treated *Variovorax* populations.
371 Although the precise role of this gene is unclear, its consistent selection after phage application
372 strongly suggests involvement in phage defence. The blocking of phage entry by resident mobile
373 genetic elements, including phage, is well-documented (Kliem & Dreiseikelmann, 1989; Berry and
374 Christie, 2011; Kamruzzaman et al., 2022; Rivard et al., 2024; Johnson et al., 2022; Taylor et al.,
375 2024). For example, the *pVII* minor capsid protein in *Pseudomonas* phage *Pf4*, interacts directly
376 with the host *PilC* protein, responsible for T4SS assembly in *Pseudomonas*, rendering it inactive
377 and preventing phage superinfection (Wang et al., 2022). We suspect that *Variovorax* developed
378 phage resistance through both chromosomal and mobile element-mediated modulation of the
379 T4SS. These mutations are likely to have existed within the *Variovorax* populations prior to phage
380 application and highlight the role of population variation in surviving phage infection.
381
382 The addition of a single or multiple phages showed little difference in *Variovorax* depletion or its
383 rate of recovery. This absence of additive effects has been observed in other studies (Pyenson
384 et al. 2024), including efforts to remove *Ralstonia* from tomato plants (Franco Ortega et al., 2024).
385 In our study, this may have occurred to all three *Variovorax*-targeting phages infecting via the
386 same receptor. In future studies, the use of multiple phages targeting different receptors would
387 likely be beneficial, as has been shown in phage-therapy research treating bacterial infections in
388 humans (Yoo et al., 2024). However, complete bacterial removal is rare and, in clinical cases, has
389 required the simultaneous use of antibiotics (Pirnay et al., 2024). Notably, in the *Ralstonia*-
390 infected tomato plant studied by Wang et al. (2019), the addition of phages resulted in the
391 enrichment of taxa that naturally suppressed the growth of *Ralstonia* through antibiotic production.
392 These studies suggest that phage application alone is unlikely to remove target bacterium long-
393 term, and that additional ecological or therapeutic pressures are required to sustain suppression
394 of the target organism. However, as we show here, even partial and temporary depletion of
395 populations is useful to test interaction hypotheses.
396
397 *Variovorax* recovered at variable rates following phage treatment, with communities in which
398 T4SS knockouts conferred positive fitness exhibiting the fastest recovery. Prior work has shown
399 that community composition can substantially impact phage-host dynamics (Blazanin and Turner,
400 2021; Alseth et al. 2019). Phage susceptibility can also be strongly influenced by the natural
401 variation in physiology of genetically identical cells, such as cell size (Schenk & Sieber, 2019) and
402 receptor protein copy numbers (Chapman-McQuiston & Wu, 2008; Igler et al., 2022). This leads
403 us to hypothesize that community- and environmental selection against T4SS expression in
404 *Variovorax* in the guar and locust bean gum communities could have led to reduced presentation
405 of this complex by *Variovorax*, thereby limiting infection by our phages targeting this system. The
406 observed variation in *Variovorax* recovery highlights how community context could impact phage

407 infection, and may be an important area of research for the application of phage-based
408 microbiome manipulations.
409
410 Here, we establish the basis of specific microbe-microbe interactions in natural enrichment-based
411 communities by isolating and using phages to selectively deplete a member of interest. This
412 approach addresses a critical challenge in microbial ecology by easily enabling the direct testing
413 of interaction hypotheses within natural consortia, including interactions involving taxa that are
414 otherwise difficult to culture or manipulate. This method is scalable, cost-effective and uses
415 standard microbiology techniques without requiring specialized equipment. Although suitable
416 phage isolation does require the isolation of the microorganism of interest, future integration of
417 phage engineering with advances in protein design (Pacesa et al., 2025) may circumvent this
418 current limitation. Overall, phage-based approaches represent valuable tools for understanding
419 the structure of microbial communities and offer promising avenues for microbiome engineering.

420 Methodology

421 Bacterial inoculum preparation

422 *Variovorax* was grown overnight from a single colony in R2A media. 100 ml of planktonic phase
423 cells were recovered from two bench-scale thiocyanate-degrading bioreactor systems, from which
424 *Variovorax* was initially isolated a year prior. Flocculated particles were allowed to settle to the
425 bottom of the container for 30 min and the top 50 ml were carefully removed. 10 ml of bioreactor
426 planktonic phase were added into 50 ml falcon tubes. 5 ml of *Variovorax* overnight culture was
427 added to this falcon tube. 15 ml of 50% glycerol was then added and mixed by swirling and
428 inverting each tube. 200 uL of each culture were distributed into cryo tubes, frozen in liquid
429 nitrogen, and stored at -80C. This approach was based on previous inoculum ratio testing
430 experiments (Gao et al., 2021), which demonstrated that a higher abundance of a member in the
431 inoculum improves its chances of establishing within the community.

432 Community passaging

433 Inoculum glycerol stocks were thawed on ice and gently mixed by pipetting. 50 uL of glycerol
434 stock was added to 10 ml of M9 minimal media in quadruplicate supplemented with 50 ug/L
435 cycloheximide, 10 mg/L pantothenic acid, and a trace metal solution described by Watts et al.
436 (2019). Culturing was performed using four independent technical replicates to provide
437 robustness for statistical tests. The electron donors used were 0.2 g/L of kojac gum, locust bean
438 gum, guar gum, and combined 0.1 g/L guar gum and 0.1 g/L arabinogalactan. Under each
439 electron donor condition, Following inoculation, cultures were incubated at 30C for 48 hours with
440 shaking at 140 rpm. Cultures were passaged using 200 uL of spent medium into 10ml of fresh
441 media. This was repeated every 48 hours for a total of seven passages over 14 days. This
442 culturing protocol was heavily informed by Goldford et al. (2018). The glycerol stocks were then
443 used to inoculate fresh tubes followed by two passages as described above. At each passaging
444 step, 8 ml of culture were centrifuged at 10 000 X g for 10 minutes and cell pellets were frozen at

445 -80C for subsequent DNA extraction. 200 uL of spent medium was used to perform OD
446 measurements using a 96-well plate reader at 600 nm.

447 Virome preparations

448 Three sources of viromes were collected: (i) soils, (ii) composts and (iii) and thiocyanate-
449 degrading bioreactors. Briefly, approximately 10 grams of soil or compost were collected in falcon
450 tubes and incubated with 25 ml of SM buffer with shaking for one hour. In the case of bioreactor
451 viromes, biofilm material was removed from the bioreactors using serological pipettes and
452 subjected to metal bead beating on a vortex at maximum speed for ten minutes to disrupt the
453 biofilm. For all three types of viromes, the particulate matter was then allowed to settle for 30
454 minutes before the supernatant was removed and centrifuged at 10 000 X g for 10 minutes and
455 the supernatant then passed through a 0.22 um filter.

456 Phage liquid enrichment

457 Bacteria were grown overnight from single colonies in 10 ml of R2A media and incubated at 30C
458 at 160 rpm. Cultures were back diluted into fresh R2A containing 2 mM MgCl2 and 2 mM CaCl2
459 to an OD600 of 0.03-0.05. Cultures were allowed to grow until reaching an OD600 of 0.15-0.3.
460 Subsequently, 100 uL of viromes were then added to the tubes and the cultures were incubated
461 for a further 24 hours. The cultures were then sampled and cells were removed by centrifugation
462 at 10 000 X g for 10 minutes. This supernatant served as the virome for the next round of
463 enrichment. This process was repeated for a total of five enrichments.

464 Phage isolation: Plaque assays

465 Plaque assays were performed on the *Variovorax* strains *V.* SCN45, *V.* CL14, *V. boronicumulans*
466 (DSM 21722), *V. ginsengisoli* DSM 25185 and *V. paradoxus* DSM 645. These strains were grown
467 overnight from single colonies in 10 ml of R2A media and incubated at 30C at 160 rpm. Cultures
468 were back diluted into fresh R2A to an OD600 of 0.03-0.05. Cultures were allowed to grow until
469 reaching an OD600 of 0.15 - 0.3. Subsequently, 100uL of log-phase cells were added to 20 uL of
470 0.2 um filter-sterilized viromes and incubated at RT for 10 minutes. These were mixed with 50C
471 R2A (2 mM MgCl2 and 2 mM CaCl2) 0.5% top agar and plated onto R2A 1.5% bottom agar plates.
472 Plates were incubated at 30C for 48 hours and inspected for plaques. Plaques were picked using
473 sterile 100 uL tips into 100 uL of SM buffer.

474 Phage genome sequencing, assembly and annotation

475 DNA from phage suspensions were extracted using Zymo Quick-DNA viral kits using
476 manufacturers instructions. Extracted DNA was then illumina shotgun sequenced on a Novaseq
477 using 150PE reads with a 300 bp insert size and yielding 1 Gbp per sample. Viral genomes were
478 assembled using Metaviral-Spades (Antipov et al., 2020) and extended using COBRA (Chen et
479 al., 2024). The genomes were then annotated using vogDB (Trgovec-Greif et al., 2024), Pfam
480 (Bateman et al., 2004) and KEGG (Kanehisa & Goto, 2000) hidden markov models using
481 hmmscan. Protein sharing of the nine phage and Refseq and was performed using Vcontact2

482 (Bin-Jang et al., 2019). Phage genome ANI was determined using Vclust (Zielezinski et al., 2024;
483 https://github.com/refresh-bio/vclust).

484 Transmission electron microscopy (TEM)

485 Virus particles were visualized using a FEI Tecnai T12 Transmission Electron Microscope at an
486 accelerating voltage of 80 kV. Carbon-coated copper grids were charged in a vacuum chamber
487 followed by application of 5uL of virus suspension and allowed to dry. The grids were washed
488 with deionised water three times before negative staining in 1.0 % ammonium acetate for 60s.
489 Grids were allowed to air dry before being visualized.
490

491 Arabidopsis inoculation and co-cultures with Variovorax CL14, Arthrobacter 492 CL28, and V.cl_11+V.cl_23 phage for root length phenotyping

493 *Arabidopsis thaliana* Col-0 seeds were surface-sterilized in 50% bleach for 20 min with 0.05%
494 Tween 20 for 20 min, removing all bleach and rinsing five times in sterile water. Sterile seeds
495 were stratified in the dark for 2 to 3 days at 4°C. A nylon mesh filter with 100 micron pore size
496 was cut to fit on the 100 mm x 100 mm square petri dish as previously described (Cole et al.
497 2017). After stratification, ~7 seeds were placed on the mesh filter on 1.0MS, 0.5% sucrose, 0.6%
498 phytagel plates and grown under long day conditions for 7d (16-h light/8-h dark regime at 22-°C).
499 Bacteria strains preparation: *Variovorax* CL14 and the root growth inhibiting strain Arthrobacter
500 CL28 were streaked on agar plates from glycerol stocks, a single colony was transferred to 3 ml
501 liquid R2A media and grown overnight. Cultures were gently pelleted then washed twice with 10
502 mM MgCl2, normalizing the volume of each culture to OD600 = 1, then adjust to OD600=0.04
503 before spreading 50 µl on each plate (Full MS, 0.6% phytagel without sucrose. Note: Full MS was
504 used instead of 1/2X as previously described to maintain the required Mg and Ca concentrations
505 for optimal phage lysis (2mM)). To prepare the phage inoculation, 5ul of a phage cocktail
506 (Siphoviridae phage V.cl_11+V.cl_23) was diluted in 500 ul 20 mM MgCl2 and 100 ul of this
507 solution was spread onto each plate. After the plants grew 7d, the mesh was transferred to each
508 of the bacteria and phage inoculated plates and returned to the growth chamber vertically grown
509 in short day conditions (16-h dark/8-h light regime at 22-°C). Plates were imaged 10 days after
510 transfer. Primary root length was measured using ImageJ software, first setting the scale to the
511 grid on the plate (13 mm) before measuring primary root elongation for each plant. There were 3
512 replicate plates (containing ~ plants each) for each control and treatment: uninoculated, no
513 bacteria + V11/V23 phage, *Variovorax* CL14, *Arthrobacter* CL28, *Variovorax* CL14 + *Arthrobacter*
514 CL28, *Variovorax* CL14 + *Arthrobacter* CL28 + V11/V23 phage.

515 Phage-perturbed community experiments

516 Stable communities grown on selected carbon sources and were exposed to *Variovorax*-specific
517 phages (V45_v66, or a three-phage cocktail of V45_v66, V.45_iii and V.45_bapic4) 8 hours after
518 the passaging of the day 8 communities (ie. following passage 5), using 50 uL of 4×10^4 PFU/uL
519 each per 10 ml culture. Experiments were conducted in quadruplicate with a no-phage control
520 included for comparison. The final varied condition involved the presence or absence of 10 mg/L

521 thiamine, administered at the time of phage addition and maintained during subsequent
522 passages.

523 DNA extraction, amplicon and shotgun sequencing

524 Genomic DNA was extracted from cell pellets using the Qiagen DNeasy PowerSoil Pro Kit
525 according to the manufacturer's instructions. V4-V5 16S rRNA gene amplicons were generated
526 by PCR using primers 515F (GTGNCAGCMGCCGCGGTAA) and 926R
527 (CCGYCAATTYMTTTRAGTTT) and cycling conditions 95C 10 min, 72C 90s, 50C 60s, 72C 90s
528 for 30 cycles and a final extension at 72C for 10 min. PCR products were cleaned using Ampure
529 XP magnetic beads. PCR products were sequenced by Innovative Genomic Institute's Next
530 Generation Sequencing Core on an Illumina MiSeq instrument using a read length of 2 x 150 bp
531 with approximately 50 000 reads per sample. Generated amplicon sequencing data was
532 processed yielding ASVs as performed by Kust et al. (2023). Whole genome community library
533 preparation and shotgun sequencing was performed by Novogene. Paired-end Illumina libraries
534 were prepared from genomic DNA with an insert size of 350 bp and sequenced on an Illumina
535 Novaseq producing approximately 6 Gbp of raw sequencing data per sample. The *Variovorax*
536 isolate was subsequently sequenced using PacBio long-read sequencing at 60x coverage, to
537 generate two circular chromosomes.
538

539 Metagenomic Read Processing, Assembly and binning

540 Illumina 150 PE reads from community shotgun sequencing were processed as documented in
541 the ggKbase data preparation pipeline (https://ggkbase-help.berkeley.edu/overview/data-
542 preparation-metagenome/). Reads were processed using BBtools (Bushnell 2020) and sickle
543 (Joshi and Fass, 2011), and were then assembled using IDBA-UD (Peng et al., 2012). Open
544 reading frames (ORFs) were predicted using Prodigal V2.6.3 (Hyatt et al., 2010). Contigs were
545 binned using CONCOCT (Alneberg et al., 2013), Maxbin2 (Wu et al., 2016), MetaBat2 (Kang et
546 al., 2019), and VAMB (Nissen et al., 2021). The best bins per sampled were selected using DAS
547 tools (Sieber et al., 2018) and a final non-redundant genome set across all enrichment
548 communities and the previously published Thiocyanate bioreactor genome set found at
549 https://ggkbase.berkeley.edu/SCN_92/organisms was selected using dRep (Olm et al., 2017) at
550 95% ANI.

551 Microbial genome annotation, classification and strain analysis

552 Microbial genomes were then annotated using hmmscan against Pfam (Bateman et al., 2004)
553 and KEGG (Kanehisa & Goto, 2000) databases and were taxonomically classified using GTDB-
554 tk (Chaumeil et al., 2020). All read mappings were performed with bowtie2 (Langmead et al.,
555 2012). Genome coverage and relative abundance across samples were determined using coverm
556 (Aroney et al., 2025). Genome-genome correlation was determined using Fastspar (Watts et al.,
557 2019) which implements the Sparcc algorithm (Friedman et al., 2012). *Variovorax* strain analysis
558 was performed using inStrain (Olm et al., 2021) and the PacBio assembled *Variovorax* genome.
559 Phage defence systems were predicted in the *Variovorax* genome using padlock (Payne et al.,

560 2022, https://padloc.otago.ac.nz/padloc/). Predicted Protein structure predictions were performed
561 using Alphafold3 (Abramson et al., 2024. https://alphafoldserver.com/)

562 Statistical analyses

563 Distributions in organism abundances were compared by two-sample t-test assuming unequal
564 variance was performed using the ttest_ind function of the scipy.stats Python package. The
565 abundance of ASVs was calculated excluding the abundance of *Variovorax* contributing to 100%
566 abundance. Relative abundance data were normalized by robust centered log-ratio (rclr)
567 transformation using the decostand function from the vegan R package (Oksanen et al., 2001).
568 Principal component analysis (PCA) was performed on the normalized abundance data using the
569 prcomp function in R to visualize variation in community composition across samples.

570 RB-TnSeq library construction

571 A DNA-barcoded Tn5 transposon mutant library of *Variovorax* SCN45 was generated as
572 described previously (Wetmore et al., 2015). Briefly, an *E. coli* strain (WM3064) carrying the
573 transposon vector library pKMW3 was conjugated with wild-type *Variovorax* SCN45 at a 1:1 ratio
574 on R2A agar plates supplemented with diaminopimelic acid (50 ug/ml). Following 6 hours of
575 incubations, the transformants were plated onto R2A agar plates containing 100 µg/ml kanamycin.
576 Several thousand *Variovorax* colonies were scraped and collected into a tube before being
577 resuspended in 100 ml of R2A with Kanamycin and incubated at 30 C for 6 hours. Glycerol stocks
578 were made at a 1:1 ratio of mid-log phase Variovorax cells and 25% glycerol, flash frozen in liquid
579 nitrogen, and stored at -80C. Simultaneously 10 ml of mid-log phase cells were pelleted through
580 centrifugation and their DNA extracted and sequenced by TnSeq-like Illumina sequencing
581 protocol.

582 RB-TnSeq library incubations

583 A 2 ml mutant library glycerol stock was thawed and inoculated into 25 ml of R2A with 50 ug/ml
584 Kanamycin and incubated at 30°C with shaking until cells entered mid log phage (approximately
585 8 hours). Four tubes of 2 ml of culture, termed time zero controls, were then recovered and cells
586 pelleted by centrifugation and frozen at -80°C. The remaining cultured cells were then washed
587 twice in relevant media (M9 media without carbon source or R2A) with spins at 3000g for 3
588 minutes. The cells were resuspended in media and inoculated into relevant or culture to a final
589 OD600 of 0.02. The cultures were grown in 10ml glass tubes and incubated at 30 °C with shaking.
590 (i) For phage-challenge experiments 10 uL of phage (4×10^4 PFU/ul) were added to the libraries
591 after 4 hours of subsequent incubation and allowed to incubate for a total of 24 h. (ii) For
592 community incubations, cultures were passaged every 48 h for four passages. Cells were
593 recovered from four replicate cultures by centrifugation and stored at -80 °C.Genomic DNA was
594 then extracted from the stored cell pellets and PCR and sequencing by BarSeq performed as
595 described previously (Wetmore et al., 2015).A 2 ml mutant library glycerol stock was thawed and
596 inoculated into 25 ml of R2A with 50 ug/ml Kanamycin and incubated at 30°C with shaking until
597 cells entered mid log phage (approximately 8 hours). Four tubes of 2 ml of culture, termed time
598 zero controls, were then recovered and cells pelleted by centrifugation and frozen at -80°C. The

599 remaining cultured cells were then washed twice in relevant media (M9 media without carbon
600 source or R2A) with spins at 3000g for 3 minutes. The cells were resuspended in media and
601 inoculated into relevant or culture to a final OD600 of 0.02. The cultures were grown in 10ml glass
602 tubes and incubated at 30 °C with shaking. (i) For phage-challenge experiments 10 uL of phage
603 (4 x10^4 PFU/ul) were added to the libraries after 4 hours of subsequent incubation and allowed to
604 incubate for a total of 24 h. (ii) For community incubations, cultures were passaged every 48 h for
605 four passages. Cells were recovered from four replicate cultures by centrifugation and stored at -
606 80 °C.Genomic DNA was then extracted from the stored cell pellets and PCR and sequencing by
607 BarSeq performed as described previously (Wetmore et al., 2015).

608 # Data availability

609 The contigs representing the Variovorax genome assembled by PacBio can be found under
610 bioproject PRJNA1212206 genome accessions CP179718-CP179720. The assembled genomes
611 of the isolated phage have been deposited in GenBank under accession numbers PV558788 -
612 PV558796. The reads associated with these genomes and assemblies can be found under
613 accession numbers SRR33449703 - SRR33449695. The 16S rRNA amplicon sequencing data
614 can be found under Bioproject PRJNA1254325, under accession numbers SRR33264975 -
615 SRR33264785. Shotgun sequencing can be found under bioproject PRJNA1254232, including
616 reads (SRR33436150-SRR33436076), assemblies (SAMN48138949 - SAMN48139025), and
617 novel MAGs (SAMN48122594 - SAMN48122609). These MAGs can also be found on ggkbase
618 https://ggkbase.berkeley.edu/Phage_treated_Variovorax_enrichments_dereplicated_MAGs/ and
619 MAGs detected in this study that had been previously published can be found on ggkbase
620 https://ggkbase.berkeley.edu/SCN_92/.
621

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632
633
634

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Supplementary materials

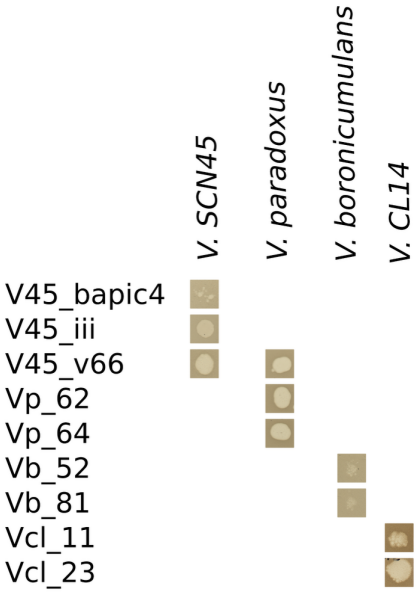


Figure S1. Plaque morphology from spot assays performed on *Variovorax* species with the nine isolated *Variovorax* phages. Only phage V45_66 was able to plaque on more than one host.

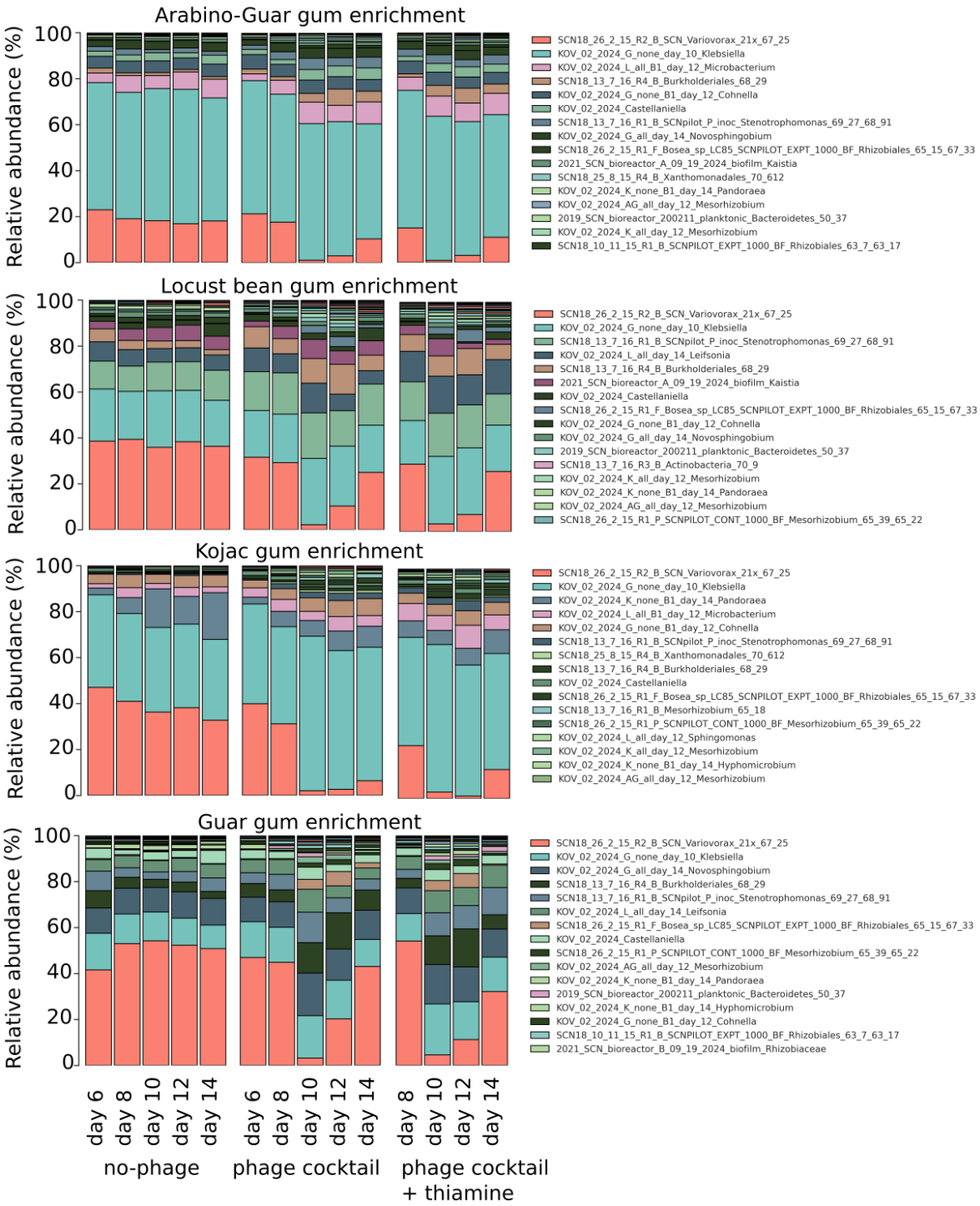


Figure S2. Genome-resolved metagenomics reveals consistent reduction in the relative abundance in Variovorax across all four enrichment cultures following phage application. The 16 most abundant organisms per enrichment culture recovered are shown.

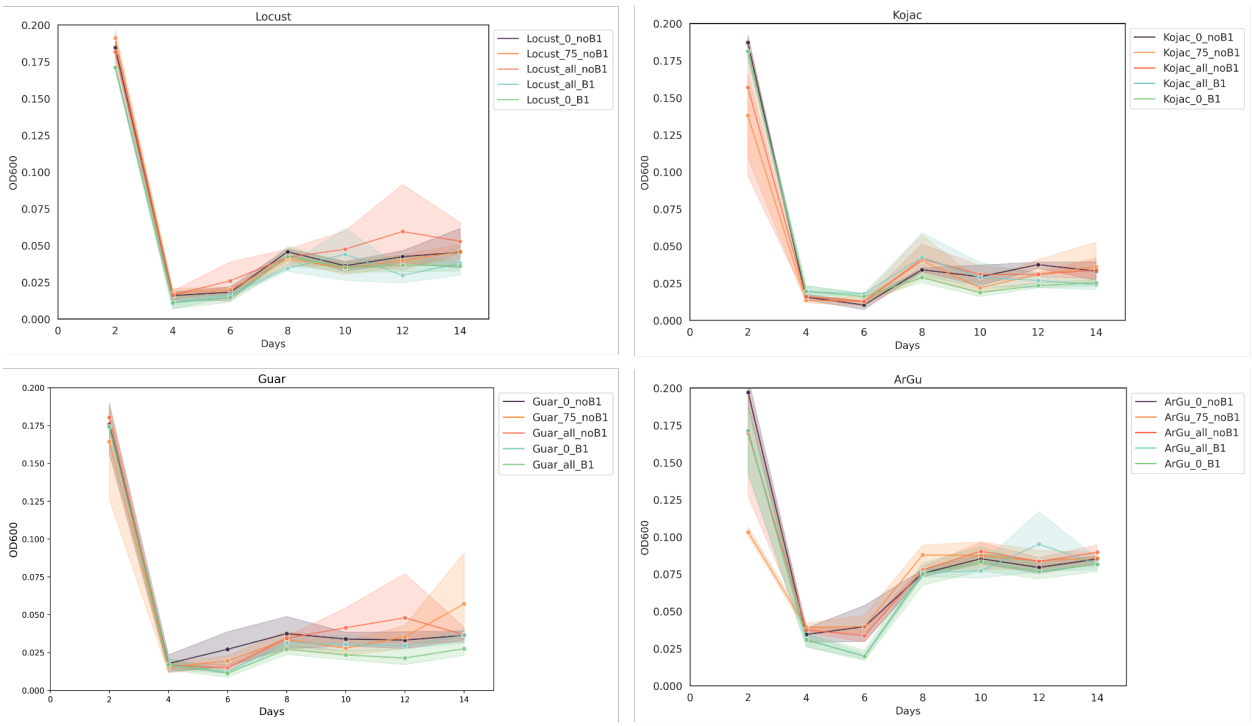


Figure S3. The optical density (600nm) of the 48 hour old communities following inoculation and each subsequent passage. All communities show a sharp reduction between day 2 and day 4 communities but then reach a stable state from day eight onwards.

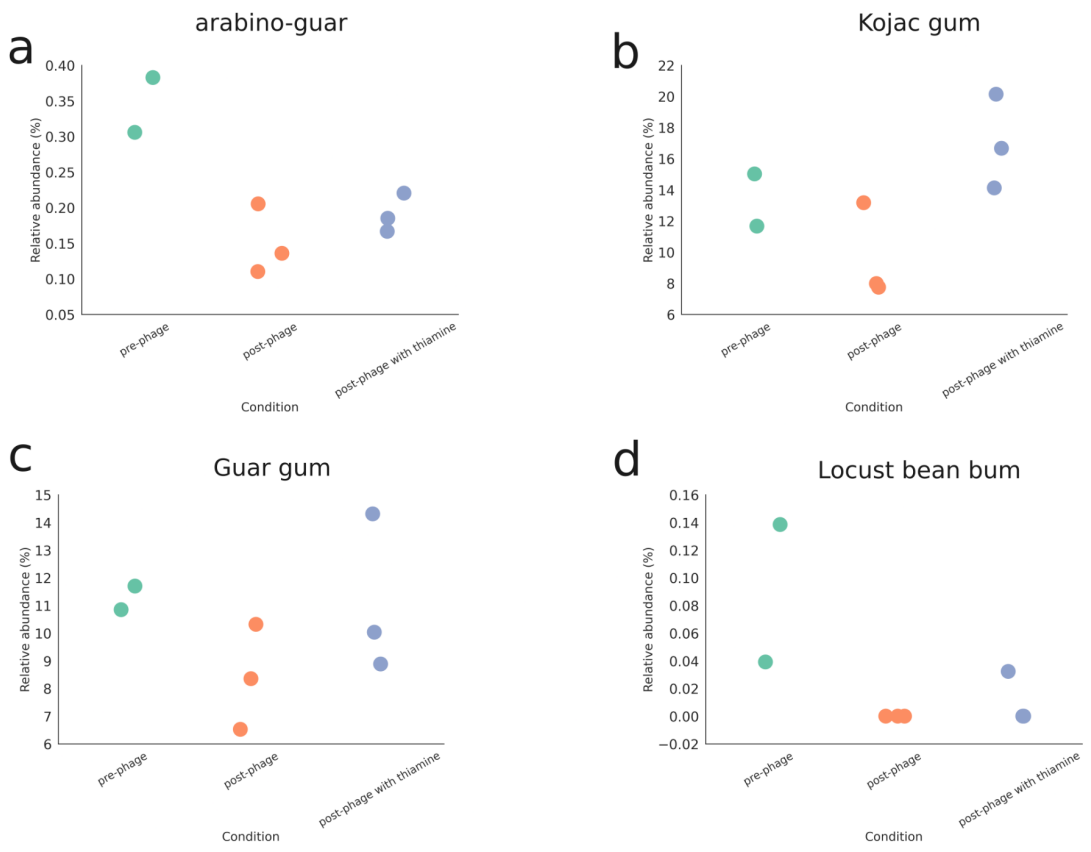
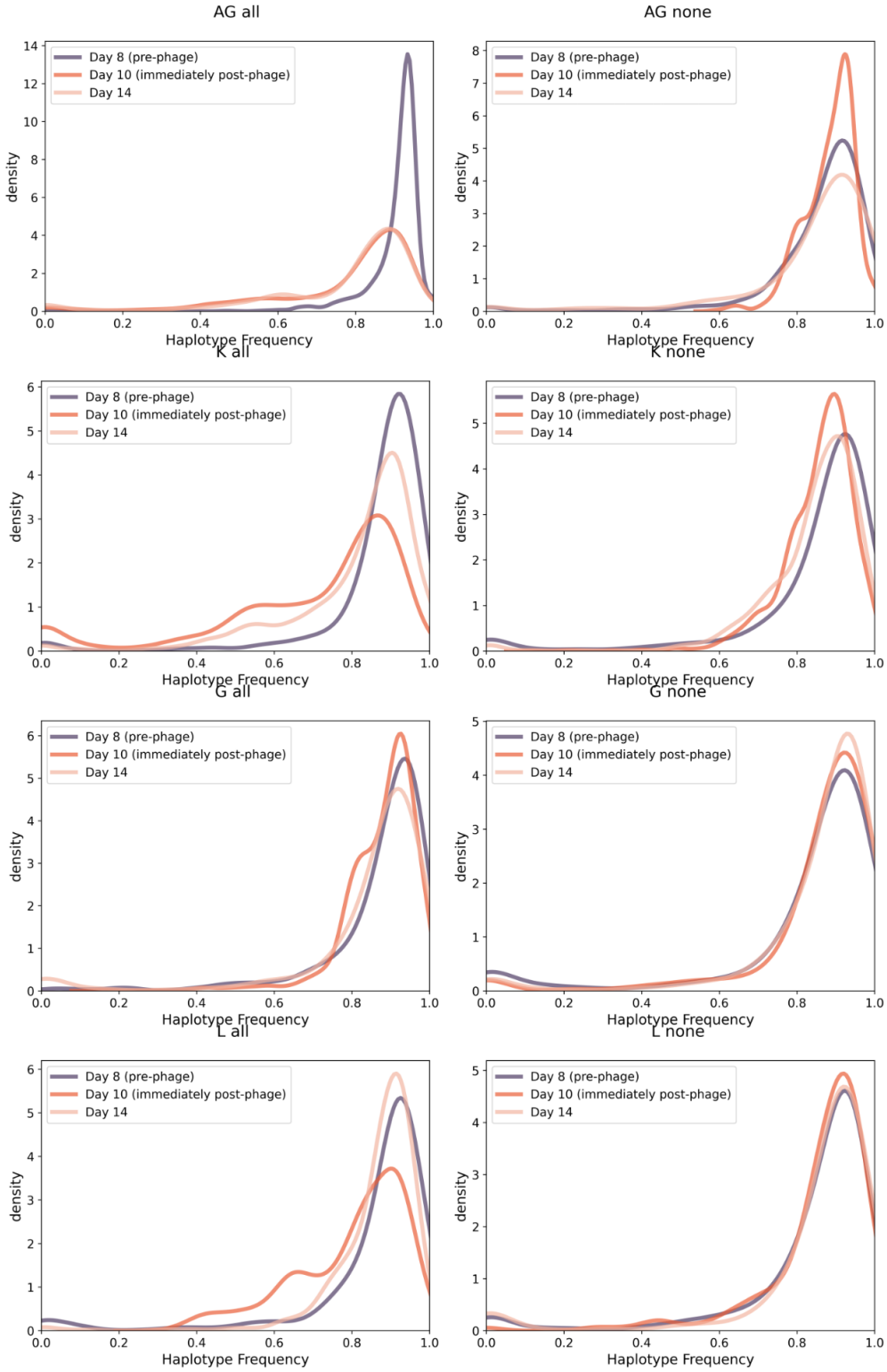


Figure S4. *Leifsonia* MAG relative abundance is consistent with the ASV data. The abundance of this MAG is shown across the four carbon source enrichment cultures before phage addition (day 6 and day 8 communities), following phage infection (day 10, day 12, day 14 communities), and following phage infection with supplementation of thiamine.



1001
1002 **Figure S5.** Haplotype frequencies associated with phage exposure to each of the four enrichment
1003 communities and their no-phage controls.

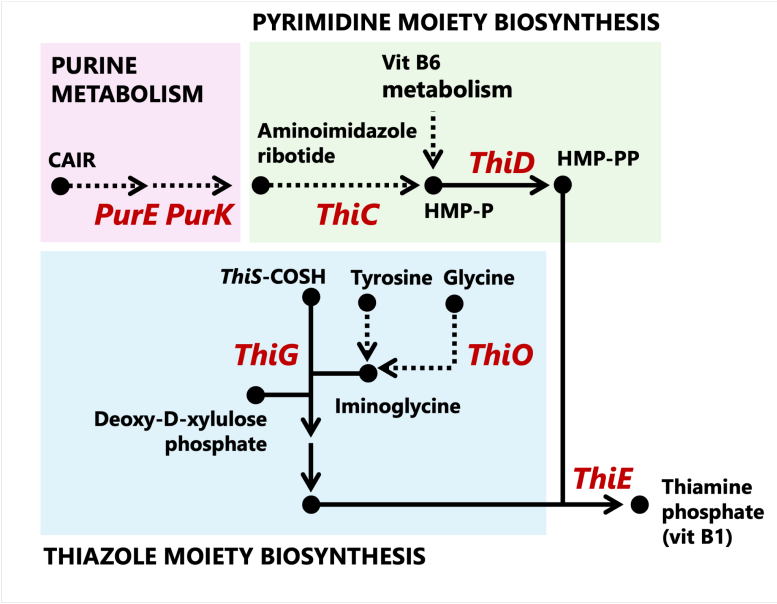


Figure S6. Thiamine biosynthesis pathway illustrating the multiple routes for the production of the thiazole and pyrimidine moieties of thiamine. Adapted from https://www.kegg.jp/ (Kanehisa & Goto, 2000).