Supplementary Information

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

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Running Title: *Methylation of tetrahydrofolate with carnitine*

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Supplementary text (Experimental procedures) Figs. S1 to S5

Supplementary Excel files for this manuscript include the following:

Dataset S1(Table S1)
Dataset S2 (Table S2)
Dataset S3 (Table S3)
Dataset S4 (Table S4)

Experimental procedures

Bacterium Strain and Growth Media. *E. limosum* ATCC 8486 was purchased from the American Type Culture Collection and cultured at 37 °C. The defined EL medium used in growth experiments contained (per liter): NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.25 g, sodium acetate, 0.83 g; biotin, 0.1 mg; folic acid, 0.2 mg; pyridoxine-HCl, 0.3 mg; thiamine-HCl, 0.2 mg; riboflavin, 0.1mg; nicotinic acid, 0.2 mg; *D*-pantothenate hemicalcium salt, 0.2 mg; hydroxocobalamin-HCl, 0.1 mg; p-aminobenzoic acid, 0.1 mg; and lipoic acid, 0.1 mg. Pfennig and Lippert's trace elements solution (1) was added at 3 ml/L and cultures were made anaerobic by flush/evacuation with 100% N_2 gas. K_2HP0_4, 0.35 g; KH_2PO_4, 0.28 g; $NaHCO_3$, 4.5 g; $Na_2S \cdot 9H_2O$, 0.48 g, cysteine-HCl, 0.47 g were added after autoclaving from sterile anoxic stock solutions. A modification of EL medium, termed LS medium, was used for fermentation balances and obtaining cell mass for cell extracts and proteomics experiments. LS medium was made as above except for the following concentration changes: $(NH_4)_2Cl_2$, 1 g; $MgSO_4 \bullet 7H_2O$, 0.1 g; $CaCl_2 \bullet 2H_2O$, 0.05 g; NaCl, 2.25 g; and $NaHCO_3$, 1.8 g. Both EL and LS defined media were supplemented with yeast extract (1 g/L) and casamino acids (2 g/L) only where indicated in text, otherwise defined EL and LS media were used. All quaternary amines were added to medium at 50 mM concentration unless otherwise indicated.

***L*-carnitine demethylation product.** To identify the demethylation product, a culture (10 ml) of *E. limosum* growing with the of *L*-carnitine was harvested approximately one day after entering stationary phase. The culture was pelleted by centrifugation at 16,100 x g for 2 min, and the supernatant was collected. Samples of the supernatant (2.5 µl) were spotted on each of two lanes of a silica gel 60 thin layer chromatography plate (EMD-Millipore) and the plate was developed in 80:20 (v/v) phenol:water. Following drying, one lane containing supernatant from *L*-carnitine-grown culture was excised and stained with 0.04% bromocresol green as previously described (2). The stained lane was aligned with the rest of the unstained plate and the upper and lower boundaries of the spot to be analyzed were marked. The region between these bounds were scraped from the unstained lanes, suspended into 1 ml of double-deionized water and incubated overnight at room temperature. The silica matrix was pelleted by centrifugation and the supernatant concentrated to dryness prior to dissolution in acetonitrile/water/acetic acid (1:1:0.001). Carnitine cultures were further analyzed by application of clarified supernatant to a Waters PAX column, followed by elution with methanol in a fraction containing the majority of remaining *L*-carnitine or produced norcarnitine in the supernatant. The methanol was dried under a stream of nitrogen and resuspended in 50% acetonitrile in water with 0.1% formic acid (v/v) before analysis. The samples were run on a Bruker MaXis ESI Q-Tof instrument (Bruker, Billerica, MA) using direct infusion. NaI was used to tune and calibrate the instrument for accurate mass measurement. The instrument was operated in positive mode with a capillary spray voltage of 4 to 4.5 kV and at a temperature of 200°C. The scan range was set between 50-1500 *m/z* for MS analysis. CID was used to fragment the targeted parent ions for further structural analysis and the collision energy was set at 15 eV.

Norcarnitine synthesis. 2.5 g [1.55 mmol] L-carnitine and 10 g [7.5 mmol] sodium thiophenolate were dissolved in 100 ml pyridine and refluxed at 115 °C under a stream of nitrogen for 6 hours. The reaction was dried on a rotary evaporator, then the residue was dissolved in 100 ml deionized water and 6.5 ml concentrated HCl. Residual thiophenol was

extracted from the resulting solution with three 50 ml portions of diethyl ether. The final aqueous phase was concentrated to an oil on a rotary evaporator, then the oil was dissolved in 100 ml n-propanol, and the resulting solution filtered to remove NaCl. The filtrate was again concentrated to oil and dissolved in deionized water. When analyzed by TLC, this solution produced a single spot that comigrated with the demethylation product of L-carnitine in the spent media of *E. limosum*. IR(neat): 1719, 1638, 1466, 1401, 1179, 1092, 972 cm^{-1}.^{1}H-NMR (in D_2O, 700 MHz) δ 4.39 (1H, m, CH-OD), 3.17 (2H, m, CH_2-N), 2.86 (6H, d, $(CH_3)_2$-N), 2.58, 2.50 (2H, dd, dd, CH_2-COO). The final product was found to have a purity of 95% when measured by quantitative ^{1}H-NMR using maleic acid as standard. This synthesis is essentially that of Stokke & Bremer (3) as verified by Rebouche (4) in spite of the comments of Ingalls et al. (5). Our starting material was *L*-carnitine instead of the hydrochloride. Hydrogen chloride was generated by dripping concentrated HCl onto anhydrous calcium chloride (6). Sodium thiophenolate was made according to the procedure of Jenden et al. (7).

Preparation of *E. limosum* cell extracts. Substrate-adapted *E. limosum* was grown on 50 mM substrate (either sodium *D,L*-lactate or *L*-carnitine) in LS medium with the addition of sodium acetate, casamino acids, and yeast extract. The atmosphere was 20% CO_2 in N_2. The cultures were incubated at 37 °C and harvested in late log phase by anaerobic centrifugation at 8,200 x g for 1 hr, resuspended in 500 ml anaerobic 75 mM potassium phosphate buffer, pH 7.2, and centrifuged again. In an anaerobic chamber, under a 98% N_2, 2% H_2 atmosphere, the cell pellets were resuspended in 2.5 ml/g wet mass of 10 mM NaCl, 10% glycerol, in 75 mM potassium phosphate, pH 7.2. The suspension was then anaerobically twice passed through a French press at 20,000 psi. The lysate was centrifuged at 48,400 x g for 30 minutes, and the resulting extract was frozen in liquid nitrogen, and stored at -80 °C until needed.

Recombinant Protein Production and Purification. Genes of interest were PCR amplified from genomic DNA for cloning into amplified pSpeedET using the PIPE method (8). Primer pairs included GTAGTAGTAGTAGTAGTGCTTTTGGACATGAAGGTCCCG and TAACGCGACTTAATTAAACGGTCTCCAGCTTGGCTGTTTTGGC (pSpeedET), CTGTACTTCCAGGGCATGATTATTATTGGAGAAAAACTGAACGG and AATTAAGTCGCGTTATTATTTCGCCTGAACTGGTCCAATCTTGCC (MtqA), CTGTACTTCCAGGGCATGATTCGTAACAGTTTAACAGATGTG and AATTAAGTCGCGTTATTAAATATCAAATTTATATTGTTCCGGCAGC (MtcB), CTGTACTTCCAGGGCATGGCAGATTGGAAAAATTTAACACAGG and AATTAAGTCGCGTTATCAGCCAACCCATGCCTGGCAGATCTTAACG (MtqC), and finally CTGTACTTCCAGGGCATGCGCGTATTGTTCCCATTACTTGAGG and AATTAAGTCGCGTTATTAAATCATCACATTATCCCCGAACATGG (RamQ). Each amplified gene and the amplified vector were gel purified before mixing and transformation into competent DH5α cells. Plasmid DNA from suitable clones was purified and the encoding sequence confirmed for each gene. All constructs except those encoding RamQ were transformed into BL21(DE3) cells for expression, while the construct with *ramQ* was transformed into *E. coli* SG13009. MtcB and MtqA were produced after induction with 1 mM IPTG in LB supplemented with 50 μg/ml kanamycin sulfate at room temperature, whereas *mtqC* was induced with 0.2 mM IPTG. Cell pellets were resuspended in buffer A (30 mM imidazole, 10% glycerol, 500 mM NaCl, 20 mM sodium phosphate, pH 7.2). A few crystals of DNAse I were added, and the cells were then passed through a French press twice at 20,000 psi. The lysate

was centrifuged at 4 °C and 48,400xg for 1 hour. The extract was loaded onto a 1 ml HiTrap nickel affinity column (GE Life Sciences, Pittsburg, PA) and the protein was eluted over a 40 ml linear gradient proceeding from 100% buffer A to 100% buffer B (composed of buffer A, except with 500 mM imidazole). The resulting protein solutions were further purified on a MonoQ column (GE Healthcare Life Sciences) equilibrated in 50 mM MOPS pH 7.2. After protein was applied the column was eluted with a linear 200 ml gradient from 0.25 to 1 M NaCl in MOPS, pH 7.2.

RamQ was expressed in *E. coli* SG13009 under a nitrogen atmosphere in LB supplemented with 80 mM D-glucose, 80 mM sodium fumarate, 50 μg/ml kanamycin sulfate, and 22 mM potassium phosphate, pH 7.2. At $OD_{600} \approx 0.4$, 0.5 mM IPTG, 0.5 mM ferrous ammonium sulfate and 3 mM cysteine-HCl were added to the culture. After the culture reached an OD_{600} of 1.0, an additional 0.5 mM ferrous ammonium sulfate and 3 mM cysteine-HCl were added, and the culture was incubated overnight. The following morning, the cells were harvested via centrifugation in sealed bottles with a N_2:H_2 (98:2) atmosphere. The cell pellet was washed with 50 mM MOPS, pH 7.2, then frozen in liquid nitrogen before storage at -80 °C until needed. RamQ was purified as described for the above proteins except the purification was performed in an anaerobe chamber (Coy Laboratory Products, Grass Lake, MI), all buffers contained 3 mM DTT, and a 1 ml HisTrap column (GE Healthcare Life Sciences) was used during the nickel affinity chromatography. The purified RamQ was stored at -80°C in 10 mM NaCl, 50% glycerol and 10 mM DTT, in 75 mM potassium phosphate, pH 7.2.

Prior to reconstitution with cobalamin, the N-terminal His-tag was removed from MtqC with recombinant TEV protease (5:1, w/w) in a solution of 0.5 mM EDTA, 20% glycerol, and 10 mM DTT in 50 mM Tris, pH 8.0 for 16 hours under a nitrogen atmosphere at room temperature. The solution was then diluted three-fold in buffer A and passed over a 1 ml HisTrap column and the flow-through containing MtqC was collected and concentrated in Amicon Ultra Centrifugal Filter Units having a 10 kDa cutoff membrane (EMD-Millipore). The cleaved MtqC was then reconstituted by incubation under a N_2:H_2 (98:2) atmosphere at 4 °C for 36 hours in a solution containing 3.5 M glycine betaine, 1 mM hydroxocobalamin, and 10 mM DTT in 50 mM Tris, pH 7.2. The mixture was then concentrated and the protein exchanged into 10 mM NaCl, 50 % glycerol in 75 mM potassium phosphate, pH 7.2 using Amicon Ultra Filter Units. The reconstituted protein was then purified on a 25 ml phenyl-sepharose column (Sigma-Aldrich) using a 100 ml gradient from 100% 1.58 M NH_4SO_4 in 50 mM MOPS, pH 7.2 to 100% of a buffer containing of 10% 1,3-propanediol, in 50 mM MOPS, pH 7.2. The MtqC peak was collected, concentrated and exchanged into 10 mM NaCl and 50 % glycerol in 75 mM potassium phosphate, pH 7.2. The resultant cobalamin content of the MtqC holoprotein was measured by the absorbance change following dicyanno derivatization employing an $\Delta\varepsilon_{368nm}$ of $1.7 \bullet 10^4$ $M^{-1}cm^{-1}$ (9).

THF methylation Assay. The assay was performed in an anaerobic chamber under red light and an atmosphere of 2% H_2 in N_2. Each reaction contained the following: 50 mM phosphate buffer, pH 7.2, 6 mM Ti(III) citrate, 0.25 mM ATP, enzymes (either cell extracts from L-carnitine-grown cells, or purified recombinant proteins), tetrahydrofolate (≥65%, Sigma-Aldrich), and L-carnitine. All reaction components, except for *L*-carnitine, were added and the reactions were incubated at room temperature for 5 minutes, and the reaction was initiated by the addition of L-carnitine. At various intervals, 100 μL reaction samples were withdrawn and mixed with 20 μL saturated trichloroacetic acid. The stopped reactions were centrifuged for 10 minutes at 16,300

rcf and the supernatants collected and stored at -80 °C under N_2 until needed. The supernatants were analyzed on an Ultimate 3000 HPLC system as previously described (2).

Spectrophotometric assays of cobalamin or MtqC methylation with quaternary amines. The formation of methylcob(III)alamin from cob(I)alamin was monitored by following the increased absorbance at 540 nm using an extinction coefficient of 4.4 $mM^{-1} \cdot cm^{-1}$.Assay with purified recombinant MtcB contained 11.7 mM Ti(III)citrate and 0.8 μM MtcB in 75 mM potassium phosphate buffer, pH 7.2. When the enzyme's substrate specificity was tested, the reactions contained 2.3 mM cob(I)alamin, and 63.4 mM substrate. Kinetic constants were estimated by varying L-carnitine concentration between 0.198 mM and 63.4 mM, while varying the cob(I)alamin concentration between 0.24 mM and 3.88 mM. Replots of the slopes and intercepts of the double reciprocal plots of these data permitted estimates of the K_m for L-carnitine and cob(I)alamin and enzyme V_{max}.

MtqC methylation assays were carried out under red light in stoppered 1 cm quartz micro-cuvettes with a 2% H_2 in nitrogen headspace and at 37 °C. Each 100 μL reaction contained 2 mM Ti(III) citrate, 2 mM MgATP, 51 μM MtqC, 0.35 μM RamQ and 10 nM (with indicated concentrations of *L*-carnitine) or 100 nM (with 100 mM of other tested substrates) MtcB. The $\Delta\varepsilon_{540}$ for the conversion of Co(I)-MtqC to methyl-Co(III)-MtqC was determined in triplicate as the MtqC methylation reaction went to completion with 40 mM L-carnitine. When the reaction reached completion an additional 10 nM MtcB and 40 mM L-carnitine were added to confirm no further absorbance change occurred at 540 nm. The $\Delta\varepsilon_{540}$ was calculated to be 3032 ± 198 $M^{-1} \bullet cm^{-1}$. Curves were fit the Michaelis-Menten equation using Prism 7 (GraphPad Software).

Proteomic sample preparations. Two sets of 4 replicate cultures (10 ml each) were grown on defined LS medium. One set was grown with 50 mM *L*-carnitine, while the second set was grown on 50 mM *D, L*-lactate. Each culture was anaerobically harvested in mid-log phase ($OD_{600} \approx 0.45$) by centrifugation. The pellets were washed once with 22 mM potassium phosphate buffer, pH 7.2, frozen in liquid nitrogen, and stored at -80 °C until needed. From each cell pellet, an aliquot was lyophilized then subjected to cyrogrinding and the subsequent addition of RIPA Buffer [50mM Tris-HCl 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF, 1x Roche Protease inhibitor cocktail, 1x HALT Phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham MA), 25U/ml Benzonase nuclease (Novagen, Madison, WI)] at a ratio of 12 μl/mg cell pellet (original wet weight). The suspension was sonicated on ice twice for 10 seconds with a 10 second interval between pulses, then centrifuged at 13,000 RPM in a microfuge, the supernatant retained, and lysis buffer (7 M Urea, 2M Thiourea, 4% CHAPS in 30 mM Tris-HCl pH 8.5) added to the remaining pellet at a ratio of 2.5 ul/mg cell pellet (original wet weight). The resuspended pellet was subjected to two five-second sonication bursts on ice, then centrifuged again at 13,000 RPM. The two supernatants were combined and the protein concentration determined by Bradford assay using BSA as standard. The protein was then precipitated with TCA (final concentration 20% v/v) and then was centrifuged at 13,000 RPM for 20 min. The pellets were washed with ice-cold acetone to remove any remaining acid and resuspended in 50 μl of 0.1% Rapigest (Waters Corp., Milford, MA) dissolved in 50 mM ammonium bicarbonate. Five μl of DTT (5ug/ μl in 50 mM ammonium bicarbonate) was added and the sample incubated at 56°C for 15 min. Five uL of iodoacetamide (15 mg/ml in 50mM ammonium bicarbonate) was added and the sample kept in dark at room temperature for 30 min. Sequencing grade-modified

trypsin (Promega, Madison WI) in 50 mM ammonium bicarbonate was added to the sample at an estimated ratio of 1:50 trypsin-substrate and incubated at 37°C overnight. This grade of trypsin is devoid of chymotrypsin activity and cleaves at the carboxylic side of lysine or arginine. Cleavage is decreased when acidic residues are present on either side of a susceptible bond. If proline is found C-terminal to either lysine or arginine, the bond is highly resistant to cleavage. After digestion, a final concentration of 0.1% TFA was added prior to incubation at 37°C for 30 min to precipitate Rapigest. The sample was centrifuged and the supernatant dried, then resuspended with 50 mM acetic acid for the measurement of peptide concentration using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham MA).

Peptide analysis with Orbitrap Fusion Mass Spectrometer. Prior to MS/MS, samples were subjected to online 2-D liquid chromatography (LC) separation using either an Acquity UPLC M-Class LC (Water's Corp., Milford, MA) or a Thermo Scientific 2D RSLC HPLC system. Peptides were separated using the same basic protocol on either instrument. Samples (12 μg) were first separated on a 5 mm x 300 μm BEH C18 column with 3 μm particle size and 130 Å pore size. Solvent A was composed of 20 mM ammonium formate, pH 10 and solvent B was 100% acetonitrile. Peptides were eluted from column in 8 successive fractions using 9.5, 12.4, 14.3, 16.0, 17.8, 19.7, 22.6 and 50% solvent B. Each eluted fraction was then online trapped, diluted, neutralized, and desalted on a μ-Precolumn Cartridge (Thermo Fisher Scientific) for the second dimensional separations performed with a 15 cm x 75 μm PepMap C18 column (ThermoFisher Scientific, Waltham, MA) with 3 μm particle size and 100 Å pore size. Mobile phase A used here was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. For M-Class LC, the flow rate for the analytical column was 500 μL/min. The gradient was: for 0 to 3 min, 3% solvent B; for 3 to 45min, 55% solvent B; for 45 to 47 min, 85% solvent B; and finally solvent B was kept at 85% for another 2 min before bought back to 3% for 10 min to equilibrate the column for the next separation. For the Thermo Scientific 2D RSLC HPLC system, the flow rate for the analytical column was 500 μl /min. The gradient was: from 0 to 5 min, 2% solvent B; from 5 to 38 min, 35% solvent B; from 38 to 46 min, 35-55% solvent B; and for 46 to 47 min, 55-90% solvent B. Solvent B was then kept at 90% for 1 min before being returned to 2% solvent B. The system was equilibrated for 11 min before the next separation. MS/MS data was acquired with a spray voltage of 1.7 KV and a capillary temperature of 275 °C. The scan sequence of the mass spectrometer was based on the preview mode data dependent TopSpeed™ method: the analysis was programmed for a full scan recorded between *m/z* 400 – 1600 and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive scans starting from the most abundant peaks in the spectrum in the next 3 seconds. To achieve high mass accuracy MS determination, the full scan was performed at FT mode and the resolution was set at 120,000. The AGC Target ion number for FT full scan was set at 2×10^5 ions, maximum ion injection time was set at 50 ms and micro scan number was set at 1. MS/MS was performed using ion trap mode to ensure the highest signal intensity of MS^2 spectra using both CID (for 2+ and 3+ charges) and ETD (for 4+ to 7+ charges) methods. The AGC Target ion number for ion trap MS^2 scan was set at 1000 ions, maximum ion injection time was set at 100 ms and micro scan number was set at 1. The CID fragmentation energy was set to 35%. Dynamic exclusion is enabled with a repeat count of 1 within 60s and a low mass width and high mass width of 10 ppm.

Sequence information from the MS/MS data was processed by converting raw files into a mgf files using MSConvert version 3.0.8738 (ProteoWizard) and then mgf files from each of the

fractions was merged into a single merged file (mgf) using an in-house program, RAW2MZXML_n_MGF_batch (merge.pl, a Perl script). The resulting mgf files were searched using Mascot Daemon (version 2.5.1) by Matrix Science (Boston, MA) and searched against the set of 3998 proteins encoded in genome of *E. limosum* ATTC8486 (10) (GenBank assembly accession GCA_000807675.2) maintained at the National Center for Biotechnology Information. The mass accuracy of the precursor ions was set to 10 ppm and an allowance for selection of one ^{13}C peak for each identified peptide was also included into the search. The fragment mass tolerance was set to 0.5 Da. Considered variable modifications were oxidation (Met), deamidation (Asn and Gln) and carbamidomethylation (Cys). Four missed cleavages for the enzyme were permitted. A decoy database was also searched to determine the false discovery rate (FDR) and peptides were filtered according to the FDR. The significance threshold for peptide identification was set at $p<0.05$, Only proteins identified with <1% FDR as well as a minimal of 2 peptides are accepted for quantitation. Label free quantitation was performed using the spectral count approach, in which the relative protein quantitation is measured by comparing the number of MS/MS spectra identified from the same protein in each of the multiple LC/MSMS datasets. Scaffold (Proteomic Software, Inc., Portland OR) was used for data analysis and calculation of emPAI values to estimate the mol% of each identified protein within the total set of identified proteins (11). Student's T-test was performed using Scaffold to evaluate if the fold change for certain proteins between growth conditions is significant ($p<0.05$).

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Supplementary Figures

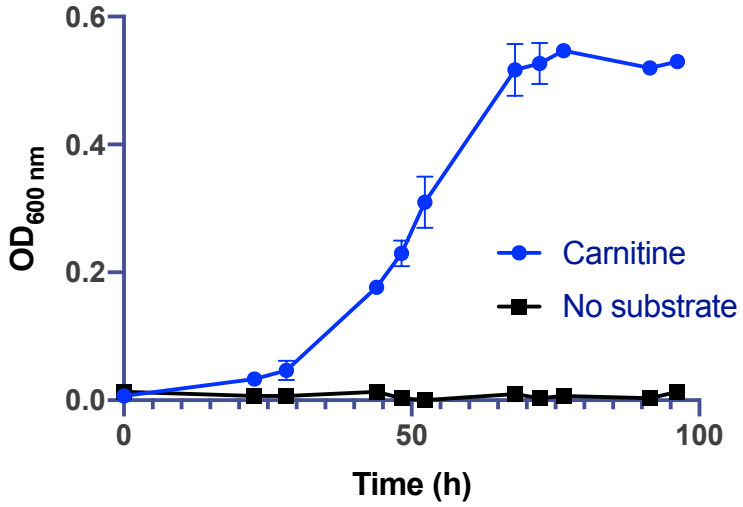

Fig. S1. Growth of *E. limosum* on *L*-carnitine in defined medium. Cultures containing LS medium were supplemented with either 30 mM *L*-carnitine or no substrate as indicated. Each point is the average from three independent cultures with vertical bars indicating standard deviation.

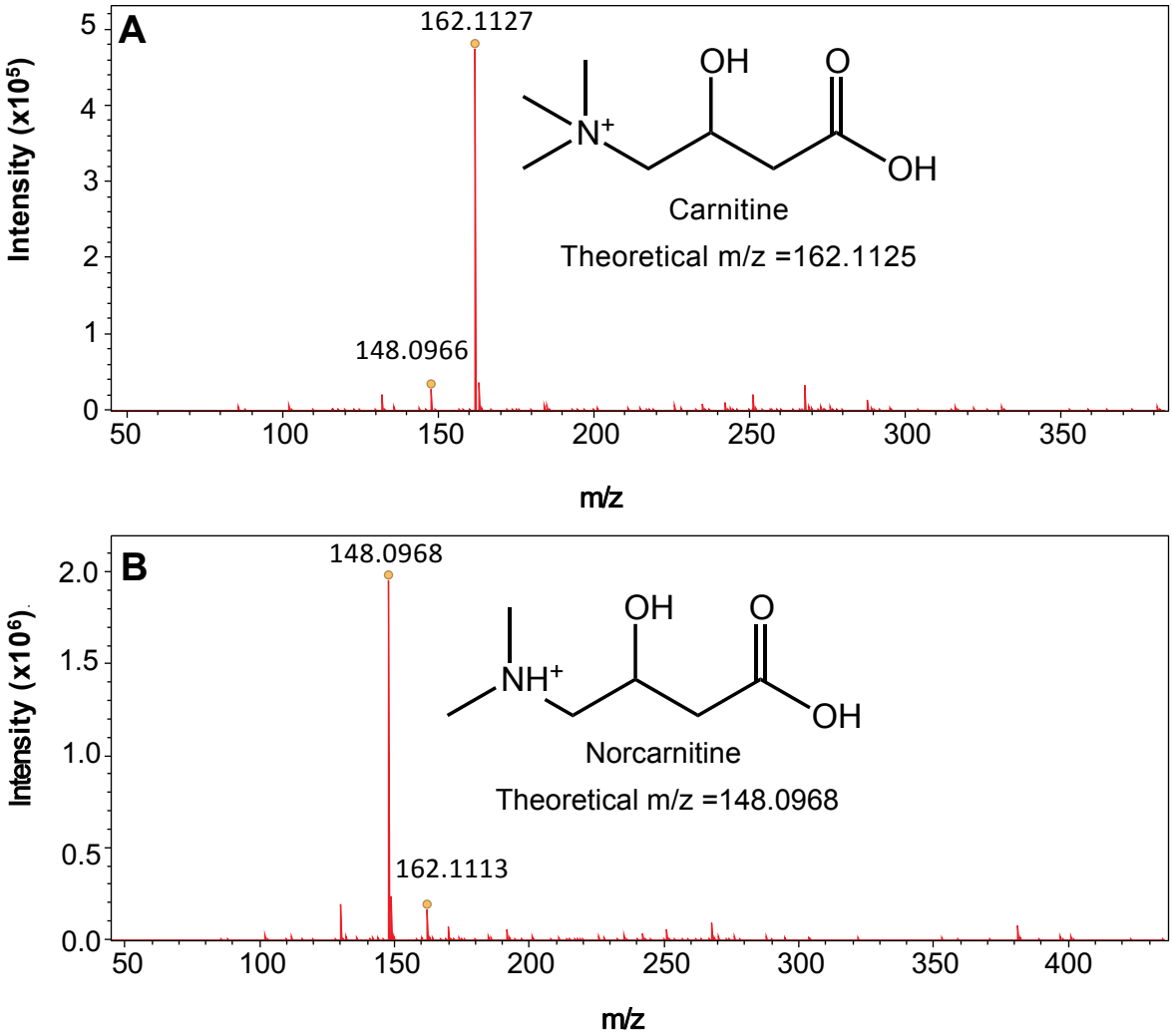

Fig. S2. Mass spectral analysis of *L*-carnitine culture supernatants. Clarified supernatants were extracted by passage over a Waters PAX cartridge followed by elution of the column with methanol which was subsequently dried under nitrogen and dissolved in 50 % acetonitrile and 0.1% formic acid in water (v/v) prior to analysis. In panel A the supernatant sample was taken after inoculation but before growth and in panel B, the sample was taken from the culture shortly after entering stationary phase.

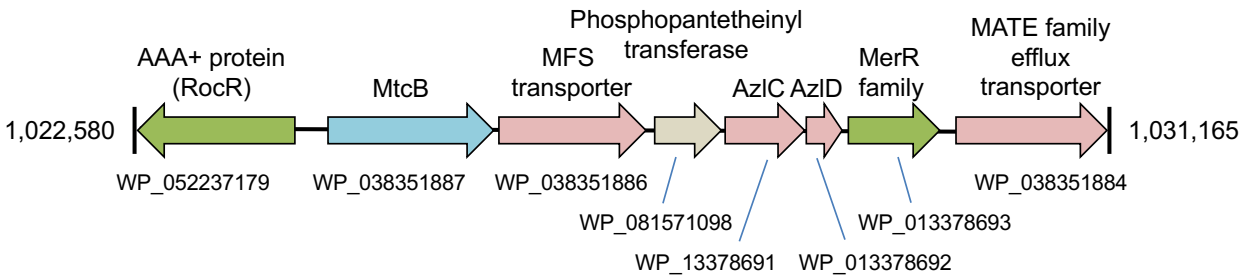

Fig. S3. Genomic context of the *mtcB* gene in the genome of *E. limosum*. The numbers on the left and right of each genomic segment are from the nucleotide numbering used for the complete *E. limosum* ATCC 8486 genome maintained at the National Center for Biotechnology Information (NCBI) under accession number NZ_CP019962.1. Above each gene is the annotation and/or possible function of the product. The numbers below each gene are annotations for the protein product. The *mtcB* gene is colored in blue, while other genes are color coded by putative metabolic roles; transporters (pink), transcriptional regulators (green), or metabolic proteins (grey).

Fig. S4

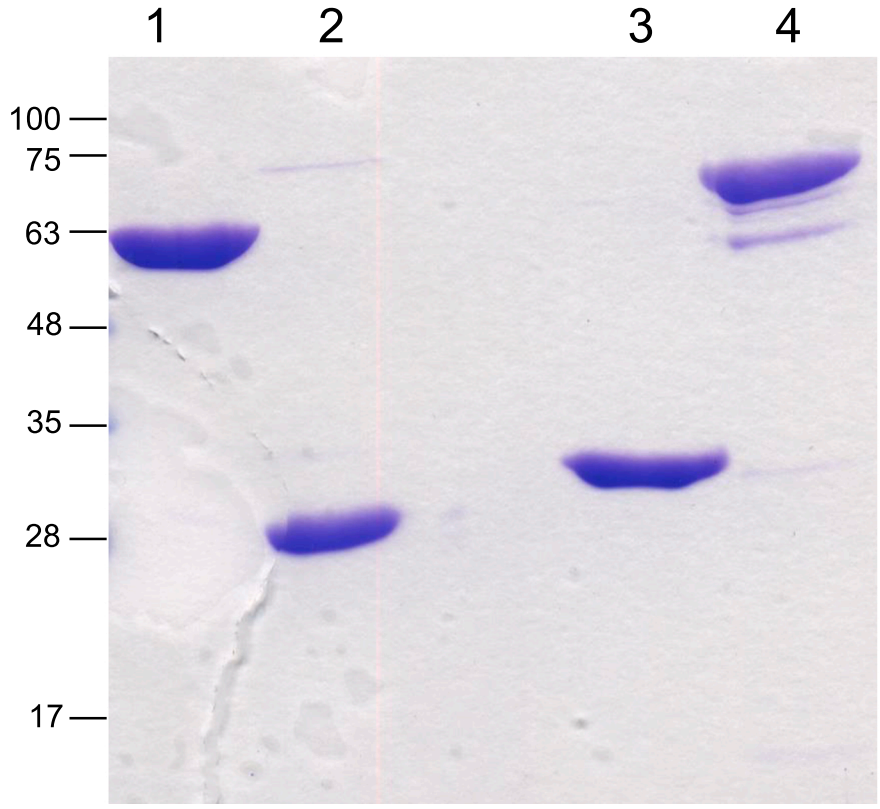

Fig. S4. SDS-PAGE analysis of the recombinant proteins used in assays for activities associated with the *L*-carnitine:THF methyltransferase system. The protein samples (5 μg) loaded were: lane 1, MtcB; lane 2: MtqC, lane 3: MtqA, and; lane 4, RamQ. Lines to the left indicate center of the migration positions of protein standards ran in the same gel with the molecular masses indicated in kDa.

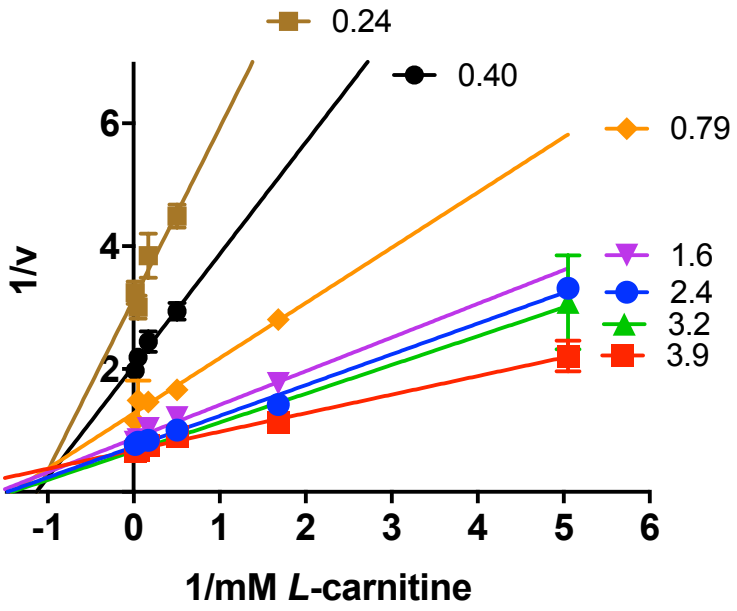

Fig. S5. Reciprocal plot of the rate of methylation of cobalamin versus *L*-carnitine concentrations as measured at increasing concentrations of cob(I)alamin. Numbers at end of line fits indicate cob(I)alamin concentration in mM. The units of enzyme velocity (v) are μmol min^{-1} mg^{1}. Each point represents the mean of three reactions with standard deviation indicated by the error bars.

Supplementary Tables supplied as separate Excel Files

Table S1. Tandem mass spectrometric analysis of *m/z* 148.0972 peak identified in the TLC spot from end-point *L*-carnitine culture supernatants confirms the presence of norcarnitine.

Table S2. Proteins identified by label free proteomics in *Eubacterium limosum* grown on *D, L*-lactate or *L*-carnitine that have confirmed or potential roles in catabolic reactions.

Table S3. Scaffold Protein Report for lactate versus carnitine grown cells.

Table S4. Averaged quantification for identified proteins in lactate- and carnitine-grown cells.