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ORIGINAL ARTICLE

Underlying mechanisms for syntrophic metabolism of essential enzyme cofactors in microbial communities

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Many microorganisms are unable to synthesize essential B vitamin-related enzyme cofactors *de novo*. The underlying mechanisms by which such microbes survive in multi-species communities are largely unknown. We previously reported the near-complete genome sequence of two ~18-member unicyanobacterial microbial consortia that maintain stable membership on defined medium lacking vitamins. Here we have used genome analysis and growth studies on isolates derived from the consortia to reconstruct pathways for biogenesis of eight essential cofactors and predict cofactor usage and precursor exchange in these communities. Our analyses revealed that all but the two *Halomonas* and cyanobacterial community members were auxotrophic for at least one cofactor. We also observed a mosaic distribution of salvage routes for a variety of cofactor precursors, including those produced by photolysis. Potentially bidirectional transporters were observed to be preferentially in prototrophs, suggesting a mechanism for controlled precursor release. Furthermore, we found that *Halomonas* sp. do not require cobalamin nor control its synthesis, supporting the hypothesis that they overproduce and export vitamins. Collectively, these observations suggest that the consortia rely on syntrophic metabolism of cofactors as a survival strategy for optimization of metabolic exchange within a shared pool of micronutrients.
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Introduction

Recent improvements in sequencing technologies have made it possible to sequence entire communities and to use genome-enabled approaches for the study of microbial interactions that lead to community-level properties (Song *et al.*, 2015). Because B vitamin-related enzyme cofactors are essential but expensive to produce, their precursors are commonly shared by community members. It is recognized that many microorganisms are unable to synthesize all required cofactors *de novo* and instead salvage select precursors to make them. The genetic basis for this salvage has been defined in many organisms, revealing a great diversity of transporters (Jaehme and Slotboom, 2015a), alternative enzymes and pathways for converting precursors to cofactors, and the tight regulation of precursor salvage and cofactor biosynthesis (Winkler *et al.*, 2002a,b; Nahvi *et al.*, 2004; Ames *et al.*, 2010; Leyn *et al.*, 2016; Suvorova and Rodionov, 2016). New genes involved in cofactor metabolism continue to be discovered, largely due to the increasing availability of whole genome sequences and the ability of comparative genomics and regulon analysis to discover new protein families that participate in this process (Sun *et al.*, 2013; Rodionova *et al.*, 2015).

At present, studies of cofactor precursor exchange in natural communities have been limited to surveys of biosynthetic and salvage potential in metagenomes (Magnusdottir *et al.*, 2015) where the sources of precursors are not defined, or in assays of binary cultures where the number of precursors exchanged are minimal (Croft *et al.*, 2005; Kazamia *et al.*, 2012; Grant *et al.*, 2014). Here we focused instead on two nearly identical consortia derived from benthic microbial mats that occur in a heliothermal saline (epsomite, $MgSO_4$) lake in northern Washington State (Lindemann *et al.*, 2013; Zachara *et al.*, 2016). These unicyanobacterial consortia (UCC) consist of a single, but distinct, cyanobacterium (*Phormidesmis priestleyi* ANA in UCC-A versus *Phormidium* sp. OSCR in UCC-O) and its associated heterotrophic cohorts (Cole *et al.*, 2014) (Supplementary Table S1).

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Although the abundances of the heterotrophs relative to the cyanobacterium differ between the consortia, they each harbor the same 16 heterotrophs. A single additional heterotroph, *Porphyrobacter* sp. HL-46, was detected in only UCC-A (Nelson *et al*., 2016). Complete or near-complete genome sequences are available for most members enabling prediction of producers of cofactors and/or consumers of cofactor precursors. Importantly, no vitamin supplements were added to the growth medium during enrichment or subsequent cultivation. Hence, these consortia are dependent entirely on endogenous production of cofactors.

Here we used the subsystems approach and comparative genomics analysis to reconstruct vitamin cofactor biosynthetic pathways and predict transport capabilities in the 19 organisms that comprise these consortia. This approach, supported by supplementary growth studies on heterotrophic isolates, revealed an extensive cofactor auxotrophy among members of these consortia and differentiation in the type of precursors salvaged and the complement of cofactor-dependent pathways present. In addition, we identified potential transporters and regulators involved in precursor salvage. Insights gained from this study support the concept of syntrophic partnership based on vitamin exchange and suggest mechanisms by which this is achieved.

Materials and methods

Growth studies on UCC heterotrophic isolates

Hot Lake strains were routinely cultured at 30 °C on Hot Lake Heterotroph broth, pH 8.0 (Cole *et al*., 2014). For testing vitamin-dependent growth, the cells were diluted to an OD_{600} of ~0.2 and inoculated into HL base medium (Hot Lake Heterotroph without yeast extract) supplemented with 5 mM carbon source and 1× Wolfe's vitamin mix (Wolin *et al*., 1963) or selected vitamins (0.74 nM cyanocobalamin, 0.015 μM thiamine and/or 0.082 μM biotin). When cultures reached mid-log phase, the cells were washed three times with HL base medium to remove residual metabolites and growth supplements. Ten replicate samples were evaluated every 1–2 h for all assays. For *Erythrobacter* HL-111, which grows slowly, the OD_{600} of replicated 20 ml cultures was measured using a SmartSpec Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA). For the remaining strains, a Norden Lab Professional-Bioscreen, C Edition was used to analyze growth (OD_{600}) of 250 μl samples in the 100-well Bioscreen plate.

Consortial composition

A combination of the species-resolved metagenome bins or isolate genome sequences (Nelson *et al*., 2016) from two unicyanobacterial consortia (UCC-A and UCC-O whose cyanobacterial members are designated *Phormidium priestleyi* ANA and *Phormidesmis* OSCR, respectively) were evaluated for the presence or absence of pathways for *de novo* biosynthesis of B vitamin-related enzyme cofactors. In addition to the cyanobacterial members, the closed genome sequences derived from 10 axenic cultures (*Algoriphagus marincola* str. HL-49, *Aliidiomarina* sp. HL-53, *Roseibaca calidilacus* HL-91, *Halomonas* sp. HL-48 and HL-93, *Marinobacter excellens* str. HL-55, *Marinobacter* sp. HL-58, *Erythrobacter* sp. HL-111, *Salinivirga fredricksonii* HL-109 and *Porphyrobacter* sp. HL-46) and binned assemblies from nine additional members (*Bacteroidetes* bin01, *Oceanicaulis* bin04 and *Rhodobacteraceae* bins 7, 8, 9, 12 and 18) were examined. Based on occurrence of conserved single-copy genes, it was estimated that the binned assemblies have a genomic coverage of at least 98% with the exception of one member, *Rhodobacteraceae* bin09, whose estimated coverage is 87.6% (Nelson *et al*., 2016).

Genomic reconstruction of metabolic pathways and regulons

Genome-based reconstruction of eight B vitamin-related cofactor biosynthesis pathways in 19 UCC genomes was performed using the subsystem-based comparative genomic approach (Osterman *et al*., 2010) implemented in SEED/RAST (Aziz *et al*., 2008; Overbeek *et al*., 2014; Brettin *et al*., 2015) combined with genomic reconstruction of vitamin-specific transcriptional regulons and identification of candidate vitamin transporters as previously described (Rodionov *et al*., 2009; Rodionova *et al*., 2015). Extensive manual pathway curation was assisted by KEGG orthology assignments from the BlastKoala annotation tool (Kanehisa *et al*., 2016). Identification of orthologs in closely related genomes and their genome neighborhood analysis were performed in the IMG workspace (Markowitz *et al*., 2014). Cofactor requirements were asserted by cataloging respective enzymes from annotated genomes and connecting them to metabolic pathways in KEGG (Kanehisa *et al*., 2016) and SEED (Overbeek *et al*., 2014). Computational searches of B_{12}, FMN and thiamine pyrophosphate (TPP) riboswitches were performed using covariance models from the Rfam database as recently reviewed in Sun and Rodionov (2014). The BirA, NrtR, NadQ regulons were reconstructed by the consistency check approach using the positional weight matrices of DNA motifs from the RegPrecise database as previously described (Leyn *et al*., 2016). A full list of reconstructed metabolic pathways and regulons is available in the Supplementary Data 1.

Genome sequence accession numbers

All sequence data were generated by the Joint Genome Institute. Assemblies derived from metagenome bins and from isolate genome sequence can

be accessed via IMGer (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi). In addition, binned metagenome assemblies have been deposited in the European Nucleotide Archive (ENA; www.ebi.ac.uk/ena) and Genbank under accession numbers LIHN00000000 (*Bacteroidetes* Bin01), LJSH00000000 (*Oceanicaulis* Bin04), LJSU00000000 (*Rhodobacterales* Bin07), LJSF00000000 (*Rhodobacterales* Bin08), LJNT00000000 (*Rhodobacterales* Bin09), LJZR00000000 (*P. priestleyi* ANA), LJSV00000000 (*Rhodobacterales* Bin12), LJZT00000000 (*Phormidum* OSCR) and LJSY00000000 (*Rhodobacterales* Bin18). Isolate genomes have been deposited under accession numbers GCA_001458075.1 (*Aliidiomarina* sp. HL-53), JMLY00000000 (*Marinobacter* sp. HL-58), GCA_001517585.1 (*R. calidilacus* sp. HL-91), NZ_JAFX00000000 (*A. marinocola* sp. HL-49), JMMC00000000 (*Halomonas* sp. HL-48), JYNR00000000 (*M. excellens* sp. HL-55), GCA_000744895.1 (*Porphyrobacter* sp. HL-46), GCA_001314745.1 (*Halomonas* sp. HL-93), GCA_001314785.1 (*Salinivirga fredricksonii* HL-109, formerly *Rhizobiales* sp. HL--109) and GCA_001314765.1 (*Erythrobacter* sp. HL-111).

Results

Composition of UCC-A and UCC-O

The unicyanobacterial consortia examined in this study are low complexity enrichments resulting from attempts to develop axenic cultures of cyanobacteria from mats found in Hot Lake Washington. The firm attachment of heterotrophs to the cyanobacteria have thus far made it impossible to isolate the autotroph, but resulted in communities that are expected to be enriched in interacting microbes because the heterotrophs cannot fix CO_2 that was provided as the sole carbon source during enrichment. Metagenomic sequence was derived from two unicyanobacterial consortia (UCC-A and UCC-O), assembled and binned into 19-member sequences (Nelson *et al*., 2016). Because the two consortia were nearly identical in taxonomic composition (16 heterotrophs were common to both consortia), co-assembly of sequencing reads lead to near-complete assembly of nearly all members of the consortia (the abundance of a 20th *Alphaproteobacterial* member was too low to produce sufficient genomic coverage to be included in this study). Further improvement of member assemblies resulted from sequencing the genomes of axenic cultures of heterotrophs derived from the consortia. In addition to the six genomic sequences from heterotrophic members of the consortia (*Porphyrobacter* sp. HL-46, *Halomonas* sp. HL-48, *Algoriphagus marincola* HL-49, *Aliidiomarinacea* HL-53, *Marinobacter excellens* HL-55 and *Marinobacter* sp. HL-58) described previously, this study includes genomic sequence from four new UCC isolates, *Roseibaca calidilacus* HL-91, *Halomonas* sp. HL-93, *Erythrobacter* sp. HL-111 and *Salinivirga fredricksonii* HL-109, bringing the total number of members with closed genomes to 10. In summary, each community is composed of a single cyanobacterium belonging to the Oscillatoriales order, two *Bacteroidetes*, five *Gammaproteobacteria* and ~10 *Alphaproteobacteria* (see Supplementary Table S1).

Exchange of cofactor precursors is necessary to support essential metabolic processes

We used a subsystems-based genomic reconstruction approach to infer biosynthesis, recycling and salvage pathways for eight B vitamin-related enzyme cofactors in the 19 members of UCC-A and UCC-O. Our analysis revealed that all members are able to synthesize PLP (pyridoxal phosphate; Supplementary Figure S1) and flavin nucleotides (Supplementary Figure S2), but only the cyanobacteria and both *Halomonas* sp. can produce the remaining six cofactors (summarized in Table 1). Four of the *Rhodobacteraceae* (bins 7, 9, 12 and 18) belonging to the *Roseobacter* group are auxotrophic for six cofactors, and the fifth (bin08) is only able to synthesize CoA *de novo*, making this group the most dependent on cofactor exchange within the consortia. By contrast, the *Gammaproteobacteria* require precursors to produce no more than two cofactors, cobalamin and/or TPP, each.

The consortia consist of 10 TPP, 11 biotin and 13 cobalamin auxotrophs, making their precursors the most widely sought after. Analysis of cofactor-dependent processes confirmed that TPP is essential for central carbon metabolism of all species in the two consortia (Supplementary Data 1 and Figure 1). Critical TPP-dependent enzymes found in all members include transketolase, a pyruvate oxidase, a TCA enzyme that degrades 2-oxogluatrate and 1-deoxy-D-xylulose-5-phosphate synthase, which produces a key metabolite needed for thiamine, pyridoxal phosphate and terpenoid biosynthesis. A single biotin-dependent enzyme, acetyl-CoA carboxylase, which is essential for the initiation of fatty acid biosynthesis, is present in all isolates.

Cobalamin is required for essential processes such as methionine biosynthesis. However, it is not universally required due to mechanisms that bypass the need for it (dashed lines, Figure 1). Examples include the use of alternative enzymes (e.g., MetE to synthesize methionine) and pathways (e.g., methyl citrate pathway to degrade propionyl-CoA) that do not require cofactors or transporters that import metabolites that would otherwise require a cofactor for synthesis (e.g., MetT or MetNIQ for methionine). The *Gammaproteobacterial* members use the most cobalamin-independent pathways, which is surprising as the two *Halomonas* spp. are among five of the only members that can synthesize this metabolically expensive cofactor. Unlike *S. fredricksonii* HL-109, the *Halomonas* spp. do not control cobalamin synthesis with a riboswitch (Supplementary Data 2

Table 1 Overview of predicted cofactor biosynthesis capability

	Est. completeness[a]	*Associated vitamin group* *Cofactor*							
		B_1 *TPP*	B_2 *FAD FMN*	B_3 *NAD*	B_5 *CoA*	B_6 *PLP*	B_7 *Biotin*	B_9 *THF*	B_{12} *Cobinamide*
Cyanobacteria									
Phormidesmis priestleyi ANA	99%	Pro+	Pro	Pro+	Pro	Pro	Pro+	Pro+	Pro
Phormidium OSCR	99%	Pro	Pro	Pro+	Pro	Pro	Pro+	Pro+	Pro+
Bacteroidetes									
Bacteroidetes sp. bin01	**99%**	**Aux**	Pro	Pro	Pro+	Pro	Pro+	Pro	**Aux**
Algoriphagus marincola HL-49	**>99%**	**Aux**	Pro	Pro+	Pro	Pro	**Aux**	Pro	**Aux**
Gammaproteobacteria									
Aliidiomarina sp. HL-53	**>99%**	**Aux**	Pro	Pro	Pro+	Pro	Pro+	Pro	—
Halomonas sp. HL-48	>99%	Pro+	Pro	Pro+	Pro+	Pro	Pro+	Pro	Pro+
Halomonas sp. HL-93	>99%	Pro+	Pro	Pro+	Pro+	Pro	Pro+	Pro	Pro+
Marinobacter excellens HL-55	>99%	Pro+	Pro	Pro	Pro	Pro	Pro+	Pro	**Aux**
Marinobacter sp. HL-58	>99%	Pro+	Pro	Pro+	Pro	Pro	Pro+	Pro	**Aux**
Alphaproteobacteria									
Erythrobacter sp. HL-111	>99%	Pro	Pro	Pro	Pro	Pro	**Aux**[b]	Pro	**Aux**
Porphyrobacter sp. HL-46	>99%	Pro	Pro	Pro	Pro	Pro	**Aux**[b]	Pro	**Aux**
Oceanicaulis bin04	99%	Pro	Pro	Pro	Pro	Pro+	**Aux**[b]	Pro	**Aux**
Salinivirga fredricksonii HL-109	**>99%**	**Aux**[c]	Pro	**Aux**	Pro	Pro+	**Aux**	**Aux**	Pro+
Roseibaca calidilacus HL-91	**>99%**	**Aux**	Pro	Pro+	Pro	Pro	**Aux**	Pro	**Aux**
Rhodobacteraceae bin12	**>99%**	**Aux**	Pro	**Aux**	**Aux**	Pro	**Aux**	**Aux**[d]	**Aux**
Rhodobacteraceae bin07	**>99%**	**Aux**	Pro	**Aux**	**Aux**	Pro	**Aux**	**Aux**[d]	**Aux**
Rhodobacteraceae bin08	**>99%**	**Aux**	Pro	**Aux**	Pro	Pro	**Aux**	**Aux**[d]	**Aux**
Rhodobacteraceae bin09	**88%**	**Aux**	Pro	**Aux**	**Aux**	Pro	**Aux**	**Aux**[d]	**Aux**
Rhodobacteraceae bin18	**>99%**	**Aux**	Pro	**Aux**	**Aux**	Pro+	**Aux**	**Aux**[d]	**Aux**

Abbreviations: Aux, auxotroph; CoA, coenzyme A; PLP, pyridoxal phosphate; Pro, prototroph; Pro+, prototroph with salvage capability; THF, tetrahydrofolate; TPP, thiamine pyrophosphate. Auxotrophies are indicated in bold.
[a]Estimated by presence/absence of 100 conserved single-copy genes (Nelson, *et al*., 2016). Organisms for which a sequenced isolate genome (improved draft) is available are listed as >99%. Metagenomic bins that contain all 100 marker genes are also listed as >99%.
[b]Can make biotin from dethiobiotin.
[c]Predicted to salvage HMP (hydroxymethylpyrimidine) and thiazole rather than thiamine.
[d]Can make folate if provided *p*ABA (*para*-aminobenzoic acid).

and Supplementary Note 1), and therefore have the potential to make excess cofactor and be important providers of B_{12} for the community.

Exchange of canonical B vitamins can complement most, but not all, deficiencies in de novo biosynthesis of respective cofactors

Although, by definition, B vitamins include a variety of different cofactor precursors (Supplementary Table S2), the chemicals included in the conventional medium supplement, Wolfe's vitamin solution (Wolin *et al*., 1963) are the standard growth supplements used to cultivate microbes and more widely assumed to be exchanged in microbial communities. Here we found, however, that pantothenate, nicotinic acid, folic acid, *para*-aminobenzoic acid (*p*ABA), and cyanocobalamin are the only standard vitamin additives that can complement UCC member deficiencies in cofactor biosynthesis.

Pantothenate, a precursor to coenzyme A (CoA), is synthesized *de novo* through the actions of (i) PanB and PanE (or IlvC), which convert 3-methyl-2-oxobutanoate to pantoate and (ii) PanC, which ligates β-alanine to pantoate to form pantothenate (Figure 2). All UCC members can convert pantothenate to CoA, but four *Rhodobacteraceae* cannot synthesize pantothenate and thus are expected to be auxotrophs even though no pantothenate transporter could be identified. An aspartate carboxylase, PanD or PanP, that is needed for the synthesis of β-alanine from aspartate is present in all members except *Phormidesmis* ANA, *R. calidilacus* HL-91, *Erythrobacter* HL-111, *Porphyobacter* HL-46 and *S. fredricksonii* HL-109. As HL-91 and HL-111 can grow in defined medium lacking CoA precursors (Supplementary Figure S7), we hypothesize that an alternative aspartate carboxylase or novel pathway to synthesize β-alanine exists.

Six heterotrophs (*S. fredricksonii* HL-109, *Rhodobacteraceae* bins 7, 8, 9, 12 and 18) are unable to synthesize NAD(P) from aspartate or tryptophan (Supplementary Figure S3) and the same six cannot produce tetrahydrofolate (Supplementary Figure S4). The presence of the nicotinate phosphoribosyltransferase (PncB and PncB2) in all auxotrophs and six

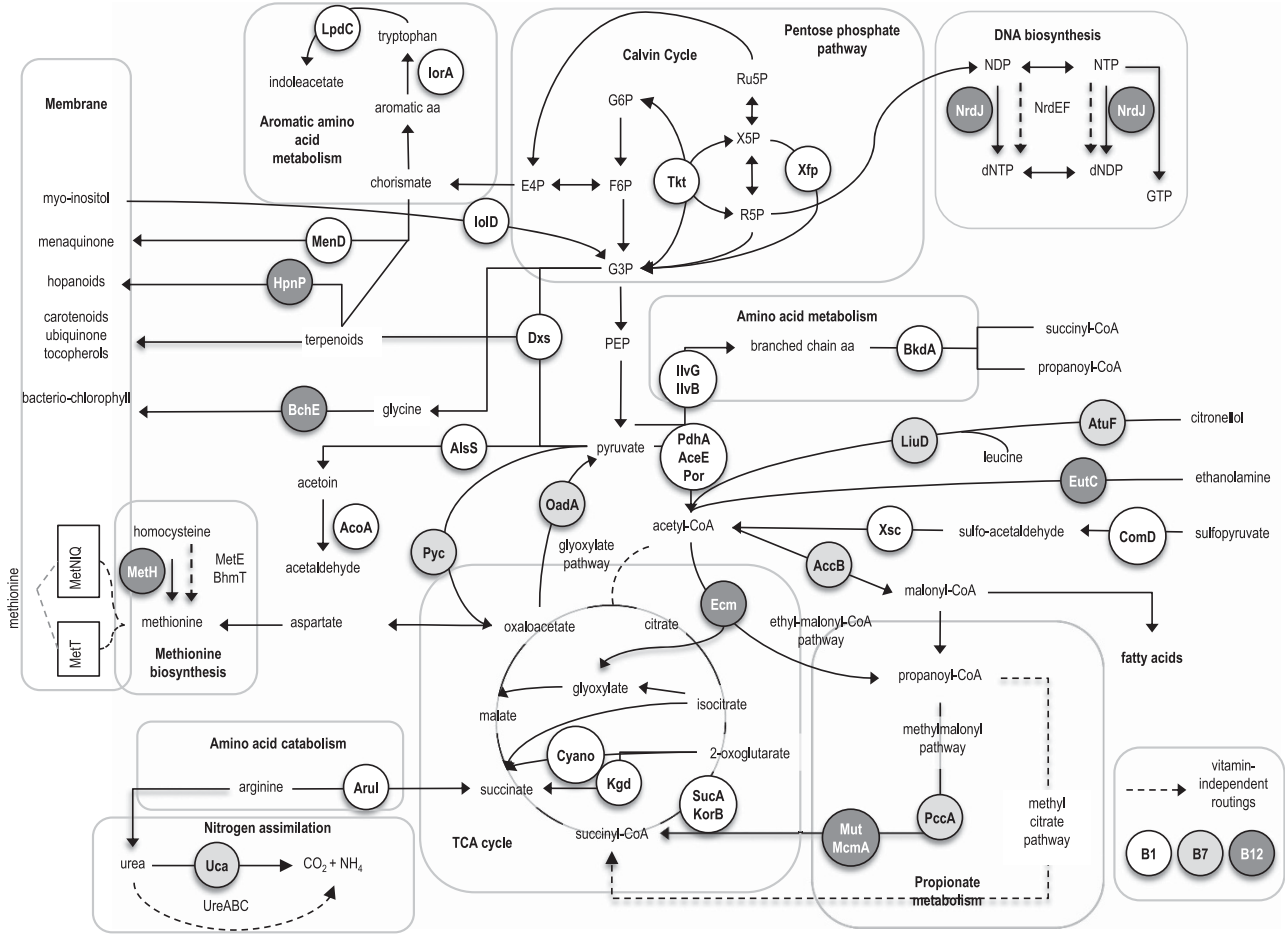

Figure 1 Overview of TPP, biotin and B_{12}-dependent processes. TPP-dependent enzymes: AceE, pyruvate dehydrogenase E1 component; AcoA, acetoin dehydrogenase complex E1 component alpha subunit; AlsS, acetolactate synthase; AruI, 2-ketoarginine decarboxylase; BkdA, 2-oxoisovalerate dehydrogenase E1 component alpha subuit; ComD, sulfopyruvate decarboxylase alpha subunit; Cyano, 2-oxoglutarate dehydrogenase; Dxs, 1-deoxy-d-xylulose-5-phosphate synthase; IlvB, biosynthetic acetolactate synthase I catalytic subunit; IlvG, biosynthetic acetolactate synthase II catalytic subunit; IolD, decyclizing 3D-(3,5/4)-trihydroxycyclohexane-1,2-dione acylhydrolase; IorA, ferredoxin oxidoreductase alpha subunit; Kgd, 2-oxoglutarate decarboxylase; KorB, 2-oxoglutarate ferredoxin oxidoreductase beta subunit; LpdC, indolepyruvate decarboxylase; MenD, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid synthase; SucA, 2-oxoglutarate dehydrogenase E1 component; Tkt, transketolase; Xfp, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase; and Xsc, sulfoacetaldehyde acetyltransferase. Biotin-dependent enzymes: AccB, acetyl-CoA carboxylase biotin carboxyl carrier protein; AtuF, geranyl-CoA carboxylase alpha subunit; LiuD, 3-methylcrotonyl-CoA carboxylase alpha subunit; OadA, oxaloacetate decarboxylase alpha subunit; PccA, propionyl-CoA carboxylase alpha subunit; Pyc, pyruvate carboxylase; and Uca, urea carboxylase. B_{12}-dependent enzymes: BchE, magnesium-protoporphyrin IX monomethyl ester cyclase; Ecm, (2R)-ethylmalonyl-CoA mutase; EutC, adenosylcobalamin-dependent ethanolamine ammonia-lyase small subunit; HpnP, hopanoid 2-methyltransferase; McmA, methylmalonyl-CoA mutase B_{12}-binding subunit; MetH, methionine synthase; Mut, methylmalonyl-CoA mutase; and NrdJ, ribonucleoside diphosphate reductase. Enzymes and transporters that obviate the need for cofactors: BhmT, betaine–homocysteine methyltransferase; MetE, methionine synthase; MetNIQ, ABC-type methionine transporter; MetT, methionine:cation antiporter; NrdEF, ribonucleoside diphosphate reductase; UreABC, ABC-type urea transporter. Additional enzymes without precise functions predictions are listed in Supplementary Data 1.

prototrophs suggest that nicotinic acid is salvaged, even though no candidate nicotinic acid transporters were identified. *S. fredricksonii* HL-109 is missing the entire pathway while five *Rhodobacteraceae* (bins 7, 8, 9, 12 and 18) lack only the ability to synthesize the precursor *p*ABA. These five members encode neither the enzymes with *p*ABA synthetase or 4-amino-4-deoxychorismate lyase activity, which are needed to convert chorismate to *p*ABA nor the recently described alternative *p*ABA-biosynthetic enzyme (Adams *et al.*, 2014; Satoh *et al.*, 2014). No candidate folate or *p*ABA transporters were detected in respective auxotrophs, but four prototrophs (*Phormidium* OSCR, *Oceanicaulis* Bin04, *Aliidiomarina* HL-53 and *Bacteroidetes* Bin01) encode AbgT family transporters, which are proposed to export *p*ABA (Delmar and Yu, 2016), suggesting that they share this resource.

A diverse set of precursors can support cobalamin auxotrophs

Although cyanocobalamin is expected to support the growth of all the UCC cobalamin auxotrophs, the

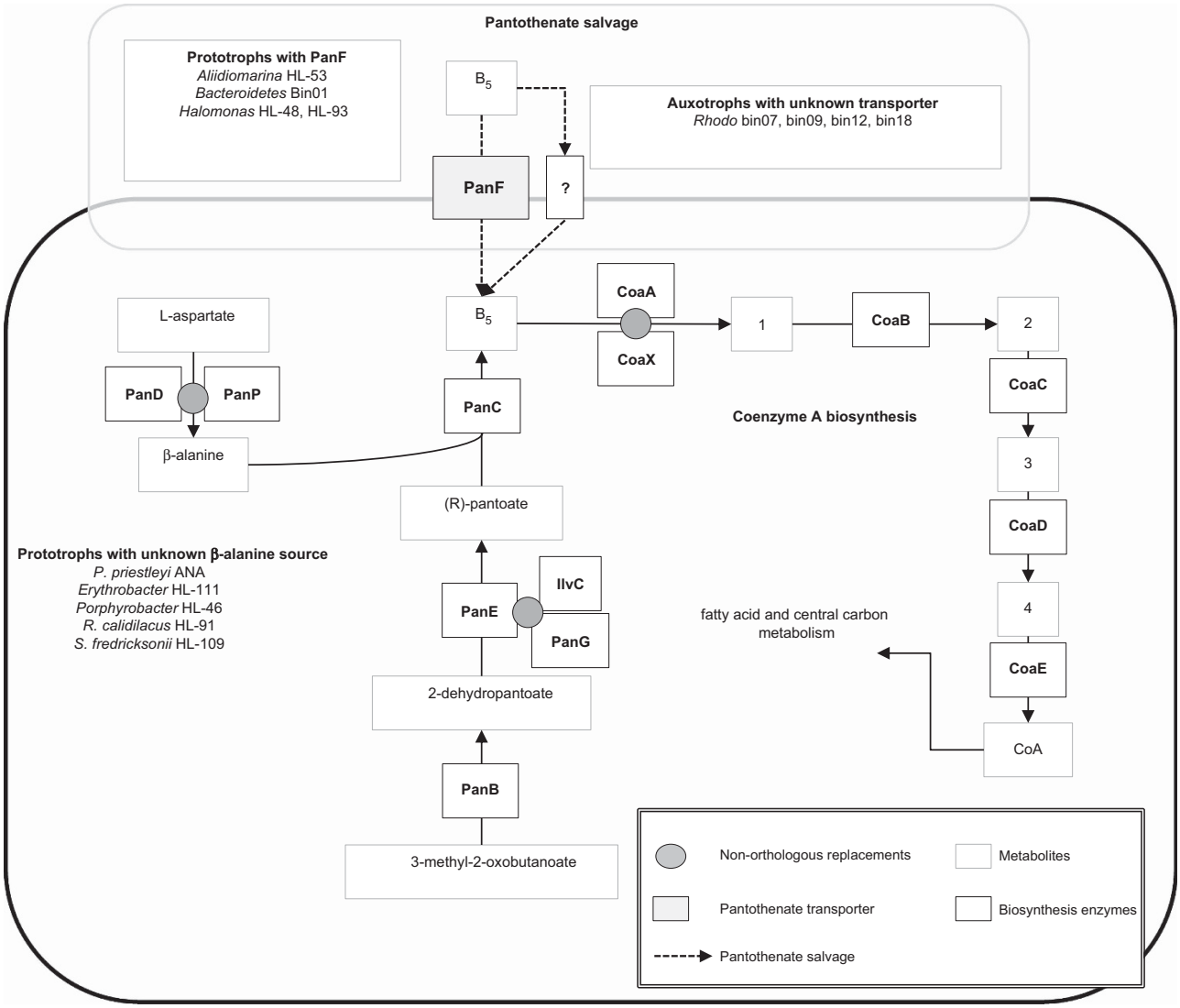

Figure 2 Coenzyme A biosynthesis and salvage of precursors. Metabolites that occur in the pathway for coenzyme A biosynthesis appear in open gray rectangles: 1, D-4′-phosphopantothenate; 2, (R)-4-phosophopantothenyoyl-L-cysteine; 3, pantetheine 4-phosphate; 4, dephospho-CoA; 5, N-carbomoyl-β-alanine; and CoA, coenzyme A. Enzymes appear in open black rectangles: CoaA, type I pantothenate kinase; CoaB, phosphopantothenate-cysteine ligase; CoaC, phosphopantothenoylcysteine decarboxylase; CoaD, pantetheine-phosphate adenylyltransferase; CoaE, dephospho-CoA kinase; CoaX, type III pantothenate kinase; IlvC, ketol-acid reductoisomerase; PanB, 3-methyl-2-oxobutanoate hydroxymethyltransferase; PanC, pantoate-β-alanine ligase; PanD, pyruvoyl-dependent aspartate 1-decarboxylase; PanE, 2-dehydropantoate 2-reductase; PanG, ketopantoate reductase; PanP, PLP-dependent aspartate 1-decarboxylase; PreA, NAD-dependent dihydropyrimidine dehydrogenase subunit; PreT, NAD-dependent dihydropyrimidine dehydrogenase subunit; PydB, dihydropyriminidase; PydC, beta-ureidopropionase. PanF refers to the Na+/pantothenate symporter and the boxed question mark to unknown transporters. Non-orthologous replacements are indicated by filled gray circles. Dashed arrows indicate precursor salvage routes for coenzyme A precursor uptake.

presence of partial biosynthetic pathways in some members suggests that additional precursors might also be salvaged. The biosynthesis of B_{12} family cofactors is initiated by synthesis of the corrin ring from uroporphyrinogen III via either an oxygen-dependent (Warren *et al.*, 2002; Heldt *et al.*, 2005) or oxygen-sensitive pathway (Warren *et al.*, 2002; Roessner and Scott, 2006; Supplementary Figure S5). Only five members (both cyanobacteria, both *Halomonas* spp. and *S. fredricksonii* HL-109) possess either of these pathways and hence synthesize cobalamin *de novo*. The second phase of B_{12} family cofactor biosynthesis involves attachment of the upper α-axial component, which is derived from aminopropanol and either 5′-deoxyadensine or a methyl group, and a lower β-axial component typically composed of a cobalt-coordinated nucleotide with DMB (5,6-dimethylbenzimidazole) as the base (Gray and Escalante-Semerena, 2007; Supplementary Figure S6). The attachment of different combinations of ligands in the upper and lower positions results in the formation of distinct cofactors (e.g., pseudo-B_{12}, methylcobalamin, adenosylcobalamin and hydroxocobalamine; Giedyk *et al.*, 2015).

Both cyanobacteria genomes encode corrin ring biosynthetic genes, but lack *cobT* that is necessary for attachment of DMB as the lower ligand and, therefore, likely produce alternative coenzymes (Supplementary Figure S6) such as adenylylcobamide (pseudo-B_{12}), which has been reported as the predominant form of B_{12} coenzyme in other cyanobacteria (Watanabe, 2007; Tanioka *et al*., 2009). Six auxotrophs (*Bacteroidetes* bin01, *A. marincola* HL-49, *M. excellens* HL-55, *Porphyrobacter* HL-46, *Oceanicaulis* bin04 and *Rhodobacteraceae* bin08) lack all biosynthetic genes and thus require an external source of cobalamin that cannot originate in the cyanobacteria. With the exception of *Bacterioidetes* bin01, the remaining auxotrophs encode partial pathways for biosynthesis of adenosylcobalamin, the TonB-dependent receptor (BtuB), and the ABC-type inner membrane translocase (BtuCDF), which are required for the uptake of cobalamin and related precursor corrinoids (Woodson *et al*., 2005; Degnan *et al*., 2014). The missing ABC transporter genes in *Bacteroidetes* bin01 are presumably in a sequencing gap. Interestingly, all three of the heterotrophic cobalamin producers lack the high affinity BtuB outer membrane receptor. Based on the gene complement present in these members, we predict that they salvage one or more of the precursors hydrogenobyrinic acid a,c-diamide, cobyrinic acid, cobyrate acid and cobinamide-guanosine diphosphate and consequently, some community members may benefit from more precursors than others (Figure 3).

S. fredricksonii HL-109 and Rhodobacteraceae bin09 are TPP auxotrophs that cannot salvage thiamine

The *de novo* biosynthesis of TPP involves the formation of phosphorylated hydroxyethylthiazole (HET) and hydroxymethylpyrimidine (HMP) intermediates, which are first joined by ThiE (thiamine-phosphate pyrophosphorylase) and then phosphorylated by ThiL (thiamine monophosphate kinase; Du *et al*., 2011; Figure 4). Only six members (each of the cyanobacteria, *Halomonas* spp. and *Marinobacter* spp.) have all the genes necessary for TPP biosynthesis. *Erythrobacter* HL-111, *Porphyrobacter* HL-46 and *Oceanicaulis* bin04 lack known enzymes for synthesis of the iminoglycine precursor. However, HL-111 can grow in defined medium lacking thiamine (Supplementary Figure S7), which suggests that an alternative iminoglycine biosynthesis pathway exists and that all three organisms are also prototrophs.

Two main salvage strategies are used by our consortia to produce TPP (Figure 5). Nine members of the consortia use the most commonly recognized strategy, which involves uptake of thiamine followed by its phosphorylation to TPP. Five different thiamine transporters were detected including ThiV2, which was discovered through our analysis (Supplementary Note 2). Five auxotrophs encode a partial TPP biosynthetic pathway (ThiD, ThiM, ThiE and ThiL), which can be used to convert salvaged HET and HMP into TPP. Two transporters were detected in auxotroph genomes; ThiXYZ that had been previously associated with HMP transport and a TRAP-type transporter that we predict to be used for HET uptake (Supplementary Note 2). The ThiV and/or TRAP transporters were also found in several TPP prototrophs, suggesting that they can reduce the cost of biosynthesis by salvaging HET and/or HMP. Salvage of both precursors is the only option for generating TPP by *S. fredricksonii* HL-109 and *Rhodobacteraceae* bin09 and thus Wolfe's vitamin solution should not support growth of these organisms.

Non-canonical vitamins can be salvaged for biotin, NAD(P) and PLP biosynthesis

Over half of the community members are biotin auxotrophs (Table 1 and Supplementary Figure S8), but all are predicted to salvage biotin or a precursor. Biotin is salvaged by either the the YigM transporter or the BioY ECF-type transporter, which can function even in the absence of the auxiliary components BioMN (Finkenwirth *et al*., 2013). Three auxotrophs lack YigM and BioY but encode the biotin synthase (BioB), which catalyzes the last step in biosynthesis. Thus, we predict that dethiobiotin is a precursor that is available for exchange in communities and that transporters other than BioY or YigM mediate its uptake.

All six NAD(P) auxotrophs (*S. fredricksonii* HL-109, *R. calidilacus* HL-91 and *Rhodobacteraceae* bins 7, 8, 9, 12 and 18) and five of the prototrophs (both cynobacteria, *A. marincola* HL-49 and both *Halomonas* spp.) encode nicotinamidase (PncA) and, therefore, are expected to salvage nicotinamide in addition to nicotinic acid. *A. marincola* HL-49 encodes the PnuC transporter and NadR kinase, which is required for the salvage of nicotinamide riboside (Supplementary Figure S3). It is recognized that pyridoxine kinase (PdxK) can convert pyridoxine, pyridoxal or pyridoxamine into PLP and, therefore, the three prototrophs that have PdxK can potentially salvage any of these three precursors (Supplementary Figure S1).

Biased distribution of one-component transporters among prototrophs and auxotrophs

We observed a consistent trend in the presence of one-component transporters in cofactor prototroph genomes and not in auxotrophs, which suggests that prototrophs might conditionally export substrates (Figure 6, Supplementary Data 4). YigM, which was found in all six of the heterotrophic biotin prototrophs, has been demonstrated to take up biotin (Ringlstetter, 2010; Finkenwirth *et al*., 2013) yet is classified as a DME family transporter (TC: 2.A.7.3; Saier *et al*., 2014). Most members of this transporter family are implicated in export functions, supporting

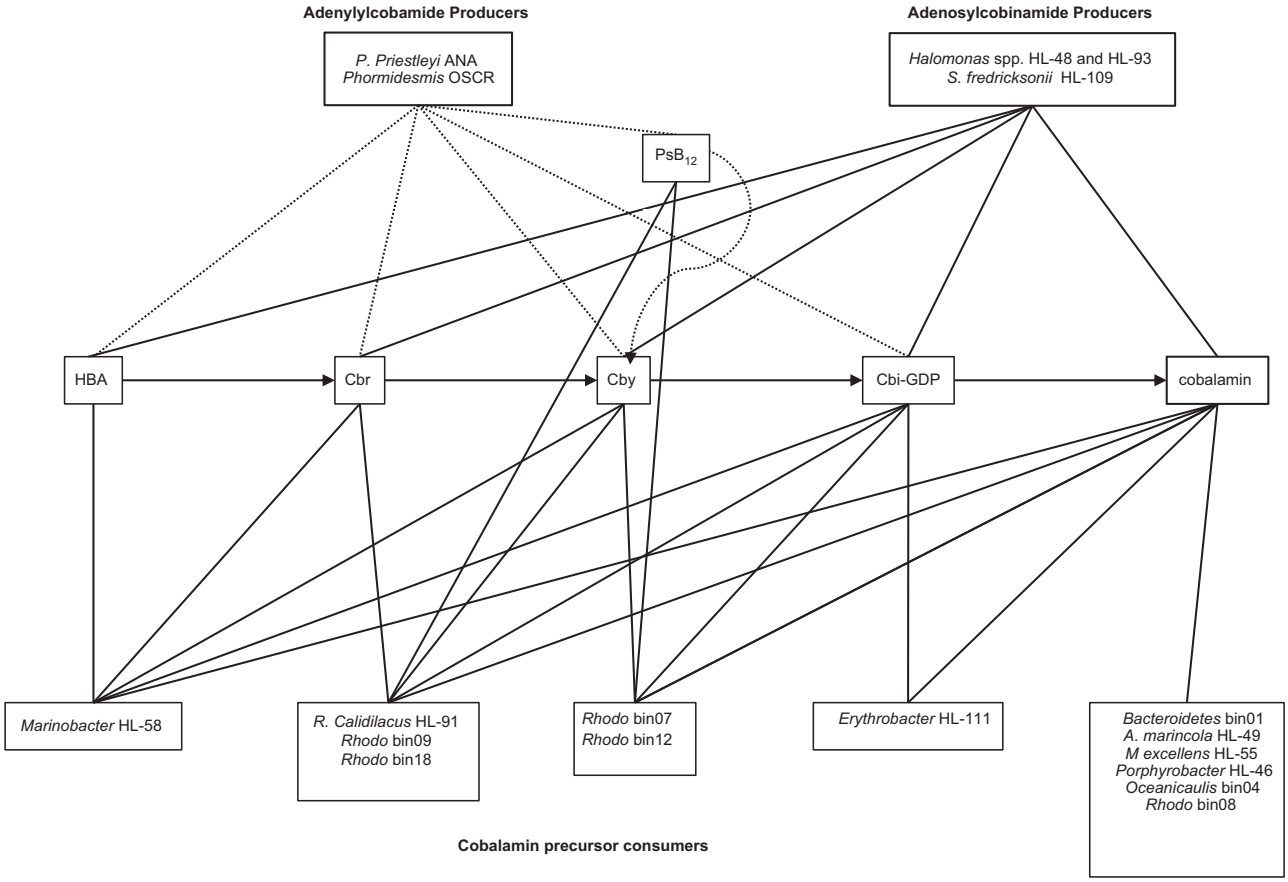

Figure 3 Model of potential cobalamin precursor exchange among UCC community members. The two types of B_{12} producers (adenylylcobamide versus adenosylcobinamide) are shown at the top and the auxotrophs are shown at the bottom. Intermediates that are predicted to be salvaged are shown in the middle; HBA, hydrogenobyrinic acid; Cbr, cobyrinate a,c-diamide; Cby, cobyrate; Cbi-GDP, cobinamide-guanosine diphosphate. Arrows between intermediates depict the order (earliest to latest from left to right) in which they enter the cobalamin biosynthetic pathway. Pseudo-B_{12} (PsB_{12}) has an adenine rather than a 5,6-dimethylbenzimidazole (DMB) at the alpha-axial ligand position. Note that auxotrophs at the far bottom right are unable to make use of precursors produced by the cyanobacteria.

the notion that this transporter operates in both directions.

Four transporters are classified as Na^+ or H^+:solute symporters and thus translocation directionality is likely controlled by pH and ionic strength. Three transporters (ThiV, ThiV2 and PanF) belong to the Na^+:solute symporter family (TC: 2.A.21) and the fourth, Fbt, belongs to the folate-biopterin transporter family (TC: 2.A.71). Among these four, ThiV2 is the only one not found exclusively in prototrophs. Two additional one-component transporters, PnuC and PnuT, catalyze transport by facilitated diffusion. Their substrates are phosphorylated by a kinase after uptake, thereby trapping them inside the cell (Jaehme and Slotboom, 2015b). Transport directionality control would, therefore, be impacted by the energy status of the host. PnuC is found in the NAD prototroph, *A. marincola* HL-49, and PnuT is found in a single auxotroph, *Aliidiomarina* sp. HL-53. Taking into account other microorganisms that encode PnuC and PnuT, it is evident that their distribution is uneven, but most occur in prototrophs.

Discussion

Our results demonstrate, for the first time, how a self-sustaining defined microbial community can retain members that have numerous essential requirements for B vitamin-related enzyme cofactor precursors (Figure 6). The analysis of member biosynthetic capabilities revealed that many of the auxotrophs encode partial biosynthetic pathways that initiate with metabolites that are precursors of canonical vitamins rather than the vitamins themselves. Diversification and specialization of precursor salvage provides a mechanism for division of labor in microbial communities and a selective advantage for partners uniquely suited for optimal syntrophic biosynthesis of cofactors (see Fredrickson, 2015). These findings also have important implications for the cultivation of microbes from environmental samples, where the naive assumption is that the metabolites in Wolfe's supplement are sufficient to support cofactor biosynthesis deficiency or to enable a desired community member to outcompete its neighbors. Although alternate salvage systems have

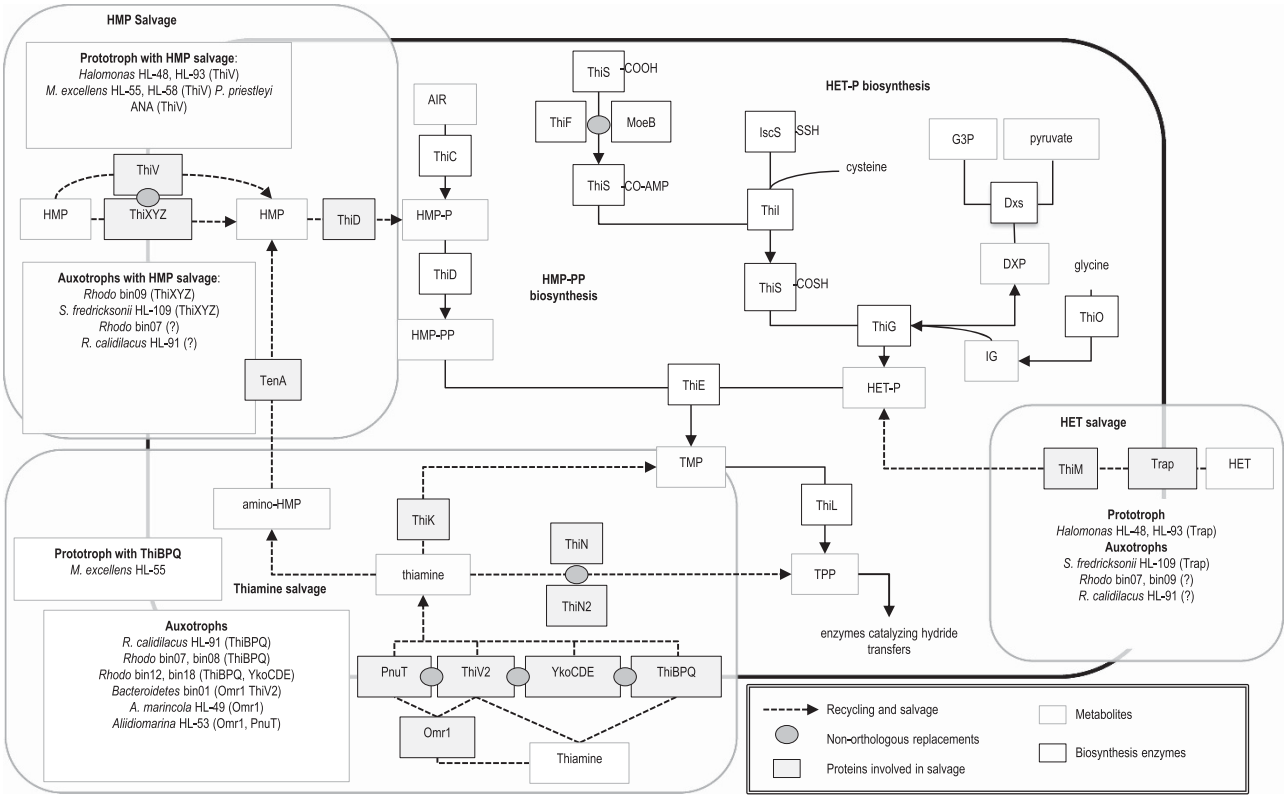

Figure 4 Thiamine, hydroxyethylthiazole and 4-amino-t-aminomethyl-2-methylpyrimidine are salvaged for thiamine pyrophosphate biosynthesis. Metabolites that occur in the thiamine pyrophosphate biosynthetic and precursor salvage pathways: AIR, 5-amino-1-(5-phospho-D-ribosyl)imidazole; amino-HMP, 4-amino-5-aminomethyl-2-methylpyrimidine; DXP, 1-deoxy-D-xylulose-5-phosphate; G3P, glyceraldehyde-3-phosphate; HET, hydroxyethylthiazole; HMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine; IG, iminoglycine; -P, phosphate; -PP, diphosphate; TMP, thiamine monophosphate; TPP, thiamine pyrophosphate. Enzymes involved in biosynthesis: CdsH, cysteine desulfhydrase; Dxs, 1-deoxy-d-xylulose-5-phosphate synthase; IscS, cysteine desulfurase; MoeB, adenylyltransferase/sulfurtransferase; ThiC, phosphomethylpyrimidine synthase; ThiD, bifunctional hydroxymethylpyrimidine/phosphomethylpyrimidine kinase; ThiE, thiamine-phosphate pyrophosphorylase; ThiF, sulfur carrier protein adenylyltransferase; ThiG, thiazole synthase; ThiL, thiamine monophosphate kinase; ThiO, glycine oxidase; and ThiS, sulfur carrier protein. Enzymes involved in salvage and recycling: TenA, thaiminase; ThiK, thiamine kinase; ThiM, 4-methyl-5-(beta-hydroxyethyl) thiazole kinase; ThiN and ThiN2, thiamine pyrophosphokinase. Thiamine transporters include the Omr1 TonB-dependent receptor, the PnuT ATP-dependent transporter, the ThiBPQ ABC-type transporter, The YkoCDE ECF-type transporter and the ThiV2 sodium-dependent transporter. Hydroxymethylpyrimidine transporters are the ThiV sodium-dependent transporter and the ThiXYZ ABC-type transporter. Trap refers to the single unnamed Trap-type hydroyethylthiazole transporter.

been described previously, (Taga and Walker, 2008; Galeazzi *et al.*, 2011), this knowledge has yet to be broadly applied to microbial physiology and cultivation. When cultivating uncharacterized microbes, we advise supplementing the standard vitamin supplements with additional precursors such as those described herein.

Precursor specialization may be driven by environmental conditions affecting precursor availability. Our consortia are grown under constant light conditions, and two cofactors, TPP and adenosylcobalamin, are susceptible to photolysis. Photolysis of thiamine yields HMP and HET (Ansari *et al.*, 2004). Six of the community members have salvage systems for HMP and HET, with four members restricted to salvage of these precursors rather than thiamine itself (Figure 4 and Supplementary Data 3). Exclusive use of these metabolites likely reflects their ready availability. Photolysis of adenosylcobalamin causes loss of its adenosyl group (Garabato *et al.*, 2016). Although it is predicted that all community members can salvage cob(II)alamin, it is likely that they do not all perform this task with the same efficiency. We predict that, *in situ*, concentrations of TPP and cobalamin precursors vary over the diel cycle and across mat depth, and that this variation may drive shifts in community function or composition.

Two mechanisms proposed for the release of vitamins to consumers are passive exchange, in which death and lysis of the producers benefits the consumers (Karl, 2002), and direct symbiosis, where producers synthesize excess vitamin and export it for use by consumers. Although passive exchange is assumed to occur in all systems, mathematical modeling of the growth dynamics of an obligate syntrophic partnership between a bacterial B_{12} producer and an algal consumer has provided strong evidence for direct symbiosis (Grant *et al.*, 2014). Our data suggest that direct symbiosis occurs in this community. The predominant member in each of our

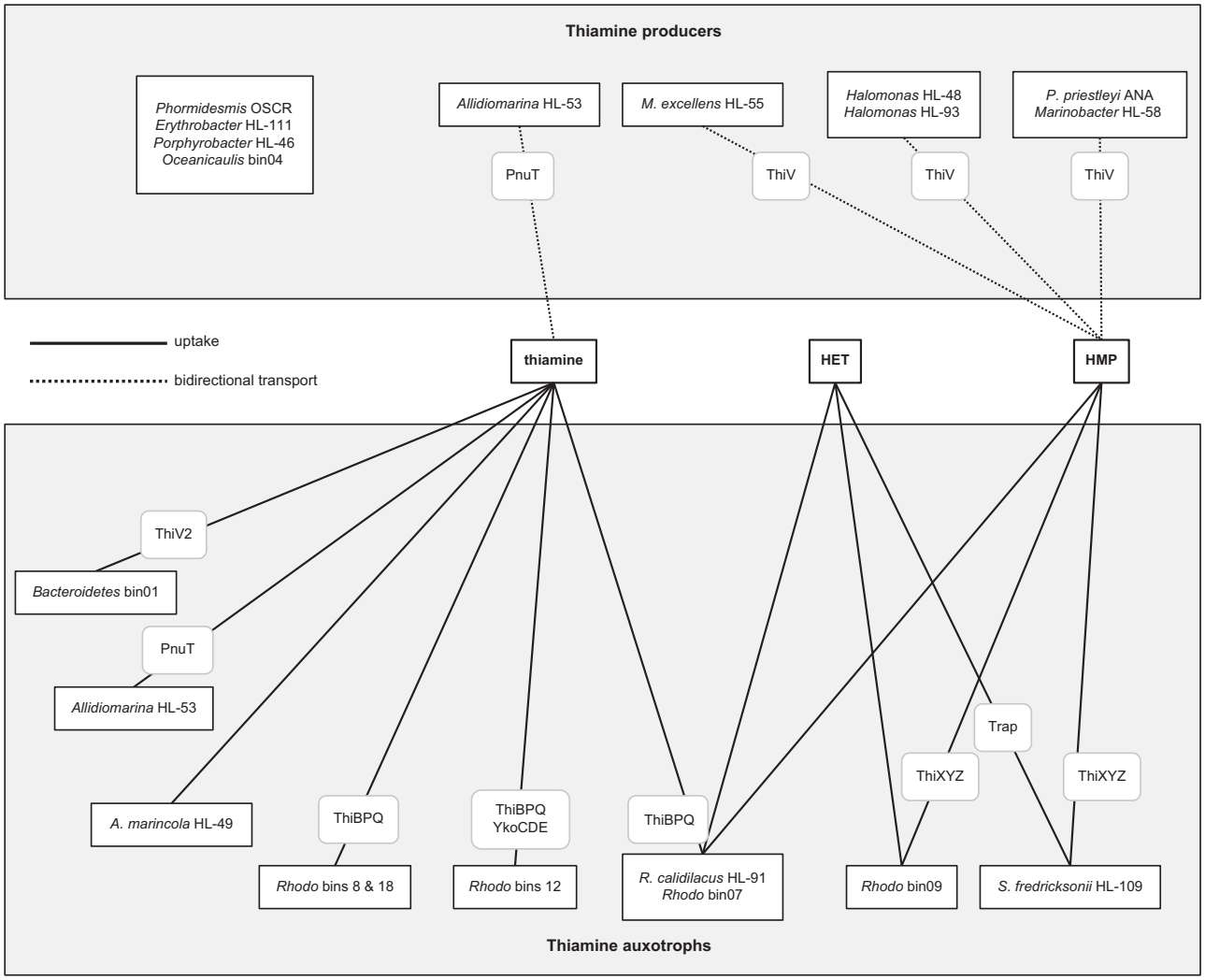

Figure 5 Overview of proposed interactions between thiamine producers and auxotrophs. TPP precursor transporters are depicted by rounded rectangles with gray edges. Precursors that are exchanged include thiamine, hydroxyethylthiazole (HET) and 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP). An exporter for HET has not been identified. Note that *M. excellens* HL-55 and both *Halomonas* spp. also have uptake transporters, but they are not shown here for clarity.

consortia is the cyanobacterium (*P. priestleyi* ANA or *Phormidium* OSCAR), which is prototrophic for all vitamins (Cole *et al*., 2014). However, the cyanobacteria are unable to produce adenosylcobalamin, which is required by six members of our community (Figure 3). Consequently, these six members must acquire cobalamin from the low-abundance producers, *Halomonas* HL-48 and HL-93 and *S. fredricksonii* HL-109. For these members to provide the needed precursors through lysis, a high turnover of cells would be necessary. In addition, we observed a preferential occurrence of energy-independent vitamin transporters of biotin, precursors to TPP, CoA, folate and NAD in respective vitamin producers in our community. We propose that these transporters are conditionally bidirectional, a hypothesis that is supported by the identification of the RibM energy-independent facilitator (transporter family 4.B.1) as a likely bidirectional translocator of riboflavin in *Streptomyces davawensis* (Hemberger *et al*., 2011). Orthologs to RibM (designated PnuX) are found in nearly 400 genomes in the SEED database (see 'Riboflavin biosynthesis and transport' subsystem in http://pubseed.theseed.org/SubsysEditor.cgi), with only 10 predicted to lack riboflavin biosynthetic genes. The occurrence of energy-independent vitamin transporters is widespread among microbes. If many are indeed bidirectional, it suggests that syntrophic partnerships that rely on them are important in shaping community behavior and structure.

Although B_{12} is assumed to be exchanged between prototrophs and auxotrophs, no B_{12} exporters were identified. We speculate that one exists because previous studies suggest that *Halomonas* forms a syntrophic partnership with algae, with the former providing B_{12} and the latter a source of carbon (Croft *et al*., 2005). The *Halomonas* spp. in our community are the only members besides *S. fredricksonii* HL-109 that can provide suitable forms of cobalamin

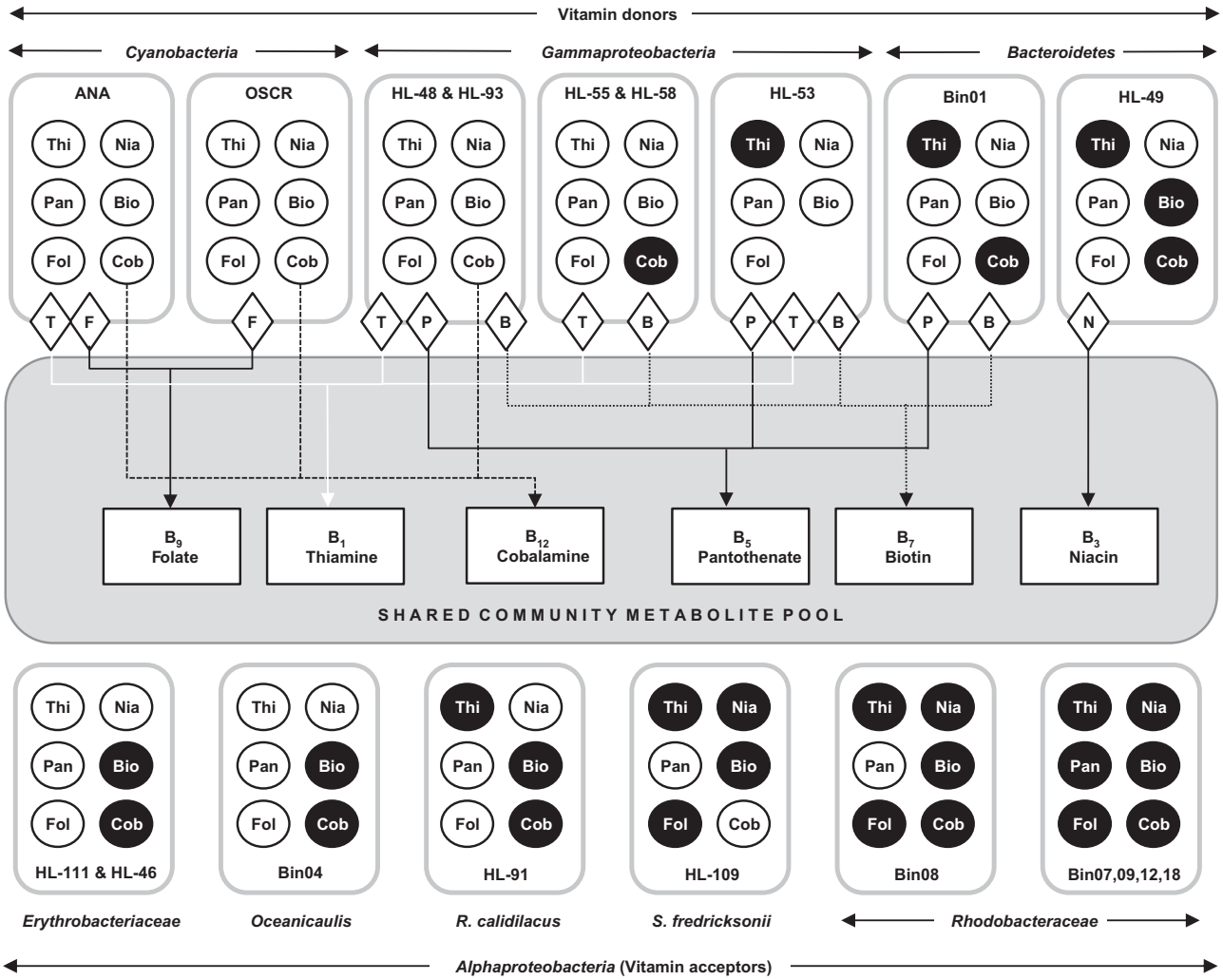

Figure 6 Reconstruction of syntrophic metabolism of B vitamin-related cofactors. Cofactor prototrophy and auxotrophy is represented by white and black ovals, respectively. Diamonds represent proposed bidirectional transporters (T = PnuT in HL-53 and ThiV in the rest, B: YigM, F: Fbt, P: PanF, N: PnuC) of cofactors or their precursors. Text within ovals and diamonds refers to precursor groups (vitamins) that contribute to the community metabolite pool: thiamine: Thi or T; niacin: Nia or N; pantothenate: Pan or P; biotin: Bio or B; folate: Fol or F; cobalamin: Cob. No cobalamin information is shown for *Aliidiomarina* HL-53 because it encodes no cobalamin-dependent enzymes. The only required precursor with no candidate exporter is cobalamin, but we speculate that one exists.

for all the members. Furthermore, unlike HL-109, their biosynthetic genes are not under control of the B_{12} riboswitch, and they encode enzymes or pathways that bypass essential requirements for this cofactor. Therefore, biosynthesis of cobalamin destined for export has reasonable potential to be achievable.

Metabolic reconstruction of cofactor biosynthesis and salvage pathways predicted the presence of precursor transporters (importers) that we were unable to identify. The most notable absence is a nicotinamide or nicotinate transporter required by 13 organisms in our system. A gene with this function has also been elusive in *Escherichia coli*; no candidate nicotinate transporter has been identified in *Escherichia coli* even though one has been known to exist for over 30 years (Rowe *et al*., 1985). One possibility is that a dedicated transporter does not exist and that available levels of precursor are sufficiently high to allow for passive transport. Other systems for which we could not identify transporters (folate, pantothenate and biotin) lack recognizable vitamin-responsive regulators, hampering efforts to use regulon analysis to identify these and other system components.

Conclusion

Our genomics-based metabolic reconstruction revealed that most members of the consortia have an essential requirement for cofactor precursors and thus must obtain them from other members of the community. We also found that some of the microbes have the potential to salvage a broader variety of precursors than are currently routinely used as growth supplements. Besides impacting strategies for the cultivation of microbes, this knowledge

suggests that an organizational principle of metabolite exchange in communities is to diversify utilization of precursors. Last, our analyses revealed that cofactor producers typically encode different classes of transporters than do cofactor auxotrophs. We hypothesize that transporters translocate cofactor precursors inward or outward depending on the metabolic status of the cell, which suggests that the realized demand for precursors is dynamic, and that by balancing metabolite production and usage, a community can reap the benefits of division of labor. Therefore, while cell lysis could contribute to the shared pool of nutrients, the availability of cofactor precursors in the pool can also be controlled by the metabolic status and export capabilities of respective prototrophs. Furthermore, because certain processes that depend on cofactors are essential, they can serve as control points for coordination of community member abundance and function, suggesting that division of labor in cofactor production is closely tied to community emergent properties. Important knowledge gaps that remain to be explored include the characterization of mechanisms by which cofactor precursors are made available to the community and the means by which levels of precursor pools are controlled.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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