Widespread B-vitamin auxotrophy in marine particle-associated bacteria

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Abstract

Microbial community assembly is governed by complex interaction networks based on the secretion and exchange of metabolites. While the importance of trophic interactions (e.g. cross-feeding of metabolic byproducts) in structuring microbial communities is well-established, the roles of myriad natural products such as vitamins, siderophores, and antibiotics remain unclear. Here, we focus on the role of B vitamins in coastal marine bacterial communities that degrade particulate organic matter. We find that natural seawater particle-associated communities are vitamin limited and almost a third of bacterial isolates from these communities are B vitamin auxotrophs. Auxotroph growth rates are limited under even maximal environmental vitamin concentrations, indicating that auxotrophs likely survive through cross-feeding with community members. We find that polysaccharide-degrading bacteria tend to be vitamin prototrophs, suggesting that the initial arrival of degraders to a particle may promote a succession to auxotrophic taxa partially through vitamin cross-feeding. However, auxotrophs with complementary vitamin requirements were generally not able to grow in co-culture, and auxotroph growth was only partially rescued by prototrophs. We conclude that while vitamin auxotrophies are important metabolic dependencies shaping community structure, vitamin cross-feeding may primarily take place through cell lysis.

Introduction

In the vast ocean, particles of organic matter serve as hubs of microbial activity and interactions.[1,2] These particles, termed marine snow, are colonized and degraded by specialized bacteria as they sink, releasing sugars and other substrates like organic acids that support the growth of cross-feeding bacteria.[3,4] Particle-associated communities harbor a dense network of interactions, through quorum sensing,[5] antibiotics,[6] and horizontal gene transfer.[7] Metabolic dependencies frequently evolve in these communities, for example through the loss of genes to produce siderophores[8] or hydrolytic enzymes.[9] More broadly, metabolic dependencies such as vitamin auxotrophies are predicted to be prevalent among marine microbes[10,11] and have been characterized in some marine isolates.[12–14]

It remains unclear how chemical interactions beyond the cross-feeding of sugars and organic acids shape marine bacterial community assembly. Vitamins are uniquely suited as a case study for chemical interactions, as these metabolites are highly conserved and required only in trace amounts, as coenzymes for central metabolic reactions. Vitamin exchange is important in algal-bacterial interactions[15] and has been explored in other biomes,[16–18] but has not been studied in the marine particle context. While some seawater amendment studies have shown that vitamins are limiting,[13,19] it has also been proposed that trace vitamin levels in seawater are sufficient for auxotroph growth.[13,20] If vitamins are indeed limiting, then biotic vitamin exchange is likely to be an important driver in community dynamics. This is especially relevant in the resource-rich particle environment, where other nutrients may not be limiting.

Here, we examine the role of vitamin exchange in the assembly of marine particle-associated bacterial communities. We combine natural seawater experiments with a large-scale characterization of vitamin requirements in 150 particle-associated isolates. We find that while auxotrophies are prevalent, vitamin cross-feeding is not, suggesting that in nature vitamin requirements are met primarily through cell lysis.

Results

Vitamins are a limiting nutrient in coastal seawater microcosms

To determine if vitamins are limiting in particle-associated communities, we examined the effect of vitamin supplementation on coastal seawater microcosm incubated with model particles. As a simplified model for natural organic matter particles, we used particulate chitin,

an abundant marine polysaccharide. We have previously shown that chitin particles are colonized by marine bacteria from the surrounding seawater in successive waves of degraders and cross-feeders.[3,4] We collected coastal seawater from Nahant, MA and incubated it with chitin particles in 50 mL aliquots over five days (see Methods for details). To test the effect of vitamins, in half of the samples we added a mixture of vitamins formulated for marine culture medium (Table 1). Every 12 hours, we harvested the particle-associated bacterial community (approximately 750 particles per sample) and sequenced the samples using shotgun metagenomics (2 replicates per condition).

Table 1. Vitamin concentrations in supplemented growth conditions.

Vitamin	**Abbreviation**	**Final conc. (M)**
Lipoic acid	ALA	4.85×10^{-7}
Thiamine hydrochloride	B1	2.97×10^{-7}
Thiamine pyrophosphate	B1	2.17×10^{-7}
Riboflavin	B2	2.66×10^{-7}
Nicotinic acid	B3	8.12×10^{-7}
Nicotinamide adenine dinucleotide (NAD)	B3	1.51×10^{-7}
Calcium d-pantothenate	B5	4.20×10^{-7}
Pyridoxine hydrochloride	B6	4.86×10^{-7}
D-Biotin	B7	1.23×10^{-7}
Folic acid	B9	2.27×10^{-7}
4-aminobenzoic acid (PABA)	B9 precursor	7.29×10^{-7}
L-ascorbic acid	C	5.68×10^{-7}
Cyanocobalamin	B12	7.38×10^{-9}

We found that adding vitamins drastically altered the community composition and functional potential of seawater microcosms, indicating that these communities are indeed vitamin limited (Fig. 1). Key taxonomic groups that typically dominate chitin communities, such as *Vibrionales*, *Alteromonadales*, and some *Pseudomonadales,* reached a higher relative abundance in the absence of vitamins (Fig. 1a, Fig. S1). This indicates that these taxa may have a competitive advantage in this condition and are likely vitamin producers (termed prototrophs). In vitamin-supplemented samples, taxa including *Rhodobacterales* and

Flavobacteriales were enriched, suggesting that these may be vitamin auxotrophs (Fig. 1a). These taxonomic shifts led to differences in functional potential: vitamin-supplemented communities had a lower fraction of chitin degraders, as inferred from the frequency of chitin-hydrolysis protein domains in the metagenomes (Fig. 1b).

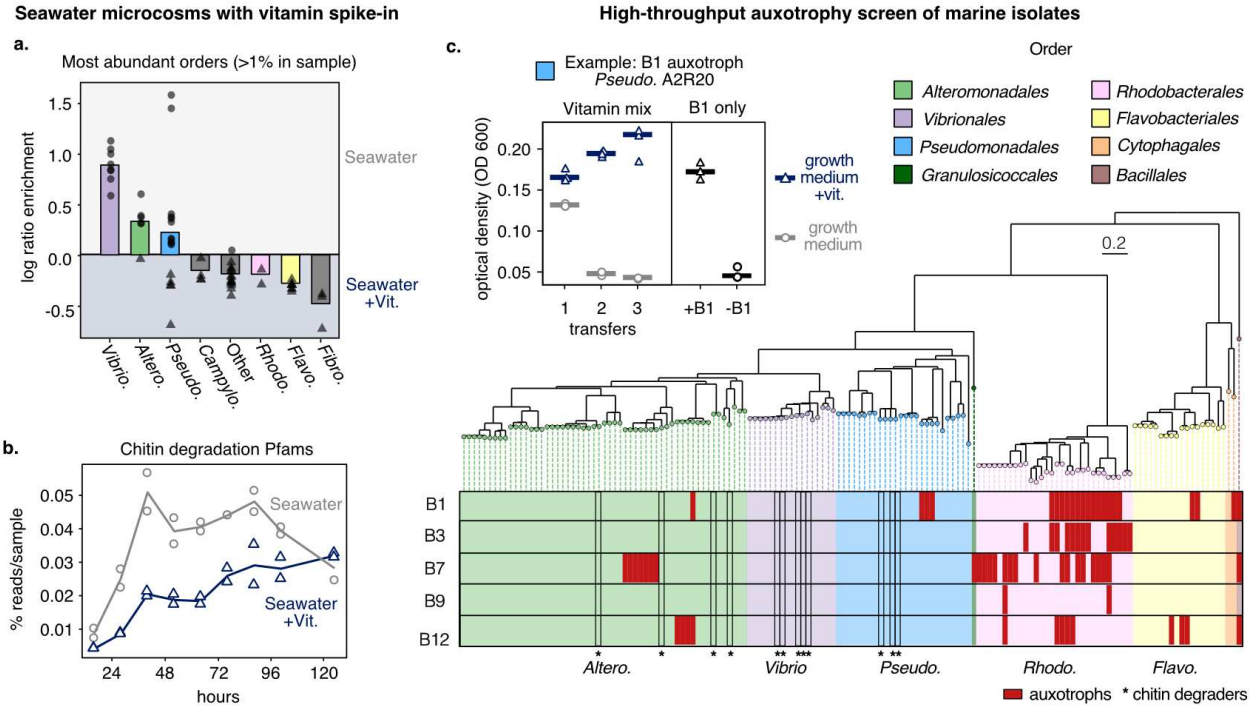

Fig. 1. Coastal seawater communities are vitamin-limited and vitamin auxotrophies are prevalent. A. Coastal seawater collected at Nahant, MA, was incubated with chitin particles, with and without a vitamin mix spike-in. The addition of vitamins led to differences in taxonomy at the order level. Each data point represents a taxon that reached at least 1% abundance in one or more time points. B. Chitin degradation proteins families (Pfams) were depleted in the vitamin-supplemented seawater condition compared to the seawater condition. C. 150 bacterial coastal marine isolates from Nahant, MA, were screened for vitamin auxotrophies in a high-throughput format. Top left, example of screen of single strain on vitamin mix and single vitamins. Chitin degraders were prototrophic (marked with *). Abbreviations: *Vibrio*. = *Vibrionales*; *Altero*. = *Alteromonadales*; *Pseudo*.= *Pseudomonadales*; *Campylo*. = *Campylobacterales*; *Rhodo*. = *Rhodobacterales*; *Flavo*. = *Flavobacteriales*; *Fibro*. = *Fibrobacterales*.

B-vitamin auxotrophies are prevalent in marine particle-associated isolates

To understand the mechanisms driving the community response to vitamins, we characterized vitamin auxotrophies in a collection of coastal, particle-associated strains isolated from the same location (Nahant, MA).[21] We assayed 150 seawater isolates representative of five of the orders affected by vitamin supplementation (Fig. 1c). To identify auxotrophies, we grew the isolates in a defined growth medium with and without vitamin supplementation (Table 1). We measured growth over three growth-dilution cycles, diluting 1:40 every 24 hours. By the third day, any vitamins from the initial seed cultures were diluted to picomolar concentrations, similar to environmental levels. Isolates with reduced growth without vitamins (Fig. S2) were then screened for each vitamin individually to identify specific auxotrophies (Fig. 1c, top left).

Consistent with the seawater microcosm experiment (Fig. 1a), all *Vibrionales* isolates were prototrophs, while 87% of the *Rhodobacterales* isolates were auxotrophic for one or more vitamins (Fig. 1c). These results indicate that the community response to vitamins in the seawater microcosms was likely driven by auxotrophies. Overall, almost one-third of the particle-associated isolates (47/150) were auxotrophic for vitamins (Fig. 1c). The auxotrophies were for one or more of five key B vitamins: thiamine (B1), niacin (B3), biotin (B7), folic acid (B9), and cobalamin (B12).

We hypothesized based on the seawater microcosm data that degraders would be likely to be prototrophic, as chitin-hydrolysis genes were more abundant in the absence of added vitamins. Indeed, in a subset of 65 isolates characterized for growth on chitin,[4,9] the 12 degraders were exclusively prototrophic, with representatives from the clades *Vibrionales*, *Alteromonadales*, and *Pseudomonadales* (Fig 1c, degraders marked with *). This was further supported by the isolate genomes, in which both chitin and alginate hydrolysis genes were enriched in prototrophs (Fig. S3; Wilcoxon rank sum test: p-value < 0.001). This data suggests that vitamin prototrophy may provide a competitive advantage for degraders in particle-associated communities in the ocean.

Auxotrophies evolve through partial loss of biosynthesis pathways

We next established the genetic basis of vitamin auxotrophies by comparing the results of the phenotypic screen to the isolate genomes. We found a strong match between the observed auxotrophies and the absence of key vitamin biosynthesis genes based on previous studies[13,14,22–25] (Fig. S4, Table S1 and S2). Based on the presence-absence of these genes, the genomes were assigned as predicted prototrophs or auxotrophs. These predictions were

generally accurate for B1, B3, and B7, with error rates between 3-7% (Table S2; B9 was excluded since there are only two B9 auxotrophs). This indicates that the observed auxotrophies were primarily the result of gene loss, as opposed to differential gene regulation or errors in the phenotypic screen. The most common mode of evolution was the partial loss of vitamin biosynthesis pathways, which occurred for all five vitamins (Fig. 1A). This results in auxotrophies for vitamin precursors to feed into the remaining biosynthetic steps. We observed fewer cases in which the entire biosynthetic pathway was lost, primarily for B7 in *Rhodobacterales* and B1 in *Flavobacteriales* (Fig. 1B).

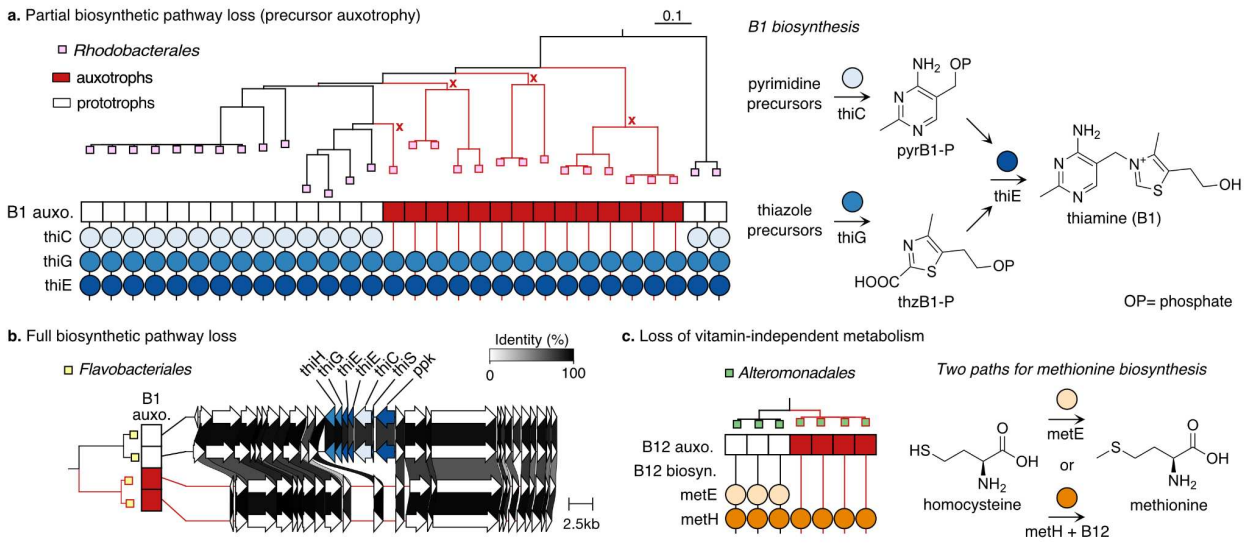

Fig 2. Three evolutionary modes of auxotroph formation. A) Partial pathway loss leading to formation of B1 pyrimidine auxotrophs in *Rhodobacterales*. Auxotrophies evolve frequently, with four putative loss events shown here (red “x”). Right, three critical genes in the thiamine biosynthesis pathway are responsible for precursor formation of the pyrimidine subunit pyrB1-P (thiC) and the thiazole subunit thzB1-P (thiG), and for their coupling to form thiamine (thiE). B. Total pathway loss. Genomic region of the thiamine biosynthesis cluster is lost in *Flavobacteriales* B1 auxotrophs compared to two closely related prototrophic isolates, with flanking regions conserved. C. Loss of vitamin-independent metabolism. Both prototrophic and auxotrophic *Alteromonadales* isolates are missing B12 biosynthesis genes and can scavenge B12. B12 auxotrophs have lost metE, retaining only the B12-dependent pathway for the biosynthesis of methionine via metH.

In contrast, there was little correlation between vitamin B12 auxotrophies and predictions based on biosynthesis genes (Fig. S4, Table S1 and S2). Vitamin B12 is not universally essential, as many bacteria and algae have alternate metabolic pathways that circumvent the need for a coenzyme. Most of these isolates are predicted to scavenge B12 but do not produce or require it, and are therefore prototrophs. However, we observed one clade of B12 auxotrophs that lost the alternate metabolic pathway (Fig. 1C). Only around 30 isolates in the collection were predicted to produce B12, primarily *Rhodobacterales* strains which were also predicted to have an obligate requirement for B12. Since the biosynthesis pathway of B12 contains over 30 genes, B12 producers are commonly classified based on percent pathway completeness rather than specific genes.[25] Here, this approach led to misclassifying some likely precursor auxotrophs with single gene losses as producers (Fig. S5).

Using the genome-based auxotrophy predictors, we expanded our analysis and found that the positive correlation between vitamin prototrophy and polysaccharide degradation is more broadly generalizable. We annotated over 11,000 diverse reference genomes from proGenomes[21] and assigned auxotrophies for vitamins B1, B3, B7, and B9. On average, vitamin prototrophs had a higher number of glycoside hydrolases (polysaccharide-degrading enzymes) than auxotrophs, even after normalizing by genome size (Fig. S6). Genomes with all four vitamin auxotrophies tended to have the fewest glycoside hydrolases. This suggests that polysaccharide degraders and vitamin auxotrophs may occupy separate ecological niches across environments.

Auxotroph growth rates are limited under environmental vitamin concentrations

How do vitamin auxotrophs survive in the open oceans? The concentration of vitamins varies widely in the coastal oceans and is undetectable (sub-picomolar levels) in many areas.[26] Since vitamins are required in low amounts and can be recycled, it has been proposed that amounts sufficient for survival can be scavenged from seawater. Alternatively, vitamin auxotrophs may require cross-feeding from more direct interactions with vitamin producers. The likelihood of either strategy depends on the amount of vitamins required to sustain growth. To determine growth requirements for vitamins, the growth of auxotrophs was measured across vitamin concentrations spanning seven orders of magnitude. We compared these values to environmental measurements of dissolved vitamins.[26–30]

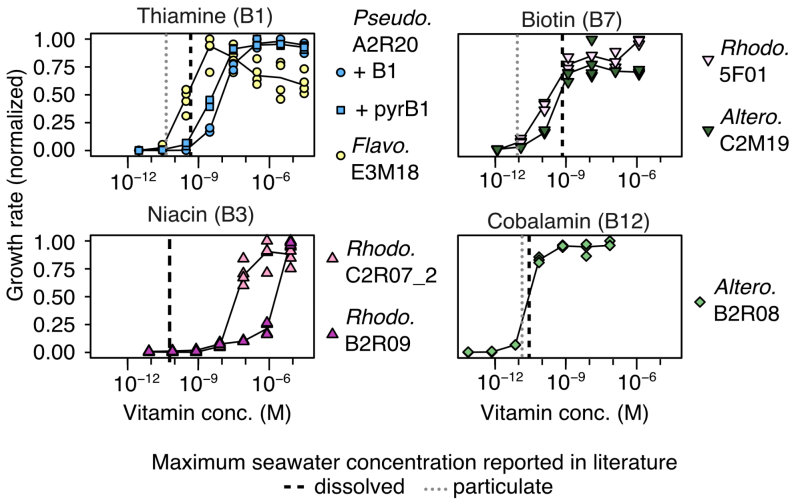

Fig. 3. **Auxotroph growth rates are limited under environmental vitamin concentrations.** Auxotrophs were grown on different vitamin or precursor concentrations for three growth-dilution cycles, diluting 1:40 every 24 hours. After the last transfer, a kinetic growth measurement was run and growth rates calculated from the exponential phase. The lines indicate maximum literature values for dissolved vitamins in seawater (black dashed line), and for particulate matter when available (gray dotted line): B1, 457 pM dissolved and 44 pM particulate, Eastern Atlantic[28]; B3, 56 pM dissolved NAD in Western Atlantic[29] and 46 pM dissolved niacin in North Sea[27]; B7, 679 pM dissolved and 9 pM particulate, Eastern Atlantic[28]; B12 (cobalamin, total for adenosyl-, hydroxy-, methyl-, and cyano-), 26 pM dissolved, North Sea[27] and 14 pM particulate, Eastern Atlantic[28]. Abbreviations: *Altero*. = *Alteromonadales*; *Pseudo*.= *Pseudomonadales*; *Rhodo*. = *Rhodobacterales*; *Flavo*. = *Flavobacteriales*; pyrB1 = B1 pyrimidine precursor.

Table 2. Estimated half-velocity constants (K_S).

Order	Genus	Strain	Vitamin	K_S (M)	Std. Error (M)
Pseudomonadales	*Marinobacter*	A2R20	B1	9.6E-09	9.0E-10
			B1 pyrimidine	3.5E-09	2.4E-10
Flavobacteriales	*Arenibacter*	E3M18	B1	1.7E-10	5.5E-11
Rhodobacterales	*Roseovarius*	B2R09	B3	4.6E-06	1.1E-06
Rhodobacterales	*Paracoccus*	C2R07_2	B3	3.7E-08	6.4E-09
Rhodobacterales	*Shimia*	5F01	B7	1.3E-10	2.6E-11
Alteromonadales	*Colwellia*	C2M19	B7	3.1E-10	6.7E-11
Alteromonadales	*Shewanella*	B2R08	B12	2.8E-11	4.6E-12

The vitamin requirements of auxotrophs ranged from approximately 30 pM for a B12 auxotroph, to 5 μM for a B3 auxotroph (Fig. 3 and Table 2). While the two B7 auxotrophs had similar requirements, there was variation between auxotrophs for both B1 and B3. We hypothesized that this is due to different affinities for vitamins and their precursors, as has been shown in SAR11.[12] However, a *Pseudomonadales* precursor auxotroph had similar affinity for the precursor and for B1, both an order of magnitude lower than a *Flavobacteriales* isolate (Fig. 3, top left). It is possible that additional metabolites or precursors may be needed, or that these differences stem from differences in transporters or physiology.

The growth rates of all auxotrophs were limited under even maximum dissolved environmental vitamin concentrations (Fig. 3, black dashed lines). This was especially stark for vitamin B3, which was recently quantified at approximately 50 pM in seawater,[27,29] 3-5 orders of magnitude lower than the requirements measured here. Vitamin levels are even lower in natural particulate organic matter when compared to dissolved (Fig. 3, gray dotted lines).[28,29] This data confirms that auxotroph growth is likely vitamin limited in the global oceans, especially in nutrient-rich particle communities where other resources are at high local concentrations. The striking similarity between the environmental concentrations and the measured bacterial affinities suggests that bacterial uptake may dictate vitamin levels in parts of the oceans, by depleting them to the lowest concentration possible.

Auxotroph co-cultures are vitamin limited

If ambient vitamins levels in seawater are not sufficient for growth, vitamin auxotrophs likely rely on cross-feeding with vitamin producers. Cross-feeding in co-culture has been shown with algae,[15,31,32] with engineered vitamin auxotrophs,[33,34] and for organic acids with isolates from this collection.[3,35] We therefore hypothesized that natural bacterial auxotrophs would complement one another's vitamin requirements during growth. To test this, we grew a selection of eight auxotrophs in co-culture, including two dual auxotrophs. Each auxotroph was initially grown in monoculture to high density in vitamin-supplemented media then combined in pairs (Fig. 4a, top). We measured co-culture growth after three growth-dilution cycles, diluting 1:40 every 24 hours, and compared it to growth in vitamin-supplemented media. Of the 28 co-cultures, 17 pairs were predicted to be able to successfully complement one another's vitamin auxotrophies, while 11 pairs were predicted not to grow.

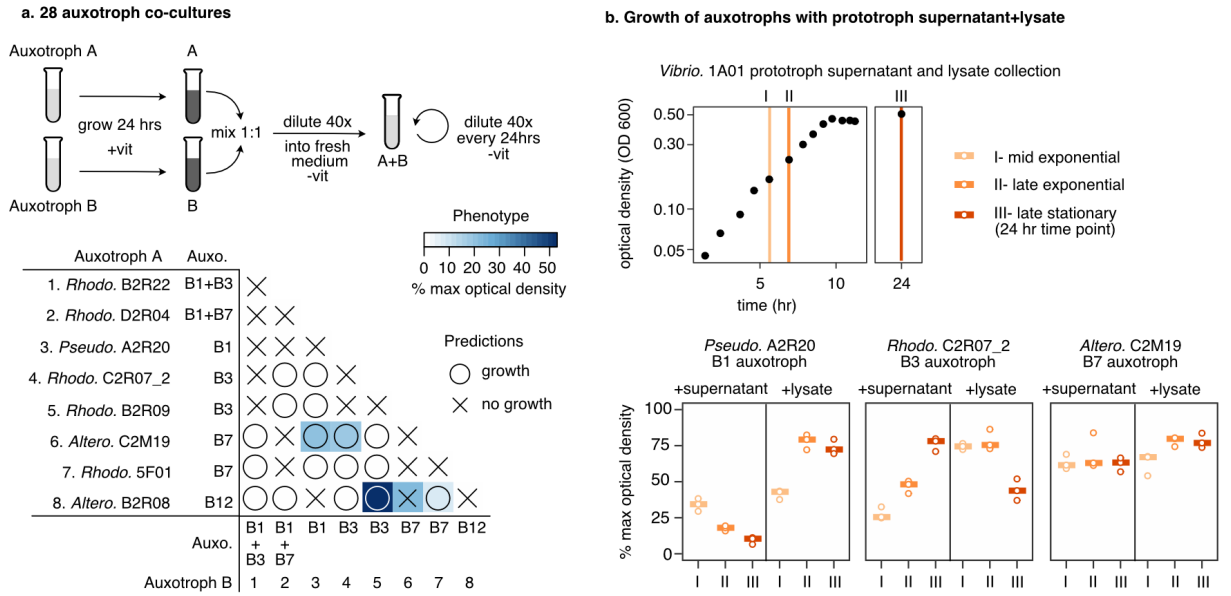

Fig. 4. **The majority of auxotrophies are not complemented in co-culture.** A. Eight auxotrophs were grown in co-culture, for a total of 28 pairs. Top, the auxotrophs were grown separately with vitamins, then combined 1:1 and transferred to fresh medium without vitamins. Growth was measured after 3 growth-dilution cycles. Bottom, only 4/17 co-cultures predicted to successfully cross-feed (circles) grew to detectable levels, as well as 1/11 of the co-cultures predicted not to grow. The co-cultures reached between 7-52% of the final optical density of co-cultures with vitamin supplementation (% max optical density, blue heatmap). The 8 monocultures appear on the diagonal. B. Three auxotrophs were further tested for growth with supernatants and lysates (containing both supernatant and lysed cells) collected from a *Vibrionales* prototroph culture in mid- and late exponential phase (peach and orange), as well as in stationary phase (red). Growth was measured after 3 growth-dilution cycles. All data is presented as a percentage of the optical density obtained when the supernatant or lysate is supplemented by vitamins (% max optical density), to correct for the effect of the depletion or release of additional nutrients in each condition (see Fig. S7 for raw data). Abbreviations: *Vibrio*. = *Vibrionales*; *Altero*. = *Alteromonadales*; *Pseudo*.= *Pseudomonadales*; *Rhodo*. = *Rhodobacterales*.

The majority of pairs of auxotrophs did not grow in co-culture (Fig. 4a). Only around one quarter (4/17) of the predicted successful pairs grew to detectable levels, and that growth was vitamin limited, ranging from just 7-52% of growth with vitamin supplementation. The extinction of the majority of these co-cultures indicates that they divided fewer than approximately 5 times over 24 hours ($\log_2$ of the dilution factor 40x). This is despite being initially combined in relatively high density after growth in vitamin-rich media to avoid Allee effects, i.e. limitations due to positive density-dependent growth[36]. While the two dual auxotrophs did not grow in any co-culture, the other six isolates each grew in at least one instance. Almost all of the 11

co-cultures predicted not to grow indeed did not, except for one: the *Alteromonadales* auxotrophs for B7 (C2M19) and B12 (B2R08). C2M19 is very unlikely to synthesize B12 based on its genome, and so the co-culture growth is likely due to methionine cross-feeding, which can alleviate the need for B12 (Fig. 2c). Overall, this data indicates that even when cross-feeding is theoretically possible, most pairs of auxotrophs are not able to grow together in co-culture.

We next hypothesized that prototrophic degraders supply vitamins to auxotrophic cross-feeders (Fig. 4b). We selected the *Vibrionales* chitin degrader 1A01 and collected supernatants and lysates during its growth, including two exponential timepoints as well as a 24 time point, which corresponds to late stationary phase (Fig. 4b, top). Lysates were generated by mechanically lysing entire cultures containing both cells and supernatants. We tested three auxotrophs from the panel, growing them with the supernatants and lysates mixed 1:1 with fresh media (Fig. 4b, bottom). All data is presented as a percentage of growth in supernatants with added vitamins, to represent the maximal possible growth given the depletion and release of other nutrients (see Fig. S7 for raw data).

In general, some auxotroph growth was supported by prototroph supernatants and lysates, depending on the vitamin and growth phase (Fig. 4b, bottom). The B7 auxotroph C2M19, which requires sub-nM levels of B7 (Fig. 3), reached between 53-87% of maximal growth on the supernatants and lysates (Fig 4b, bottom right). The B1 and B3 auxotrophs, which both have higher vitamin requirements, were generally more vitamin limited, depending on the growth phase (Fig. 4b, bottom left and center). In almost all cases, growth on supernatants was more limited than on lysates. This data indicates that cross-feeding may be more easily facilitated by prototrophic bacteria that are not vitamin-limited, and raises the possibility that degraders may play a role as vitamin providers in particle communities.

Discussion

We found that B vitamin auxotrophies are prevalent in marine particle-associated communities. In coastal seawater microcosms, the addition of vitamins altered community composition and reduced the community chitin degradation potential (Fig 1). These effects were likely due to vitamin auxotrophies, which we found in almost a third of particle-associated isolates in a high-throughput screen. Vitamin auxotrophies for B1, B3, and B7 are generally correctly predicted from genomes, and most often evolve through partial pathway loss (Fig 2). The growth rates of all auxotrophs were limited under even maximum dissolved environmental

vitamin concentrations (Fig 3), leading us to hypothesize that vitamin requirements are met through interactions. However, when auxotrophs were grown in pairs, the majority of co-cultures did not grow (Fig. 4a). Some auxotroph growth was supported by supernatants and lysates collected from a prototroph degrader, depending on the vitamin and growth phase (Fig. 4b).

Vitamin auxotrophy in particle-associated communities was found in cross-feeders, not degraders. We found that all of the degrader isolates in our collection were prototrophs (Fig. 1c), and that hydrolytic enzyme genes were more abundant in prototrophs than auxotrophs (Fig. S3). This was further supported by the seawater microcosm data, in which chitinase genes were more abundant in samples without vitamin supplementation (Fig. 1b). These degraders may also play an additional role as vitamin providers to auxotrophic cross-feeders, as is suggested by our supernatant experiments (Fig. 4b). This data fits well with previous findings that degraders are the first to arrive on particles, followed by a wave of cross-feeders.[37] It may therefore be more advantageous for pioneering degraders to be self-sufficient prototrophs than for cross-feeders, which are reliant on other community members regardless. We observed the same pattern in a broad analysis of vitamin auxotrophies and polysaccharide degradation genes in over 11,000 genomes, indicating that this division into separate ecological niches may be more broadly applicable (Fig. S6).

The patchy distribution of auxotrophies suggests that vitamin biosynthesis genes are frequently lost in particle-associated communities. The auxotrophies were generally well-correlated to gene losses of key vitamin biosynthesis genes, especially for vitamins B1, B3, and B7 (Table S2). In the majority of cases, only part of the biosynthetic pathway was lost, as has been recently observed in many marine bacteria.[12–14] This partial pathway loss leads to auxotrophies for vitamin precursors, and also may make it possible to regain vitamin biosynthesis through horizontal gene transfer. We conclude that vitamin biosynthesis is heterogeneously distributed in natural communities and that auxotrophies have evolved multiple times through independent loss events, as has been shown in algae[38].

How are auxotrophs meeting their vitamin needs? We found that auxotroph growth was limited under environmental vitamin concentrations, and therefore likely requires cross-feeding with other bacteria. However, the majority of auxotrophs could not grow in co-culture, and growth was limited in some cases from prototroph supernatants as well (Fig. 4). Vitamin secretion during growth has been linked to overproduction and secretion via transporters.[39] This data indicates that vitamin secretion is relatively rare and strain-specific, as has also been

recently found in *Rhodobacterales* isolates for B12.[40] Therefore vitamins are not necessarily shared public goods, in contrast to sugars released from particle degradation,[9] excreted organic acids,[35] or siderophores.[8]

If vitamins are only rarely secreted during growth, a likely route for cross-feeding in particle communities is through cell lysis. We have recently found that phage-mediated lysis, coinciding with bacterial metabolite release, structures particle-associated communities especially through predation of degraders.[41] Phage predation[42] and defense mechanisms[43] can also remodel metabolism, leading to higher levels of released nutrients.[44] Bacterial antagonism has also been found in particle-associated isolates, including antibiotic production.[6] We conclude that cell death may be a more important driver for bacterial cross-feeding in natural communities than has been previously recognized.

Materials and methods

Media and Growth Conditions

All experiments with isolates were performed at room temperature or 25 °C incubators in liquid culture using minimal media (MBL, see below). For co-cultures and kinetic experiments, frozen individual environmental isolates were streaked onto marine broth (Marine Broth 2216, Difco) agar plates, and single colonies were inoculated into marine broth liquid medium.

MBL media is a defined marine minimal media adapted from a protocol from the Marine Biological Laboratory Microbial Diversity course. The standard recipe contains the following components: 4x sea salt mix (1L water containing 80 g NaCl, 12 g $MgCl_2 \cdot 6H_2O$, 0.60 g $CaCl_2 \cdot 2H_2O$, 2.0 g KCl); 1000x trace mineral mix (1 L 20 mM HCl containing: 2100 mg $FeSO_4 \cdot 7H_2O$, 30 mg H_3BO_3, 100 mg $MnCl_2 \cdot 4H_2O$, 190 mg $CoCl_2 \cdot 6H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, 2 mg $CuCl_2 \cdot 2H_2O$, 144 mg $ZnSO_4 \cdot 7H_2O$, 36 mg $Na_2MoO_4 \cdot 2H_2O$; 25 mg $NaVO_3$, 25 mg $NaWO_4 \cdot 2H_2O$, 6 mg $Na_2SeO_3 \cdot 5H_2O$; note: $NaVO_3$ and $NaWO_4 \cdot 2H_2O$ solids were handled in the chemical hood); 100x nitrogen source (1 M NH_4Cl); 500x phosphorus source (0.5 M Na_2HPO_4); 1000x sulfur source (1 M Na_2SO_4); 20x HEPES buffer (1 M HEPES sodium salt, pH 8.2), and a carbon source as detailed below. All components were filter sterilized with 0.2 µm filters. Sea salt mix was stored at room temperature; sulfur, nitrogen, carbon, and phosphorus sources were stored in 4 °C; vitamin, trace metals, and HEPES were aliquoted into single use aliquots and frozen at -20 °C.

For the phenotype screen, four carbon sources (glucose, glutamine, glycerol, and pyruvate) were added to the MBL media to support the growth of isolates with different nutrient preferences. Each carbon source was normalized by its number of carbon atoms to achieve a final concentration of 15 mM carbon each (2.5 mM glucose; 3 mM glutamine; 5 mM sodium pyruvate; 5 mM glycerol). For the kinetic growth curves as well as the supernatant/lysate experiments, a single carbon source (glucose for prototroph 1A01, pyruvate for auxotrophs) was used at 30 mM carbon.

Seawater microcosms

Nearshore coastal seawater was collected from Nahant, MA on Oct 21, 2021 and filtered sequentially with 63 µm and 5 µm filters. Hydrogel chitin particles (New England Biolabs, #E8036L) were washed in sterile artificial seawater (Sigma-Aldrich, #S9883) and filtered through 100 µm mesh filters. The flowthrough was then passed through a 40 µm filter, and the particles captured on the filter were resuspended in artificial seawater. The particles were added to 50 mL aliquots of filtered seawater, for a final concentration of approximately 750 particles per tube (15 particles/mL). To half of the tubes, vitamins were added using the vitamin stock from the MBL media (Table 1). The tubes were rotated end over end at room temperature over five days. Every 12 hours, we harvested 3 replicate tubes from each condition and separated the chitin particles and associated bacterial community using a neodymium magnet. The seawater supernatant was removed with a serological pipette, leaving approximately 1 mL seawater and beads. The seawater+beads fraction was transferred to an Eppendorf tube and 0.5 mL of artificial sea salt solution was added. The samples were frozen at -20 °C.

Genomic DNA was extracted from 2 replicate tubes per condition using the Qiagen DNeasy Blood & Tissue Kit. The DNA yield was quantified using the Invitrogen™Quant-iT™ PicoGreen™ dsDNA Assay, with yields ranging between 0.01-0.04 ng/µl. The samples were processed and sequenced by the Microbial Genome Sequencing Center using the Illumina DNA Prep kit and protocol, modified for low concentrations. Paired end sequencing was run on an Illumina NextSeq2000, yielding 2x151bp reads. Raw sequencing reads were quality trimmed with Trimmomatic v0.36[45]. The metagenomes were annotated for taxonomy using phyloFlash[46] and using a custom database of profile hidden Markov models of proteins involved in growth on chitin[41].

Vitamin auxotrophy screen

For a full list of strains, including their taxonomy and isolation details, see Supplementary File 1. Apart from the Vibrionales strains YB2[47] and 1A06, 12B01 and 13B01[48], all strains were isolated from enrichments of coastal seawater on different polysaccharides[3,4,21]. A total of 187 strains were originally double-streaked at the time of isolation and double-streaked again before being arrayed in two 96-well plates and stored in 5% dimethylsulfoxide at −80 °C.

For the phenotypic screen, the arrayed isolate collection was defrosted and inoculated 1:10 into marine broth liquid medium in 1 mL 96-well plates. After 72 hours, the plates were transferred 1:100 into MBL medium. After 24 hours, the culture was transferred 1:40 into standard 96-well plates in two conditions, each in triplicate: standard MBL medium containing vitamins (MBL^{+vit}; see vitamin list in Table 1) and MBL without the addition of vitamins (MBL^{-vit}). Isolates that did not grow consistently upon being transferred to MBL^{+vit} were dropped from the screen, leaving 150 isolates in total.

Every 24 hours, bacterial growth was estimated by measuring the optical density at 600 nm (OD_{600}) using a TecanSpark plate reader with a stacker module. Then the plates were transferred 1:40 into fresh medium (MBL^{+vit} or MBL^{-vit}). Plates were incubated at room temperature without shaking.

The isolates were classified as vitamin auxotrophs using the growth data from the third transfer (72 hours). The median OD_{600} value was calculated for both conditions and the growth deficit calculated as follows: $(MBL^{-vit} - MBL^{+vit})/(MBL^{-vit} + MBL^{+vit})$. A growth deficit value of -0.4 was chosen as the cutoff for putative auxotrophs based on the distribution of values, with 52 isolates passing this threshold (Fig. S2). The putative auxotrophs were then grown individually and assayed for growth with each vitamin separately, with supplementation of single vitamins as well as mixes of all vitamins but one. Each putative auxotroph was tested at least twice and the results compared. 47 of the putative auxotrophs were confirmed, with the remaining five removed as their growth was not consistent.

Genome analysis

We established the genetic basis of vitamin auxotrophies by comparing the results of the phenotypic screen to the isolate genomes. The sequenced genomes[3,4,21] were annotated using eggnog v4.5 (mmseqs mode with default parameters)[49] and dbCAN v2 (diamond mode)[50] for CAZymes. Taxonomy assignment and the creation of phylogenetic trees were performed using

the standard workflow in GTDB-tk[51], followed by renaming of taxa falling into the NCBI clades Vibrionales and Alteromonadales (both assigned Enterobacterales by GTDB-tk) to use the more familiar names for those clades[52]. The eggnog annotations were searched for vitamin biosynthesis genes based on previous studies[13,14,22–25] (Fig. S4, Table S1 and S2).

Growth curves

To determine growth requirements for vitamins, the growth of auxotrophs were measured across vitamin concentrations spanning seven orders of magnitude. A selection of auxotrophs (Fig. 3) for different vitamins were chosen and grown in the same transfer protocol described above in the phenotypic screen, with two key changes. 1. Each auxotroph was initially grown in marine broth and then MBL^{+vit} as specified above, then transferred to eight conditions in total: MBL^{-vit}, and seven ten-fold dilutions of a single vitamin in MBL^{-vit}. The vitamin conditions were present in each transfer. 2. After the third transfer, the OD_{600} was measured continuously in the plate reader for 24-36 hours. The resulting growth curves were visualized and the growth rate calculated for each vitamin concentration from the exponential region using the lm function in R.

Co-culture and supernatant experiments

We grew a selection of eight auxotrophs in co-culture, including two dual auxotrophs. The same general growth protocol was used as described above in the phenotypic screen, with the following modifications. Each auxotroph was initially grown in monoculture to high density in marine broth and then MBL^{+vit}. The monocultures were all normalized to OD_{600} 0.2, then combined in pairs and diluted 1:40 into MBL^{-vit} medium. The co-cultures were grown as described above, with daily 1:40 transfers and OD_{600} measurements. As a control, the same co-cultures were grown in MBL^{+vit} medium.

For the supernatant and lysis experiments, exponentially growing cultures of 1A01 were grown in replicate 4 mL tubes. At the collection timepoints, tubes were collected for both supernatants and lysates. For the supernatants, cells were pelleted by centrifugation (6000g for 10 minutes at room temperature), and the supernatant carefully removed and sterilized by 0.2 μm filtration to remove any remaining cells. The supernatants were immediately frozen at −80 °C. For the lysates, the cultures (containing cells suspended in supernatant) were immediately frozen at −80 °C. On the day of the experiment, all lysate samples were defrosted and lysed with a microtip probe sonicator on ice (Misonix Sonicator 3000, 2 minutes total of sonication at power setting 5, in intervals of 30 seconds on/60 seconds off). The supernatants were also defrosted

on the first day, and both supernatants and lysates were stored at 4 °C throughout the experiment. The experiments were performed using the standard protocol described above. The supernatants and lysates were mixed in a 1:1 ratio with fresh MBL^{-vit} media, with MBL^{+vit} media serving as a control. The cultures were grown as described above, with daily 1:40 transfers and OD_{600} measurements for three days.

Statistical analysis and data visualization

All statistical analyses were done using R v4.0.2[53]. Plots were created using the ggplot2 package v3.3.3[54] and ggtree v2.4.1[55]. Genomes were visualized using clinker [56].

Data availability

Metagenomic reads from seawater microcosms are accessible through NCBI BioProject (accession number PRJNA1028615).

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