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Exchange of Vitamin B_1 and Its Biosynthesis Intermediates Shapes the Composition of Synthetic Microbial Cocultures and Reveals Complexities of Nutrient Sharing

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ABSTRACT Microbial communities occupy diverse niches in nature, and community members routinely exchange a variety of nutrients among themselves. While large-scale metagenomic and metabolomic studies shed some light on these exchanges, the contribution of individual species and the molecular details of specific interactions are difficult to track. In this study, we follow the exchange of vitamin B_1 (thiamin) and its intermediates between microbes within synthetic cocultures of *Escherichia coli* and *Vibrio anguillarum*. Thiamin contains two moieties, 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and 4-methyl-5-(2-hydroxyethyl)thiazole (THZ), which are synthesized by distinct pathways using enzymes ThiC and ThiG, respectively, and then coupled by ThiE to form thiamin. Even though *E. coli* Δ*thiC*, Δ*thiE*, and Δ*thiG* mutants are thiamin auxotrophs, we observed that cocultures of Δ*thiC*-Δ*thiE* and Δ*thiC*-Δ*thiG* mutants are able to grow in a thiamin-deficient medium, whereas the Δ*thiE*-Δ*thiG* coculture does not. Further, the exchange of thiamin and its intermediates in *V. anguillarum* cocultures and in mixed cocultures of *V. anguillarum* and *E. coli* revealed that there exist specific patterns for thiamin metabolism and exchange among these microbes. Our findings show that HMP is shared more frequently than THZ, concurrent with previous observations that free HMP and HMP auxotrophy is commonly found in various environments. Furthermore, we observe that the availability of exogenous thiamin in the media affects whether these strains interact with each other or grow independently. These findings collectively underscore the importance of the exchange of essential metabolites as a defining factor in building and modulating synthetic or natural microbial communities.

IMPORTANCE Vitamin B_1 (thiamin) is an essential nutrient for cellular metabolism. Microorganisms that are unable to synthesize thiamin either fully or in part exogenously obtain it from their environment or via exchanges with other microbial members in their community. In this study, we created synthetic microbial cocultures that rely on sharing thiamin and its biosynthesis intermediates and observed that some of them are preferentially exchanged. We also observed that the coculture composition is dictated by the production and/or availability of thiamin and its intermediates. Our studies with synthetic cocultures provide the molecular basis for understanding thiamin sharing among microorganisms and lay out broad guidelines for setting up synthetic microbial cocultures by using the exchange of an essential metabolite as their foundation.

KEYWORDS HMP, nutrient exchange, synthetic microbial coculture, thiamin, thiazole, vitamin B_1

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Microorganisms inhabit diverse natural habitats and ecosystems and are engaged in a multitude of interactions, including sharing and competing for essential nutrients. Microbial communities or consortia are shaped via these positive and/or negative interactions and have their own unique metabolic network that is defined by the spatial distribution, physiology, and availability of nutrients among the microbial participants (1–3). The exchange of biomolecules such as sugars, nucleobases, amino acids, vitamins, electron acceptors, fermentation by-products, and metal-chelating siderophores is found to occur between members of natural and synthetic microbial consortia (1, 4–8). Some members of a microbial consortia may stop synthesizing a metabolite that is readily available in their environment and eventually become auxotrophic for that nutrient (8, 9). Auxotrophy is beneficial for an individual organism, as it allows for the reduction in metabolic burden and/or genome size (10, 11). For example, in experiments with *Escherichia coli*, about 13% of mutants auxotrophic for vitamins, amino acids, and nucleotides show a higher fitness than the wild-type strain when the missing nutrient is provided exogenously in sufficient quantities in the growth medium (12). Another study shows that the coevolution of a coculture of the sulfate-reducing bacterium *Desulfovibrio vulgaris* and the archaeon *Methanococcus maripaludis* over 10^3 generations leads to loss-of-function mutations in the sulfur-reducing genes in *D. vulgaris*. Further, deleting these genes increases the yield of the corresponding *D. vulgaris* strains in coculture with *M. maripaludis* by an optical density at 600 nm (OD_{600}) of ~0.05 in comparison to the wild-type strain (13). Conversely, microorganisms that produce a metabolite to share or exchange with their fellow community dwellers (commensalism or mutualism, respectively) secure

a position of prominence as they become indispensable for the consortium (14). *Ruminococcus bromii*, a starch-degrading bacterium associated with the human gut, converts starch to sugars that are substrates for other gut bacteria, thus playing the role of a keystone species (15). Thus, auxotrophy and metabolite sharing are important features for the formation and sustenance of microbial communities, and synthetic communities developed for biotechnological applications or for studying microbe-microbe interactions are often designed using these principles.

Among the commonly shared metabolites in microbial communities are vitamins (1, 16, 17). The activated forms of vitamins play an indispensable role as cofactors for numerous enzymes in primary metabolism across all domains of life. Several metagenomic analyses reveal that the water-soluble B vitamins are readily exchanged in marine microbial communities, the human gut microbiota, and communities associated with insects and other hosts (17–20). Of these, vitamin B_1 (thiamin) is an important member of the B vitamins that plays an essential role in carbohydrate, amino acid, and lipid metabolism by assisting enzymes in conducting "impossible" decarboxylations (21). In the human gut microbiome, B_1 auxotrophy appears to be most widespread at both the genus and family level, indicating that it is a commonly shared metabolite (17).

Even though most bacteria, plants, and eukaryotes such as fungi are capable of thiamin biosynthesis, many other organisms are unable to produce it and instead acquire it from their surroundings or their diet. The structure of thiamin consists of a 5-membered 4-methyl-5-(2-hydroxyethyl)thiazole ring (THZ) and a 6-membered 4-amino-5-hydroxymethyl-2-methylpyrimidine ring (HMP), which are synthesized in the natural world in their phosphorylated forms, THZ-P and HMP-P, respectively, in two distinct branches of the thiamin biosynthesis pathway (Fig. 1A) (22–26). The HMP-P is further phosphorylated to HMP-PP, following which the diphosphate is displaced by an attack via the THZ-P ring nitrogen to form a methylene bridge between the two rings to yield thiamin monophosphate (TMP) (Fig. 1A) (27). A final phosphorylation of TMP yields thiamin diphosphate (TDP), which is used as a cofactor by enzymes for cellular metabolism (28, 29). In some bacteria and yeast, thiamin is pyrophosphorylated directly to form TDP with the help of thiamin pyrophosphokinase (30, 31).

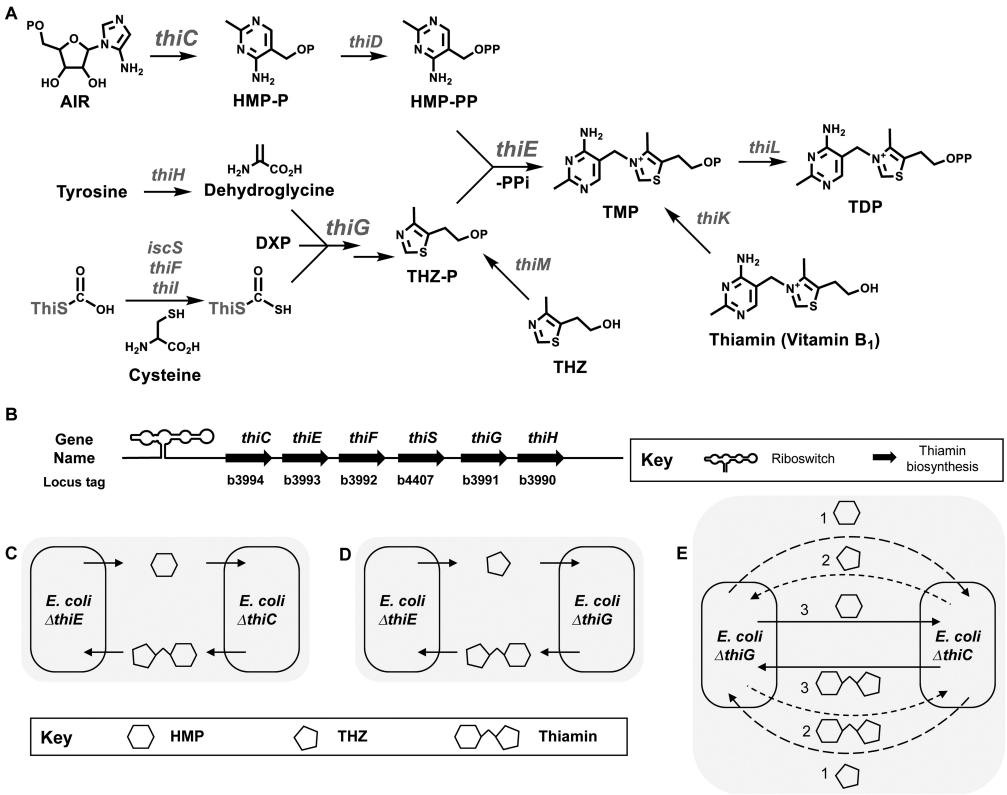

FIG 1 Overview of the thiamin biosynthesis pathway in *E. coli* strain K-12 substrain MG1655. (A) Major steps in the thiamin biosynthesis pathway are depicted. All phosphate groups ($-PO_3^{2-}$) are indicated as P. The 4-amino-5-hydroxy-2-methylpyrimidine phosphate (HMP-P) ring is formed by rearrangement of its precursor AIR by the enzyme ThiC. 4-Methyl-5-(2-hydroxyethyl)thiazole (THZ) is formed by the enzyme ThiG. The sulfur in the THZ ring is transferred via enzymes IscS, ThiI, and ThiF to form a thiocarboxylate moiety on the C terminus of the enzyme ThiS. The 1-deoxyxylulose-5-phosphate is synthesized by Dxs, and the ThiH enzyme converts tyrosine to dehydroglycine. ThiD, ThiM, and ThiK act as kinases for HMP-P, THZ, and thiamin, respectively. The enzyme ThiE attaches the HMP-PP and the THZ-P rings together in the final steps of the pathway to form thiamin monophosphate (TMP), and ThiL phosphorylates it to form the active form of the cofactor, thiamin pyrophosphate/diphosphate (TDP). Abbreviations: AIR, 5′-phosphoribosyl-5-aminoimidazole; DXP, 1-deoxyxylulose-5-phosphate; HMP-P, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate; HMP-PP, 4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate; THZ-P, 4-methyl-5-(2-hydroxyethyl)thiazole phosphate; THZ, 4-methyl-5-(2-hydroxyethyl)thiazole. Gene names are shown in gray. (B) Arrangement of the genes involved in the *de novo* biosynthesis pathway for thiamin in *E. coli* K-12 MG1655. (C to E) We hypothesize that the coculture of *Ec*Δ*thiC*-*Ec*Δ*thiE* strains can survive by exchanging HMP and thiamin (C), the coculture of *Ec*Δ*thiE*-*Ec*Δ*thiG* strains can survive by exchanging THZ and thiamin (D), and the coculture of *Ec*Δ*thiC*-*Ec*Δ*thiG* strains can survive in three possible scenarios where metabolites are exchanged in the following pairs (E): exchange of HMP and THZ (1), exchange of THZ and thiamin (2), and exchange of HMP and thiamin (3).

Thiamin and its intermediates THZ and HMP are stable under physiological conditions and are salvaged from the environment by organisms for producing thiamin. Metagenomic analysis of the human gut microbiome reveals that the thiamin biosynthesis and salvage pathways display the largest variety of intermediates and noncanonical metabolic precursors (17). Recent findings implicate HMP as an important metabolite in shaping marine algal and bacterial consortia (32). Additionally, studies show that there exist thiamin auxotrophs that lack thiamin transporters but instead contain putative transporters for the uptake of HMP and/or THZ, which

permit the salvage of these intermediates to produce thiamin (16, 33, 34). Examples of HMP and thiamin transporters and their uptake have been reported widely in literature (16, 35–37). On the other hand, information on THZ uptake and exchange is limited to only a handful of studies that predict a THZ transporter and show the uptake of the precursor carboxythiazole (16, 35, 36, 38, 39).

The modular nature of thiamin biosynthesis, where HMP and THZ are found to be independently synthesized and salvaged, makes this pathway a unique candidate for studying metabolic cross talk within microbial cocultures. To experimentally validate some of these findings, we require a simple model system whose members are engaged in thiamin, THZ, and HMP exchange. Such a system will allow us to (i) understand the molecular principles of thiamin biosynthesis occurring beyond an individual organism within a community and (ii) establish the design principles of building synthetic communities sustained by thiamin biosynthesis and uptake with diverse biotechnological applications.

This study involves a series of thiamin-dependent synthetic cocultures using *E. coli*, a Gram-negative bacterium that is capable of *de novo* thiamin synthesis and salvage and that is a member of several environmental and enteric microbial communities. In *E. coli*, the formation of the HMP-P ring is catalyzed by the enzyme ThiC, the THZ-P ring is synthesized by a host of enzymes including ThiG, and subsequently, these rings are coupled together by ThiE to form TMP. Also, no known transporters and salvage enzymes of HMP or THZ or their analogues are found in *E. coli*, and only one known transporter, ThiBPQ, exists to facilitate thiamin transport (32, 35, 36, 39, 40). We generated *E. coli* strain K-12 substrain MG1655 thiamin biosynthesis mutants—the Δ*thiC*, Δ*thiE* and Δ*thiG* mutants—which are impaired in *de novo* thiamin biosynthesis and thus are thiamin auxotrophs. We then set up pairwise synthetic cocultures of these three *E. coli* mutants to study their growth over short time periods. We also analyzed the exchange of thiamin, THZ, and HMP at a molecular level and its effect on the coculture composition. Further, we studied similar cocultures of another gammaproteobacterium, *Vibrio anguillarum*, and finally, mixed cocultures of *E. coli* and *V. anguillarum* to understand the extent to which our findings on thiamin metabolism within the *E. coli* cocultures hold true for other bacteria.

A unique property of the thiamin-based synthetic cocultures we have devised is that these are reliant on the exchange of precursors and intermediates within a single metabolic pathway, in comparison to other synthetic coculture studies in literature which involve exchange of molecules derived from two or more metabolic pathways (1, 4, 6). The advantages of a coculture system which is based on the biosynthesis of a single metabolite are the following: (i) the growth conditions of individual strains are similar, as they are auxotrophs for the same metabolite, (ii) the regulation of the biosynthesis and uptake of individual intermediates along the pathway can be studied, and (iii) coupling the results we observe from our system with genetic data from isolates and metagenomes has the potential to improve predictions and hypotheses of B_1-related auxotrophy and metabolite exchange in natural systems.

Our results indicate that the intermediates in a single biochemical pathway can be exchanged with different efficiencies. In addition, we show that the exchange of nutrients in the thiamin biosynthesis pathway can also modulate the ratios of the organisms that are engaged in the cross talk.

RESULTS

***E. coli* thiamin biosynthesis auxotrophic mutants show concentration-dependent increases in growth when supplemented with thiamin or its biosynthesis intermediates.** *E. coli* is capable of producing TDP *de novo* and also contains genes to salvage thiamin from its environment (Fig. 1A). All the major genes for thiamin synthesis and salvage are found in three operons: (i) *thiCEFSGH*, which conducts *de novo* thiamin biosynthesis (Fig. 1B), (ii) *thiMD*, which codes for kinases in the salvage pathway, and (iii) *thiBPQ*, which codes for an ABC-type thiamin transporter (25) (see Fig. S1A in the supplemental material). All three operons are regulated by TDP-dependent riboswitches (41).

To begin our studies, we created three knockout *E. coli* K-12 MG1655 strains, *Ec*Δ*thiC*, *Ec*Δ*thiE*, and *Ec*Δ*thiG* (referred to as the *thi* mutant strains), and noted that all three strains grew without any growth disadvantage in a nutrient-rich medium (Fig. S2A). Next, we tested their growth in a thiamin-deficient minimal medium containing M9 salts with glucose and NH_4Cl as the carbon and nitrogen sources, respectively. We expected that they would require exogenously added thiamin for growth in this medium, but to our surprise, all three strains survived well in the first passage (P1) from the nutrient-rich to the minimal medium (Fig. S2C). Analysis of the M9 medium without any cells as a control showed the absence of thiamin and its phosphorylated analogues, indicating that thiamin is indeed carried over by the cells (Fig. S3). A second passage (P2) of the mutants in the thiamin-deficient minimal medium showed significantly lesser growth than that of the wild-type strain, and the third passage (P3) showed no growth, indicating that the *thi* mutant strains were indeed thiamin auxotrophs (Fig. 2A to D, no thiamin added trace; see also Fig. S2E). Our results match similar observations in the literature, which note that thiamin stored inside the cells during their growth in rich medium is carried over into a few generations of cell growth (42, 43). For all future experiments, the P2 cells were used, as this allowed us to have some cells from the controls for thiamin quantitation experiments while yet showing a sufficient difference in optical density (OD_{600}) between the single-culture and coculture growth experiments.

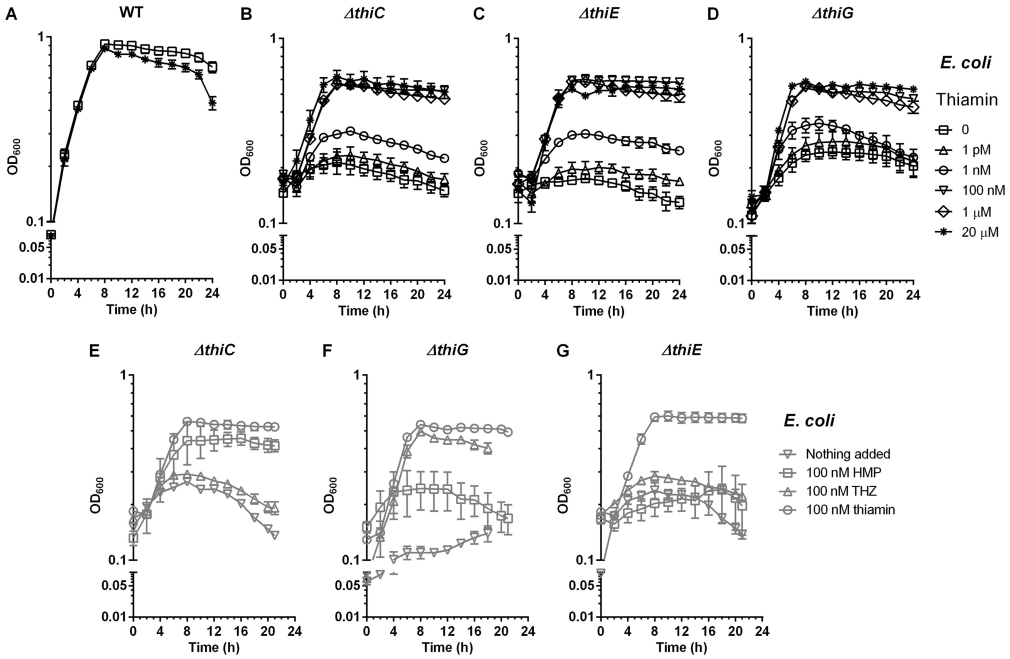

FIG 2 Supplementation of the thiamin mutants of *E. coli* K-12 MG1655 with thiamin, HMP, and THZ in M9 medium. (A to D) Growth phenotype of the wild-type strain (A), the *Ec*Δ*thiC* strain (B), the *Ec*Δ*thiE* strain (C), and the *Ec*Δ*thiG* strain (D). The following symbols in panels A to D represent the indicated concentrations of thiamin: ▫, nothing added; ○, 1 pM; Δ, 1 nM; ∇, 100 nM; ◊, 1 μM; *, 20 μM. (E to G) Supplementation of the *Ec*Δ*thiC* mutant (E), the *Ec*Δ*thiG* mutant (F), and the *Ec*Δ*thiE* mutant (G). Symbols in panels E to G: ∇, no HMP/THZ; ▫, 100 nM HMP; Δ, 100 nM THZ; ○, 100 nM thiamin. Means ± standard errors of the means of results from three independent experiments are plotted.

Next, to determine the minimum thiamin concentration required by the *thi* mutant strains, we tested their growth in minimal medium supplemented with thiamin concentrations ranging from 0 to 20 μM. We found that while these strains show low growth with up to 1 nM thiamin, they are able to achieve an OD_{600} of ~0.6 with 100 nM, 1 μM, and 20 μM thiamin (Fig. 2B to D). This shows that thiamin is the growth-limiting nutrient for the *thi* mutants. To ensure that thiamin is not limiting in our assays, all further experiments were conducted with 20 μM thiamin unless otherwise stated. We further complemented each knockout strain with a plasmid containing the deleted gene and confirmed that growth can be restored in these strains in minimal medium in the absence of thiamin, as also observed in previous studies (Fig. S4) (44–46).

We expect the *Ec*Δ*thiC* and *Ec*Δ*thiG* strains to be impaired in the biosynthesis of the intermediates HMP and THZ, respectively, while the *Ec*Δ*thiE* mutant should not be able to link them together to synthesize thiamin. To test this, we fed HMP and THZ to the *thi* mutants in various concentrations ranging from 0 to 1 μM. The *Ec*Δ*thiC* strain survived only when supplemented with HMP, not with THZ (Fig. 2E; Fig. S5), as did the *Ec*Δ*thiG* strain when supplemented with THZ but not with HMP (Fig. 2F; Fig. S5), and the *Ec*Δ*thiE* strain was unable to survive with either HMP or THZ alone (Fig. 2G; Fig. S5). This confirms that the metabolic phenotypes of the *thi* mutants are correlated with their genotypes.

Specific cocultures of the thiamin biosynthesis mutants grow in minimal medium with no exogenously added thiamin. Next, we constructed three pairwise cocultures, *Ec*Δ*thiC*-*Ec*Δ*thiE* (*Ec-CE*), *Ec*Δ*thiC*-*Ec*Δ*thiG* (*Ec-CG*), and *Ec*Δ*thiE*-*Ec*Δ*thiG* (*Ec-EG*), and studied their growth in thiamin-deficient minimal medium. We hypothesized that if the *thi* mutant strains can share thiamin biosynthesis intermediates among themselves and produce thiamin, the cocultures will survive, as opposed to the single cultures, which are auxotrophic and perish under similar growth conditions. We started these cocultures with 9:1, 1:1, and 1:9 ratios of the two strains within the coculture and observed their growth over a period of 24 h. We observed that both the *Ec-CE* and the *Ec-CG* co-cultures showed increased survival at a 1:9 ratio of the Ec deltathiC to Ec deltathiE and Ec deltathiC to Ec deltathiG strains, respectively, compared to the individual pure strains (Fig. 3A and B, data for the 9:1 and 1:1 ratios not shown). On the other hand, the *Ec-EG* coculture showed no difference in growth in comparison to its individual pure cultures (Fig. 3C).

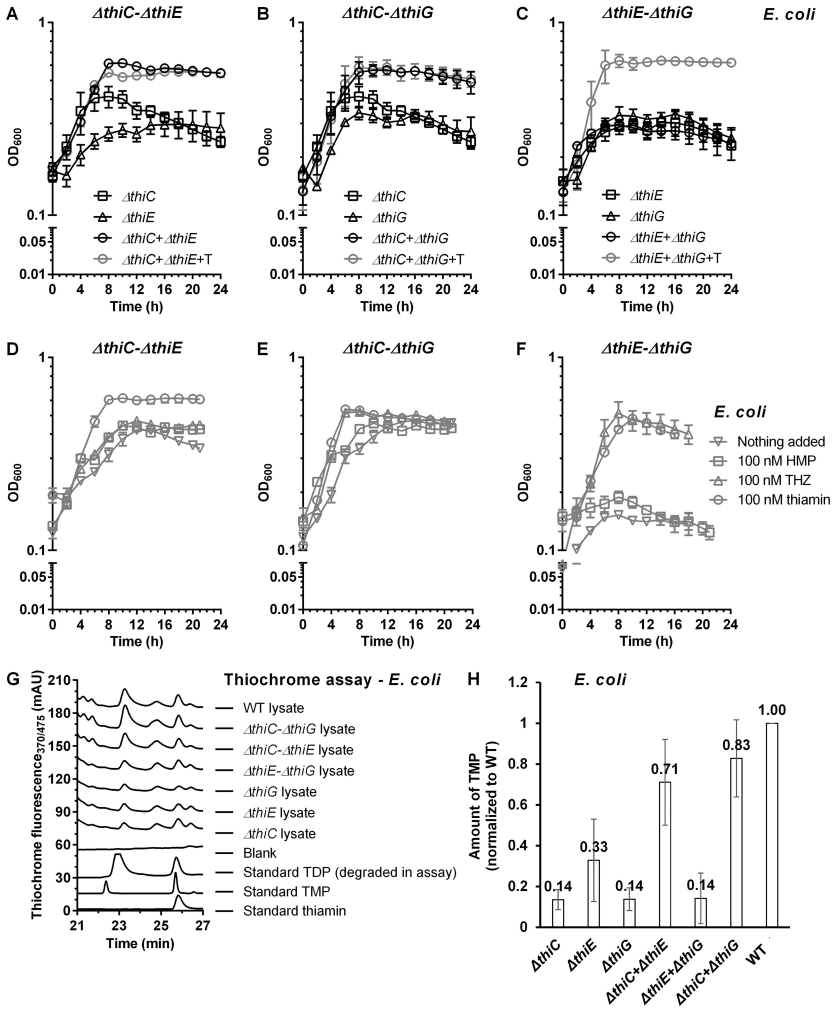

FIG 3 Thiamin biosynthesis mutants of *E. coli* K-12 MG1655 grow in pairwise cocultures in thiamin-deficient M9 medium. (A to C) Coculture of the *Ec*Δ*thiC*-*Ec*Δ*thiE* strains (A), the *Ec*Δ*thiE*-*Ec*Δ*thiG* strains (B), and the *Ec*Δ*thiC*-*Ec*Δ*thiG* strains (C). (D to F) Supplementation of the Δ*thiC*-Δ*thiE* coculture (D), the Δ*thiE*-Δ*thiG* coculture (E), and the Δ*thiC*-Δ*thiG* coculture (F). Symbols in panels D to F represent the following: ∇, no HMP/THZ; ▫, 100 nM HMP; Δ, 100 nM THZ; ○, 100 nM thiamin. (G) HPLC of thiochrome assay samples to detect thiamin from coculture lysates. (H) Amount of thiochrome monophosphate (TMP), normalized to that in the WT strain, detected in lysates of monocultures or cocultures of the thiamin biosynthesis mutants grown in P2. Means ± standard errors of the means of results from three independent experiments are plotted.

There are several possibilities of exchange of thiamin and its intermediates that account for the survival of the *Ec-CE* and *Ec-CG* cocultures (Fig. 1C to E). The *Ec*Δ*thiC* strain cannot synthesize HMP, but if it can acquire it from its environment, it can combine the HMP with the THZ it synthesizes to form thiamin. Alternately, it can acquire thiamin directly from its environment. Similarly, the *Ec*Δ*thiG* strain cannot synthesize THZ but it can grow if it acquires THZ or thiamin from its surrounding. On the other hand, the *Ec*Δ*thiE* strain can synthesize both the HMP and the THZ intermediates but is unable to combine them to form thiamin and needs to acquire it from its growth medium. The growth observed in the *Ec-CE* coculture can be explained only if the *Ec*Δ*thiE* strain supplemented the *Ec*Δ*thiC* strain with HMP and the *Ec*Δ*thiC* strain in return supplemented the *Ec*Δ*thiE* strain with thiamin (Fig. 1C and 3A). This indicates that both HMP and thiamin are likely being exchanged in the medium. Along similar lines, the *Ec-EG* coculture would grow if the *Ec*Δ*thiE* strain supplemented the *Ec*Δ*thiG* strain with THZ and the *Ec*Δ*thiG* strain, in return, supplemented the *Ec*Δ*thiE* strain with thiamin (Fig. 1D and 3C). Since the *Ec-EG* coculture does not grow, and we know that THZ and thiamin are salvaged by the *E. coli* cells based on our feeding studies and that thiamin is also exchanged as per the results of the *Ec-CE* coculture, this result suggests that THZ is not released by *Ec*Δ*thiE* mutant at sufficient concentrations and thus does not support the growth of the *Ec*Δ*thiG* mutant. The absence of any annotated THZ transporters in *E. coli* also supports this hypothesis. Interestingly, the *Ec-CG* coculture shows a higher OD_{600} than the individual pure cultures grown in thiamin-deficient medium (Fig. 3B). The *Ec-CG* coculture can grow in three scenarios: (i) the *Ec*Δ*thiC* strain and the *Ec*Δ*thiG* strain provided the other with THZ and HMP, respectively, (ii) the *Ec*Δ*thiC* strain provided the *Ec*Δ*thiG* strain with THZ and the *Ec*Δ*thiG* strain synthesized thiamin and provided it back to the *Ec*Δ*thiC* strain, or (iii) the *Ec*Δ*thiG* strain provided the *Ec*Δ*thiC* strain with HMP and the *Ec*Δ*thiC* strain synthesized thiamin and provided it back to the *Ec*Δ*thiG* strain (Fig. 1E). Since the data for the *Ec-EG* coculture indicates that THZ is likely not being exchanged, only the third possibility remains for the *Ec-CG* coculture, that is, HMP and thiamin are exchanged among the thiamin biosynthesis mutants. Incidentally, several reports in literature note the exchange or release of HMP among microbial communities, confirming our observation (33, 47).

Next, we compared carbon sources to understand whether these results hold true across different growth conditions. In addition to glucose, we chose pyruvate and succinate, since their utilization as carbon sources via the Kreb's cycle is linked to thiamin availability. Similar to what we observed with glucose, the *Ec-CE* and *Ec-CG* cocultures showed growth in pyruvate and succinate minimal media without thiamin while the *Ec-EG* did not, and the growth of the *Ec-CG* coculture was highest among the three (Fig. S6). Since glucose, pyruvate, and succinate metabolism in cells require thiamin-utilizing enzymes, our growth studies imply that the

Ec-CE and *Ec-CG* cocultures are able to synthesize thiamin. The *Ec-CE* coculture showed lower growth in the presence of pyruvate and succinate than in the presence of glucose, and thus we continued with glucose as the carbon source for all further experiments.

Analysis of the cocultures demonstrates that the exchange of HMP and thiamin aids their survival. To probe the growth patterns observed for the *Ec-CE*, *Ec-CG*, and *Ec-EG* cocultures, we conducted a supplementation study with a range of HMP and THZ concentrations (Fig. 3D to F; Fig. S5). We observed that while the *Ec-CE* and *Ec-CG* cocultures each show growth without or with supplementation with both molecules, the *Ec-EG* coculture survives only when fed with THZ, not with HMP (Fig. 3F). Interestingly, the *EcΔthiG* mutant can survive with 1 nM THZ, whereas the *EcΔthiC* mutant requires 100 nM HMP to survive (Fig. S5A, I, and K). This indicates that *E. coli* differs in its ability to either acquire and/or utilize the thiamin biosynthesis intermediates THZ and HMP. This result also sheds light on one of our preliminary observations that when the *Ec-CE* and *Ec-CG* cocultures were started at a total OD_{600} of 0.01 in the P2 passage instead of 0.1, they were unable to survive (data not shown). We attribute this to the lack of an adequate pool of thiamin intermediates at the start that would allow the coculture strains to begin dividing and cooperating, thus ensuring their survival.

***De novo* biosynthesis of thiamin occurs within cocultures.** To verify that the growth of the cocultures is due to the *de novo* biosynthesis of thiamin, we analyzed the lysates of the cells grown in thiamin-deficient medium from the second passage, P2, for single cultures and cocultures for the presence of thiamin and its phosphorylated versions TMP and TDP. To do so, we used the thiochrome assay, which employs an oxidation reaction under alkaline conditions to generate a fluorescent derivative of thiamin (23). First, we noted that under the thiochrome assay conditions we used, the standard thiochrome diphosphate formed is unstable and undergoes dephosphorylation as demonstrated by high-performance liquid chromatography (HPLC) analysis (Fig. 3G). Next, we analyzed the lysates of the *Ec-CE* cocultures and the *Ec-CG* cocultures and noted that the levels of thiochrome monophosphate in them were significantly higher than those in their respective single cultures when measured at 24 h and similar to those in the wild-type *E. coli* cell lysate (Fig. 3G and H). In contrast, the amounts of thiochrome monophosphate detected from the lysates of the *Ec-EG* cocultures and their respective single cultures were similar and were significantly lower than those of the wild-type lysate (Fig. 3G and H). This implies that thiamin is synthesized *de novo* in the *Ec-CE* and *Ec-CG* cocultures. Further, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of these samples confirmed the presence of thiamin in the lysates of the cocultures (Fig. S7). Taken together, these results show that the *Ec-CE* and the *Ec-CG* cocultures grow due to *de novo* thiamin synthesis, whereas the *Ec-EG* cocultures do not survive, as they are unable to produce thiamin.

***Vibrio anguillarum* thiamin mutants follow a similar pattern of exchange as *E. coli*.** In order to determine whether the pattern of exchange of thiamin biosynthesis intermediates observed in *E. coli* is conserved across other microbes, we analyzed another gammaproteobacterium, *Vibrio anguillarum* strain PF430-3, which is capable of *de novo* thiamin biosynthesis and salvage (Fig. S1B). Similar to the previous experiment, *V. anguillarum* Δ*thiC*, Δ*thiE*, and Δ*thiG* mutant strains were grown individually and in pairwise cocultures in thiamin-deficient M9 medium (Fig. 4).

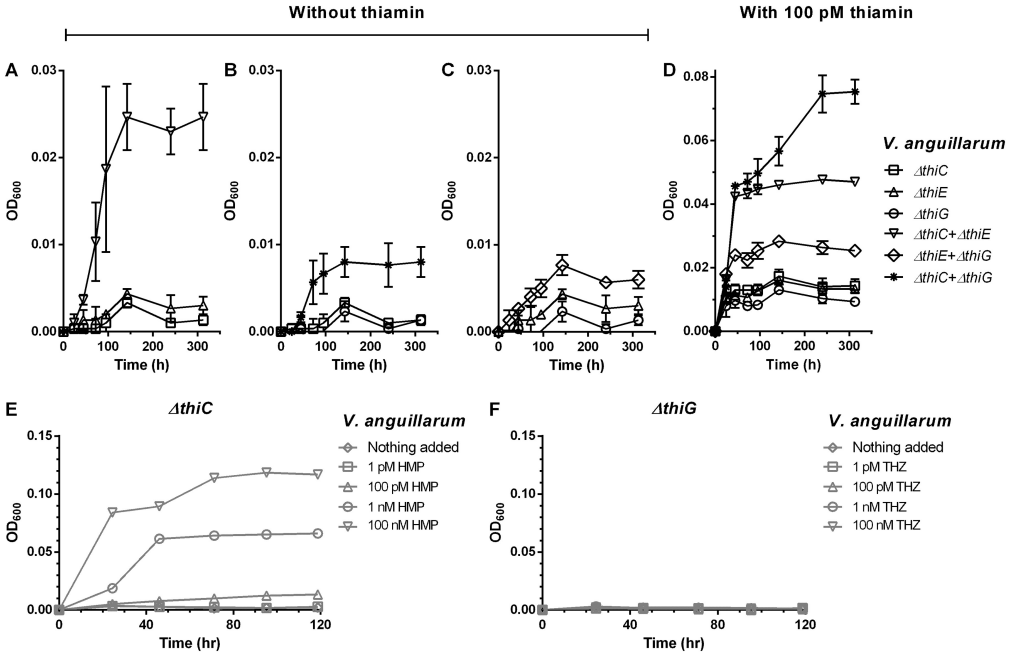

FIG 4 Thiamin biosynthesis mutants of *V. anguillarum* strain PF430-3 grow in pairwise cocultures in thiamin-deficient M9 medium. (A to C) Cocultures of the *VaΔthiC-VaΔthiE* strains (A), the *VaΔthiC-VaΔthiG* strains (B), and the *VaΔthiE-VaΔthiG* strains (C) without thiamin supplementation. (D) Cocultures of the *VaΔthiC*, *VaΔthiE*, and *VaΔthiG* strains supplemented with 100 pM thiamin. (E) HMP supplementation of the *VaΔthiC* mutant. (F) THZ supplementation of the *VaΔthiG* mutant. The following symbols in panels E and F represent the indicated concentrations of HMP and THZ, respectively: ◊, nothing added; □, 1 pM; Δ, 100 pM; ○, 1 nM; ∇, 100 nM. Means ± standard deviations of results from three independent experiments are plotted.

Since *V. anguillarum* and *E. coli* are both gammaproteobacteria containing the same set of thiamin genes except the kinase gene *thiM*, we expect *V. anguillarum* to show a similar pattern of exchange (Fig. S1). The *V. anguillarum* Δ*thiC*, Δ*thiE*, and ΔthiG single cultures showed no background growth in the P1 passage and a concentration-dependent increase in growth starting with nanomolar concentrations of supplemented thiamin (Fig. S8). For the cocultures grown without supplemented thiamin, we observed that the *V. anguillarum CE* (*Va-CE*) coculture showed significant growth, followed by the *Va-CG* coculture, while the *Va-EG* coculture

showed background growth similar to what we observed in *E. coli* (Fig. 4A to C). When supplemented with 100 pM thiamin, the *Va-CE*, *Va-CG*, and *Va-EG* cocultures grew significantly better than the single cultures, reiterating that the cocultures were likely producing thiamin (Fig. 4D). Interestingly, even though the *V. anguillarum* Δ*thiC* strain shows a concentration-dependent increase in growth when supplemented with HMP similar to its *E. coli* counterpart, the *Va*Δ*thiG* strain does not grow with exogenously added THZ (Fig. 4E and F). This indicates that unlike the *E. coli* Δ*thiG* mutant, whose growth can be complemented by thiamin and THZ, the *V. anguillarum* Δ*thiG* mutant can be complemented only by thiamin. This may be attributed to the absence of the thiazole kinase gene *thiM* in *V. anguillarum*, annotated as a salvage enzyme that phosphorylates THZ to produce THZ-P for incorporation in thiamin biosynthesis in *E. coli* and other organisms (Fig. 1; Fig. S1) (48).

***V. anguillarum* and *E. coli* thiamin mutants exchange thiamin and its biosynthesis intermediates among themselves.** Finally, to test whether the pattern of exchange that we observe occurs between different species, mixed cocultures of the thiamin biosynthesis mutants of *E. coli* and *V. anguillarum* in a pairwise manner were studied (Fig. 1C, D, and E describe all possible combinations of exchange). We observed that the *V. anguillarum* Δ*thiC*-*E. coli* Δ*thiE* (*VaC-EcE*) mixed cocultures grow in the absence of thiamin (Fig. 5A). This observation, along with the results obtained so far, shows that HMP is provided by *EcE* to *VaC* cells, which synthesize thiamin and provide it back to the *EcE* cells, thus leading to the growth of the coculture. The *VaE-EcC*, *VaC-EcG*, and *VaG-EcC* mixed cocultures also survived in the absence of thiamin as expected, further corroborating the exchange of HMP and thiamin (Fig. 5B to D). We noted that the final OD_{600} achieved by the *VaC-EcE* and *VaC-EcG* cocultures is greater than that of their corresponding reverse combinations, *VaE-EcC* and *VaG-EcC* cocultures, respectively. Since *V. anguillarum* has lesser growth in the thiamin-deficient medium than *E. coli*, it is likely that the amount of HMP provided by the *VaE* or the *VaG* strain to the *EcC* strain is lesser, leading to less growth of *VaE-EcC* and *VaG-EcC* cocultures.

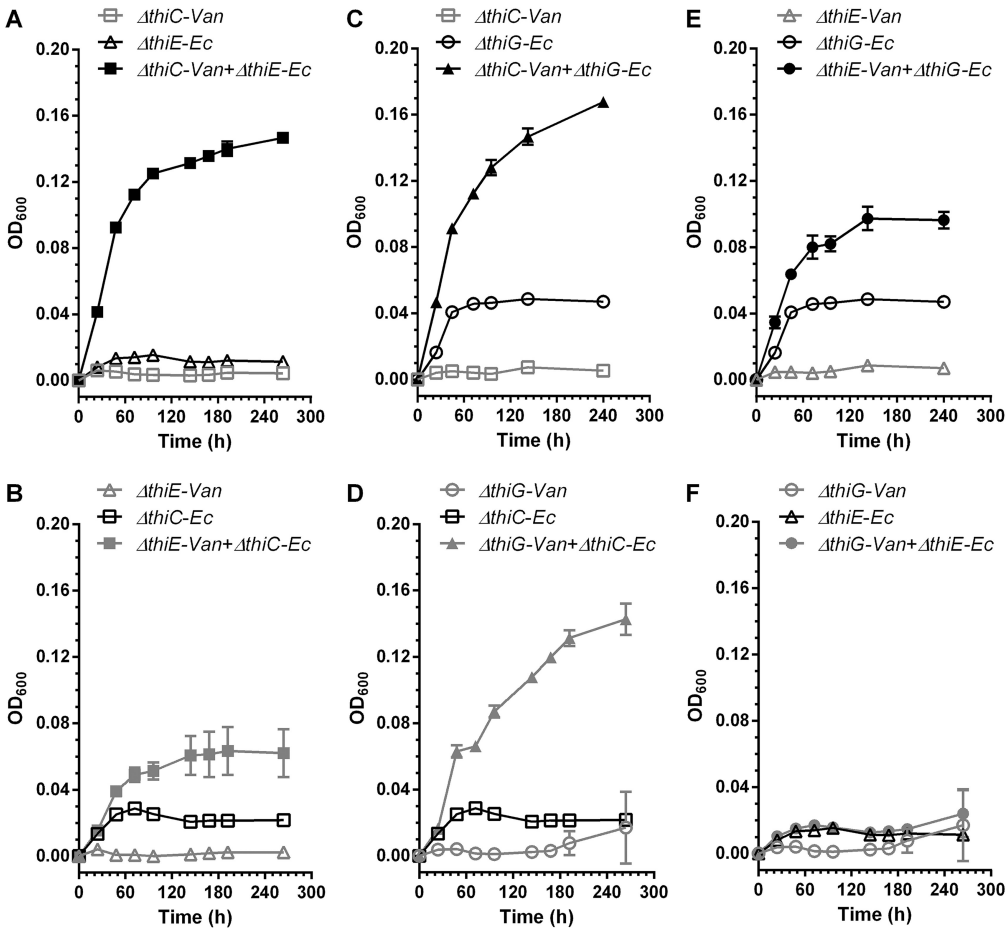

FIG 5 Mixed-species cocultures of the thiamin biosynthesis mutants of *V. anguillarum* PF430-3 and *E. coli* K-12 MG1655 in thiamin-deficient M9 medium. Mixed cocultures of the *Va*Δ*thiC*-*Ec*Δ*thiE* strains (A), the *Va*Δ*thiE*-*Ec*Δ*thiC* strains (B), the *Va*Δ*thiC*-*Ec*Δ*thiG* strains (C), the *Va*Δ*thiG*-*Ec*Δ*thiC* strains (D), the *Va*Δ*thiE*-*Ec*Δ*thiG* strains (E), and the *Va*Δ*thiG*-*Ec*Δ*thiE* strains (F) without thiamin supplementation. All empty gray and black symbols represent single cultures of *V. anguillarum* and *E. coli*, respectively. All filled symbols represent the cocultures, with gray and black colors representing the corresponding reverse combinations. Means ± standard deviations of results from three independent experiments are plotted.

Surprisingly, we observed that the *VaE-EcG* coculture also grew significantly better than the individual strains without thiamin supplementation (Fig. 5E), even though its overall growth was lower than that of the *VaC-EcG* coculture. This result is in contrast to what was observed in the *Va-EG* or *Ec-EG* cocultures, which did not grow beyond the background level. This indicates that THZ synthesized by the *V. anguillarum* Δ*thiE* strain is available in the medium at a concentration that allows *E. coli* Δ*thiG* to grow and produce thiamin and share it in return with *V. anguillarum* Δ*thiE*. The reverse combination coculture *VaG-EcE* does not survive in the absence of thiamin. This may be attributed to *V. anguillarum* lacking ThiM as mentioned previously, which restricts its ability to salvage THZ (Fig. 4F and 5F). All mixed cocultures of *V. anguillarum* and *E. coli* were able to survive with 100 pM of supplemented thiamin, as expected (Fig. S9).

To summarize, the *CE* and *CG* cocultures of *E. coli* or *V. anguillarum* strains can survive in the absence of externally supplemented thiamin, whereas the *EG* cocultures cannot. These experiments confirm that HMP and thiamin are shared within the synthetic cocultures but that THZ may not be as easily shared. Further, results obtained from the mixed cocultures of *E. coli* and *V. anguillarum* strains suggest that even though THZ is picked up when present at higher concentrations, it might not be readily shared among microorganisms, as the concentrations produced are too low to be salvaged.

The ratio of the individual strains within the coculture is determined by the exchange of thiamin and its biosynthesis intermediates. Our observations of the coculture experiments thus far are based on the total OD_{600} of the cocultures. To understand and quantify the contribution of each individual strain, we created the *thi* mutants fluorescently labeled with green fluorescent protein (GFP) and set up the pairwise *Ec-CE* and *Ec-CG* cocultures in thiamin-deficient minimal medium, where one of the strains in each coculture was fluorescently labeled (Fig. S2B and D). This approach allows us to quantify the amount of each strain in the coculture by using two parameters, i.e., (i) the total OD_{600} and (ii) the fluorescence of the coculture, which indicates the growth of the GFP-tagged strain. Briefly, we generated a standard curve of fluorescence versus OD_{600} for each individual strain, after which the cocultures (GFP strain indicated with an asterisk)—*Ec-C*E* and *Ec-CE** with the controls *Ec-C*E** and *Ec-CE* and a similar set for the *Ec-CG* cocultures—were set up. We then noted the increase in the OD_{600} and fluorescence values over time and mapped the fluorescence signal of the coculture to the standard curve of the corresponding GFP-tagged strain, allowing us to quantify its OD_{600} in the coculture (Fig. S10). The remaining untagged strain numbers were then calculated by subtracting this number from the total OD_{600}, eventually yielding the ratios of the two strains over the course of the coculture growth.

Our experiments and subsequent calculations showed that the quantities of the strains in the cocultures change over a period of 24 h when no thiamin is exogenously provided (Fig. 6; Fig. S11). The OD_{600} of the *Ec-C*E* coculture increases over time as expected (Fig. 6A). The fluorescence of the coculture also increased, indicating that the quantity of the GFP-marked *EcΔthiC** strain increased over time (Fig. 6B). Next, we observed that for the *Ec-CE** coculture in the absence of thiamin, the fluorescence did not increase even though the OD_{600} value increased over time, reiterating the result that the *EcΔthiC* strain increased in numbers in the coculture (Fig. 6C and D).

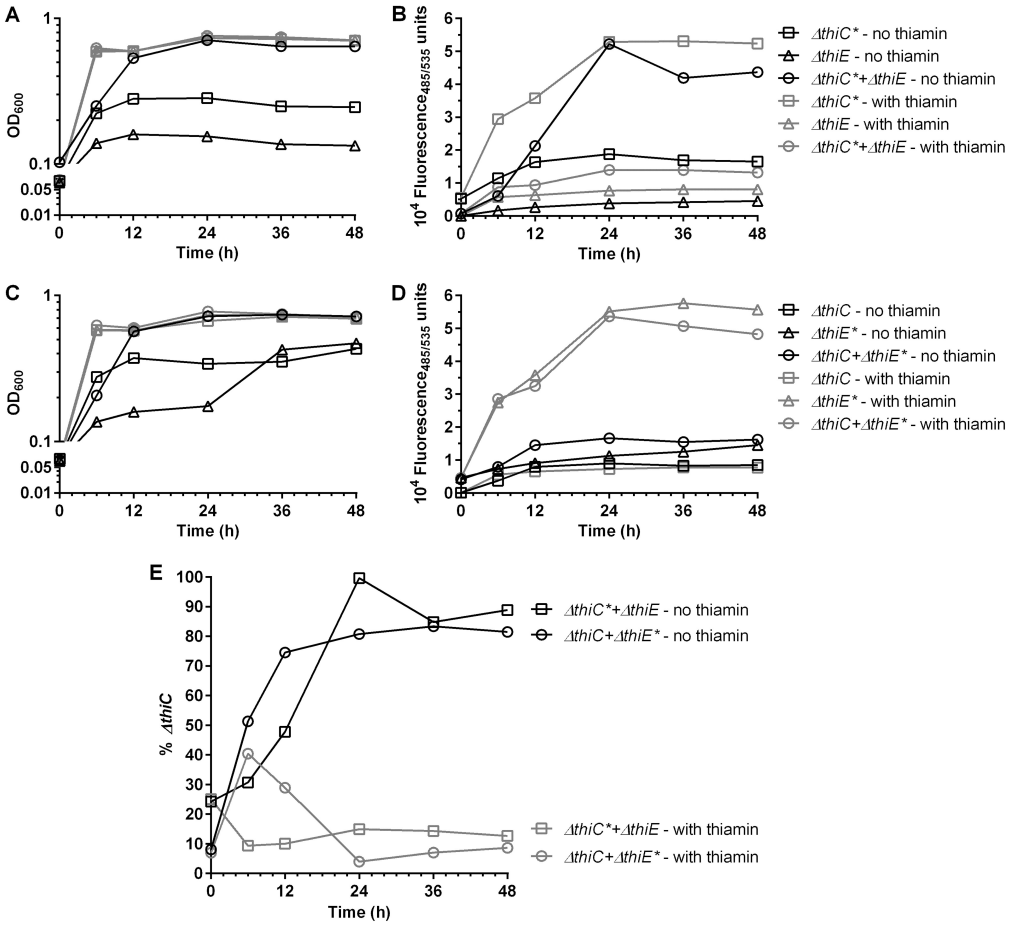

FIG 6 Growth phenotypes and fluorescence of the monocultures and cocultures of the thiamin mutant strains. The strains containing the *GFPmut2* cassette are marked with an asterisk. Black symbols, without thiamin; gray symbols, with thiamin. (A and B) OD_{600} (A) and fluorescence (B) of *EcΔthiC*-EcΔthiE* cocultures. (C and D) OD_{600} (C) and fluorescence (D) of *EcΔthiC-EcΔthiE** cocultures. (E) Percentage of *EcΔthiC* cells in the *EcΔthiC*-EcΔthiE* cocultures and the *EcΔthiC-EcΔthiE** cocultures. Average values of results from two independent experiments are plotted.

Interestingly, in the presence of thiamin, even though the OD_{600} of the *Ec-C*E* coculture increased over time, and the fluorescence increase in the *EcΔthiC** single culture cells was proportional to its growth as expected, the total fluorescence of the *Ec-C*E* coculture increased very slightly over time (Fig. 6A and B). This indicates that the ratio of the individual strains remains constant over time with respect to the starting ratio. Also, both the OD_{600} and the fluorescence of the *Ec-CE** coculture and the

*Ec*Δ*thiE** single culture increased over time, confirming that the numbers of the two participating strains do not deviate in the coculture in the presence of thiamin (Fig. 6C and D).

Upon quantifying the *Ec-C*E* and *Ec-CE** coculture results, we found that in the absence of thiamin, the percentage of the *Ec*Δ*thiC* cells in the cocultures increased over time to attain an average ratio of ~8:2 of *Ec*Δ*thiC* to *Ec*Δ*thiE* cells at 24 h (Fig. 6E). Also, the presence of GFP does not alter the final ratios of the strains in the cocultures, as illustrated by the *Ec-C*E* and *Ec-CE** cocultures showing similar ratios. Comparable ratios were obtained when the *Ec-C*G* cocultures were similarly analyzed (Fig. S9B). When the cocultures were further transferred at the end of 24 h of growth to a fresh thiamin-deficient M9 medium in passage P3, the new ratios held constant over a period of 24 h (Fig. S11A and C). Additionally, even after the continued growth of the P2 cocultures for another ~24 h, the ratios attained stayed constant (Fig. 6E; Fig. S11B). We hypothesize that this change in the ratio of the two strains results from the exchange of HMP and thiamin, which equilibrates after ~24 h and subsequently stabilizes. However, in the presence of exogenously added thiamin, the exchange is no longer necessary and hence the ratios of the two strains remain mostly unaltered.

DISCUSSION

Thiamin, an essential nutrient for living organisms, assists enzymes in executing key decarboxylation reactions in primary metabolism. Several studies based on metagenomic analyses predict that thiamin and its building blocks HMP and THZ can be salvaged by both thiamin auxotrophs and prototrophs (16, 17, 47). In this study, we investigated the mechanism of thiamin synthesis and exchange within microbial cocultures through a molecular lens.

It has been reported that secondary transporters such as PnuT, which facilitate bidirectional transport of thiamin, are found more often in prototrophs, whereas the ABC family primary transporters such as ThiT, which promote thiamin uptake, are found more often in auxotrophs (16, 17). It has also been observed for both marine and gut microbial communities that some organisms in the community might be auxotrophic for the biosynthesis of both THZ and HMP, whereas certain others in the same community can produce both these intermediates but lack the ability to combine them to form thiamin (16, 17). These observations reiterate that thiamin sharing is common among microorganisms.

To better understand the specifics of the exchange of thiamin and its intermediates in a community, we created synthetic cocultures with bacterial strains with defined thiamin auxotrophy patterns. Our results from the *E. coli* and *V. anguillarum* cocultures as well as their mixed cocultures suggest that thiamin and HMP are commonly exchanged among microorganisms, whereas the exchange of THZ may occur less frequently and under specific conditions (Fig. 7A). These results corroborate previous observations, where (i) marine organisms *Synechococcus* sp. strain WH8102 and *Dunaliella tertiolecta* have been shown to release HMP in their growth medium, and thiamin and HMP concentrations in seawater show day-night variation suggesting changes in their release and consumption, (ii) the haptophyte *Emiliania huxleyi* has been shown to utilize HMP and its analogue more efficiently than thiamin, and (iii) most aquatic microbes are pyrimidine (thiamin) auxotrophs lacking *thiC*, and other prevalent taxa require intact thiamin (17, 32, 33, 47). Our results show that the *Ec-EG* and *Va-EG* cocultures do not grow, and we attribute this to the inability of THZ to be shared (illustrated in the schematic shown in Fig. 7A and 1D). However, the the mixed cocultures of *VaC-EcG* and *VaE-EcG* cocultures both show growth, which may be a result of the exchange of THZ between these organisms (Fig. 5C and E and 1D and E). Of these, the growth of the *VaE-EcG* coculture was surprising and unexpected based on our previous results, and we reason that there is only one possibility for how these two thiamin auxotroph strains may support one another's growth—*V. anguillarum* Δ*thiE* supplies THZ to *E. coli* Δ*thiG*, which produces thiamin and in turn returns it to *V. anguillarum* Δ*thiE*, enabling it to grow and the coculture to be sustained over 12 days (~300 h) (Fig. 5E and 1D). In the *VaC-EcG* coculture, there are three possibilities, as illustrated in the schematic in Fig. 1E showing the potential exchanges, briefly, (i) *Va*Δ*thiC* → THZ → *Ec*Δ*thiG*, *Ec*Δ*thiG* → thiamin → *Va*Δ*thiC*, (ii) *Va*Δ*thiC* → THZ → *Ec*Δ*thiG*, *Ec*Δ*thiG* →HMP → *Va*Δ*thiC*, or (iii) *Ec*Δ*thiG* → HMP → *Va*Δ*thiC*, *Va*Δ*thiC* → thiamin → *Ec*Δ*thiG*. Based on the observation that the *VaE-EcG* coculture is able to grow, it opens up the possibility for any of these to occur. However, as the OD_{600} of the *VaC-EcG* coculture is significantly higher than that of the *VaE-EcG* coculture, it is likely that the two cocultures have different patterns of exchanges (Fig. 5C and E). Based on this observation, we hypothesize that the *VaC-EcG* coculture may follow possibilities (ii) and (iii), and this needs to be investigated further.

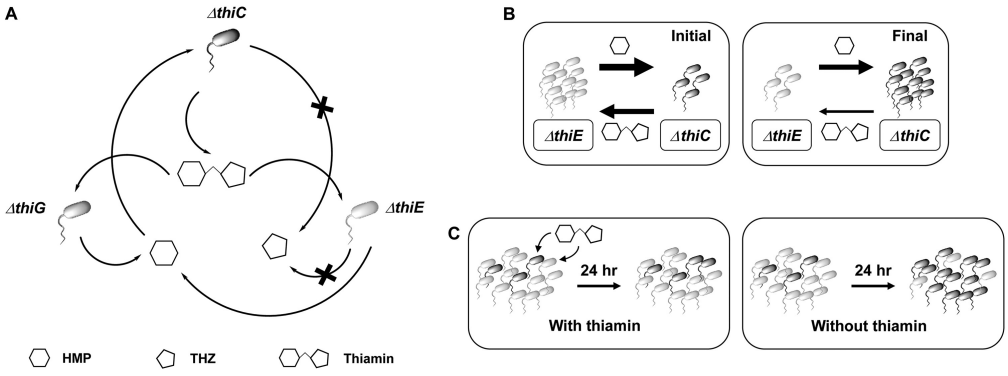

FIG 7 Proposed model for the exchange of thiamin biosynthesis intermediates in the cocultures and effects of the exchange on the coculture dynamics. (A) Proposed molecular exchanges among the thiamin mutants. (B) Model for exchanges in the coculture of the Δ*thiC*-Δ*thiE* strains without thiamin at the initial and final stages (after 24 h) of coculture. The thickness of the arrows is proportional to the amounts of the respective nutrients being released. (C) Ratios of the two strains in the coculture differ based on the presence or absence of thiamin. Black cells, Δ*thiC* strain; gray cells, Δ*thiE* strain.

The unexpected growth of the *VaE-EcG* mixed coculture in thiamin-deficient medium might be explained based on some of the characteristics of the coculture inhabitants. We hypothesize that the cells of *Va*Δ*thiE* lyse owing to the longer incubation time of ~300 h, as opposed to *Ec*Δ*thiE* cells in the *EcE-EcG* cocultures, which are grown for only 24 h. This results in the release of THZ in the medium, a sufficient amount of which then accumulates and is salvaged by the *Ec*Δ*thiG* cells, and thus the *VaE-EcG* coculture survives. But had this been the case, the *Va-EG* coculture, which showed no growth for ~300 h, should have also survived (Fig. 4C). We hypothesize that this inability to grow is because unlike *E. coli,* which harbors the thiazole kinase ThiM, *V. anguillarum* lacks this enzyme and is hence unable to convert exogenous THZ to THZ-P, which is subsequently routed into thiamin biosynthesis (Fig. 1A). This hypothesis is further supported by two additional observations: (i) the *Va*Δ*thiG* strain does not grow when supplemented with up to 100 nM THZ (Fig. 4F), and (ii) the *VaG-EcE* coculture is unable to survive in thiamin-deficient medium (Fig. 5F). It is also possible that instead of or alongside lysis, *V. anguillarum* exports THZ into the medium using yet-unannotated transporters, which enables *Ec*Δ*thiG* to grow in the *VaE-EcG* coculture.

When calculating the ratios of the two strains in the coculture, we noted that the *Ec*Δ*thiC* strain increases in the coculture over time and the ratios of *Ec*Δ*thiC* to *Ec*Δ*thiG* and *Ec*Δ*thiC* to *Ec*Δ*thiE* finally stabilize at ~8:2. The role of the *Ec*Δ*thiG* or *Ec*Δ*thiE* strains in both cocultures is to provide HMP, whereas that of *Ec*Δ*thiC* is to produce thiazole and subsequently thiamin. Let us take the instance of the *Ec-CE* coculture. The *Ec*Δ*thiE* strain (present in a higher amount at the start) produces HMP and supplies it to the *Ec*Δ*thiC* strain. The *Ec*Δ*thiC* strain produces THZ, combines it with the HMP that it obtains, and provides *Ec*Δ*thiE* with thiamin in return. However, since producing THZ appears to be the rate-determining step in thiamin biosynthesis, the numbers of the *Ec*Δ*thiC* strain need to be higher to fulfill the thiamin requirement of the coculture (49). An alternate explanation may be that as the thiamin-producing *Ec*Δ*thiC* strain grows and replicates, it will require more thiamin, and hence, it needs a small but continuous supply of HMP. Thus, just enough thiamin to aid the survival of some *Ec*Δ*thiE* cells to ensure the availability of HMP is released from the *Ec*Δ*thiC* strain in the coculture (Fig. 7B). In conclusion, our results strongly suggest that the strain that produces thiamin or both thiamin and THZ numerically dominates the coculture once a cross talk has been established.

We also observed that when the cocultures of the *E. coli thi* mutants are supplemented with thiamin, the ratios of the two strains in the cocultures do not deviate much from the starting ratios (Fig. 7C). This suggests that when a nutrient is available in plenty in a community of auxotrophs, they may not interact with each other. But when the nutrient is unavailable or scarce, a cross talk that allows for the microorganisms to share it may evolve, which includes enhancing or limiting certain interactions and microbial populations. This subsequently shapes the community composition and relative abundance of its members, and these principles may be utilized to design synthetic microbial cocultures. Indeed, the seasonal blooms of marine microorganisms which either produce or utilize thiamin alter the concentrations of thiamin biosynthesis intermediates in seawater, and when the microbial numbers are low, the overall concentrations of the intermediates remain at an equilibrium (50). Such changes in the community composition have also been reported earlier for synthetic cocultures based on their differential abilities of nutrient exchange or uptake (4, 5, 45).

Finally, we hypothesize that the reason for HMP being exchanged more readily than THZ among auxotrophs is that the biosynthesis of THZ involves more steps and intermediates than the biosynthesis of HMP and is also perhaps metabolically more expensive (49). THZ is assembled by *thiG* (or *THI4* in eukaryotes) using three intermediates, ThiS-thiocarboxylate, dehydroglycine, and 1-deoxyxylulose-5-phosphate (DXP), each of which has intriguing properties. The sulfur which is installed in the THZ ring is transferred from cysteine onto the carboxylate end of ThiS, a small protein synthesized solely for this purpose, to form ThiS-thiocarboxylate (51). Dehydroglycine is unstable under aqueous conditions and needs to be protected in the active site of the enzyme that synthesizes it (52). Finally, DXP, an isoprenoid biosynthesis intermediate, requires thiamin for its own biosynthesis. Also, THI4, the eukaryotic homolog of ThiG, is a single turnover enzyme that itself provides the S atom of the THZ ring that it synthesizes (53). In contrast, HMP is synthesized by a single-step rearrangement of the substrate aminoimidazole ribotide (AIR) by ThiC, an intermediate common to purine, vitamin B_1, and vitamin B_{12} biosynthesis pathways (24, 54, 55). Thus, the biosynthesis of HMP may have a lower metabolic expense than that of THZ, making it easier and less costly in a competitive sense for organisms to share HMP rather than THZ. Interestingly, one study reports that the ratio of the *thiC* gene to the total number of *thiG* and *thi4* genes in marine microbes is in the 0.06 to 0.28 range, always less than 1 (47). Congruently, another study reported higher concentrations of HMP than thiamin in surface waters of the Sargasso Sea and that the abundance of *thiC* genes was lesser than that of the *thiG* genes at depths ranging from 0 to 80 m (33). Even beyond marine ecosystems, there is a propensity for HMP exchange within the human gut microbiome (HGM) as well, wherein out of the 2,228 reference genomes studied, 199 were HMP auxotrophs, whereas only 114 were THZ auxotrophs (17). These studies, taken together with our observations, point to HMP and possibly other pyrimidine intermediates as key nutrients in determining the dynamics of nutrient exchange and subsequently microbial abundance (32, 40, 50).

Our results indicate that the rules of exchange of thiamin and its intermediates are broadly similar across organisms, and variations may be predicted based on growth conditions and the genome sequences of the interacting species. Variation in thiamin-related genotypes is evident among natural populations, but characterizing the behavior of model organisms as done here is expected to aid predictions of exchange among natural communities (33, 47). We also observe temporal changes in the ratios of the *thi* mutants in our cocultures based on the ability of the strains to either make B_1 or a B_1 biosynthesis intermediate, or use exogenously added B_1. Our findings inform the physiology of single microbial members with regard to thiamin metabolism within the context of a microbial community. Finally, our study highlights the nature of interdependencies that arise from relying on acquiring essential metabolites from the environment or from fellow community members.

Conclusions. In this study, we designed a unique coculture system based on the exchange of intermediates derived from a single metabolic pathway, i.e., vitamin B_1 and its biosynthesis intermediates. We conclude that the sharing of vitamin B_1 and its intermediates is modulated by their availability and the presence of biosynthesis, salvage, and transporter proteins in cells.

Exchange forms the basis of building an interacting community of microbes but may also be a feasible mechanism to halt interactions or limit the success of portions of a community, e.g., provision of thiamin rather than HMP to prevent dominance of pyrimidine auxotrophs. Finally, our investigations at the molecular level underscore the specific role of metabolite exchange in determining, stabilizing, and sustaining the collective metabolism and composition of our microbial cocultures.

MATERIALS AND METHODS

Chemicals and reagents. All the chemicals used were obtained either from TCI, HiMedia, or Sigma unless otherwise specified. The enzymes used were obtained from TaKaRa.

Strains and plasmids. The *E. coli* K-12 MG1655 strain containing pKD46 and the plasmids pKD3 and pProEX-Hta were a gift from Nishad Matange at IISER Pune, India. The plasmids pCA24N-*EcthiC*, pCA24N-*EcthiG*, and pCA24N-*EccobT* were obtained from the ASKA collection hosted at IISER Pune, India. The *E. coli* KL-16 strain harboring the *GFPmut2-kan*^{R} cassette was a gift from Deepa Agashe at NCBS, Bangalore, India.

Generating single-gene knockouts in *E. coli*. All the single-knockout mutants of *E. coli* K-12 MG1655 used in the study were generated using recombination by the λ Red recombineering system (42, 56). The primer sequences used for generating the gene knockouts and for their verification are listed in Table S1 in the supplemental material. For generating the strains marked with GFP, we flipped out the *kan*^{r} cassette from the *thi* mutants of *E. coli* K-12 MG1655. We then cloned and inserted the *GFPmut2*::*kan*^{R} cassette from the *E. coli* KL-16 strain into the *thi* mutants, after the *aidB* gene, in the reverse orientation with respect to the *aidB* gene. This gave us the following *E. coli* mutants: *thiC aidB1633*::*GFPmut2-kan*^{R} (*EcΔthiC**), *thiE aidB1633*::*GFPmut2-kan*^{R} (*EcΔthiE**), and *thiG aidB1633*::*GFPmut2-kan*^{R} (*EcΔthiG**). The *GFPmut2-kan*^{R} insertions were carried out using the same λ Red recombineering system mentioned above.

Primary culture setup (LB and P1 cultures) for *E. coli*. *E. coli* K-12 MG1655 wild type (WT) and Δ*thiC*, Δ*thiE*, and Δ*thiG* mutants were grown in LB aerobically at 37°C, 180 rpm, for 6 to 8 h. The cultures were centrifuged at 6,500 rpm for 1 min, and the pellets were washed three times with 1× M9 salts by resuspending them using a vortex for each wash. This step was used to make sure that the cells do not carry over any residual nutrients from LB. These cells were used to start passage 1 (P1) cultures (first subcultures in minimal medium) in M9 medium containing NH_4Cl, glucose, and inosine (50 μM), in 4 mL of medium in 25-mL test tubes, at a starting OD_{600} of 0.05 and were incubated aerobically at 37°C, 180 rpm, for 16 to 18 h. Cells grown in P1 were centrifuged at 6,500 rpm for 1 min, and the pellets were washed three times with 1× M9 salts. For the *thi* mutant rescue experiments, the mutants with or without pProEx-Hta or pCA24N plasmids harboring the genes mentioned were grown similarly in P1, supplemented with or without thiamin (20 μM).

Pairwise coculture setup of *E. coli* mutants in P2. *E. coli* cells washed after P1 were used to start their cocultures in P2 (second subcultures in minimal salts medium with composition as described above) at a starting OD_{600} of 0.1, in a 96-well plate with a lid, with 200 μL medium in each well, and were incubated aerobically at 37°C, with ~240 rpm orbital shaking, for 24 to 96 h, with OD_{600} reading and fluorescence reading at excitation/emission values of 485/535 after every shaking cycle of ~300 s, with the upper lid at a temperature 2°C higher than 37°C (in EnSight) to avoid condensation, inside a plate reader (either Tecan or EnSight, respectively). Alternately, the cells were grown in 25-mL test tubes with 4 mL medium each at 37°C, 180 rpm, in a shaker incubator. The media used were supplemented with various nutrients as and when required at the concentrations mentioned for both the thiamin requirement and the HMP and THZ feeding studies. The two different mutants used for cocultures were inoculated either singly as a control or in their coculture at ratios of 1:9, 1:1, or 9:1. Both the single cultures and the cocultures were inoculated at a starting OD_{600} of 0.1; that is, e.g., for the cocultures with the 1:9 ratio, the two mutants were mixed at a starting OD_{600} of 0.01 and 0.09 of the individual mutants, respectively. When the alternate carbon sources were used, glucose (final concentration in the medium, 22.2 mM) was replaced with 33.3 mM Na succinate or 44.4 mM Na pyruvate, to keep the amount of carbon fed the same for all media.

***V. anguillarum* cultures and experiments.** *Vibrio anguillarum* PF430-3 (57, 58) wild type and Δ*thiC*, Δ*thiE*, and Δ*thiG* mutants (47) were used in experiments. All were reisolated from cryopreserved stock using marine broth agar plates and liquid medium (59) with estuarine surface water from the ModMon Neuse River Estuary monitoring station 180 (60) as the base medium. Cells from liquid ZoBell cultures in late exponential or early stationary phase were washed and centrifuged (9,000 × *g*, 3 min) three times with 1× M9 medium without thiamin and then resuspended in M9 medium without thiamin. Absorbance at 600 nm (i.e., optical density [OD]) was measured using a spectrophotometer (Genesys 30; Thermo Scientific). Based on the OD of resuspended cell cultures, 0.001 OD of washed cells was added (final density) at the start of each experiment.

Cocultures of PF430-3 strains were started by adding 0.001 OD (final concentration) of each strain to M9 medium. Cultures were grown in clear sterile polystyrene tubes and incubated in the dark at 20°C with daily homogenization by repetitive inversion. Thiamin hydrochloride, HMP, and THZ used in experiments were purchased from Fisher Scientific, TCI, and Alfa Aesar, respectively, at ≥98% HPLC purity. Fresh solutions of vitamins were prepared under reduced light in a laminar flow hood with autoclaved MilliQ water as the diluent. Solutions were kept on ice while the experiments were being set up.

E. coli JW5549 Δ*thiG761*::*kan* (Keio Collection) was reisolated as described for PF430-3 but using M9 as the base for ZoBell solid and liquid media. Cells were washed and resuspended in M9 medium without B_1 as for PF430-3. Cocultures of *V. anguillarum* and *E. coli* thi mutants were initiated by adding 0.001 OD of each cell type to M9 medium without amended B_1. Growth conditions were the same as for PF430-3.

Additional methods pertaining to medium and cloning techniques used, the thiochrome assay, and fluorescence versus OD_{600} correlation can be found in the supplemental material.

SUPPLEMENTARY MATERIAL

Supplemental material is available online only.

Supplemental file 1

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