Elucidation of an anaerobic pathway for metabolism of L-carnitine-derived γ-butyrobetaine to trimethylamine in human gut bacteria

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ABSTRACT

Trimethylamine (TMA) is an important gut microbial metabolite strongly associated with human disease. There are prominent gaps in our understanding of how TMA is produced from the essential dietary nutrient L-carnitine, particularly in the anoxic environment of the human gut where oxygen-dependent L-carnitine-metabolizing enzymes are likely inactive. Here, we elucidate the chemical and genetic basis for anaerobic TMA generation from the L-carnitine-derived metabolite γ-butyrobetaine (γbb) by the human gut bacterium Emergencia timonensis. We identify a set of genes upregulated by γbb and demonstrate that the enzymes encoded by the induced γbb utilization (bbu) gene cluster convert γbb to TMA. The key TMA-generating step is catalyzed by a previously unknown type of TMA-lyase enzyme that utilizes a flavin cofactor to catalyze a redox neutral transformation. We identify additional cultured and uncultured host-associated bacteria that possess the bbu gene cluster, providing insights into the distribution of anaerobic γbb metabolism. Lastly, we present genetic, transcriptional, and metabolomic evidence that confirms the relevance of this metabolic pathway in the human gut microbiota. These analyses indicate that the anaerobic pathway is a more substantial contributor to TMA generation from L-carnitine in the human gut than the previously proposed aerobic pathway. The discovery and characterization of the bbu pathway provides the critical missing link in anaerobic metabolism of L-carnitine to TMA, enabling investigation into the connection between this microbial function and human disease.
SIGNIFICANCE

Trimethylamine (TMA) is a disease-associated metabolite produced in the human body exclusively by microbes. Gut microbes generate TMA from essential nutrients consumed in the human diet, including L-carnitine. However, our understanding of the biochemical mechanisms involved in these transformations is incomplete. In this work, we define the biochemical pathway and genetic components in gut bacteria required for anaerobic production of TMA from \( \gamma \)-butyrobetaine, a metabolite derived from L-carnitine. This discovery identifies a new type of TMA-producing enzyme and fills a critical gap in our knowledge of L-carnitine metabolism to TMA in the anaerobic environment of the human gut. This knowledge will enable evaluation of the link between L-carnitine metabolism and human disease, and the design of potential therapeutics.
INTRODUCTION

The human gut microbiota collectively synthesizes an array of small molecule metabolites. The metabolic output of this microbial community varies substantially between individual human subjects, and specific metabolites are strongly associated with health and disease (1-3). In many cases, however, we lack both a molecular understanding of how gut microbial metabolites influence human physiology and how the metabolites themselves are produced. These gaps in knowledge limit our ability to establish causative effects of microbial metabolites in human disease and to develop microbiota-based strategies to improve human health. Identification of the specific organisms, genes, and enzymes responsible for metabolite production is needed to accurately profile specific metabolic functions in microbial communities, to experimentally investigate links to disease, and to modulate the metabolic output of the gut microbiota.

Trimethylamine (TMA) is a gut microbial metabolite that has been strongly associated with human disease. It is derived from gut microbial transformations of dietary nutrients including phosphatidylcholine, choline, L-carnitine, betaine, and trimethylamine N-oxide (TMAO) (4-9). Microbially produced TMA is absorbed by the host in the gastrointestinal tract, enters hepatic circulation, and is oxidized to TMAO in the liver by the flavin-dependent monooxygenase FMO3 (10). Genetic mutations in the human FMO3 gene lead to accumulation of TMA in the body, causing the metabolic disorder trimethylaminuria or fish malodor syndrome (11). In addition, elevated plasma levels of TMA and TMAO have been associated with multiple human diseases, including cardiovascular, chronic kidney, and non-alcoholic fatty liver diseases, obesity and type II diabetes, and colorectal cancer (12). Especially strong correlations between TMAO and its precursors have been demonstrated for cardiovascular disease. Elevated plasma levels of dietary TMA precursors were also associated with disease risk, but only when co-occurring with elevated
TMAO levels (6, 7, 9). Furthermore, administration of the TMA precursors phosphatidylcholine, choline, and carnitine to atherosclerosis-prone mice resulted in development of atherosclerotic plaques in a gut microbiota-dependent fashion (6, 7). Direct oral administration of TMAO in these mice similarly resulted in phenotypes of atherosclerosis (6). These observations suggest a causal role for TMAO in animal models of cardiovascular disease and that gut microbial metabolism is a crucial factor contributing to pathogenesis. However, a causative role of TMA or TMAO in the development or exacerbation of complex diseases has not yet been definitively established in humans. Deciphering the contribution of TMA production to human disease clearly necessitates a better understanding of the gut microbial metabolic pathways that generate this small molecule.

L-Carnitine is an important dietary precursor to TMA, and its metabolism by gut microbes is associated with cardiovascular disease (13). An essential nutrient for the host, L-carnitine plays a key role in fatty acid β-oxidation by transporting fatty acids across the mitochondrial membrane for metabolism (5, 14). Although it is produced endogenously, humans must uptake additional L-carnitine in the diet to support cellular function (5, 14). The major sources of L-carnitine are animal-based products, especially red meat (15), but it is also ingested as a supplement for enhanced physical performance (16, 17). Early studies of L-carnitine metabolism in rats and human subjects demonstrated its conversion to TMA, the former showing dependence on the gut microbiota (18, 19). More recent studies in humans confirmed the involvement of the gut microbiota in this transformation (7). These studies also noted accumulation of an intermediate metabolite identified as γ-butyrobetaine (γbb) that was also produced by the gut microbiota (18-20). Furthermore, γbb was shown to be a pro-atherogenic metabolite in mouse models like its precursor L-carnitine (20). The well-characterized metabolic pathway that converts L-carnitine to γbb is encoded by the cai gene operon (Figure 1) and is used during anaerobic respiration by
facultative anaerobic Proteobacteria such as *Escherichia coli*, *Salmonella typhimurium*, and *Proteus mirabilis* (5). The microbial genes and enzymes that are responsible for generating TMA from L-carnitine-derived γbb, however, are not fully elucidated.

Specifically, there is a significant gap in our understanding of the molecular basis for TMA production from L-carnitine and γbb precursors under anaerobic conditions. Select facultative anaerobic Proteobacteria and Actinobacteria possess an iron-dependent Rieske-type monooxygenase (CntA) that can directly convert carnitine to TMA (20-22). This enzyme uses dioxygen to hydroxylate L-carnitine at the C4 position, followed by non-enzymatic formation of an aldehyde through elimination of TMA (Figure 1). Whereas L-carnitine was the only substrate tested for activity with CntA from *Acinetobacter baumannii* (21), the *E. coli* homolog, also known as Yeaw (71% amino acid identity), was shown to produce low levels of TMA from both L-carnitine and γbb (20). Although CntA activity was originally proposed to represent the major mechanism for conversion of L-carnitine to TMA by the human gut microbiota, this conclusion has been called into question. This enzyme strictly requires dioxygen for catalysis, however, dioxygen levels in the colon lumen are <1 mm Hg (23). In addition, a study in humans showed that plasma TMAO levels after a carnitine challenge were not correlated with *cntA* gene abundance in gut microbiomes (24). Notably, TMA production from L-carnitine and γbb was demonstrated in anaerobic ex vivo incubations of mice cecal and colon tissues (20). Finally, the human gut isolate *Emergencia timonensis*, an obligate anaerobe that does not encode a CntA homolog, was recently found to metabolize γbb to TMA under strictly anaerobic growth conditions (13). Collectively, this information indicates the existence of an as-yet-uncharacterized anaerobic pathway for γbb metabolism in the human gut microbiota.
We present here the identification and characterization of the metabolic pathway, genes, and enzymes responsible for anaerobic TMA production from γbb in *E. timonensis*. The enzyme catalyzing the key C–N bond cleavage reaction that generates TMA is a flavin-dependent, acyl-CoA dehydrogenase-like enzyme that uses the activated CoA thioester of γbb as its substrate. This chemically challenging reaction generates the intermediate crotonyl-CoA, which is further metabolized by *E. timonensis* for anaerobic respiration and as a source of carbon and energy. Homologous gene clusters for γbb metabolism are present in other cultured and uncultured host-associated bacteria from the Clostridiales order. We find that anaerobic γbb metabolism is prevalent in human gut microbiomes and is likely a major, underappreciated contributor of L-carnitine-derived TMA. Together, this work expands our knowledge of TMA-producing enzymes, pathways, and organisms, providing a more complete understanding of microbial TMA production in the anoxic human gut. These findings identify potential new targets for manipulation of this microbial function and will help resolve the major dietary and microbial contributors to TMA production.
Figure 1. Anaerobic and aerobic bacterial metabolic pathways producing TMA from L-carnitine.

RESULTS

γ-Butyrobetaine induces expression of a candidate gene cluster in E. timonensis SN18.

Only a single cultured bacterium, a human fecal isolate of E. timonensis, has been reported to produce TMA from γbb under anaerobic conditions (13). We began our efforts to identify the genes and enzymes responsible for γbb metabolism by testing the type strain E. timonensis SN18 for this activity. Indeed, anaerobic cultures of E. timonensis SN18 supplemented with γbb demonstrated complete consumption of this substrate and production of a stoichiometric amount of TMA (Figure S1). When the cultures were supplemented with [N-(CD₃)₃]-γbb, deuterium-labeled TMA was detected by liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Figure S1), confirming its origin from γbb. Resting cell suspensions of E. timonensis SN18 also fully converted γbb to TMA (Figure 2A). However, this activity was only observed when the cells had been cultured in medium containing γbb; resting cell suspensions of E. timonensis SN18 cultured in the absence of γbb were unable to consume γbb and did not generate TMA (Figure 2A). Together, these results suggest that γbb induces expression of the genes involved in its metabolism.

We also evaluated the ability of E. timonensis SN18 to metabolize DL-carnitine and γ-aminobutyric acid (GABA), close structural homologs of γbb. Neither of these substrates was consumed during growth (Figure S1) nor in resting cell suspensions of cultures supplemented with γbb (Figure S1). Next, we tested whether these substrate analogs could induce expression of the γbb metabolic enzymes. Resting cell suspensions of cultures supplemented with GABA did not exhibit γbb metabolism; however, resting cell suspensions from cultures supplemented with DL-
carnitine did convert γbb to TMA (Figure 2A). Thus, while DL-carnitine appears to induce expression of the genes responsible for γbb metabolism in *E. timonensis*, the metabolic enzymes are unable to use DL-carnitine as a substrate for TMA generation.

The inducible metabolism of γbb in *E. timonensis* SN18 suggested that the genes involved in this pathway could be discovered using RNA-sequencing (RNA-seq). Only two sets of genes were both highly and differentially expressed in γbb-induced cultures compared to control cultures grown in the absence of γbb (Figure 2B, Dataset S1). As expected from our previous cell-based activity experiments, expression of these genes was also upregulated in cells grown with DL-carnitine, but not with GABA (Figure S2, Dataset S1). The most differentially upregulated genes are colocalized in the *E. timonensis* SN18 genome within a region we designate the γ-butyrobetaine utilization (bbu) gene cluster (Figure 2C). This six-gene cluster encodes a predicted flavin-dependent acyl-CoA dehydrogenase (bbuA), two class III CoA-transferases (bbuB and bbuC), a di-[4Fe-4S]-cluster ferredoxin (bbuD), a betaine/carnitine/choline transporter (bbuE), and an acyl-CoA thioesterase (bbuF). The second set of genes upregulated in γbb-induced cells encodes for predicted riboflavrin biosynthesis enzymes (Dataset S1), which likely support cofactor biosynthesis for the enzyme encoded by bbuA. The highly specific transcriptional response to γbb suggested that the *bbu* gene cluster was involved in γbb metabolism. In addition, the presence of a gene encoding a transporter for trimethylammonium-containing compounds (bbuE) was consistent with this hypothesis.
Figure 2. γbb induces expression of the bbu gene cluster in E. timonensis SN18. (A) Stacked bar plots of γbb and TMA concentrations after a 3 h incubation of 1 mM γbb in resting cell suspensions of E. timonensis SN18 previously cultured in media supplemented with 1 mM γbb, DL-carnitine, GABA, or 1× PBS (vehicle). Error bars represent the standard deviation from the mean of three biological replicates. (B) Differential gene expression from E. timonensis SN18 cultures supplemented with γbb or treated with a vehicle at an OD = 0.7, plotted against the mean of normalized counts from three biological replicates. The two sets of differentially upregulated genes are circled in yellow. Genes with an adjusted p-value >0.01 (Wald test) comparing γbb- and vehicle-induced cells are represented by grey circles. (C) The bbu gene cluster and automated protein function annotations.

The CoA transferases BbuB/C initiate γbb metabolism via formation of a γbb-CoA thioester.

Based on the predicted annotations of the bbu genes, we hypothesized that γbb would be initially activated for subsequent TMA elimination through formation of a CoA thioester. The conversion of a carboxylic acid to a thioester is a common first step in many metabolic pathways as it lowers the pKₐ of the Cα-protons, facilitating further reactions (25, 26). Indeed, in resting cell suspensions incubated with γbb, a metabolite was detected by LC–MS with a peak retention time and mass-to-charge ratio (895.5 m/z, [M+H]⁺) matching those of a γbb-CoA standard (Figure 3A, Figure S3). The origin of this metabolite was confirmed by incubating cell suspensions with deuterium-labeled 2,2,3,3,4,4-D₆-γbb (D₆-γbb), which led to an increase of 6 Da (901.5 m/z,
[M+H]$^+$ for the assigned γbb-CoA peak (Figure 3A, Figure S3). These results support the hypothesis that γbb metabolism proceeds via a CoA thioester intermediate.

We next investigated whether two CoA transferases encoded by the bbu gene cluster, BbuB and BbuC, were directly involved in γbb-CoA production. Indeed, γbb-CoA was detected from incubations of γbb with crude lysate of E. coli cells constitutively expressing the first three genes of the cluster (bbuABC) (Figure 3B). Interestingly, γbb-CoA was not detected when either of the two CoA transferase genes was expressed alone (Figure 3B). Production of γbb-CoA was also observed when recombinant, purified BbuB and BbuC were added together with γbb to lysate of E. coli transformed with empty vector (Figure S3), but only when both proteins were present. The requirement for both CoA transferase proteins was confirmed in vitro, suggesting that the two proteins may form an active complex. In these experiments, the BbuB and BbuC proteins together generated γbb-CoA from γbb and an appropriate acyl-CoA donor substrate (Figure 3C). Various short-chain fatty acyl-CoAs (acetyl-, butyryl-, propionyl-, and crotonyl-CoA) were tested as CoA-donating substrates in the reaction, and while all compounds resulted in production of γbb-CoA, acetyl-CoA was the preferred co-substrate (Figure S3). We also tested dL-carnitine and GABA as alternative CoA acceptors in vitro with BbuB/C and acetyl-CoA as the donor. However, neither of the presumed products, carnitinyll-CoA or γ-aminobutyryl-CoA, was detected by LC–MS/MS after a 3 h incubation (Figure S3). The high selectivity of the CoA transferases BbuB and BbuC for γbb directly connects the bbu gene cluster to γbb metabolism.
Figure 3. γbb-CoA is the first intermediate in γbb metabolism. (A) Chemical reaction catalyzed by the BbuB and BbuC proteins. (B) LC–MS extracted ion chromatograms (EIC) of γbb-CoA ([M+H]^+ = 895.5 m/z) and D₆-γbb-CoA ([M+H]^+ = 905.5 m/z) from extracts of E. timonensis cell suspensions incubated for 40 min with γbb (black) or D₆-γbb (red), respectively, compared to a γbb-CoA standard (blue). (C) LC–MS/MS selected ion chromatograms (SIC) of the 136.0 m/z fragment ion of γbb-CoA produced from a 1 h incubation of γbb and acetyl-CoA with crude lysate of E. coli constitutively expressing bbu genes or empty vector. (D) LC–MS/MS SIC of the 136.0 m/z fragment ion of γbb-CoA produced from a 1 h incubation of γbb and acetyl-CoA with purified recombinant proteins. Figure S3 shows relative amounts of γbb-CoA from three biological replicates of experiments described for panels C and D.

The flavoenzyme BbuA catalyzes elimination of TMA from γbb-CoA.

The key step in TMA production from γbb is breaking an unactivated C–N bond. None of the bbu genes is predicted to encode homologs of known enzymes that catalyze C–N bond cleavage and release TMA (e.g. GRE choline TMA-lyase (27), glycine betaine reductase (28), ergothionase (29)), indicating that this pathway uses a distinct enzyme for this critical step. We identified the predicted acyl-CoA dehydrogenase-like enzyme BbuA as the most likely candidate for this reaction. Enzymes belonging to the acyl-CoA dehydrogenase family are flavin-dependent oxidoreductases that act on acyl-CoA thioester substrates, typically installing or removing an α,β-unsaturation through two-electron oxidation or reduction reactions, respectively (30). We envisioned that BbuA might instead use its flavin cofactor in a cryptic radical mechanism to
activate γbb-CoA for TMA elimination from the C4 position. This proposed reaction resembles the C4 elimination of water from 4-hydroxybutyryl-CoA catalyzed by the FAD-dependent enzyme 4-hydroxybutyryl-CoA dehydratase (4HBD) involved in bacterial GABA and succinate metabolism (31). While BbuA and 4HBD are predicted to have a similar structure and flavin cofactor, they share very minimal sequence identity (~10%).

Initial attempts to access soluble BbuA protein via heterologous expression in *E. coli* were unsuccessful. Soluble BbuA protein was only obtained under the following conditions: 1) co-expression with the native GroEL-ES proteins from *E. timonensis* SN18, 2) addition of exogenous riboflavin to the growth medium, and 3) addition of excess FAD during cell lysis. Crude lysates of *E. coli* cells over-expressing the *bbuABC* genes under these conditions demonstrated TMA production when incubated anaerobically with γbb and acetyl-CoA (*Figure S4*). Purified BbuA was then tested for activity *in vitro* when incubated with γbb, acetyl-CoA, and the CoA transferases BbuBC. Robust TMA production was observed after a 1 h incubation without redox mediators (*Figure 4B*), demonstrating that these reaction components are sufficient to catalyze multiple turnovers of γbb. Finally, γbb-CoA was tested directly as the substrate for BbuA activity *in vitro*. This reaction also resulted in TMA production (*Figure 4C*). These results conclusively demonstrate that BbuA is the critical TMA-generating enzyme in anaerobic γbb metabolism.

Next, we sought to determine the co-product of the BbuA-catalyzed reaction. We predicted that crotonyl-CoA would be generated upon elimination of TMA from γbb-CoA by analogy to 4-hydroxybutyryl-CoA dehydration in GABA/succinate metabolism (31). Indeed, crotonyl-CoA was detected by LC–MS/MS in both the *E. coli* crude lyse incubations (*Figure S4*) and the *in vitro* assays with purified BbuA (*Figure 4*). Both the retention time and *m/z* of the detected metabolite were consistent with a crotonyl-CoA standard (*Figure S4*). The production of crotonyl-CoA
supports the proposed redox-neutral TMA elimination from γbb-CoA (**Figure 4**). Together, the gain-of-function experiments in *E. coli* and *in vitro* activity assays demonstrate that the *bbu* gene cluster is responsible for TMA production from γbb, and that the three genes *bbuABC* are necessary and sufficient to confer this function in *E. coli* and *in vitro*.

![Diagram](image)

**Figure 4.** The FAD-dependent enzyme BbuA generates TMA. (A) Chemical reaction catalyzed by BbuA. (B) LC–MS/MS SIC of the precursor-fragment ion pairs of TMA (left) and crotonyl-CoA (right) produced from 1 h reactions containing γbb, acetyl-CoA, BbuB, BbuC, and FAD with or without addition of BbuA. (C) LC–MS/MS SIC of the precursor-fragment ion pairs of TMA (left) and crotonyl-CoA (right) produced from 1 h reactions containing γbb-CoA and FAD with or without addition of BbuA. **Figure S4** shows quantification of TMA concentration and relative amounts of crotonyl-CoA from three biological replicates of experiments described for panels B and C.

*E. timonensis* metabolizes γbb for anaerobic respiration and carbon acquisition.

Although crotonyl-CoA was produced in lysate and *in vitro* reactions containing BbuA, crotonyl-CoA did not accumulate in resting cell suspensions of *E. timonensis* SN18 incubated with γbb. Instead, we detected the known products of bacterial crotonyl-CoA metabolism: 3-hydroxybutyryl-CoA, acetyl-CoA, and butyryl-CoA (**Figure 5B**). Inspection of their mass spectra
showed deuterium incorporation when D_6-γbb was used, confirming their origin from γbb (Figure S5). These results suggest that crotonyl-CoA generated from γbb is further metabolized by E. timonensis. Notably, the bbu gene cluster does not encode any enzymes known to be involved in crotonyl-CoA metabolism (32). However, protein homologs are encoded by constitutively expressed genes located elsewhere in the E. timonensis SN18 genome (Dataset S2). To test whether they could account for the observed crotonyl-CoA metabolism, crotonyl-CoA was incubated with cell lysates of E. timonensis SN18 grown in the presence or absence of γbb. In both cases, crotonyl-CoA was converted to 3-hydroxybutyryl-CoA during an hour incubation (Figure S6). Longer incubations led to accumulation of acetyl-CoA and low levels of butyryl-CoA (Figure S6). These results demonstrate that the final steps of γbb metabolism in E. timonensis SN18 are known transformations of crotonyl-CoA, catalyzed by constitutively produced enzymes encoded outside of the bbu gene cluster (Figure 5A).

Some species of Clostridia can use crotonyl-CoA metabolism for anaerobic respiration via reduction to butyryl-CoA, and as a carbon and energy source through conversion to acetyl-CoA (Figure 5A) (33, 34). We examined whether γbb metabolism via crotonyl-CoA would enhance the growth of E. timonensis SN18 in media conditions with limited electron acceptors or carbon sources. In both culture conditions, the growth yield was markedly enhanced with γbb supplementation (Figure 5C, 5D) and the growth rate was consistent with the rates of γbb depletion and TMA production (Figure S7). These results support a key role for crotonyl-CoA as an intermediate in γbb metabolism and indicate a physiological role for this activity in E. timonensis.

Due to its prominent roles in the human gut, we next investigated whether free butyrate was produced from E. timonensis γbb metabolism. Using a carboxylate-derivatization method and
LC–MS, elevated levels of butyrate were detected in suspensions of γbb-induced cells incubated with γbb compared to suspensions of non-induced cells (Figure 5E, Figure S8). In addition, deuterium incorporation into butyrate was observed when D₆-γbb was used (Figure 5F). Analysis of the mass spectrum revealed that the D₆-isotopolog was not present; rather the +4 Da species was the primary isotopolog detected (Figure 5F, Figure S8). The loss of two deuteria in the butyrate product is consistent with a crotonyl-CoA precursor. To rule out the possibility that the loss of label resulted from exchange of the acidic Cα-deuterons with protons from solution, a control experiment was performed incubating perdeuterated D₇-butyrate with cell suspensions. The mass spectrum of butyrate from this experiment showed minimal deuterium exchange with solvent protons during the same incubation period (Figure S8). In addition, γbb-CoA detected from incubations with D₆-γbb showed minimal deuterium loss (Figure S8). These control reactions support the proposal that γbb metabolism in E. timonensis proceeds through the α,β-unsaturated crotonyl-CoA intermediate and generates free butyrate, an important metabolite in the human gut environment.

Figure 5. γbb-derived crotonyl-CoA is further metabolized by E. timonensis for carbon and respiration. (A) Complete metabolic pathway using γbb as a carbon source and electron acceptor.
(B) LC–MS/MS SICs of parent ions with 136.0 m/z fragments that correspond to expected products of crotonyl-CoA metabolism from extracts of *E. timonensis* SN18 cell suspensions incubated with γbb for 40 min. Chromatogram magnification is indicated next to each trace. (C-D) Anaerobic growth curves of *E. timonensis* SN18 in minimal media lacking (E) carbon sources or (F) electron acceptors with (red) or without (black) γbb. Error bars represent standard deviation from the mean of three biological replicates. (E) Concentrations of derivatized butyrate detected from extracts of *E. timonensis* cell suspensions incubated for 0 (pink) or 40 (red) min in the presence or absence of 1 mM γbb. Bars show the average of two biological replicates. (F) Mass spectra of derivatized extracts (unlabeled butyrate [M+H]+ = 222 m/z) of *E. timonensis* cell suspensions incubated for 40 min with γbb (black) or D6-γbb (red).

The *bbu* gene cluster predicts γbb metabolism in bacterial isolates.

As noted previously, *E. timonensis* is the only bacterial species known to metabolize γbb to TMA under anaerobic conditions. Having discovered the gene cluster responsible for this activity, we searched for homologous gene clusters in other sequenced bacterial genomes. We identified *bbu*-like gene clusters in four host-associated, cultured bacteria belonging to the order Clostridiales. To examine whether the *bbu* genes are diagnostic of γbb metabolism, two commercially available strains, the human oral isolate *Eubacterium minutum* ATCC 700079 and the feline gut isolate *Agathobaculum desmolans* ATCC 43058 (previously *Eubacterium desmolans* or *Butyricoccus desmolans*), were tested for this activity. Both strains converted γbb to TMA in anaerobic cultures (Figure 6A), demonstrating that the presence of the *bbu* gene cluster predicts the capacity for anaerobic γbb metabolism. The rarity of this gene cluster in sequenced genomes of cultured bacteria motivated us to search uncultured bacterial genomes that had been assembled from human metagenomes (*i.e.* metagenome-assembled genomes, MAGs) (35, 36). We identified homologous *bbu* gene clusters in 11 uncultured, human-associated MAGs, all of which belong to the order Clostridiales (Figure 6B, Dataset S3). Two of the uncultured bacterial genomes were assembled from human oral samples, while the remaining genomes harboring the *bbu* gene cluster were assembled from human stool samples. The presence of the *bbu* gene cluster in multiple
uncultured gut bacteria suggests that γbb metabolism is underrepresented among cultured isolates and that *E. timonensis* may not be solely responsible for this metabolism in the human gut. Finally, we searched for the presence of the *cai* genes responsible for metabolizing L-carnitine to γbb in these *bbu*-containing bacterial genomes. However, none of these genomes possess homologs of known *cai* genes, indicating that these γbb-metabolizing bacteria are incapable of the full conversion of L-carnitine to TMA.

**Figure 6.** Cultured and uncultured host-associated Clostridiales bacteria possess *bbu*-like gene clusters indicative of γbb metabolism. (A) TMA and γbb detected by LC–MS/MS from pure cultures of *A. desmolans* and *E. minutum* grown in rich media supplemented with γbb. Error bars represent the standard deviation from the mean of three biological replicates. (B) BbuA clade of a maximum likelihood phylogenetic tree (Figure Sx) and genomic context of BbuA homologs. aBayes branch supports are shaded based on the legend. Red labels indicate experimentally validated γbb-metabolizing bacteria. Blue branches indicate an isolate or MAG originating from an oral sample.

**Presence of bbu genes in human gut metagenomes is correlated with lower γbb levels.**

Motivated by the presence of the *bbu* gene cluster in uncultured gut bacteria and the implications for γbb metabolism in human health, we assessed the prevalence of the *bbu* gene cluster in publicly available human metagenomic datasets. Our analysis used the *bbuA* gene as a
representative of the full cluster in stool metagenomes from healthy adult populations. We detected hits for the \textit{bbuA} gene in samples from every cohort, but the proportion of \textit{bbuA}-positive samples varied substantially between cohorts, ranging from 15–85% positive (\textbf{Figure 7A}). We also quantified the abundance of the \textit{bbuA} gene in \textit{bbuA}-positive samples and found that it was consistent across cohorts at 1:1000 genes per microbial genome, with a range of 1:10^2 to 1:10^4 genes per microbial genome (\textbf{Figure S9}). Since diet has been reported as an important factor in TMA production capacity of the microbiota, we examined whether \textit{bbuA} gene presence or abundance was correlated with diet. However, neither \textit{bbuA} gene presence nor abundance was correlated with self-reported omnivorous or vegetarian donors from the BIO-ML cohort (\textbf{Figure S10}). Overall, the distribution profile of \textit{bbu} genes suggests that \textgamma{bb} metabolizing bacteria are frequently present in the human gut microbiota but are low-abundance members of the community.

To provide further support for the relevance of the \textit{bbu} pathway in the human gut, we evaluated gene expression using metatranscriptomic data from three cohorts – HMP2 (37), iHMP (38), and MLVS (39). We detected \textit{bbuA} transcripts in 27–56% of samples in these cohorts (\textbf{Figure 7B}), suggesting that the \textit{bbu} pathway is expressed in the human gut. Transcripts of the \textit{bbuA} gene were detected in samples that are positive for the \textit{bbuA} gene, but also a subset (16–26%) of \textit{bbuA}-negative samples (\textbf{Figure S11}). This observation suggests that the \textit{bbuA} gene abundance was too low to be detected by the metagenomic sequencing, but that the transcript levels were high enough to be captured by the read depth of the metatranscriptomic sequencing. Notably, very few samples in these cohorts (0–4%) were positive for \textit{cntA} transcripts (\textbf{Figure 7B, Figure S11}), consistent with previously reported analyses (40). Thus, the metatranscriptomic data suggest that the \textit{bbu} pathway is more likely to be functionally operant in the human gut than the \textit{O_2}-dependent pathway mediated by \textit{cntA}.  

Next, we examined three cohorts with corresponding stool metabolomic data (HMP2 (37), BIO-ML (41), and PRISM (42)) to evaluate whether the presence of bbuA gene or transcripts is associated with changes in metabolite levels. In each of the three independent cohorts, the levels of γbb were lower in samples that were positive for the bbuA gene compared to those that were negative (Figure 7C, Figure S12). In the HMP2 cohort, the samples positive for bbuA transcripts were also correlated with lower γbb levels (Figure S13). The bbuA gene, however, was not associated with stool carnitine or butyrate levels (Figure S14). In contrast to bbuA, the presence of the cntA gene was correlated with higher or equivalent γbb levels compared to cntA-negative samples rather than lower (Figure 6C, Figure S12). These results suggest that the bbu gene cluster may be primarily responsible for γbb consumption in the human gut.

Finally, we evaluated whether differences in the presence or abundance of the bbuA gene are correlated with human disease. Although TMA production is not known to be a factor in inflammatory bowel disease (IBD), we noticed that the bbuA gene was detected in fewer samples from donors with Crohn’s disease and ulcerative colitis compared to healthy controls in both HMP2 (37) and PRISM (42) cohorts (Figure 7D, Figure S15). The same trend was observed when analyzing bbuA transcripts in the HMP2 study (Figure 7D). However, there was no difference in gene abundance between the IBD and healthy cohorts (Figure S15). Next, we analyzed metagenomes of a cardiovascular disease (CVD) cohort from China (43). The proportion of bbuA-positive samples was not different between healthy and CVD groups (Figure 7E), but the gene abundance in bbuA-positive samples was modestly higher in CVD donors compared to healthy controls (Figure 7F). A more significant elevation was found in the caiA gene abundance in the CVD cohort compared to the healthy group (Figure 7F), suggesting that the conversion of L-carnitine to γbb may be a more relevant biomarker for CVD than the bbu gene cluster. Consistent
with previous reports (40), we also detected a higher prevalence and abundance of the cntA gene in CVD samples compared with healthy controls from this study (Figure 7E-F). However, the lack of correlation between cntA gene presence and expression observed in other cohorts raises questions about the functional relevance of this finding.

**Figure 7.** Anaerobic γbb metabolism genes are widely distributed and expressed in the human gut microbiota. (A) Fraction of stool metagenomes that are positive (red) and negative (grey) for the bbuA gene from healthy donors in human studies (Dataset S4). Study sample size (N) is indicated above each bar. Asterisks indicate that only healthy donors from those projects were included in this analysis. (B) Fraction of stool metatranscriptomes that are positive and negative for bbuA or cntA. Study sample size (N) is indicated above each bar. (C) Correlation between the presence of bbuA or cntA genes in metagenomes and γbb metabolite levels in HMP2 stool samples. Mean values are represented by yellow diamonds and p-values were determined using the Mann-Whitney U-test. (D) Fraction of samples positive for the bbuA gene or transcripts in HMP2 IBD cohorts compared to healthy controls. Study sample size (N) is indicated above each bar. (E) Fraction of samples positive for the bbuA or cntA gene in a CVD cohort compared to healthy controls. Study sample size (N) is indicated above each bar. (F) Correlations between the presence of the bbuA, caiA, or cntA genes and CVD compared to healthy controls. Mean values are represented by yellow diamonds and p-values were determined using the Mann-Whitney U-test.
DISCUSSION

Gut microbial production of TMA from dietary L-carnitine has been recognized for decades, but our knowledge of the reactions, enzymes, and organisms involved in the O₂-independent pathway has remained incomplete. We used RNA-seq to identify a substrate-inducible bbu gene cluster that is responsible for anaerobic γbb metabolism in the gut microbe *E. timonensis*. We demonstrated that the enzymes encoded within the bbu gene cluster are necessary and sufficient to convert γbb to TMA. With the discovery of the genetic basis for anaerobic γbb metabolism, we then determined the chemical steps involved, identified other gut microbes with this function, and evaluated the relevance of this microbial activity in human populations.

The pathway for anaerobic metabolism of γbb provides an interesting parallel to GABA metabolism by gut microbes (44). Given the structural similarity of the two molecules, which differ by only the N-methylation of γbb, the use of comparable pathways for their catabolism is perhaps not surprising. Analogous chemical steps, including substrate activation via CoA formation and C4 elimination, occur in both pathways to yield the common product crotonyl-CoA. This intermediate is then processed by the bacteria as a carbon and energy source or as an electron acceptor. Yet there are notable distinctions in the substrate activation steps and key lyase enzymes of each pathway. Conversion of the carboxylate to the thioester lowers the pKₐ of the Cα-protons (25, 26), facilitating elimination of the C4 hydroxyl or trimethylammonium group in the 4HBD- or BbuA-catalyzed reaction, respectively. Due to its positive charge, the trimethylammonium moiety of γbb-CoA is a good leaving group for C4 elimination. At physiological pH, the primary amine of GABA would also be protonated and positively charged (45), which could enable an analogous direct elimination from a hypothetical γ-aminobutyryl-CoA intermediate. Interestingly, GABA-metabolizing bacteria instead convert the amine into a hydroxyl group, which is a worse
leaving group, prior to formation of the CoA thioester and C4 elimination. This biochemical pathway may be the result of repurposing the 4HBD enzyme from succinate metabolism in bacteria and archaea (46-48), which proceeds via a 4-hydroxybutyryl-CoA intermediate. The flavin-dependent lyase enzymes, 4HBD and BbuA, that catalyze the key C4 elimination step in these pathways both belong to the broad structural superfamily of acyl-CoA dehydrogenases (30). This generic functional annotation implies two-electron redox chemistry using a flavin cofactor. We hypothesize instead that BbuA may use a radical-based mechanism similar to that proposed for 4HBD (49), wherein the flavin cofactor enables single-electron chemistry to mediate TMA elimination. Interestingly, however, these two enzymes do not appear to be closely related homologs based on the negligible sequence identity to one another. Therefore, if 4HBD and BbuA use similar mechanistic strategies, these enzymes may be an interesting example of convergent evolution to achieve a chemically challenging elimination reaction. The mechanism of the TMA-lyase reaction catalyzed by BbuA will be the subject of future studies.

The BbuA enzymatic reaction is unique compared to other known TMA-producing enzymes. The l-carnitine monooxygenase CntA uses a metallocofactor to activate dioxygen for substrate hydroxylation, generating a chemically unstable product that breaks down to eliminate TMA. Conversely, BbuA directly catalyzes the C–N bond cleavage step. The enzyme glycine betaine reductase catalyzes an overall two-electron reduction of glycine betaine to produce TMA, employing protein-substrate covalent adducts via (seleno)cysteine residues (28). In contrast, BbuA catalyzes a redox-neutral transformation and uses an organic flavin cofactor. The choline TMA-lyase CutC is a glycyl radical enzyme which uses protein-based radicals to initiate catalysis (27). CutC is proposed to generate a carbon-centered substrate radical intermediate that undergoes a spin center shift and promotes direct 1,2 elimination of TMA (50). The BbuA enzyme could share
a similar radical-based mechanistic logic with CutC but, instead of protein-based radicals, uses a flavin cofactor for radical generation. However, the chemical differences between the γbb and choline substrates – the unactivated carbon chain attached to the trimethylammonium in γbb compared to the vicinal hydroxyl group in choline that actively participates in TMA elimination – will necessitate mechanistic divergence. Intriguingly, the mechanism employed by CutC could theoretically operate using L-carnitine as the substrate, as it also has a vicinal alcohol, yet such a hypothetical reaction is not known to exist.

The metabolic pathways that produce TMA from both choline and γbb are used by gut bacteria to access a source of carbon and energy; in addition, γbb metabolism is a mode of anaerobic respiration. This latter pathway is yet another example of reductive chemistry performed by microbes in the anaerobic gut environment. The product of γbb respiration is the short-chain fatty acid butyrate, which has many established roles in host-microbiota interactions (51). It is a major source of energy for intestinal epithelial cells (52) and is an important signaling molecule as a GPCR ligand (51). The physiological roles of butyrate generally promote host health, for example, by enhancing gut barrier function and reducing inflammation (51). Therefore, the juxtaposition of butyrate and TMA production from γbb highlights the complexity of microbial functions in the context of the human host and the need to fully understand the chemistry underlying metabolite production in order to evaluate the potential impact.

Anaerobic γbb metabolism is a microbial function found in only a select group of human gut bacteria. Prior to this work, E. timonensis was the only organism known to perform this transformation and, correspondingly, was found in high abundance in high-TMAO producers from a human carnitine-challenge study (53). However, E. timonensis was only detected in 25% of individuals from that group (53), suggesting that additional gut bacteria possess this metabolic
activity. Indeed, we have discovered that the bbu gene cluster is present in other related gut bacteria. The bbu gene cluster was found exclusively in obligate anaerobic bacteria of the Clostridiales order, but it is not ubiquitous within this taxonomic group. This observation contrasts with choline metabolism, which is widely distributed across different gut microbial phyla and classes (54). In addition, the bbu gene cluster is only found in host-associated bacteria. While most bbu-encoding bacteria were isolated from human stool, a few bacteria originated from the human oral cavity. Bacteria that inhabit the gastrointestinal tract of other animals (i.e., cat and fish) were also found to possess bbu gene clusters, suggesting a broader range of hosts. Anaerobic TMA production from γbb has not been reported outside of host-associated contexts, consistent with our finding that the bbu gene cluster is not found in environmental bacteria. Thus, a select group of anaerobic gut bacteria appear to possess a specialized metabolism that supports their growth in this niche.

Our discovery of the genetic basis for γbb metabolism confirms the proposal that the anaerobic conversion of L-carnitine to TMA is an interspecies metabolic pathway. Our analyses show that bacteria possessing the bbu pathway to produce TMA from γbb do not have the cai operon required to convert L-carnitine to γbb, and vice versa. This observation could explain why γbb-metabolizers inhabit host environments where L-carnitine is abundant due to dietary consumption and where other bacteria can convert it to γbb. The inability of γbb-metabolizing bacteria to generate γbb from L-carnitine highlights the importance of cross-feeding in the gut microbiota. There are now many examples of microbial co-metabolism in the gut (55), including polysaccharide utilization (56), lignin degradation (57), and even drug metabolism (58). Since TMA can only be produced anaerobically when both L-carnitine and γbb utilizing organisms are
present, an investigation into their co-occurrence will be important to deconvolute the contribution of anaerobic L-carnitine metabolism to TMA generation in the human gut.

Our initial bioinformatic analyses indicate that the \textit{bbu} pathway producing TMA is likely functionally important in the human gut. Using the \textit{bbuA} gene as a marker for metabolism, we find that this activity is widely distributed in human stool metagenomes. We also detect \textit{bbuA} transcripts in human stool sequencing data, demonstrating that these genes are expressed in the gut environment. Furthermore, the presence of the \textit{bbuA} gene and transcripts is correlated with lower levels of \(\gamma\)bb in stool, suggestive of active \(\gamma\)bb consumption in subjects possessing this gut bacterial pathway. Conversely, the \textit{cntA} gene is not correlated with changes in stool metabolite levels, nor is it highly expressed in the human gut. This lack of expression could perhaps indicate dioxygen-dependent regulation of \textit{cntA}. Intriguingly, 79\% of the HMP2 samples (30/38) that were positive for \textit{cntA} transcripts were from donors with IBD, suggesting that factors associated with inflammation, such as increased \(O_2\) levels, may induce expression of \textit{cntA}. An independent study also concluded that the \textit{cntA} gene is not expressed in healthy human gut microbiomes (40). Our analysis replicates their reported results using two of the same cohorts (MLVS and iHMP) and adds the larger HMP2 cohort that was since published. These results suggest that the \(O_2\)-independent \textit{bbu} pathway, rather than the \(O_2\)-dependent pathway, is more relevant for TMA production from L-carnitine in the anoxic gut environment. This conclusion warrants caution when inferring functional relevance from gene presence alone and highlights the importance of considering how environmental factors can influence microbial metabolic functions.

Our evaluation of anaerobic \(\gamma\)bb metabolism in the human gut microbiota suggests that this pathway may be associated with disease states. In IBD cohorts, the lower prevalence of the \textit{bbuA} gene and transcripts could reflect a decrease in \(O_2\)-intolerant bacteria, such as \textit{bbeu}}
Clostridiales, due to elevated O\textsubscript{2} levels caused by inflammation. This trend has been noted for other microbial functions that are restricted to obligate anaerobes, for example metabolism of cholesterol to coprostanol by uncultured \textit{Clostridia} (59). Although TMA production has not been linked to IBD pathology, this observation demonstrates how environmental conditions can influence metabolic functions. On the other hand, the strong connection between TMA production from L-carnitine and CVD motivated our targeted analysis of γbb metabolism in this disease. We did not find a significant difference in the prevalence of \textit{bbu} genes in CVD donors compared to healthy controls, but we did observe that the abundance of \textit{bbu} and \textit{cai} genes was elevated in CVD donors. However, our analysis of paired metagenomic and metatranscriptomic datasets (not associated with CVD) highlights challenges in drawing conclusions from metagenomic data alone. We found that the presence or absence of the \textit{bbu} genes in metagenomic data does not always reflect the presence or absence of \textit{bbu}-encoding organisms. For example, samples that are negative for \textit{bbu} genes can be positive for \textit{bbu} transcripts. Furthermore, we conclusively demonstrate that expression of the \textit{bbu} gene cluster is regulated by γbb and L-carnitine in \textit{E. timonensis}. These results together indicate that transcriptomics is a better reporter of metabolic function, particularly for low-abundance members of the microbiota like γbb-metabolizing bacteria.

A more comprehensive analysis of the contribution of anaerobic γbb metabolism to TMA production in human populations should integrate dietary information and host serum metabolomics with meta-omics analyses. Dietary studies have demonstrated a greater capacity for the gut microbiota from omnivorous donors to produce TMA from L-carnitine compared to vegans or vegetarians (7, 13, 24, 53). Our analysis of the \textit{bbuA} gene in self-reported dietary groups did not reveal differences in prevalence or abundance. However, a more thorough analysis of co-occurrence of L-carnitine and γbb metabolism in controlled dietary cohorts may reveal differences.
Diet may also be a complicating factor to consider in meta-analyses of disease cohorts. Finally, although our results show that the *bbuA* gene and transcripts are correlated with lower γbb levels, implying increased production of TMA from this substrate, we were unable to evaluate TMA and TMAO levels from stool metabolomics in these human populations because they are most reliably detected in serum, plasma, or urine. Demonstrating this correlation will be important to conclusively link anaerobic metabolism of L-carnitine-derived γbb to elevated systemic levels of TMA(O) in healthy and disease populations. Our discoveries set the groundwork for subsequent analyses to address this connection.

This work highlights the importance of enzyme discovery for understanding the chemical basis of gut microbial metabolite production, connecting metabolic activities with specific microbes, and analyzing gut microbial functions in human populations. We have uncovered a new enzymatic strategy to generate TMA in an O2-independent manner. The BbuA enzyme acts in a pathway that leads to the production of metabolites that are independently associated with human disease and health. In addition, we have discovered a new group of TMA-producing bacteria in the human gut microbiota. The identification of the *bbu* gene cluster in many uncultured bacteria highlights this portion of the microbiota as a rich source of new chemistry that can have important roles in human health. Finally, the discovery of the *bbu* pathway fills an important gap in our knowledge of TMA production from L-carnitine in the anoxic human gut. With a more complete understanding of the metabolic network involved in TMA production, we can begin to dissect the dietary, microbial, and metabolic factors that lead to its generation and connection to human disease.

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**MATERIALS AND METHODS**

**Cloning and plasmids.** Genomic DNA (gDNA) was extracted from *E. timonensis* SN18 cultures using the DNeasy UltraClean Microbial Kit (Qiagen, Germantown, MD) according to the manufacturer’s protocol. Oligonucleotide primers were synthesized by Sigma-Aldrich. Genes from the *bbu* gene cluster were amplified from 10 ng *E. timonensis* SN18 gDNA in a 50 μL reaction containing 0.5 μM of forward and reverse primers (**Dataset S5**) and Phusion High-Fidelity PCR Master Mix with HF buffer (New England Biolabs, Ipswich, MA). A plasmid based on the pET28a(+) vector for constitutive expression was constructed using the following protocol and verified by Sanger sequencing. The empty vector pET28a(+) was digested with the restriction enzymes FspI and BamHI (New England Biolabs, Ipswich, MA). After PCR cleanup using the Zymo DNA Clean and Concentrator Kit (Zymo, Irvine, CA), the linearized vector was assembled with a GBlock (**Dataset S5**) carrying the proD promoter (60) using HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA). Vectors used to construct plasmids containing the *bbu* genes were linearized by PCR amplification from 10 ng vector template DNA in a 50 μL reaction containing 0.5 μM of forward and reverse primers (**Dataset S5**) and Phusion High-Fidelity PCR Master Mix with HF buffer (New England Biolabs, Ipswich, MA). Thermocycling was carried out
in a Bio-Rad C100 thermal cycler using the following parameters: denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min/kb, and a final extension at 72 °C for 5 min. PCR reactions were analyzed by agarose gel electrophoresis with ethidium bromide staining and purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Chicago, IL). Reactions (10 μL) containing 50 ng linearized vector, 4 molar equivalents of gene insert, and Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA) were incubated at 50 °C for 1 h and transformed into E. coli TOP10 chemically competent cells (Thermo Fischer Scientific, Waltham, MA). Transformants were screened by antibiotic selection. Plasmids were purified using the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-tek, Norcross, GA) according to the manufacturer’s protocol and confirmed by Sanger sequencing by Eton Bioscience (Charlestown, MA).

**Bacterial strains and culture conditions.** *E. timonensis* SN18 was purchased from Leibniz Institute DSMZ. *E. timonensis* SN18 culturing was performed in Hungate or Balch tubes (Chemglass Life Sciences, Vineland, NJ) at 37 °C and set up in an anaerobic chamber (Coy Laboratory Products, Grasslake, MI) under an atmosphere of 2–4% H₂, 2–4% CO₂, and N₂ as the balance. Standard cultures were grown in PYG medium, modified (medium recipe DSM 104) that was sparged with N₂ after autoclaving. Basal medium lacking electron acceptors was prepared as described previously (58), containing 1 g/L tryptone (trypsinase peptone; BD Biosciences, San Jose, CA), 1 g/L yeast extract (BD Biosciences, San Jose, CA), 0.4 mM L-cysteine, 2.5 g/L NaHCO₃, 1 g/L NaCl, 0.5 g/L MgCl₂•6H₂O, 0.2 g/L KH₂PO₄, 0.3 g/L NH₄Cl, 0.3 g/L KCl, 0.015 g/L CaCl₂•2H₂O, 0.25 mL/L of 0.1% resazurin, and 1% ATCC vitamins and trace mineral solutions (American Type Culture Collection, Manassas, VA). NCE medium lacking carbon sources was prepared as described previously (61), containing 4 g/L KH₂PO₄, 5 g/L K₂HPO₄, 3.5
g/L NaNH₄PO₄ 40 mM sodium fumarate dibasic, 1 mM MgSO₄•7H₂O, 0.1% casamino acids (VWR Life Science, Radnor, PA), and 1% ATCC vitamin and trace mineral solutions (American Type Culture Collection, Manassas, VA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

**Chemical synthesis of [N-(CD₃)]-γbb and D₆-γbb.** The following general procedure was used for synthesis of both [N-(CD₃)]-γbb and D₆-γbb, based on a published protocol (62). In an oven-dried round bottom flask under nitrogen, 1 molar equivalent of GABA (1 g, 9.7 mmol) or D₆-GABA (92 mg, 0.8 mmol) and 4.6 molar equivalents of K₂CO₃ were dissolved in anhydrous methanol (0.1 M) and stirred at room temperature for 15 min. Next, 5.1 molar equivalents of CD₃I (3.1 mL, 49.4 mmol) or CH₃I (0.42 mL, 4.3 mmol) was added to the solution, which was stirred at room temperature for 3 days. The reaction mixture was then concentrated under vacuum. The residue was resuspended in chloroform. The solid was filtered under vacuum and washed twice with chloroform. The solid was dissolved in 10% aqueous HCl (0.5 M). The resulting solution was concentrated under vacuum and further dried under vacuum. The residue was triturated three times with acetone. The combined organic layers were concentrated under vacuum. Anhydrous THF (0.1 M) was added and the solution was cooled to 0 °C. The solid was filtered under vacuum and washed with fresh THF, to afford the desired products.

[N-(CD₃)]-γbb (white solid; 160 mg, 1 mmol, 10%): ¹H NMR (400 MHz, D₂O) δ ppm 2.07-2.16 (m, 2H), 2.53 (q, 2H, J = 7.3 Hz), 3.37 (td, 2H, J = 8.3, 3.9 Hz); ¹³C NMR (100 MHz, D₂O) δ ppm 75.2, 87.3, 109.8, 122.4, 232.4. The NMR data were in accordance with literature values (62).

D₆-γbb (yellow solid; 76 mg, 0.4 mmol, 48%): ¹H NMR (400 MHz, D₂O) δ ppm 3.07 (s, 9H).
**Assays in *E. timonensis* whole cell suspensions.** *E. timonensis* SN18 was cultured anaerobically at 37 °C in 10 mL PYG-modified medium supplemented with 1 mM γbb, DL-carnitine, or GABA or with an equivalent volume of 1× PBS. When the cultures reached an OD$_{600} = 0.5$, they were centrifuged at 1500 × g for 10 min at 4 °C. In an anerobic chamber (Coy Laboratory Products, Grasslake, MI) under an atmosphere of 2-4% H$_2$, 2-4% CO$_2$, and N$_2$ as the balance, the cell pellets were resuspended in 1 mL anoxic 1× PBS and centrifuged at 1500 × g for 10 min at 4 °C. The cell pellets were resuspended in 0.5 mL anoxic 1× PBS and incubated at room temperature for 1 h with 1 mM γbb, [N-(CD$_3$)$_3$]-γbb, D$_6$-γbb, DL-carnitine, or GABA or with an equivalent volume of 1× PBS. Reactions were analyzed by LC–MS as described below using three different methods for γbb/TMA, CoA, and fatty acid detection.

**RNA sample preparation, sequencing, and data analysis.** *E. timonensis* SN18 cultures were prepared in an anerobic chamber (Coy Laboratory Products, Grasslake, MI), under an atmosphere of 2-4% H$_2$, 2-4% CO$_2$, and N$_2$ as the balance, in Balch tubes containing 15 mL PYG-modified medium. Cultures were grown anaerobically at 37 °C to an OD$_{600} = 0.7$, when 0.15 mL of a 1 M O$_2$-free stock solution of γbb prepared in 1× PBS was added (1 mM final concentration) or an equivalent volume of O$_2$-free 1× PBS was added to triplicate cultures for each condition. In a separate experiment (Figure S2), when cultures reached an OD$_{600} = 0.5$, 0.15 mL of 1 M O$_2$-free stock solutions of γbb, DL-carnitine, or GABA prepared in 1× PBS were added (1 mM final concentration) or an equivalent volume of O$_2$-free 1× PBS was added to triplicate cultures for each condition. Cultures were grown anaerobically at 37 °C for an additional 45 min and then centrifuged at 3,320 × g for 10 min at 4 °C. Keeping the samples cold on ice, the supernatant was decanted, then removed entirely using a micropipette. Cell pellets were immediately resuspended...
in 0.5 mL cold Trizol reagent (ThermoFisher, Waltham, MA) and flash frozen in liquid N₂. Samples were stored at –80 °C until further processing.

Total RNA was isolated by bead beating using 0.1 mm dia. Zirconia/Silica Beads (BioSpec Products, Bartlesville, OK) at 10 m/s for 90 s to lyse cells and then using the Zymo Research Direct-Zol RNA MiniPrep Plus kit (Zymo, Irvine, CA) according to the manufacturer’s protocol. Illumina cDNA libraries were generated using a modified version of the RNAtag-Seq protocol (63). Briefly, 500 ng of total RNA was fragmented, depleted of genomic DNA, and dephosphorylated prior to its ligation to DNA adapters carrying 5’-AN8-3’ barcodes with a 5’ phosphate and a 3’ blocking group. Barcoded RNAs were pooled and depleted of rRNA using the RiboZero rRNA depletion kit (Epicentre, Madison, WI). These pools of barcoded RNAs were converted to Illumina cDNA libraries in three main steps: (i) reverse transcription of the RNA using a primer designed to the constant region of the barcoded adaptor; (ii) addition of a second adapter on the 3’ end of the cDNA during reverse transcription using SmartScribe RT (Clontech Biotechnologies, Mountain View, CA) as previously described (63); (iii) PCR amplification using primers that target the constant regions of the 3’ and 5’ ligated adaptors and contain the full sequence of the Illumina sequencing adaptors. cDNA libraries were sequenced on Illumina HiSeq 2500.

For the analysis of RNAtag-Seq data, reads from each sample in the pool were identified based on their associated barcode using custom scripts, and up to one mismatch in the barcode was allowed with the caveat that it did not enable assignment to more than one barcode. Barcode sequences were removed from the first read as were terminal G’s from the second read that may have been added by SMARTScribe during template switching. Reads were aligned to the *E. timonensis* SN18 genome (NCBI GenBank: FLKM0000000.1) using BWA (64) and read counts were assigned to
genes and other genomic features using custom scripts. Differential expression analysis was conducted with DESeq2 (65).

**Heterologous overexpression in E. coli and protein purification.** Plasmids (Dataset S5) containing the *E. timonensis* *bbuB* gene or the *bbuC* gene in a pET28a(+) vector or the *Sinorhizobium meliloti* *bcoAB* gene in a pET22 vector (66) were used to transform *E. coli* BL21 Codon-Plus (DE3-pLysS) chemically competent cells (Invitrogen, Carlsbad, CA). Transformed cells with kanamycin and chloramphenicol resistance were cultured at 37 °C with shaking (180 rpm) in rich Luria Broth (LB) medium with 50 mg/L kanamycin and 25 mg/L chloramphenicol. When cultures reached an OD$_{600}$ between 0.6-0.8, protein expression was induced by addition of IPTG to a final concentration of 0.25 mM. The cultures were then incubated at 15 °C with shaking (180 rpm) for ~18 h and harvested by centrifugation at 6,000 × g for 15 min at 4 °C. Cell pellets were flash frozen in liquid N$_2$ and stored at −80 °C until further use. Cells were resuspended in lysis buffer [50 mM Tris-(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5) buffer, 150 mM NaCl, and 10% glycerol] at a ratio of approximately 10 mL buffer per 1 g of wet cell mass. The suspension was lysed by passing twice through a cell disrupter at 12,000 psi and centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant was loaded onto a Ni$_{2+}$-NTA immobilized affinity chromatography column (~10 mL resin per 100 mL lysate) pre-equilibrated with lysis buffer. After loading, 3 column volumes of lysis buffer were passed through the column, followed by 5 column volumes of wash buffer (lysis buffer with 50 mM imidazole). Protein elution was achieved by passing elution buffer (lysis buffer with 250 mM imidazole) over the column. Fractions containing the protein of interest were combined and concentrated at 3,500 × g using a 10K MWCO Amicon Ultra-15 Centrifugal Filter Unit (Millipore Sigma, Burlington, MA). The concentrated protein was then dialyzed three times for 4 h each against 100 equivalent volumes of lysis buffer. The protein
was frozen in liquid N\textsubscript{2} and stored at \(-80\) °C. Protein purity was assessed by SDS-PAGE with Coomassie staining, and protein concentration was determined by using the molar absorption coefficient at 280 nm.

\textit{E. coli} BL21 competent cells (Invitrogen, Carlsbad, CA) were co-transformed with a pET28a plasmid containing the \textit{E. timonensis} \textit{bbuA} gene and a pACYC-Duet1 plasmid containing the \textit{E. timonensis} \textit{groEL-ES} genes (Dataset S5). Transformed cells with kanamycin and chloramphenicol resistance were cultured at 37 °C with shaking (180 rpm) in LB medium with 50 mg/L kanamycin, 25 mg/L chloramphenicol, and 0.2 mM riboflavin. When cultures reached an OD\textsubscript{600} of 0.6, protein expression was induced by addition of IPTG to a final concentration of 0.25 mM. The cultures were then incubated at 15 °C with shaking (180 rpm) for \(-18\) h and harvested by centrifugation at 6,000 \(\times\) \(g\) for 15 min at 4 °C. Cell pellets were flash frozen in liquid N\textsubscript{2} and stored at \(-80\) °C until further use. Cells were resuspended in lysis buffer [50 mM potassium phosphate (pH 7.5) buffer, 300 mM NaCl, 10\% glycerol, and 1 mM FAD] at a ratio of approximately 10 mL buffer per 1 g of wet cell mass. The suspension was lysed by passing twice through a cell disrupter at 12,000 psi and centrifuged at 20,000 \(\times\) \(g\) for 30 min at 4 °C. The supernatant was loaded onto a Ni\textsuperscript{2+}-NTA immobilized affinity chromatography column (~10 mL resin per 100 mL lysate) pre-equilibrated with lysis buffer without FAD. After loading the lysate, 3 column volumes of lysis buffer without FAD were passed through the column, followed by 3 column volumes of wash buffer A [50 mM potassium phosphate (pH 7.5) buffer, 300 mM NaCl, 10\% glycerol, and 50 mM imidazole] and 3 column volumes of wash buffer B [50 mM potassium phosphate (pH 7.5) buffer, 300 mM NaCl, 10\% glycerol, and 75 mM imidazole]. Protein elution was achieved by passing elution buffer [50 mM potassium phosphate (pH 7.5) buffer, 300 mM NaCl, 10\% glycerol, and 250 mM imidazole] over the column. Fractions containing the protein of interest were combined and immediately
concentrated at 3,500 × g using a 30K MWCO Amicon Ultra-15 Centrifugal Filter Unit (Millipore Sigma, Burlington, MA). The protein was frozen in liquid N₂ without buffer exchange and stored at −80 °C. Protein purity was assessed by SDS-PAGE with Coomassie staining, and protein concentration was determined by using the molar absorption coefficient at 280 nm. FAD-bound protein concentration was determined by using a molar absorption coefficient of 11,300 M⁻¹cm⁻¹ at 450 nm.

**Chemical synthesis of crotonyl-CoA.** Crotonyl-CoA was synthesized according to a published protocol (67) with the following modifications. One molar equivalent of solid Coenzyme A trilithium salt (CoAla Biosciences; 35 mg, 0.046 mmol) was dissolved in 3 mL of ice-cold water. To this solution, 1.5 molar equivalents of crotonic anhydride (Sigma Aldrich; 10 μL, 0.068 mmol) was added and the pH was adjusted to pH 7.0 using a saturated solution of NaHCO₃. The reaction was stirred in an ice bath for 1.5 h. Reaction progress was monitored using the DTNB “Ellman’s” assay to detect free CoA. The reaction was quenched by adding 48 μL formic acid to lower the pH below 3 and was kept frozen at −20 °C prior to purification. The crotonyl-CoA product was purified using an Thermo Scientific Dionex UltiMate 3000 HPLC instrument and a Thermo Scientific Hypersil GOLD aQ C18 preparative column (250 × 20 mm, 5 μM). The reaction solution was injected onto the column equilibrated with 100% solvent A (10 mM ammonium acetate) and the following gradient method was applied over 21 min with a flow rate of 8 mL/min: 1) solvent B was increased to 40% from 0 to 14 min, 2) solvent B was increased to 95% from 14 to 15 min, 3) solvent B was maintained at 95% from 15 to 16 min, 4) solvent B was decreased to 0% from 16 to 16.5 min, and 5) the column was re-equilibrated with 100% solvent A from 16 to 21 min for subsequent injections. Fractions with absorption at 260 nm were collected and lyophilized to afford crotonyl-CoA as a white solid (10 mg, 0.012 mmol, 26%). ¹H NMR (400 MHz, D₂O) δ ppm 0.78
(s, 3H), 0.91 (s 3H), 1.87 (d, 3H, J = 6.7 Hz), 2.44 (t, 2H, J = 6.1 Hz), 3.03 (t, 2H, J = 6.1 Hz), 3.35 (t, 2H, J = 6.2 Hz), 3.46 (t, 2H, J = 6.2 Hz), 3.58 (dd, 1H, J = 4.3 Hz, J = 9.3 Hz), 3.85 (dd, 1H, J = 4.3 Hz, J = 9.6 Hz), 4.04 (s, 1H), 4.26 (br s, 2H), 4.61 (br s, 1H), 4.84-4.87 (m, 2H), 6.17-6.21 (m, 2H), 6.95 (sextet, 1H, J = 7.2 Hz), 8.27 (s, 1H), 8.56 (s, 1H).

**Enzymatic synthesis of γbb-CoA.** A 1 mL solution of 0.02 mM *S. meliloti* BcoAB, 0.2 M MgCl$_2$, 0.2 M ATP, 0.02 M CoA, 0.05 M γbb in 0.5 M Tris-HCl (pH 8.0) was incubated at room temperature for 30 min. The reaction was quenched with 1% acetic acid (final concentration) and incubated at –20 °C for 30 min. The solution was centrifuged at 16,000 × g for 30 min. The γbb-CoA product was purified by preparative HPLC using the same method as described above for crotonyl-CoA. Fractions with absorption at 260 nm were collected and lyophilized. The product identity was confirmed by MS.

**CoA transferase assays in *E. coli* lysates.** *E. coli* MG1655 chemically competent cells were transformed with pET-proD plasmids containing *E. timonensis* bbu genes (Dataset S5). Transformed cells with kanamycin resistance were grown in 50 mL LB medium with 50 mg/L kanamycin at 37 °C with shaking (180 rpm) for 20 h, reaching an OD$_{600}$ of 1-1.4. Culture aliquots of 15 mL were harvested by centrifugation at 3,220 × g for 15 min at 4 °C and cell pellets were frozen at –80 °C until further use. Cell pellets were resuspended with a volume of 50 mM potassium phosphate (pH 7.5) buffer, 300 mM NaCl, 10% glycerol to achieve a normalized OD$_{600}$ of 20. The cells were lysed on ice by sonication for a total of 1 min, with cycles of 2.5 s on and 10 s off. Reactions (0.05 mL total) containing 0.01 mL of crude lysate, 10 mM acetyl-CoA, 25 mM γbb in 100 mM Tris-HCl (pH 8.0) buffer were incubated at room temperature for 1 h and analyzed by LC–MS using the CoA detection method.
TMA-lyase assays in *E. coli* lysates. *E. coli* MG1655 (DE3) chemically competent cells were transformed with a pET28a plasmid containing *E. timonensis bbu* genes and the pACYC-Duet1 plasmid containing the *E. timonensis groEL-ES* genes (Dataset S5). Transformed cells with kanamycin and chloramphenicol resistance were grown in 50 mL LB medium with 50 mg/L kanamycin, 25 mg/L chloramphenicol, and 0.2 mM riboflavin at 37 °C with shaking (180 rpm). When cultures reached an OD$_{600}$ of 0.6, protein expression was induced by addition of IPTG to a final concentration of 0.25 mM. The cultures were then incubated at 15 °C with shaking (180 rpm) for ~15 h. Cultures aliquots of 15 mL were harvested by centrifugation at 3,220 × g for 15 min at 4 °C and cell pellets were frozen at −80 °C until further use. Cell pellets were transferred and thawed in an anaerobic chamber (Coy Laboratory Products; Grasslake, MI) located in a cold room at 4 °C under an atmosphere of ~3% H$_2$ and N$_2$ as the balance. The pellets were resuspended with a volume of O$_2$-free 50 mM potassium phosphate (pH 7.5) buffer, 300 mM NaCl, 10% glycerol to achieve an OD$_{600}$ = 10. The cells were lysed by sonication for a total of 1 min, with cycles of 2.5 s on and 10 s off. In an anaerobic chamber (Coy Laboratory Products; Grasslake, MI) under an atmosphere of ~3% H$_2$ and N$_2$ as the balance, reactions (0.05 mL) containing 0.02 mL of crude lysate, 10 mM acetyl-CoA, 10 mM γbb in O$_2$-free 100 mM Tris-HCl (pH 8.0) buffer were incubated at room temperature for 1 h and analyzed by LC–MS using the methods for CoA and TMA detection.

**CoA transferase in vitro activity assay.** Reactions (50 μL) containing 0.01 mM BbuB, 0.01 mM BbuC, 10 mM acetyl-CoA, and 10 mM γbb in 100 mM Tris-HCl (pH 8.0) buffer were incubated at room temperature for 1 h and analyzed by LC–MS using the CoA detection method.

**TMA-lyase in vitro activity assay.** Protein solutions of BbuA, BbuB, and BbuC were deoxygenated on ice by eight rapid cycles of vacuum and N$_2$ gas for a total of four times. In an
anaerobic chamber (Coy Laboratory Products; Grasslake, MI) under an atmosphere of ~3% H\textsubscript{2} and N\textsubscript{2} as the balance, reactions (0.1 mL) containing 0.2 mM BbuA, 0.01 mM BbuB, 0.01 mM BbuC, 10 mM acetyl-CoA, and 10 mM \(\gamma\text{bb}\) in O\textsubscript{2}-free 100 mM Tris-HCl (pH 8.0) buffer were incubated at room temperature for 1 h and analyzed by LC–MS using the methods for CoA and TMA detection.

**Liquid chromatography – mass spectrometry sample preparation and analytical methods**

\(\gamma\text{bb}\) and TMA analysis. Samples were diluted 1:10 (v/v) in solvent A composed of 95:5 acetonitrile (ACN):100 mM ammonium formate (MS grade) in H\textsubscript{2}O with 0.02% formic acid (MS grade) and centrifuged at 3,220 \(\times\) g for 10 min. The extracted supernatant samples were further diluted 1:4 (v/v) in solvent A and kept at 4 \(^\circ\)C until analysis (performed within 24 h).

Liquid chromatography was conducted using an Agilent 1200 Series G1312B Binary Pump (Agilent Technologies, Santa Clara, CA) instrument and a Phenomenex HILIC column (30 mm \(\times\) 2.1 mm, 2.5 \(\mu\)m). Samples (2 \(\mu\)L) were injected onto the column equilibrated with 100% solvent A and the following gradient method was applied over 7.5 min with a flow rate of 0.6 mL/min: 1) Solvent A was maintained at 100% from 0 to 1 min, 2) solvent B was increased to 90% from 1 to 3 min, 3) solvent B was maintained at 90% from 3 to 4.5 min, 4) solvent B was decreased to 0% from 4.5 to 6 min, and 5) the column was re-equilibrated with 100% solvent A from 6 to 7.5 min. Tandem MS/MS detection was performed with an Agilent 6410 Triple Quadrupole (Agilent Technologies, Santa Clara, CA) instrument with electron spray ionization in positive mode (ESI+). The source parameters were gas temperature of 200 \(^\circ\)C, gas flow of 10 L/min, nebulizer pressure of 45 psi, and capillary voltage of 4000 V. Conditions for MS/MS fragmentation for the metabolites of interest are found in Table 1. Standards were prepared according to the same
methods used for experimental samples and standard curves were used to quantify metabolite concentrations.

**Table 1.** MS/MS parameters used to detect metabolites of interest.

| Metabolite | Parent ion [M+H]+ (m/z) | Daughter ion [M+H]+ (m/z) | Dwell (ms) | Fragmentor (V) | CE (V) |
|------------|-------------------------|---------------------------|------------|----------------|--------|
| γbb        | 146                     | 87                        | 200        | 110            | 21     |
| TMA        | 60                      | 45                        | 200        | 110            | 26     |
| DL-Carnitine | 162                   | 103                       | 200        | 110            | 21     |
| GABA       | 104                     | 87                        | 200        | 110            | 21     |

*Derivatization of fatty acids with 2-nitrophenylhydrazine/1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and analysis.* Samples were diluted 1:10 (v/v) in 95:5 ACN:100 mM ammonium formate (MS grade) in H₂O with 0.02% formic acid (MS grade) and centrifuged at 3,220 × g for 10 min. The extracted supernatant samples (10 μL) were diluted 1:10 (v/v) in a solution of 10% 1:1 pyridine:HCl (pH 3.5-5) in H₂O. A pre-mixed derivatization solution consisting of equal parts 0.29 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) hydrochloride (dissolved in water) and 0.12 M 2-nitrophenylhydrazine (2-NPH; dissolved in 0.25 M HCl) was prepared fresh and 20 μL of this solution was added to 100 μL of 100-fold diluted sample. The derivatization reactions were heated at 60 °C for >15 min and then diluted 1:10 (v/v) in H₂O prior to LC–MS analysis.

Liquid chromatography was conducted using an Agilent 1200 Series G1312B Binary Pump (Agilent Technologies, Santa Clara, CA) instrument and an Agilent Extend C18 column (50 mm × 2.1 mm, 1.8 μm). The derivatized samples (2 μL) were injected onto the column equilibrated with 95% H₂O (solvent A) and 5% ACN (solvent B). The following gradient method was applied over 13 min with a flow rate of 0.3 mL/min: 1) solvent A was maintained at 95% from 0 to 4 min, 2) solvent B was increased to 100% from 0 to 4 min, 3) solvent B was maintained at 100% from 4
to 7 min, 4) solvent B was decreased to 5% from 7 to 9 min, and 5) the column was re-equilibrated with 95% solvent A from 9 to 13 min. An MS scan was performed with an Agilent 6410 Triple Quadrupole (Agilent Technologies, Santa Clara, CA) instrument with electron spray ionization in negative mode (ESI–). The source parameters were a gas temperature of 325 °C, a gas flow of 12 L/min, a nebulizer pressure of 40 psi, and a capillary voltage of 4000 V. The detection mode was set to a scan range of 100–400 m/z and a scan time of 500 ms with a fragmentor voltage of 135 V and a step size of 0.1 amu. The parent [M-H]− ions for derivatized short-chain fatty acids were monitored, including formate (180 m/z), acetate (194 m/z), propionate (208 m/z), and butyrate (222 m/z). Butyrate concentrations were determined by comparison to a 10 μM sodium 13C4-butyrate internal standard added to the derivatization solution.

CoA-thioester analysis. Cell cultures (5 mL) or whole cell suspensions (1.5 mL) were centrifuged at 3,220 × g or 16,000 × g, respectively, for 10 min at 4 °C. Cell pellets were resuspended in 0.2 mL of cold solution of 40:20:20 ACN:MeOH:H2O. Resuspended cells were incubated at −20 °C for 30 min and then centrifuged at 16,000 × g for 30 min. The supernatant was transferred to a new tube and dried using a Genevac EZ-2.3 Elite Evaporation System (Genevac Ltd, Ipswich, UK) for 1 h. The dried samples were resuspended in 80 μL H2O for LC–MS analysis. Samples (20 μL) from lysate or in vitro reactions were quenched in 2 μL concentrated acetic acid, diluted 1:10 in H2O, and incubated at −20 °C for 30 min before centrifugation at 16,000 × g for 10 min. Samples were further diluted 1:4 in H2O and kept at 4 °C prior to LC–MS analysis. Standards were prepared according to the same methods used for experimental samples.

Liquid chromatography was conducted using a Waters Acquity UPLC H-Class System (Waters Corporation, Milford, MA) instrument and a Waters Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm). The samples (2 μL) were injected onto the column equilibrated with 100% 30 mM
ammonium acetate (solvent A). The following gradient method was applied over 5 min with a flow rate of 0.8 mL/min: 1) solvent A was maintained at 100% from 0 to 0.5 min, 2) solvent B (acetonitrile + 0.1 % formic acid) was increased to 20% from 0.5 to 4 min, 3) solvent B was increased to 100% from 4.0 to 4.1 min, 4) solvent B was maintained at 100% from 4.1 to 4.4 min, 5) solvent B was decreased to 0% from 4.4 to 4.5 min, and 5) the column was re-equilibrated with 100% solvent A from 4.5 to 5 min. MS detection was performed with a Waters Xevo TQ-S (Waters Corporation, Milford, MA) instrument with electron spray ionization in positive mode (ESI+). The source parameters were gas temperature of 500 °C, desolvation gas flow of 1000 L/h, cone gas flow of 150 L/h nebulizer pressure of 7 bar, capillary voltage of 0.5 kV and cone voltage of 3 V.

Conditions for tandem MS/MS detection were optimized using an acetyl-CoA standard. Conditions for a parent MS/MS scan of the 136 m/z mass fragment (corresponding to adenine loss) were a scan range of 800–910 m/z, scan time of 0.15 s, collision energy of 53 V, and cone voltage of 70 V. Conditions for an MS scan were a scan range of 800–910 m/z, cone voltage of 70 V, and scan time of 0.15 s. Conditions for MS/MS fragmentation for the metabolites of interest are found in Table 2.

Table 2. MS/MS parameters used to detect metabolites of interest.

| Metabolite          | Parent ion [M+H]+ (m/z) | Daughter ion [M+H]+ (m/z) | Dwell (ms) | Fragmentor (V) | CE (V) |
|---------------------|------------------------|--------------------------|------------|----------------|--------|
| Acetyl-CoA          | 810.1957               | 135.9617                 | 52         | 70             | 54     |
| Crotonyl-CoA        | 836.5                  | 135.9617                 | 52         | 70             | 54     |
| Butyryl-CoA         | 838.12                 | 135.9617                 | 52         | 70             | 54     |
| 3-Hydroxybutyryl-CoA| 854.0                  | 135.9617                 | 52         | 70             | 54     |
| γbb-CoA             | 895.5                  | 135.9617                 | 52         | 70             | 54     |

Bioinformatic analyses

Genome Searches. The E. timonensis BbuA protein sequence was used in a Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI) non-
redundant protein database, the UniProt database (release 2019_07), and the Joint Genome Institute-Integrated Microbial Genomes (JGI-IMG) database of all isolates. Hits with >70% amino acid sequence identity were considered BbuA homologs. Searches were also conducted of the HMP1 reference genomes and genomes from the human gut bacteria culture collections described in Dataset S3. No additional hits were identified from these searches. Next, collections of human metagenome-assembled genomes (MAGs) (Dataset S3) were performed using a tblastn search and an e-value cutoff of <0.0001. Hits were manually evaluated and further filtered using a >70% amino acid sequence identity cutoff. Contigs containing hits were annotated using the Galaxy webtool Prokka (68, 69).

**Phylogenetics.** A multiple sequence alignment was generated using MAFT v7.455 (70) of the BbuA homolog protein sequences and 2,388 representatives of protein clusters with >80% amino acid sequence ID from the top 10,000 hits of a BLAST search of the UniProt database (release 2019_07) using the *E. timonensis* SN18 BbuA protein as a query. A maximum-likelihood phylogenetic tree was constructed using IQ-TREE v1.6.12 (71) with the LG+F0+G12 model and visualized using FigTree v1.4.4 (72). Branch supports were calculated using the aBayes method (73).

**Metagenome and Metatranscriptome Searches & Quantification.** BbuA homolog protein sequences (Dataset S6) were used to generate a protein database. A negative control protein database was made using protein sequences acquired from the follow steps: (1) the top 10,000 results were obtained from a BLAST search of the UniProt database (release 2019_07) using *E. timonensis* SN18 BbuA as the query, excluding BbuA homologs described above, (2) these sequences were clustered using UniRef50 (release 2019_07), resulting in 288 representative proteins, and (3) each unique sequence from step 1 between 50-70% sequence identity to *E.
timonensis SN18 BbuA was added to the list from step 2. The CntA protein database consisted of *E. coli* YeaW (UniProt P0ABR8), *A. baumannii* CntA (UniProt D0C9N6), and *Klebsiella pneumoniae* CntA (UniProt A0A377WGT7).

Human studies reporting the stool meta’omic data that were analyzed in this work are listed in **Dataset S4**. A blastx DIAMOND(74) search with an e-value cut-off of <0.0001 and a percent amino acid sequence identity of >50% was performed using the raw shotgun metagenome or metatranscriptome sequencing reads against the BbuA, CntA, and negative control databases. If the highest sequence identity hit for the read to a BbuA protein was greater than or equal to that of a negative control protein and the sequence identity to the BbuA protein was >70%, then that read was considered a positive hit for a *bbuA* gene or transcript. The positive hits for each metagenome sample were summed and then normalized by RPKM and average genome size (AGS) using the following equations.

\[
RPKM = \frac{\text{reads}}{\text{total reads}} / 10^6 \\
\text{Abundance} = RPKM \times AGS \times 10^{-9}
\]

Average genome size for each sample was calculated using MicrobeCensus (75). Plots and statistical analyses were performed using the ggplot2 package v3.3.2 (76) and R v3.6.0 (77).

**SUPPORTING INFORMATION**

The Supporting Information includes additional figures (S1-S15) and legends for datasets (S1-S6) that are provided as separate files.

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