MtcB, a member of the MttB superfamily from the human gut acetogen Eubacterium limosum, is a cobalamin-dependent carnitine demethylase

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The trimethylamine methyltransferase MttB is the first described member of a superfamily comprising thousands of microbial proteins. Most members of the MttB superfamily are encoded by genes that lack the codon for pyrrolysine characteristic of trimethylamine methyltransferases, raising questions about the activities of these proteins. The superfamily member MtcB is found in the human intestinal isolate Eubacterium limosum ATCC 8486, an acetogen that can grow by demethylation of l-carnitine. Here, we demonstrate that MtcB catalyzes l-carnitine demethylation. When growing on l-carnitine, E. limosum excreted the unusual biological product norcarnitine as well as acetyl, butyrate, and caproate. Cellular extracts of E. limosum grown on l-carnitine, but not lactate, methylated cobalamin or tetrahydrofolate using l-carnitine as methyl donor. MtcB, along with the corrinoid protein MtqC and the methylcorrinoid: tetrahydrofolate methyltransferase MtqA, were much more abundant in E. limosum cells grown on l-carnitine than on lactate. Recombinant MtcB methylates either cobalamin or Co(I)-MtqC in the presence of l-carnitine and, to a much lesser extent, γ-butyrobetaine. Other quaternary amines were not substrates. Recombinant MtcB, MtqC, and MtqA methylated tetrahydrofolate via l-carnitine, forming a key intermediate in the acetogenic Wood–Ljungdahl pathway. To our knowledge, MtcB methylation of cobalamin or Co(I)-MtqC represents the first described mechanism of biological l-carnitine demethylation. The conversion of l-carnitine and its derivative γ-butyrobetaine to trimethylamine by the gut microbiome has been linked to cardiovascular disease. The activities of MtcB and related proteins in E. limosum might demethylate proatherogenic quaternary amines and contribute to the perceived health benefits of this human gut symbiont.

At present, ~10,000 representatives of the MttB protein superfamily can be found in nearly 2000 different archaeal and bacterial genomes maintained at the National Center for Biotechnology. The first-described member of this large and well-distributed superfamily is the trimethylamine methyltransferase MttB, which catalyzes the corrinoid-dependent demethylation of trimethylamine (TMA) (1, 2). MttB is one of the few proteins known to possess the rare genetically encoded amino acid pyrrolysine (1, 3, 4). However, the genes encoding the vast majority of the superfamily lack the amber codon necessary for co-translational insertion of the pyrrolysine residue that is characteristic of verified TMA methyltransferases (5), leaving the function of their gene products an open question. This conundrum was in part resolved by the discovery of MtgB, a nonpyrrolysine MttB homolog from nitrite-respiring Desulfotobacterium hafniense Y51 (5). MtgB initiates the corrinoid-dependent demethylation of glycine betaine to dimethylglycine as part of a multicomponent glycine betaine:THF methyltransferase system that also requires MtgC, a corrinoid-binding protein, and MtgA, a methylcorrinoid:THF methyltransferase (see Fig. 1 for a schematic of reactions involved in quaternary amine and TMA metabolism). Highly similar homologs of MtgB, MtgC, and MtgA were recently implicated in glycine betaine demethylation catalyzed by Acetobacterium woodii, an acetogen (6). The notable sequence divergence among MttB superfamily members led Ticak et al. (5) to hypothesize that different members of the family may have evolved specificity for other quaternary amines beyond glycine betaine. If so, the impact of the MttB superfamily could be significant in environments where organisms encoding nonpyrrolysine MttB family members are found. One such environment is the human intestine (5), where the metabolism of quaternary amines by members of the microbiome is now known to have a significant effect on human health (7).

In this work, we demonstrate for the first time that an MttB family member is an l-carnitine methyltransferase. l-Carnitine is widely used by eukaryotic cells for transport of fatty acids into the mitochondria. As a result, l-carnitine is commonly found in many foodstuffs as a component of an omnivorous diet. Following ingestion, l-carnitine, as well as other quaternary amines such as choline and glycine betaine (8, 9), enter the intestines, where they are either absorbed by the host or converted by members of the gut microbiota into TMA (10). In the gut, l-carnitine is primarily dehydrated and reduced to γ-butyrobetaine, which is then converted to TMA (11, 12). Once in the bloodstream, TMA is transported to the liver and converted into trimethylamine N-oxide (TMAO), predominantly by flavin monooxygenase 3 (13). High serum levels of TMAO have been shown to promote formation of atherosclerotic plaques in a mouse model (8). Furthermore, serum levels of TMAO were significantly correlated with the incidence of heart attack, stroke, and death in a clinical population (14). TMAO,

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as well as choline and L-carnitine, are further associated with increased risk for atherosclerosis (9). Loss-of-function mutations of FMO3 itself can lead to trimethylaminuria, whose sufferers emit the odor of unmetabolized TMA (15).

The health effects of TMA and TMAO have led to renewed interest in understanding quaternary amine degradation by members of the gut microbiome. Choline and L-carnitine have long been known to be converted to glycine betaine (16, 17), which can be then cleaved by betaine reductase to form TMA (18). It is indicative of our relative lack of knowledge concerning the potential for methylamine metabolism by the microbiota that only recently have other enzymes been found that act directly on quaternary amines to generate TMA. Choline was shown to be directly converted to TMA by CutC, a glycyl radical enzyme that acts as a choline-TMA lyase (19). TMA may be produced from L-carnitine in a single step by the L-carnitine monooxygenase CntAB (20). L-Carnitine can be converted to γ-butyrobetaine, which the oxygenase YeaWX can convert to TMA (11). YeaWX also has some activity with L-carnitine and choline. Recent work has indicated that an anoxic uncharacterized pathway for TMA production from γ-butyrobetaine also exists in the gut microbiome (12). Inhibitors of the major enzymes of quaternary amine degradation have been proposed as drugs to potentially decrease the net production of TMA in the gastrointestinal tract (21, 22).

Microbes have also been proposed to control net gut TMA production, and thus net TMAO levels, in humans. Supporting the idea that microbiota influence TMAO levels, introduction of “humanized” gut microbiomes into germ-free mice led to differential production of TMAO (23), and atherosclerosis susceptibility could be transmitted via transplantation of gut microbiota (24). Different microbes thus might contribute to or interfere with the net synthesis of TMA (25). Only one group of microbes that might diminish TMA production has been previously identified (26, 27); the methanogenic archaea inhabiting the gut, whose genomes encode the pyrrolysyl-protein MttB, the TMA methyltransferase (1, 2). Another route that might conceivably limit TMA production would be competition for the quaternary amines that are precursors to TMA. However, such routes of quaternary amine degradation that would not eventually yield TMA under anaerobic conditions have been unknown, save for one, the demethylation of glycine betaine by the nonpyrrolysine (nonPyl) MttB family member, MtgB (5).

Here we show that Eubacterium limosum ATCC 8486, an acetogenic and butyrogenic human gut isolate, consumes L-carnitine to produce norcarnitine. The latter is, to our knowledge, a novel biological product. The nonPyl MttB family member MtcB, along with a corrinoid protein and a corrinoid-dependent THF methyltransferase, were significantly more abundant in cells grown on L-carnitine than on lactate. These three proteins together catalyzed the methylation of THF with L-carnitine, thus providing a key intermediate toward the catabolic synthesis of acetate, butyrate, and propionate. MtcB initiates THF methylation by methylation of an abundant corrinoid protein specifically with L-carnitine. These results expand the known substrates of the MttB superfamily to include a pro-atherogenic dietary component and reveal a novel anoxic mechanism of L-carnitine degradation via demethylation.

**Results**

**Demethylation of L-carnitine during growth of E. limosum**

E. limosum strains have previously been reported to grow utilizing glycine betaine or choline (28). We found that E. limosum ATCC 8486 can also utilize L-carnitine as a growth substrate. Growth with L-carnitine was best in a medium supplemented with yeast extract and casamino acids with a doubling time of ~6–7 h. Little or no growth was observed when E. limosum was inoculated into the same medium not supplemented with L-carnitine (Fig. 2A). E. limosum grew more slowly on L-carnitine when in a completely defined medium with a doubling time of ~16–18 h, indicating that yeast extract and casamino acids were stimulatory but not necessary for growth with L-carnitine (Fig. S1).

E. limosum strains were reported to demethylate glycine betaine and choline during growth (28). Therefore, we examined L-carnitine–grown cultures to determine whether L-carnitine was also demethylated during growth. Culture supernatants taken before and after growth were analyzed by TLC, followed by staining with bromocresol green (Fig. 2B). L-Carnitine was...
and 10 mmol of caproate from acetyl-CoA (30). Demethylation of fatty acid production, as evidenced by the stoichiometry of L-carnitine degradation in defined medium in which L-carnitine was omitted (Fig. S3). Given this and assuming a 1:1 stoichiometry between the L-carnitine consumed and norcarnitine produced, the carbon recovery of the methyl group of L-carnitine and CO₂ in acid products was 91 ± 11%. Overall, our data support the following idealized equation for L-carnitine metabolism by E. limosum: 64 L-carnitine + 17 CO₂ → 64 nor-carnitine + 12 acetate + 12 butyrate + 1.5 caproate.

Corrinoid-dependent methyltransferase activities in cell extracts

Acetogens such as E. limosum form methyl-THF as an obligate step in the synthesis of acetate (29, 31). During growth on methylated substrates, methyl-THF is synthesized by substrate-specific multicomponent methyltransferase systems (32–35). Methyl transfer to THF consists of three components: 1) a methylated substrate:corrinoid protein methyltransferase, 2) a corrinoid-binding protein homologous to the cobalamin-binding domain of methionine synthase (36, 37), and 3) a methylcorrinoid protein:THF methyltransferase. During catalysis, the corrinoid protein cofactor undergoes multiple cycles, alternating between the unmethylated Co(I) state and the methyl-Co(III) form. Adventitious oxidation of the Co(I)-corrinoid necessitates reactivation by an ATP-dependent reductive activation protein (sometimes referred to as a fourth component), which reduces the Co(II)-corrinoid back to the Co(I) state (38–40). However, not all corrinoid proteins require the activating protein for in vitro activity and instead can be reduced to Co(I) using a low potential chemical reductant (32, 41).

To determine whether a corrinoid-dependent methyltransferase system might underlie L-carnitine demethylation by E. limosum, we first examined L-carnitine–grown cell extracts for L-carnitine:THF methyltransferase activity and found an L-carnitine–dependent rate of 82.3 ± 4.9 nmol of methyl-THF min⁻¹ mg of protein⁻¹ (average of three preparations). Methylation of THF depended on the addition of extract, THF, and Ti(III)citrate, but ATP did not stimulate the reaction. The requirement of Ti(III)citrate for THF methylation by extracts is consistent with involvement of Co(I)-corrinoid–dependent methyltransferase in the reaction, so we further tested extracts for this activity by using UV-visible spectroscopy (Fig. 3A). Extracts catalyzed L-carnitine–dependent methylation of Ti(III)citrate-reduced cob(I)alamin at a rate of 94 ± 27 nmol min⁻¹ mg of protein⁻¹ (average of three preparations) when supplemented with L-carnitine (Fig. 3B). We did not observe methylation of cob(I)alamin when L-carnitine was omitted (Fig. 3B). Activity with choline, γ-butyrobetaine, glycine betaine, or tetramethylammonium ion was not detectable when tested as described under “Experimental procedures.” Additionally, we did not detect L-carnitine:cob(I)alamin methyltransferase activity in the extracts of cells grown on lactate (Fig. 3B).
Catabolic proteins are generally present at high levels in anaerobes, and high-abundance proteins involved in acetate and butyrate formation were readily identified in cells grown on either lactate or \( L \)-carnitine. Abundant catabolic proteins specific to growth on each substrate were also identified (Table S2). For example, in lactate-grown cells, a lactate dehydrogenase and associated proteins (44, 45) were abundant among the \( \sim 1630 \) proteins identified. These same proteins were at very low abundance or not detectable among the \( \sim 1400 \) proteins identified from cells grown on \( L \)-carnitine. Conversely, homologs of the two methyltransferases and corrinoid proteins involved in multicomponent systems for THF methylation were abundant when cells were grown on \( L \)-carnitine, but much less so when grown on lactate. Therefore, these proteins were considered as candidates for a \( L \)-carnitine:THF methyltransferase system. The proteins included an MttB superfamily member (WP_038351887.1) that we designated MtcB, which constituted \( 2.9 \pm 0.87 \) mol % of detected proteins from \( L \)-carnitine–grown cells. MtcB was not detectable in lactate–grown cells, and a \( t \) test indicated that the difference in MtcB abundance between lactate– and \( L \)-carnitine–grown cells had a low probability of occurring randomly (\( p = 0.00058 \)). Of the genes located near \( mtcB \) in the \( E.\ limosum \) genome (Fig. S3), only the product of the adjacent downstream gene (WP_038351886.1) was detectable in cells grown on \( L \)-carnitine, but not on lactate (Table S2). This protein, a member of the major facilitator family (MF) of transporters, was present at low abundance in the \( L \)-carnitine proteome. It should be noted that membrane proteins are generally underrepresented by the proteomic protocol employed here.

The corrinoid cofactor methylated by MttB superfamily members is bound to a discrete corrinoid-binding protein. Three homologs of methylocoronorhoic corrinoid proteins were detected in \( L \)-carnitine–grown cells. Two of these proteins were not abundant (<0.001 mol %). The third (WP_038352545.1) was present at a relative abundance of 0.64 ± 0.38 mol % but was only 0.0083 ± 0.004 mol % of protein in lactate–grown cells, indicating a \( 76 \)–fold increase (\( p \) = 0.016) increase in abundance when \( L \)-carnitine was the growth substrate. We have designated this corrinoid protein MtqC. Additionally, an ATP-dependent reductive activation protein belonging to a superfamily defined largely as COG3894 was identified (WP_038351874.1, designated RamQ) was 14-fold (\( p \) = 0.0001) more abundant during growth on \( L \)-carnitine (0.13 ± 0.02% of protein) when compared with growth on lactate (0.009 ± 0.003% of protein).

The \( E.\ limosum \) ATCC 8486 genome (46) encodes five proteins homologous to the Meth domain that carries out the methylcobalamin:THF methyltransferase subreaction of methionine synthase (47). We detected two of these enzymes in our proteomic data sets. The first (WP_013381869.1) is encoded in a gene cluster that contains the components of carbon monoxide dehydrogenase/acetyl-CoA synthase and is therefore most likely the corrinoid iron-sulfur protein methyltransferase (AcS) in \( E.\ limosum \). Although quite abundant in \( L \)-carnitine–grown cells (3.5± 0.7% of detected protein), its abundance was also high during growth on lactate (6.2 ± 1.6% of detected protein). The second Meth-like enzyme detected in \( L \)-carnitine–grown cells is WP_038351870.1, which we have

![Figure 3. A carnitine:cob(II)alamin methyltransferase activity is present in extracts of carnitine-grown, but not lactate-grown, \( E.\ limosum \). A, UV-visible spectra were collected every 30 s during a single carnitine:cob(II)alamin methyltransferase reaction initiated by the addition of extract from carnitine-grown cells. The arrow indicates the direction of increased absorbance at 540 nm with time indicative of methylcob(II)alamin formation. The sharp isosbestic point (*) at 578 nm indicates that other cobalamin species did not accumulate appreciably during the reaction. The complete UV-visible spectrum is not shown due to the intense absorbance of Ti(III)citrate and cob(II)alamin below 425 nm.](Image)

**Candidates for mediating \( L \)-carnitine:THF methyl transfer in \( E.\ limosum \)**

Cell extracts from \( L \)-carnitine– but not lactate–grown cells catalyzed formation of methyl-cob(III)alamin with \( L \)-carnitine. Therefore, to identify candidate proteins that might mediate this activity and couple it to THF methylation, we identified proteins found in cells grown with \( L \)-carnitine using label-free proteomic analysis (see Tables S2–S4). We compared this data set with one we described previously from cells growing on lactate (42), focusing on proteins potentially involved in catabolism (Table S2). For each substrate, four separate cultures were grown to mid-log phase prior to individual harvest, lysis, tryptic digestion, and peptide analysis by LC–MS/MS using the same methodology. The relative abundances of proteins in each sample were determined from the percentage of summed emPAI values (43).
expressed the robust L-carnitine:cob(I)alamin recombinant MtcB (Fig. S4), which we found did carry out a L-carnitine:corrinoid methyltransferase. Therefore, we hypothesized that the MttB family member MtcB might be an L-carnitine methyltransferase domain, was also significantly increased when cells were grown on L-carnitine, and therefore we purified recombinantly produced MtqA (Fig. S4) to test whether this protein might participate in methyl transfer between L-carnitine and THF. The addition of MtqA to MtcB and apparent Km values for L-carnitine and cob(I)alamin corresponding to 0.40 (95% CI, 0.20–0.60) mM and 1.34 (95% CI, 1.24–1.45) mM, respectively.

We next tested to see whether MtcB could use L-carnitine to methylate MtqC, the corrinoid protein of increased abundance in L-carnitine–grown cells. Recombinant MtqC was produced as an apoprotein from E. coli; therefore, we reconstituted the purified protein (Fig. S4) with cobalamin prior to further purification. Incubation of the MtqC holoprotein with recombinant RamQ (Fig. S4), Ti(III)citrate, and ATP generated a stable pool of Co(I)-MtqC. Upon subsequent addition of L-carnitine and MtcB, methylation of Co(I)-MtqC occurred as indicated by the disappearance of the 387 nm peak corresponding to Co(I)-MtqC and appearance of a peak at 532 nm corresponding to methyl-Co(III)-MtqC (Fig. S5A). The reaction depended on both L-carnitine and MtcB (Fig. S5B). During the reaction, we observed an isosbestic point near 585 nm indicating that the conversion of Co(I)-MtqC to methyl-Co(III)-MtqC occurred without accumulation of an appreciable amount of a spectrally distinct intermediate. MtcB methylated MtqC with 4 mM L-carnitine at an average rate of 14 μmol min⁻¹ mg⁻¹ (n = 2).

No MtcB-dependent methylation of Co(I)-MtqC was detectable with 100 mM choline, glycine betaine, proline betaine, TMA, or tetramethylammonium as methyl donors. However, we found that the purified enzyme did use γ-butyrobetaine (an analog of L-carnitine in which the β-hydroxy group is absent) as a substrate for methylation of MtqC. However, the rate of methylation of MtqC by MtcB with 65 mM γ-butyrobetaine was 0.5 μmol min⁻¹ mg⁻¹, a rate considerably lower than the rate of the enzyme with L-carnitine.

**In vitro reconstitution of the L-carnitine:THF methyltransferase reaction with recombinant MtcB, MtqC, and MtqA**

The preceding experiments revealed that MtcB could methylate MtqC in an L-carnitine–dependent manner and that both proteins were more abundant when cells were grown on L-carnitine. The abundance of MtqA, a homolog of the MetH methylcobalamin:THF methyltransferase domain, was also significantly increased when cells were grown on L-carnitine, and therefore we purified recombinantly produced MtqA (Fig. S4) to test whether this protein might participate in methyl transfer between L-carnitine and THF. The addition of MtqA to MtcB and MtqC allowed methylation of THF with carnitine. Equimolar (2 μM) amounts of MtcB and MtqA incubated with a 12.5-fold molar excess of MtqC catalyzed L-carnitine–dependent THF methylation at a rate of 188 ± 38 nmol min⁻¹ mg⁻¹ total protein (Fig. 6). Methyl-THF formation was not detectable in reactions lacking either MtcB, MtqC, or MtqA. As MtcB can methylate MtqC independently of MtqA, the requirement of MtqA for THF methylation indicates that methyl-Co(III)-MtqC is utilized as a substrate to methylate THF at a rate of at least 2.8 μmol min⁻¹ mg⁻¹ MtqA under the conditions of the assay. In contrast to the two methyltransferases and the corrinoid protein, RamQ was not required for the reaction but.

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**Figure 4. Purified recombinant MtcB-dependent methylation of cob(I)alamin with L-carnitine. A, methylation was followed by the increase at 540 nm in the complete reaction (●) as described under “Experimental procedures.” Methylation was not observed in the absence of MtcB (▲) or L-carnitine (■). B, kinetic analysis of MtcB in which the L-carnitine concentration was varied at different cob(I)alamin concentrations. The numbers beside each curve are the millimolar concentration of cob(I)alamin used for that data set. Each point is the average of three determinations. Error bars, S.D. Some error bars are not visible due to size of data point markers.**

**MtcB is an L-carnitine–dependent corrinoid methyltransferase**

Due to its abundance in L-carnitine–grown cells, we hypothesized that the MttB family member MtcB might be an L-carnitine:corrinoid methyltransferase. Therefore, we expressed the mtcB gene in *Escherichia coli* and purified recombinant MtcB (Fig. S4), which we found did carry out a robust L-carnitine: cob(I)alamin–dependent methyltransferase reaction with spectral changes identical to those observed for the same reaction mediated by cell extract. We monitored the reaction at 540 nm and found that the reaction was completely dependent on L-carnitine and MtcB (Fig. 4A). The enzyme displayed saturation kinetics with both substrates with varying L-carnitine and cob(I)alamin concentrations (Fig. 4B). A series of double reciprocal plots (1/v against 1/[L-carnitine]) at different cob(I)alamin concentrations (Fig. S5) generated a pattern typical of a sequen-
stimulated the rate of THF methylation. Controls lacking RamQ or ATP produced 38 and 39%, respectively, of the methyl-THF formed in assays supplemented with RamQ. This again indicates that Ti(III)citrate was capable of reducing sufficient MtqC to the active Co(I) form in the absence of RamQ-mediated reduction.

Discussion

A major stumbling block to precise annotation of microbial genomes and subsequent fuller understanding of the metabolic potential within metagenomes remains our yet incomplete understanding of metabolism. This is especially true for genes encoding members of protein superfamilies that have undergone functional diversification and are therefore sometimes subject to overly specific assignments during annotation that are not justified by retention of essential residues (48). The MttB superfamily is proving to be a case in point. Genes encoding superfamily members are often annotated as TMA methyltransferases due to their similarity to MttB, despite the lack of a pyrrolysine codon that distinguishes the gene encoding MttB, the first member of the superfamily with a described function (1, 3, 4). Many intestinal isolates bear genes encoding nonPyl MttB superfamily members in their genomes. Here we show that a fecal isolate of E. limosum, whose genome encodes 42 nonPyl MttB superfamily members, can grow by demethylation of L-carnitine. MtqC is the only one of these nonPyl MttB homologs that becomes abundant during growth on carnitine, and it acts as an L-carnitine:cobalamin methyltransferase.

MtcB is a strong candidate as a primary entry point of methyl groups from L-carnitine into the acetogenic metabolism of E. limosum. This idea is supported by the relative abundance of MtcB in cells grown on L-carnitine relative to lactate, as well as the favorable kinetics for the L-carnitine–dependent methylation of MtqC by MtcB. Upon the addition of MtqA, MtcB initiates the rapid in vitro methylation of THF relative to the abundance of the methyltransferase (Fig. 7). L-Carnitine–dependent methylation of THF would be crucial for methylotrophic acetogenesis with L-carnitine as the major growth substrate but would not be required for the proposed pathway of acetogenesis from lactate (45) in which lactate is initially catalyzed by a regulated lactate dehydrogenase (44). Correspondingly, MtcB is only abundant in cells grown on L-carnitine and not on lactate.

MtcB is also not abundant in cells grown on another methylotrophic substrate, proline betaine. Recently our laboratory described the ability of MtpB, another E. limosum MttB superfamily member, to demethylate proline betaine (42). MtqC is undetectable in the proteome of proline betaine-grown cells (42). Conversely, MtpB was 3.8 mol % protein in cells grown with proline betaine (42) but 1500-fold less abundant in the L-carnitine–grown cells examined here. MtcB and MtpB also display distinct substrate specificity toward their respective quaternary amines. MtcB’s robust activity with L-carnitine contrasts with its inability to detectably demethylate proline betaine. In comparison, MtpB has only trace activity with L-
The majority of the l-carnitine consumed by *E. limosum* was recoverable as the singly demethylated product, norcarnitine. Excretion of singly demethylated products has been typical of demethylation of quaternary amines by this acetogen (5, 28, 42). However, with l-carnitine, the end product is to our knowledge a novel catabolite. Most references in the literature refer to norcarnitine as a synthetic compound tested as an alternative substrate for l-carnitine–dependent enzymes, such as l-carnitine acetyltransferase (55) or palmitoyltransferase (56). However, among other l-carnitine derivatives, norcarnitine was shown to serve as sole carbon and nitrogen source for the facultative anaerobe *Pseudomonas putida* (57), suggesting that the norcarnitine produced by *E. limosum* during growth on l-carnitine is likely to be degraded by other members of the gut microbiota.

One of the most important implications of this work is that it reveals for the first time the potential of gut microbes having l-carnitine–corrinoid methyltransferase activity to positively affect human health. Work over the past decade has revealed that serum TMAO levels correlate with an increased risk of cardiovascular disease (58) and serum TMAO originates in the TMA excreted by gut microbes metabolizing quaternary amines, such as l-carnitine and its derivative γ-butyrobetaine (9, 11, 12). The demethylation of l-carnitine by MtcB represents the first instance of l-carnitine metabolism producing a catabolic product that cannot be readily converted into TMA. Indeed, quaternary amine demethylation by MtcB and perhaps other MttB superfamily members in different microbes might serve as a natural or therapeutic route to limit the production of TMA from dietary quaternary amines in the gut and thereby limit proatherogenic TMAO accumulation in the serum. It is interesting in this regard that *E. limosum* itself is widely regarded as a beneficial organism (33, 59, 60) and has been proposed as a marker of human longevity due to its significantly increased abundance in the microbiota of centenarians relative to younger groups (61). This leads us to speculate that demethylation of proatherogenic quaternary amines by MtcB, and perhaps other MttB family members, may be a factor underlying the perceived health benefits of *E. limosum* as a human gut symbiont.

**Experimental procedures**

**l-Carnitine demethylation during growth**

*E. limosum* ATCC 8486 was cultivated at 37 °C on the E. limosum defined medium supplemented with yeast extract, casamino acids, and sodium acetate as indicated (supplemental Experimental procedures). Supernatant from a culture was separated on silica gel 60 TLC plates followed by detection of quaternary amines and demethylation product(s) by bromocresol green as described previously (5). Norcarnitine used as a standard was synthesized via demethylation of l-carnitine with thio-phenolate (supplemental Experimental procedures). To confirm the identity of demethylation products, chromatographed supernatant was left unstained, and the area with an Rf corresponding to the demethylation product was extracted for mass spectral analysis on a Bruker MaXis ESI Q-TOF mass spectrometer.
Protein preparations

Cell extracts were prepared from *E. limosum* grown on either DL-lactate or L-carnitine by lysis with a French pressure cell followed by centrifugation at 48,000 g. The genes encoding MtcB, MtqC, MtqA, and RamQ were cloned into pSpeed using PIPE techniques. MtcB and MtqA were produced by iso-propyl-1-thio-β-D-galactopyranoside induction in *E. coli* BL21 (DE3) and purified by nickel affinity column followed by purification on a MonoQ column (GE Healthcare Life Sciences). RamQ was produced anaerobically in *E. coli* SG13009 (supporting Experimental procedures). The harvested cells were stored anaerobically at −80 °C until needed. RamQ was purified as described for MtcB and MtqA except in a Coy anaerobe chamber with buffers that contained 3 mM DTT for both nickel affinity and monoQ columns. MtqC was produced as an apoprotein in *E. coli* BL21 (DE3) and was reconstituted with hydroxycobalamin following removal of the N-terminal His tag using tobacco etch virus protease. The cleaved MtqC was then incubated under N₂/H₂ (98:2) at 4 °C for 36 h in a solution containing 3.5 M glycine betaine, 1 mM hydroxocobalamin, 10 mM DTT in 50 mM potassium phosphate buffer, pH 7.2. The reconstituted protein was then further purified on phenyl-Sepharose. The cobalamin content was assayed by measuring the absorbance change following dicyano derivatization and using a ∆ε₄₀₈ nm of 1.7·10⁴ M⁻¹ cm⁻¹ (66). Further details are available in the supporting Experimental procedures. Protein was assayed using the Bradford method with BSA as a standard.

Enzyme assays

All reactions were carried out under anoxic conditions and dim red light to prevent photolysis of the methyl-Co bond in corrinoid derivatives. All assays were conducted at 37 °C and the indicated concentration of phosphate buffer, pH 7.2. Methylation of cob(I)alamin by L-carnitine or other quaternary amines was measured in 2-mm cuvettes essentially as described previously for the glycine betaine:cob(I)alamin methyltransferase (5) except for use of the 75 mM potassium phosphate buffer, pH 7.2 (supporting Experimental procedures), and the substrate was L-carnitine or other quaternary amines as methylating substrates. Reactions were initiated with either cell extract (0.7-1.0 mg of total protein) or purified MtcB (0.8 μM). When the substrate specificity was tested, the reactions contained 2.3 mM cob(I)alamin, and a 63.4 mM concentration of the potential quaternary amine substrate. Kinetic parameters for MtcB L-carnitine:cob(I)alamin methyltransferase activity were collected by varying L-carnitine concentration between 0.2 and 63.4 mM and the cob(I)alamin concentration between 0.24 and 3.88 mM. Replots of the slopes and y intercepts of the double reciprocal plots of these data were used to calculate Kₘ and V_max values.

Proteomics

The experimental L-carnitine data set obtained here is compared with the same lactate data set described in a previous study (42). Both data sets were obtained with identical procedures. Two sets of four replicate cultures were grown in defined media on either 50 mM DL-lactate or L-carnitine in LS medium to mid-log phase (OD₆₀₀ ≈ 0.45). The harvested cell pellets were independently subjected to lysis and protein extraction prior to tryptic digestion. Peptides (12 μg) were separated on a two-dimensional liquid chromatography system prior to introduction of the fractionated peptides into an Orbitrap Fusion Mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) for MS/MS peptide sequencing and identification. The merged data were searched against the closed *E. limosum* ATCC 8486 genome (46) using Mascot Daemon 2.5.1 (Matrix Science, Boston, MA, USA). Scaffold (Proteomic Software, Inc., Portland, OR, USA) was used to compile data and assign emPAI values (43) based on peptide counts for estimation of the mol % of each protein relative to the total set of identified proteins in each proteome. For more details, see the supporting Experimental procedures.
**Data availability**

The MS proteomics data have been deposited to the ProteomExchange Consortium via the PRIDE partner repository (67) with the data set identifier PXD013806 (for the lactate data set) and with the identifier PXD013961 (for the 1-carnitine data set). All other data cited can be found in the article.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: TMA, trimethylamine; TMAO, trimethylamine N-oxide; nonPyl, nonpyrolysin; THF, tetrahydrofolate; emPAI, exponentially modified protein abundance index; PIPE, polymerase incomplete primer extension; OD, optical density; MFS, major facilitator family; CI, confidence interval.

**References**


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