(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 December 2017 (21.12.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/218889 A1

(51) International Patent Classification:
A61K 35/74 (2015.01)

(21) International Application Number:
PCT/US2017/037869

(22) International Filing Date:
16 June 2017 (16.06.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/351,124 16 June 2016 (16.06.2016) US

(71) Applicants: **OHIO STATE INNOVATION FOUNDATION** [US/US]; 1524 North High Street, Columbus, Ohio 43201 (US). **MIAMI UNIVERSITY** [US/US]; 501 E. High Street, Oxford, Ohio 45056 (US).

(72) Inventors: **KRZYCKI, Joseph A.**; 340 Arden Rd., Columbus, Ohio 43214 (US). **KOUNTZ, Duncan**; 698 Patrick Henry Circle, West Chester, Pennsylvania 19382 (US). **FERGUSON, JR., Donald J.**; 90 Pearson Hall, Department of Microbiology, Oxford, Ohio 45056 (US).

(74) Agent: **PRATHER, Donald M.** et al.; MEUNIER CARLIN & CURFMAN LLC, 999 Peachtree St. NE, Suite 1300, Atlanta, Georgia 30309 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*

(54) Title: EUBACTERIUM PROBIOTICS AND METHODS OF TREATING OR PREVENTING HEART DISEASE

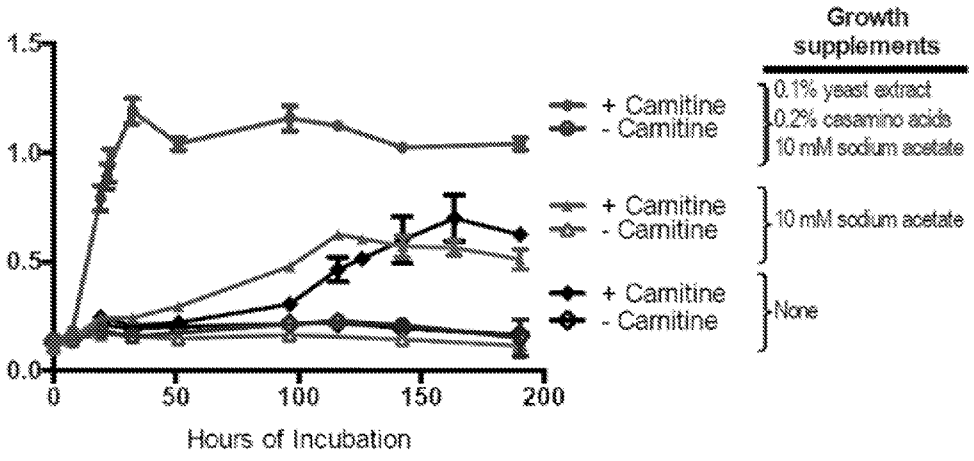


FIG. 4

(57) Abstract: The present disclosure relates to probiotic compositions and methods of treating or preventing heart disease.

WO 2017/218889 A1

EUBACTERIUM PROBIOTICS AND METHODS OF TREATING OR PREVENTING HEART DISEASE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/351,124 filed June 16, 2016, which is expressly incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with Government Support under Grant No. GRT8275000 awarded by the National Institutes of Health and Grant No. DEFG0291ER20042 awarded by the Department of Energy. The Government has certain rights in the invention.

FIELD

The present disclosure relates to probiotic compositions and methods of treating or preventing heart disease.

BACKGROUND

In the last few years it has been found that certain human intestinal microbes contribute to cardiovascular disease, thereby increasing the risk of heart attack, stroke, and death. Gut microbes convert quaternary amines (QAs), e.g. carnitine, butyrobetaine, choline, and glycine betaine, to trimethylamine (TMA). TMA enters the bloodstream and once converted by liver enzymes to trimethylamine-N-oxide (TMAO), can trigger macrophage mediated lipid deposition in the vascular system. Serum TMAO levels accordingly correlate with arteriosclerosis and the above catastrophic health events. What is needed are novel compositions and methods for reducing levels of trimethylamine (TMA) to improve heart health.

The compositions and methods disclosed herein address these and other needs.

SUMMARY

The inventors have found that *Eubacterium* strains can demethylate each quaternary amine (QA) that would otherwise form trimethylamine (TMA) and/or trimethylamine-N-oxide (TMAO). Therefore, *Eubacterium limosum* and members of Clostridial Clade XV, which are close relatives of *E. limosum* including E. aggregans, E. barkeri, and E. callanderi, can be taken as a probiotic supplement to treat or prevent against heart disease.

In one aspect, disclosed herein is a probiotic composition comprising an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier.

In one embodiment, the isolated *Eubacterium* spp. (species) strain is selected from *Eubacterium limosum, Eubacterium aggregans, Eubacterium barkeri, Eubacterium callanderi.* In one embodiment, the isolated *Eubacterium* spp. is *Eubacterium limosum.* In one embodiment, the isolated *Eubacterium* spp. is *Eubacterium limosum* ATCC 8486.

In one embodiment, the isolated *Eubacterium* spp. is present at 10^5 to 10^{13} cfu *Eubacterium* per gram.

In one embodiment, the probiotic composition is in the form of a suspension, capsule, tablets or a microencapsulated product.

In one embodiment, the probiotic composition further comprises an additional probiotic strain.

In one aspect, disclosed herein is a method of treating or preventing heart disease, comprising administering to a subject in need thereof an effective amount of a probiotic composition, wherein the probiotic composition comprises an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier.

In another aspect, disclosed herein is a method of reducing trimethylamine or trimethylamine-N-oxide levels in a subject, comprising administering to the subject an effective amount of a probiotic composition, wherein the probiotic composition comprises an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier.

In one embodiment, the isolated *Eubacterium* spp. is selected from *Eubacterium limosum, Eubacterium aggregans, Eubacterium barkeri, Eubacterium callanderi.* In one embodiment, the isolated *Eubacterium* spp. is *Eubacterium limosum.* In one embodiment, the isolated *Eubacterium* spp. is *Eubacterium limosum* ATCC 8486.

In one embodiment, the isolated *Eubacterium* spp. is present at 10^5 to 10^{13} cfu *Eubacterium* per gram.

In one embodiment, the probiotic composition is in the form of a suspension, capsule, tablets or a microencapsulated product.

In one embodiment, the probiotic composition further comprises an additional probiotic strain.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several aspects described below.

Fig. 1. Flux of quaternary amines (QAs) in the gut towards (black arrows) and away (white arrows) from trimethylamine-N-oxide (TMAO). Known trimethylamine (TMA) generating enzymes found in microbes are indicated over the black arrows. The QA demethylating reactions as typified by *E. limosum* 8486 are indicated by white arrows. Glycine betaine is demethylated by MtgB, a TMA MT family member without pyrrolysine (non-Pyl MttB). The data herein indicating the carnitine and choline demethylations carried out by *E. limosum* are QA demethylases of the TMA MT family. The remaining QA demethylation reactions can be carried out by other non-Pyl TMA MT family members. The ratio of QA demethylating microbes and TMA generating microbes present in the gut may predispose an individual towards or away from TMAO production and heart disease. This further shows that QA demethylating organisms such as *E. limosum* can serve as a microbial therapeutics (probiotics) to ameliorate the risk of heart disease.

Figs. 2A-2B. Example three component systems. Fig. 2A) Three component systems in methanogenic archaea. Fig. 2B) Three component systems in acetogens such as *Eubacterium limosum*. MT I methylates the corrinoid protein (CP) with substrate. MT II demethylates CP and methylates Coenzyme M or THF for subsequent catabolism. Only 3-component systems are known to initiate metabolism of methanogens or acetogens on N-methyl or O-methyl substrates. For example, the methanogen methanol MT I is also found in the genome of *E. limosum*. Members of the TMA MT family could mediate the demethylation of quaternary amines such as choline, carnitine, butyrobetaine, and glycine betaine by a human gut isolate of *E. limosum*.

Fig. 3. Demethylation can divert compounds away from trimethylamine (TMA) production. Several members of the human intestinal microflora encode MtgB homologs. L-carnitine is another substrate for some members of the MttB superfamily.

Fig. 4. Growth of *E. limosum* on L-carnitine (50 mM), demonstrating that L-carnitine can serve as the sole fixed carbon and energy source for *E. limosum*. Points represent the means of three cultures.

Fig. 5. Catabolic stoichiometry. In a simple defined medium, *E. limosum* coverst L-carnitine and carbon dioxide to norcarnitine and a mixture of even-chain short fatty acids. Although the fermentation is complex, it is consistent with knowledge that *E. limosum* is an acetogen that also produces butyrate.

Figs. 6A-6C. Carnitine corrinoid methyltransferase activity in *E. limosum* cell extracts. Fig. 6A) Time course of free cob(I)alamin methylation dependent on the addition of L-carnitine and

extract from carnitine-grown *E. limosum*. Specific activity was 94.2 ± 26.9 nmol/min/mg protein (n = 3 preparations). Fig. 6B) Spectral profile of the reaction. Each line represents a timepoint and unlabeled arrows indicate the direction of spectral shift over time. The distinct isosbestic point at 578 nm is indicative of cob(I)alamin methylation. Fig. 6C) Schematic of the reaction catalyzed.

Figs. 7A-7B. Extract catalysis of L-carnitine: tetrahydrofolate (THF) methyl transfer. Fig. 7A) Time course of the methylation of THF measured by HPLC. The reaction was dependent on the presence of extract, carnitine, THF, ATP and Ti(III)citrate. Specific activity was 12.6 nmol/min/mg protein (average of two preparations). Fig. 7B) Schematic of the reaction catalyzed.

Figs. 8A-8C. Demethylation reaction catalyzed by MtcB. Fig. 8A) Time course of cob(I)alamin spectral changes catalyzed by MtcB. Fig. 8B) Double reciprocal plot showing the Michaelis-Menten kinetics of MtcB. Each point represents a mean of at least three reactions. Fig. 8C) Schematic of the reaction catalyzed by MtcB.

Figs. 9A-9B. Demethylation reaction catalyzed by MtcA. Fig. 9A) Time course of methylcobalamin spectral changes catalyzed by MtcA. A decrease in absorption at 526 nm with an apparent isosbestic point at 487.5 nm is indicative of conversion of methylcob(III)alamin to cob(II)alamin. 5 mM DTT is included in the reaction to convert cob(I)alamin (the direct product of demethylation) to cob(II)alamin. Specific activity was 7.88 nmol/min/mg protein. Fig. 9B) Schematic of the probable reaction catalyzed by MtcA.

Fig. 10. Schematic of a 3-component methyl transfer pathway.

Fig. 11. Growth of *E. limosum* on quaternary amines (QAs). Also scored by letter is growth on each substrate for: (a), *E. aggregans*, (b), *E. barkeri*; and (c), *E. callanderi*.

Fig. 12. MttB methylation of Co(I)-MttC. Top: Spectra after TMA added. Arrows show movement of spectra with time. Bottom:Rxn traces for conversion of Co(I) to methyl-Co(III) MttC. (I.P.) Isosbestic point, (A) Absorbance.

Figs. 13A-13B. *In* vitro demonstration of the carnitine:tetrahydrofolate (THF) methyltransferase reaction mediated by MtcB (MttB family member), corrinoid protein MtcC, and methyl-corrinoid:THF methyltransferase MtcA. (Fig. 13A) Methylation of THF by reactions containing the indicated components (inset box) in 50 mM potassium phosphate buffer, pH 7.2 incubated at 37°C. At the indicated times, aliquots were removed from the reaction vial and acidified prior to analysis by liquid chromatography. Each data point is an average of 3 independent assays with standard deviation represented by error bars. Separate control experiments demonstrated methylation of THF was dependent on addition of MtcB, MtcC, MtcA, and carnitine. Addition of RamC stimulated the reaction by approximately 50%, but was not necessary, suggesting Ti(III)citrate is sufficient for activation of the corrinoid protein. (Fig. 13B)

Schematic of the reaction, the roles of the two methyltransferases MtcB and MtcA were demonstrated in separate reactions.

Figs. 14A-C. Carnitine:MtcC methyltransferase is mediated by MtcB, an MttB family member. Fig. 14A) UV-Vis spectroscopic time course of L-carnitine:MtcC methyl transfer reaction recorded at 37°C. The complete reaction contains 50 μM MttC (reduced to the Co(I) form usingTi(III)citrate, 10 mM ATP, and 0.3 μM RamC), as well as 0.1 μM MtcB and 10 mM carnitine. Traces were taken 1 min apart. Arrows indicate the direction of peak movement during the reaction. Fig. 14B) Absorbance traces at 540 nM indicative of Co(I)-MttC methylation, while 585 nm is the isosbestic point between Co(I)-MtcC and methyl-Co(III)-MtcC. Methylation was dependent on both carnitine and MtcB. MtcB has an apparent Vmax of 16.6 μmol min^{-1} mg^{-1} and Km of 100 μM carnitine. Fig. 14C) Schematic of L-carnitine:Co(I)-MtcC methyl transfer reaction catalyzed by MtcB.

Fig. 15. Schematic of the initial steps of quaternary amine catabolism in *E. limosum*. Each MttB family member shown is not detectable in cells grown on lactate, but upregulated specifically in cells grown on the indicated quaternary amine. However, MtcC, MtcA, and RamC are upregulated on all quaternary amines. The biochemical roles of MtcB, MtcC, and MtcA in carrying out carnitine:THF methyl transfer have been shown by *in vitro* assays. In addition, MtpB can methylate MtcC with proline betaine.

Fig. 16. Strain adaptation of *E. limosum* cultures to growth on choline. Cultures were successively transferred to fresh medium on the dates indicated and adaptation to growth by demethylation of choline was examined.

DETAILED DESCRIPTION

The inventors have found that *Eubacterium* strains can demethylate each quaternary amine (QA) that would otherwise form trimethylamine (TMA) and/or trimethylamine-N-oxide (TMAO). Therefore, *Eubacterium limosum* and members of Clostridial Clade XV, which are close relatives of *E. limosum* including *Eubacterium aggregans*, *Eubacterium barkeri*, and *Eubacterium callanderi*, can be taken as a probiotic supplement to treat or prevent against heart disease.

Reference will now be made in detail to the embodiments of the invention, examples of which are illustrated in the drawings and the examples. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention

belongs. The following definitions are provided for the full understanding of terms used in this specification.

Terminology

As used in the specification and claims, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

As used herein, the terms "may," "optionally," and "may optionally" are used interchangeably and are meant to include cases in which the condition occurs as well as cases in which the condition does not occur. Thus, for example, the statement that a formulation "may include an excipient" is meant to include cases in which the formulation includes an excipient as well as cases in which the formulation does not include an excipient.

As used here, the terms "beneficial agent" and "active agent" are used interchangeably herein to refer to a chemical compound or composition that has a beneficial biological effect. Beneficial biological effects include both therapeutic effects, i.e., treatment of a disorder or other undesirable physiological condition, and prophylactic effects, i.e., prevention of a disorder or other undesirable physiological condition. The terms also encompass pharmaceutically acceptable, pharmacologically active derivatives of beneficial agents specifically mentioned herein, including, but not limited to, salts, esters, amides, prodrugs, active metabolites, isomers, fragments, analogs, and the like. When the terms "beneficial agent" or "active agent" are used, then, or when a particular agent is specifically identified, it is to be understood that the term includes the agent per se as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, prodrugs, conjugates, active metabolites, isomers, fragments, analogs, etc.

As used herein, the terms "treating" or "treatment" of a subject includes the administration of a drug to a subject with the purpose of preventing, curing, healing, alleviating, relieving, altering, remedying, ameliorating, improving, stabilizing or affecting a disease or disorder, or a symptom of a disease or disorder. The terms "treating" and "treatment" can also refer to reduction in severity and/or frequency of symptoms, elimination of symptoms and/or underlying cause, prevention of the occurrence of symptoms and/or their underlying cause, and improvement or remediation of damage.

As used herein, the term "preventing" a disorder or unwanted physiological event in a subject refers specifically to the prevention of the occurrence of symptoms and/or their underlying cause, wherein the subject may or may not exhibit heightened susceptibility to the disorder or event.

By the term "effective amount" of a therapeutic agent is meant a nontoxic but sufficient amount of a beneficial agent to provide the desired effect. The amount of beneficial agent that is "effective" will vary from subject to subject, depending on the age and general condition of the subject, the particular beneficial agent or agents, and the like. Thus, it is not always possible to specify an exact "effective amount." However, an appropriate "effective" amount in any subject case may be determined by one of ordinary skill in the art using routine experimentation. Also, as used herein, and unless specifically stated otherwise, an "effective amount" of a beneficial can also refer to an amount covering both therapeutically effective amounts and prophylactically effective amounts.

An "effective amount" of a drug necessary to achieve a therapeutic effect may vary according to factors such as the age, sex, and weight of the subject. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

As used herein, a "therapeutically effective amount" of a therapeutic agent refers to an amount that is effective to achieve a desired therapeutic result, and a "prophylactically effective amount" of a therapeutic agent refers to an amount that is effective to prevent an unwanted physiological condition. Therapeutically effective and prophylactically effective amounts of a given therapeutic agent will typically vary with respect to factors such as the type and severity of the disorder or disease being treated and the age, gender, and weight of the subject.

The term "therapeutically effective amount" can also refer to an amount of a therapeutic agent, or a rate of delivery of a therapeutic agent (e.g., amount over time), effective to facilitate a desired therapeutic effect. The precise desired therapeutic effect will vary according to the condition to be treated, the tolerance of the subject, the drug and/or drug formulation to be administered (e.g., the potency of the therapeutic agent (drug), the concentration of drug in the formulation, and the like), and a variety of other factors that are appreciated by those of ordinary skill in the art.

As used herein, the term "pharmaceutically acceptable" component can refer to a component that is not biologically or otherwise undesirable, i.e., the component may be incorporated into a pharmaceutical formulation of the invention and administered to a subject as described herein without causing any significant undesirable biological effects or interacting in a deleterious manner with any of the other components of the formulation in which it is contained. When the term "pharmaceutically acceptable" is used to refer to an excipient, it is generally implied that the component has met the required standards of toxicological and manufacturing

testing or that it is included on the Inactive Ingredient Guide prepared by the U.S. Food and Drug Administration.

Also, as used herein, the term "pharmacologically active" (or simply "active"), as in a "pharmacologically active" derivative or analog, can refer to a derivative or analog (e.g., a salt, ester, amide, conjugate, metabolite, isomer, fragment, etc.) having the same type of pharmacological activity as the parent compound and approximately equivalent in degree.

As used herein, the term "mixture" can include solutions in which the components of the mixture are completely miscible, as well as suspensions and emulsions, in which the components of the mixture are not completely miscible.

As used herein, the term "subject" or "host" can refer to living organisms such as mammals, including, but not limited to humans, livestock, dogs, cats, and other mammals. Administration of the therapeutic agents can be carried out at dosages and for periods of time effective for treatment of a subject. In some embodiments, the subject is a human. In some embodiments, the pharmacokinetic profiles of the systems of the present invention are similar for male and female subjects.

As used herein, the term "controlled-release" or "controlled-release drug delivery" or "extended release" refers to release or administration of a drug from a given dosage form in a controlled fashion in order to achieve the desired pharmacokinetic profile in vivo. An aspect of "controlled" drug delivery is the ability to manipulate the formulation and/or dosage form in order to establish the desired kinetics of drug release.

The phrases "concurrent administration", "administration in combination", "simultaneous administration" or "administered simultaneously" as used herein, means that the compounds are administered at the same point in time or immediately following one another. In the latter case, the two compounds are administered at times sufficiently close that the results observed are indistinguishable from those achieved when the compounds are administered at the same point in time.

***Eubacterium* Probiotics**

Disclosed herein are human intestinal isolates that catabolically remove the N-methyl groups of quaternary amines (QAs) during growth. Importantly the demethylated QA products are not known to generate trimethylamine (TMA). This suggests that the total amount of TMA produced in the gut may depend on competition between two groups of microbes for a common pool of QAs (see Fig. 1). One group produces TMA from QA, while the other (typified by *E. limosum*) demethylates QAs, thus removing the precursor from pathways that could produce proarteriosclerotic TMA. *Eubacterium limosum* ATCC 8486 (a human gut isolate) was determined

herein to demethylate each of the known QA precursors that would otherwise form TMA. This makes *E. limosum* an excellent choice for a microbial therapeutic (probiotic). Properly formulated, *E. limosum,* or other bacteria with similar QA demethylation activities carried out by MttB family members, can be taken as a microbial therapeutic (probiotic) for those at high risk for atherosclerosis and resultant heart disease.

As disclosed herein, *E. limosum* can utilize carnitine, choline, glycine betaine, gammabutyrobetaine, or proline betaine as growth substrates. Thus, *E. limosum* can demethylate each quaternary amine (QA) that would otherwise form trimethylamine (TMA) and trimethylamine-N-oxide (TMAO). During growth, it produces the demethylated products of each of these QAs as shown using TLC and/or mass spectrometry. *E. limosum* demethylates QAs in complex medium, supplemented with Yeast Extract and casamino acids; or in a defined medium (Fig. 4). Demethylation in these diverse nutrient conditions suggests *E. limosum* demethylation can be robust in the nutrient rich environment of the human intestine. Only the demethylation of glycine betaine by strains of *E. limosum* was previously demonstrated in a 1987 paper. The same paper claimed choline demethylation but this was not documented with data figures, and the study was never reproduced in the literature.

Demethylation of carnitine or gamma butyrobetaine by *E. limosum* has not been previously asserted in the literature. The demethylation of all known precursors of TMA (and TMAO) by gut strains of *E. limosum* was previously unsuspected. The use of *E. limosum* as a probiotic for prevention of heart disease has never before been proposed.

Additional experiments were performed with carnitine as a sole growth and energy substrate, and demonstrated that norcarnitine (demethylated carnitine) is produced, concomitant with production of acetic, butyric, and caproic acids. Such small acids are known to lower intestinal inflammation, which underlie the widespread belief that *E. limosum* is a beneficial organism. Indeed, one study found that *E. limosum* is more widespread among centenarians than the general population and suggested this microbe was associated with long life. These positive traits allow use of *Eubacterium limosum* a probiotic for prevention of heart disease. In addition, members of Clostridial Clade XV, which are close relatives of *E. limosum* including *E. aggregans*, *E. barkeri*, and *E. callanderi*, are also capable of demethylating examples of dietary QAs that would otherwise form TMA (Fig. 11). Thus, *E. limosum* and/or close relatives can be taken as a supplement to defend against heart disease.

Additionally, it has been demonstrated that members of the TMA methyltransferase (TMA MT) family of proteins underlie demethylation of quaternary amines (QAs) by *Eubacterium limosum*. The first described representative of this family of proteins was MttB, shown to be a

TMA methyltransferase in the mid-1990s. MttB begins metabolism in methanogens and other anaerobes demethylating TMA. The MttB homolog is teamed with a methyl-carrying corrinoid protein and a tetrahydrofolate (in acetogens) or Coenzyme M (in methanogens) methylating protein (see Fig. 2). The methyltransferase methylates the corrinoid protein with the methylated amine, the methylated corrinoid protein then donates a methyl group to the cofactor methylating protein. Recently, for the first time, it was demonstrated that not all TMA MT family members are specific for TMA, and that a TMA MT family member uses glycine betaine as substrate.

Disclosed herein is an MttB family member that is a specific carnitine:corrinoid methyltransferase, which is made when *E. limosum* grows on carnitine. Further, another MttB homolog, corrinoid protein, and tetrahydrofolate methylating enzymes have been identified that are induced in cells grown on choline. These proteins act to mobilize the methyl group of QAs into the central pathways of this acetogenic bacterium. Thus, TMA MT family members underlie the molecular mechanism by which *Eubacterium limosum* can demethylate proatheriosclerotic QAs. TMA MT family members, corrinoid proteins, and tetrahydrofolate methyltransferases can underlie demethylation of butyrobetaine as well. These newly discovered abilities of the MttB family members and associated proteins are advantageous to using an organism as a probiotic for prevention of arteriosclerosis and subsequent heart disease.

The inventors have found that *Eubacterium* strains can demethylate each quaternary amine (QA) that would otherwise form trimethylamine (TMA) and trimethylamine-N-oxide (TMAO).

Therefore, *Eubacterium limosum* and members of Clostridial Clade XV, which are close relatives of *E. limosum* including *E. aggregans*, *E. barkeri*, and *E. callanderi*, can be taken as a probiotic supplement to treat or prevent against heart disease. Other nonlimiting examples of Eubacterium spp. (species) strains are known in the art, for example, in US Patent No. 6,056,978. *Eubacterium* is a genus of Gram-positive bacteria in the family Eubacteriaceae. These bacteria have a rigid cell wall and may either be motile or nonmotile.

In one aspect, disclosed herein is a probiotic composition comprising an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier.

In one embodiment, the isolated *Eubacterium* spp. is selected from *Eubacterium limosum*, *Eubacterium aggregans*, *Eubacterium barkeri*, *Eubacterium callanderi*. In one embodiment, the isolated *Eubacterium* spp. is *Eubacterium limosum*. In one embodiment, the isolated *Eubacterium* spp. is *Eubacterium limosum* ATCC 8486. In one embodiment, the isolated *Eubacterium* spp. is *Eubacterium limosum* ATCC 10825. As used herein, the term "*Eubacterium* spp." simply refers to the species that fall within the genus Eubacterium. See Wade, W. Prokaryotes (2006) 4:823 (Chapter 1.2.28 "The Genus Eubacterium and Related Genera").

In one embodiment, the isolated *Eubacterium* spp. is present at 10^5 to 10^{13} cfu *Eubacterium* per gram.

In one embodiment, the probiotic composition is in the form of a suspension, capsule, tablets or a microencapsulated product.

In one embodiment, the probiotic composition further comprises an additional probiotic strain.

In one aspect, disclosed herein is a method of treating or preventing heart disease, comprising administering to a subject in need thereof an effective amount of a probiotic composition, wherein the probiotic composition comprises an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier.

In another aspect, disclosed herein is a method of reducing trimethylamine or trimethylamine-N-oxide levels in a subject, comprising administering to the subject an effective amount of a probiotic composition, wherein the probiotic composition comprises an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier.

In one embodiment, the isolated *Eubacterium* spp. is selected from *Eubacterium limosum, Eubacterium aggregans, Eubacterium barkeri, Eubacterium callanderi*. In one embodiment, the isolated *Eubacterium* spp. is *Eubacterium limosum*. In one embodiment, the isolated *Eubacterium* spp. is *Eubacterium limosum* ATCC 8486.

In one embodiment, the isolated *Eubacterium* spp. is present at 10^5 to 10^{13} cfu *Eubacterium* per gram.

In one embodiment, the probiotic composition is in the form of a suspension, capsule, tablets or a microencapsulated product. In one embodiment, the microbial culture is lyophilized or freeze dried. In one embodiment, the microbial culture is in the form of a spray-dried powder. In one embodiment, the composition can be administered to the subject as a feedstuff, food product, dietary supplement, nutritional supplement or food additive.

In one embodiment, the isolated *Eubacterium* spp. is a biologically pure microbial culture. In one embodiment, the composition comprises a biologically pure microbial culture of *Eubacterium limosum*. In one embodiment, the composition comprises a biologically pure microbial culture of *Eubacterium limosum* ATCC 8486.

Combination Therapy with Additional Probiotics

In one embodiment, the Eubacterium probiotic composition disclosed herein further comprises an additional probiotic strain.

Probiotics are a class of microorganisms defined as live microbial organisms that beneficially affect the animal and human hosts. The beneficial effects include improvement of the microbial balance of the intestinal microflora or improving the properties of the indigenous microflora. The beneficial effects of probiotics may be mediated by a direct antagonistic effect against specific groups of organisms, resulting in a decrease in numbers, by an effect on their metabolism or by stimulation of immunity. The mechanisms underlying the proposed actions remain vastly unknown, partly as a consequence of the complexity of the gastro-intestinal ecosystem with which these biotherapeutic agents are expected to interact. Probiotics may suppress viable counts of an undesired organism by producing antibacterial compounds, by competing for nutrients or for adhesion sites. Further, they may alter microbial metabolism by increasing or decreasing enzyme activity or they may stimulate the immune system by increasing antibody levels or increasing macrophage activity. Probiotics may have antimicrobial, immunomodulatory, anti-carcinogenic, anti- diarrheal, anti-allergenic and antioxidant activities. Probiotic strains known in the art include, for example, Bifidobacteria, Lactobacillus, Lactococcus, Saccharomyces, Streptococcus thermophilus, Enterococcus and E. coli.

Non-limiting examples of additional probiotics for use in combination with the *Eubacterium* spp. probiotic strain for the treatment or prevention of heart disease include, but are not limited to species of: *Lactobacillus, Bifidobacterium,* Non-*difficile Clostridium,* Non-toxigenic *Clostridium difficile*, *Saccharomyces, Streptococcus Propionibacterium, or Bifidus.*

In one embodiment, the probiotic is selected from the group consisting of *Lactobacillus* species, *Bacillus* species, *Bifidobacterium* species, *Saccharomyces* species, and *Streptococcus* species. In one embodiment, the probiotic is *Lactobacillus* species. In one embodiment, the probiotic is *Bacillus* species. In one embodiment, the probiotic is *Bifidobacterium* species. In one embodiment, the probiotic is *Saccharomyces.* In one embodiment, the probiotic is *Streptococcus.* In some embodiments, the *Eubacterium* spp. probiotic strain and the additional probiotic are combined in a single dosage form (for example, in a single oral tablet).

Compositions

Compositions, as described herein, comprising a *Eubacterium* spp. probiotic composition and an excipient of some sort may be useful in a variety of applications, for example, in treating or preventing heart disease.

"Excipients" include any and all solvents, diluents or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired.

General considerations in formulation and/or manufacture can be found, for example, in *Remington's Pharmaceutical Sciences*, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980), and *Remington: The Science and Practice of Pharmacy,* 21st Edition (Lippincott Williams & Wilkins, 2005). The pharmaceutically acceptable excipients may also include one or more of fillers, binders, lubricants, glidants, disintegrants, and the like.

Exemplary excipients include, but are not limited to, any non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Some examples of materials which can serve as excipients include, but are not limited to, sugars such as lactose, glucose, and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; detergents such as Tween 80; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator. As would be appreciated by one of skill in this art, the excipients may be chosen based on what the composition is useful for. For example, with a pharmaceutical composition or cosmetic composition, the choice of the excipient will depend on the route of administration, the agent being delivered, time course of delivery of the agent, etc., and can be administered to humans and/or to animals, orally, rectally, parenterally, intracisternally, intravaginally, intranasally, intraperitoneally, topically (as by powders, creams, ointments, or drops), bucally, or as an oral or nasal spray.

Exemplary diluents include calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, etc., and combinations thereof.

Exemplary granulating and/or dispersing agents include potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose

(croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (Veegum), sodium lauryl sulfate, quaternary ammonium compounds, etc., and combinations thereof.

Exemplary surface active agents and/or emulsifiers include natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (e.g. bentonite [aluminum silicate] and Veegum [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers (e.g. carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [Tween 20], polyoxyethylene sorbitan [Tween 60], polyoxyethylene sorbitan monooleate [Tween 80], sorbitan monopalmitate [Span 40], sorbitan monostearate [Span 60], sorbitan tristearate [Span 65], glyceryl monooleate, sorbitan monooleate [Span 80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [Myrj 45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and Solutol), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. Cremophor), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [Brij 30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, Pluronic F 68, Poloxamer 188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, etc. and/or combinations thereof.

Exemplary binding agents include starch (e.g. cornstarch and starch paste), gelatin, sugars (e.g. sucrose, glucose, dextrose, dextrin, molasses, lactose, lactitol, mannitol, etc.), natural and synthetic gums (e.g. acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, poly(vinyl-pyrrolidone), magnesium aluminum silicate (Veegum), and larch arabogalactan), alginates, polyethylene oxide, polyethylene glycol, inorganic calcium salts, silicic acid, polymethacrylates, waxes, water, alcohol, etc., and/or combinations thereof.

Exemplary preservatives include antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and other preservatives.

Exemplary antioxidants include alpha tocopherol, ascorbic acid, acorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium metabisulfite, and sodium sulfite.

Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA) and salts and hydrates thereof (e.g., sodium edetate, disodium edetate, trisodium edetate, calcium disodium edetate, dipotassium edetate, and the like), citric acid and salts and hydrates thereof (e.g., citric acid monohydrate), fumaric acid and salts and hydrates thereof, malic acid and salts and hydrates thereof, phosphoric acid and salts and hydrates thereof, and tartaric acid and salts and hydrates thereof. Exemplary antimicrobial preservatives include benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and thimerosal.

Exemplary antifungal preservatives include butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and sorbic acid.

Exemplary alcohol preservatives include ethanol, polyethylene glycol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and phenylethyl alcohol.

Exemplary acidic preservatives include vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroacetic acid, ascorbic acid, sorbic acid, and phytic acid.

Other preservatives include tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisol (BHA), butylated hydroxytoluened (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite, Glydant Plus, Phenonip, methylparaben, Germall 115, Germaben II, Neolone, Kathon, and Euxyl. In certain embodiments, the preservative is an anti-oxidant. In other embodiments, the preservative is a chelating agent.

Exemplary buffering agents include citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glubionate, calcium gluceptate, calcium gluconate, D-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic

calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, etc., and combinations thereof.

Exemplary lubricating agents include magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behanate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, etc., and combinations thereof.

Exemplary natural oils include almond, apricot kernel, avocado, babassu, bergamot, black current seed, borage, cade, camomile, canola, caraway, carnauba, castor, cinnamon, cocoa butter, coconut, cod liver, coffee, corn, cotton seed, emu, eucalyptus, evening primrose, fish, flaxseed, geraniol, gourd, grape seed, hazel nut, hyssop, isopropyl myristate, jojoba, kukui nut, lavandin, lavender, lemon, litsea cubeba, macademia nut, mallow, mango seed, meadowfoam seed, mink, nutmeg, olive, orange, orange roughy, palm, palm kernel, peach kernel, peanut, poppy seed, pumpkin seed, rapeseed, rice bran, rosemary, safflower, sandalwood, sasquana, savoury, sea buckthorn, sesame, shea butter, silicone, soybean, sunflower, tea tree, thistle, tsubaki, vetiver, walnut, and wheat germ oils. Exemplary synthetic oils include, but are not limited to, butyl stearate, caprylic triglyceride, capric triglyceride, cyclomethicone, diethyl sebacate, dimethicone 360, isopropyl myristate, mineral oil, octyldodecanol, oleyl alcohol, silicone oil, and combinations thereof.

Additionally, the composition may further comprise a polymer. Exemplary polymers contemplated herein include, but are not limited to, cellulosic polymers and copolymers, for example, cellulose ethers such as methylcellulose (MC), hydroxyethylcellulose (HEC), hydroxypropyl cellulose (HPC), hydroxypropyl methyl cellulose (HPMC), methylhydroxyethylcellulose (MHEC), methylhydroxypropylcellulose (MHPC), carboxymethyl cellulose (CMC) and its various salts, including, e.g., the sodium salt, hydroxyethylcarboxymethylcellulose (HECMC) and its various salts, carboxymethylhydroxyethylcellulose (CMHEC) and its various salts, other polysaccharides and polysaccharide derivatives such as starch, dextran, dextran derivatives, chitosan, and alginic acid and its various salts, carageenan, varoius gums, including xanthan gum, guar gum, gum arabic, gum karaya, gum ghatti, konjac and gum tragacanth, glycosaminoglycans and proteoglycans such

as hyaluronic acid and its salts, proteins such as gelatin, collagen, albumin, and fibrin, other polymers, for example, polyhydroxyacids such as polylactide, polyglycolide, polyl(lactide-co-glycolide) and poly(.epsilon.-caprolactone-co-glycolide)-, carboxyvinyl polymers and their salts (e.g., carbomer), polyvinylpyrrolidone (PVP), polyacrylic acid and its salts, polyacrylamide, polyacilic acid/acrylamide copolymer, polyalkylene oxides such as polyethylene oxide, polypropylene oxide, poly(ethylene oxide-propylene oxide), and a Pluronic polymer, polyoxyethylene (polyethylene glycol), polyanhydrides, polyvinylalchol, polyethyleneamine and polypyrridine, polyethylene glycol (PEG) polymers, such as PEGylated lipids (e.g., PEG-stearate, 1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-1000], 1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000], and 1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-5000]), copolymers and salts thereof.

Additionally, the composition may further comprise an emulsifying agent. Exemplary emulsifying agents include, but are not limited to, a polyethylene glycol (PEG), a polypropylene glycol, a polyvinyl alcohol, a poly-N-vinyl pyrrolidone and copolymers thereof, poloxamer nonionic surfactants, neutral water-soluble polysaccharides (e.g., dextran, Ficoll, celluloses), non-cationic poly(meth)acrylates, non-cationic polyacrylates, such as poly(meth)acrylic acid, and esters amide and hydroxyalkyl amides thereof, natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (e.g. bentonite [aluminum silicate] and Veegum [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers (e.g. carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [Tween 20], polyoxyethylene sorbitan [Tween 60], polyoxyethylene sorbitan monooleate [Tween 80], sorbitan monopalmitate [Span 40], sorbitan monostearate [Span 60], sorbitan tristearate [Span 65], glyceryl monooleate, sorbitan monooleate [Span 80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [Myrj 45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and Solutol), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. Cremophor), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [Brij 30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate,

triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, Pluronic F 68, Poloxamer 188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, etc. and/or combinations thereof. In certain embodiments, the emulsifying agent is cholesterol.

Liquid compositions include emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active compound, the liquid composition may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Solid compositions include capsules, tablets, pills, powders, and granules. In such solid compositions, the particles are mixed with at least one excipient and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets, and pills, the dosage form may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Tablets, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Heart Disease/Cardiovascular Disease

In one aspect, disclosed herein is a method of treating or preventing heart disease, comprising administering to a subject in need thereof an effective amount of a probiotic composition, wherein the probiotic composition comprises an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier. The probiotic compositions of the present invention are useful in preventing, inhibiting or reducing risk of heart diseases resulting from arteriosclerosis and/or atherosclerosis, such as cardiac and/or cerebral ischemia, myocardial infarction, angina, peripheral vascular disease, or stroke. The compositions of the present invention are also useful in preventing, inhibiting or reducing risk of heart diseases such as congestive heart disease, coronary heart disease, carotid artery disease, chronic kidney disease, which can arise from atherosclerosis.

In one embodiment, a method is provided for preventing, inhibiting or reducing risk of heart diseases resulting from arteriosclerosis or atherosclerosis in a host by administering to a subject in need thereof an effective amount of a probiotic composition, wherein the probiotic composition comprises an isolated *Eubacterium* spp. strain.

In one embodiment, a method is provided for preventing, inhibiting or reducing risk of heart diseases resulting from arteriosclerosis or atherosclerosis in a host by administering to a subject in need thereof an effective amount of a probiotic composition, wherein the probiotic composition comprises an isolated *Eubacterium* spp. strain, in combination or alteration with an anti-atherosclerotic agent.

Examples of anti-atherosclerotic agents include, but are not limited to, HMG CoA reductase inhibitors, microsomal triglyceride transfer protein (MTP) inhibitors, fibric acid derivatives, squalene synthetase inhibitors and other known cholesterol lowering agents, lipoxygenase inhibitors, ACAT inhibitors, PPAR α/γ dual agonists, anti-platelet medications, beta-blockers, angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers, and diuretics. The anti-atherosclerotic agent may be an HMG CoA reductase inhibitor, which includes, but is not limited to, mevastatin, pravastatin, simvastatin, lovastatin, fluvastatin, cerivastatin, and atorvastatin. The squalene synthetase inhibitors suitable for use herein include, but are not limited to, a-phosphono-sulfonates disclosed in U.S. Pat. No. 5,712,396. Other cholesterol lowering drugs suitable for use herein include, but are not limited to, antihyperlipoproteinemic agents such as

fibric acid derivatives, such as fenofibrate, gemfibrozil, clofibrate, bezafibrate, ciprofibrate, clinofibrate, probucol, probucol and gemfibrozil.

EXAMPLES

The following examples are set forth below to illustrate the compositions, methods, and results according to the disclosed subject matter. These examples are not intended to be inclusive of all aspects of the subject matter disclosed herein, but rather to illustrate representative methods and results. These examples are not intended to exclude equivalents and variations of the present invention which are apparent to one skilled in the art.

Example 1. Demethylation of quarternary amines by *Eubacterium limosum* to diminish the risk of arteriosclerosis and subsequent heart disease.

Evidence is accumulating that certain human intestinal microbes contribute to cardiovascular disease, and increase the risk of heart attack, stroke, and death. Gut microbes convert quaternary amines (QAs), e.g. carnitine, butyrobetaine, choline, and glycine betaine, to trimethylamine. Trimethylamine (TMA) enters the bloodstream and once converted by liver enzymes to trimethylamine-N-oxide (TMAO), can trigger macrophage mediated lipid deposition in the vascular system. Serum TMAO levels accordingly correlate with arteriosclerosis and the above catastrophic health events. TMA production by QA lyases or reductases has long been considered the sole route of microbial degradation of QAs under the anaerobic condition prevalent in the gut; but recent evidence indicates a more complex microbial ecology of QAs exists. In this example, *Eubacterium limosum* intestinal isolates have been shown to catabolically remove the N-methyl groups of QAs during growth; and the demethylated QA products do not generate TMA.

As disclosed herein, QA demethylation serves to moderate microbial TMA production in the human intestine, providing a mechanism for homeostasis or probiotic control of TMAO levels; and thereby a means to decrease the risk of cardiovascular disease. As disclosed herein, the gut isolate *Eubacterium limosum* can grow by demethylating each QAs known to otherwise form TMA.

Quaternary amines (QAs) such as glycine betaine, choline, carnitine, and γ–butyrobetaine are found in a variety of foods (1-4). Once QAs enter the intestine, they can be absorbed by the host, or converted by certain gut microbiota to trimethylamine (TMA) via known enzymes (Fig. 1) (5-8). TMA enters the bloodstream and is oxidized to TMAO by liver monoxygenases (4, 9). Higher levels of circulating TMAO correlate with adverse cardiovascular events including heart attack, stroke, and death in large clinical populations (10, 11). The involvement of the gut

microbiota was demonstrated with germ-free (or antibiotic treated) ApoE-/- mice, which produced little TMAO upon dietary challenge with choline, carnitine, butyrobetaine, or glycine betaine. However, the same strain with an introduced or normal gut microbiome produced TMAO and had a concomitant increase in atherosclerotic lesions upon QA challenge (3, 8, 10). These experiments have supported the idea that gut microbiota greatly contribute to heart disease by converting QAs to TMA (3, 12). How TMAO may cause disease is yet unknown, but several proatherogenic pathways are up-regulated by TMAO, which may increase lipid deposition by foam cell macrophages on vascular tissue (12).

Carnitine or choline levels predict increased risk for adverse cardiac events, but only in the presence of increased levels of TMAO. TMAO independently has the strongest predictive risk, suggesting that the flux of QAs to TMA can be modulated (10, 11). Diet can influence the fate of QAs, as vegetarians fed carnitine have resultant lower serum levels of TMAO than meat-eaters (10). Supporting the idea that microbiota influence TMAO levels, introduction of 'humanized' gut microbiomes into germ-free mice led to differential production of TMAO (13). This observation led to the idea that different microbes might hinder or contribute to net synthesis of TMA (14). Only one 'hindering' group of microbes have been previously proposed (15), methanogenic archaea having MttB with the 22nd amino acid, pyrrolysine (Pyl) (16-18). MttB demethylates TMA to methylate a corrinoid protein as the first step of methane formation (16, 19). However, other groups of gut microbes that would act to hinder TMA (or TMAO) formation have been unknown.

Disclosed herein, the inventors have identified certain intestinal gut anaerobes that can demethylate QAs as part of their catabolic pathways. Importantly, these products of demethylation are not converted to TMA (Fig.1). As most anaerobes turn over large amounts of substrate to extract energy for growth, relatively large amounts of QA may be demethylated by a relatively small number of bacteria. QA demethylation can keep in check the amount of TMA generated by other gut microbes, and serve as a mechanism of serum TMAO homeostasis. Indeed, QA demethylating organisms can thus provide a means to therapeutically control TMAO levels in at-risk individuals.

QA demethylation products are found in mammalian urine (20-22). For example, proline betaine is associated with citrus intake in humans (2), and the demethylation product, *N*-methyl-proline has been detected in serum and the colonic lumen from conventional, but not germ-free mice (21, 22). Serum *N*-methyl-proline is associated with changes in physical functioning in older humans (22). Breakdown of phosphatidylcholine from meat or dairy products generates choline, which can lead to glycine betaine (23) (Fig. 1, dotted arrows). Carnitine is involved in fatty acid transport into the mitochrondria and abundant in dairy products and meats (1). Carnitine can be

microbially converted to γ-butyrobetaine (Fig. 1, dotted arrows) (1) and what may be the complete demethylation product, γ–aminobutyrate is found at higher concentrations in the gut lumen of conventional, but not germ free mice (21). Via γ–butyrobetaine, carnitine can also lead to glycine betaine (Fig. 1, dotted arrows) (1). Glycine betaine (trimethylglycine) itself is a common osmolyte and found at high concentration in seafoods, wheat germ, or bran (20). The demethylation products of glycine betaine, dimethylglycine and monomethylglycine, have been noted in human urine, but had been attributed to the actions of host enzymes such as betaine:homocysteine methyltransferase or betaine dehydrogenase (2). However, in recent years it has been demonstrated that monomethylglycine and dimethylglycine are formed from microbiome replete, but not germ-free, mice (21, 24).

Aerobes typically oxidatively demethylate C-1 substrates. Anaerobes (e.g. methanogens, acetogens, and other anaerobic respirers) instead employ 3-component methyltransferase systems to initiate the catabolism of methylated compounds (16, 17, 25-33). At the heart of 3-component systems lies a family of corrinoid-binding proteins interacting with two discrete methyltransferases. The first methyltransferase (MT I) methylates the corrinoid protein with the growth substrate. The second methyltransferase (MT II) demethylates the corrinoid protein and methylates a cellular one-carbon carrier, e.g. coenzyme M (CoM) in methanogenic archaea, and tetrahydrofolate (THF) in acetogenic bacteria (Fig. 2). The corrinoid protein and MT II proteins belong to a few recognizable families (27, 30, 34-36). In contrast, MT I come from a number of different families (17, 27, 31), but not always. A single family comprises MT I proteins for different methoxylated aromatics (37). Similarly, members of the MttB family can be MT I proteins with active sites that have diversified to bind different QAs.

The inventors and others have discovered and characterized the three 3-component systems that methanogens use to demethylate TMA (16, 17), dimethylamine (17, 28), or monomethylamine (25-27, 36). Although each MT I is specific for its own methylamine and cognate corrinoid protein, all three methyl-corrinoid proteins are demethylated by a single CoM methylase, the MT II protein MtbA. In vivo, the 3-component system requires substoichiometric RamA, an activase that reduces the corrinoid proteins to the active cobalt (I) state (38). MttB is the pyrrolysine (Pyl) containing MT I protein of the TMA methyltransferase system (termed Pyl-MttB) (17, 18). The Pyl imine bond is thought to form a covalent bond with TMA, converting TMA from a *tertiary* amine to a *quaternary* amine. The TMA-Pyl adduct is thought to position TMA for methyl transfer to the docked cognate corrinoid protein, MttC (33, 39, 40). Yet, genes for most MttB family members lack Pyl codons (termed non-Pyl MttBs). This suggested that Pyl-MttB is actually part of a family of quaternary amine (QA)-dependent methyltransferases. Most QAs have multiple functional

groups, and would not require Pyl for proper binding and orientation in the active site for methyl transfer. This idea was applied to *Desulfitobacterium hafniense*, which encodes four non-Pyl MttBs. It was found that *D. hafniense* grew by demethylating glycine betaine, then coupling oxidation of the methyl group to reduction of nitrate (41). It was found the nonPyl-MttB gene *mtgB* was upregulated during growth on glycine betaine. Recombinant MtgB demethylated glycine betaine (but not TMA) while methylating free cobalamin and producing dimethylglycine (41). MtgA, a possible MT II enzyme, is encoded next to MtgB along with a corrinoid protein. MtgA proved a methylcobalamin:THF methyltransferase; and it was concluded that MtgB was the MT I of the first known 3-component system specific for demethylation of a QA (41). Subsequent analysis of the active site residues showed complete conservation within the MtgB clade, but not in other clades, supporting the idea of expanded functional diversification within the MttB family.

A number of isolated human gut microbes were found to encode non-Pyl MttB homologs. Of particular interest is *Eubacterium limosum* 8486 (42-44), which encodes 42 non-Pyl MttBs (Table 1).

Table 1. MttB homologs

Organism	# MttB Homologs
Butyricicoccus pullicaecorum	1
Intestinibacter bartlettii DSM 16795	1
Pseudoflavonifractor capillosus	1
Kineosphaera limosa	1
Bilophila wadsworthia 3_1_6	3
Blautia product DSM 2950	4
Ruminococcus gauvreauii	4
Desulfitobacterium hafniense DP7	4
Eubacterium limosum ATCC 8486	42

E. limosum was known to grow by demethylating glycine betaine or choline (45), and the inventors have now found that *E. limosum* also grows by demethylating carnitine, γ–butyrobetaine, and proline betaine. The only QAs known to generate TMA in the gut are shown in Fig. 1, and *E. limosum* grows on each, making demethylated products incapable of serving as precursors to TMA by known reactions. *E. limosum* is an acetogen (45) and considered a beneficial gut microbe (46-48). The acetate and butyrate it produces from methylated substrates are thought to underlie the microbe's ability to quiet gut inflammation in model systems (Fig. 5) (48). A metagenomic study

found that *E. limosum* and close relatives of Clostridium cluster XV are enhanced in the gut microbiome of centenarians. The authors correlated abundance of gut *E. limosum* with longetivity (47). In a study identifying microbes associated with TMAO levels and atherosclerosis, *Eubacterium* spp. were noted as significantly increased in fecal samples from healthy human controls relative to those with high TMAO levels and onset of cardiovascular disease (49).

Gut microbes can grow by demethylation of QAs. This finding reveals a novel understanding of the ecology of methylamines in the gut. The fate of QAs during gut microbial metabolism is more complex than previously appreciated. While some members of the gut community can produce TMA from QAs, other members might decrease net TMA production by QA demethylation. The ratios of organisms mediating these two alternative routes of QA degradation in an individual gut could determine the net level of TMA produced from QAs, and thereby an individual's proclivity for hepatic TMAO production and subsequent risk for cardiovascular disease. In some embodiments, the individual's predisposition towards heart disease is modulation of the ratios of these two different groups of organisms. Thus, a rational probiotic can diminish the risk of heart disease.

E. limosum uses many QA substrates (Fig. 11). Growth was negligible in the absence of QAs, and growth on QA did not require yeast extract or trypticase. The slower growth on 50 mM choline may reflect toxicity, and faster growth was observed at lower concentrations. No growth was obtained on any QA demethylation product. Other members of Clostridium cluster XV also use QAs (Fig. 11).

The demethylation of QAs using silica gel TLC stained with bromocresol green (41). The identity of the carnitine and butyrobetaine demethylation products has also been confirmed by MS/MS analysis at the OSU Campus Chemical Instrumentation Center. The measured m/z values were within 3 ppm of theoretical. This marks the first time that *L*-norcarnitine has been identified as a microbial product.

Sporomusa ovata is an acetogen (54) encoding five non-Pyl MttBs, one is in a gene cluster with a corrinoid protein, a THF methylase homolog, and a homolog of the proline/betaine transporter PutP. *E. limosum* has a close homolog of the same non-Pyl MttB. Proline betaine was not known to be a substrate of either organism, but is expensive. *S. ovata* grew on *L*-proline betaine, which it converted to *N*-methyl-proline. It was also found that *E. limosum* grows just as robustly on proline betaine.

A round of label-free proteomics (a single culture extract in a single lane) was performed with *E. limosum* grown on choline versus lactate. Members of a 3-component system were among the most abundant proteins detectable after growth on choline. A corrinoid protein was

approximately 8% of the detected proteins, while a MetH-like THF methylase was 4%. A non-Pyl MttB homolog was 0.12%. However, inspection of genomic context showed these same three proteins were encoded next to each other, along with a putative choline/betaine transporter. Each member of this putative 3-component system was not detectable in lactate-grown cells.

In this same experiment, carnitine-grown cells were also analyzed. The putative choline 3-component system was not detected. Instead, in carnitine-grown cells, a distinct corrinoid protein, THF methylase, and non-Pyl MttB were upregulated. The non-Pyl MttB was not detectable in lactate-grown cells, while the corrinoid protein and THF protein were 20 and 7 fold more abundant in carnitine-grown versus lactate-grown cells. These three proteins were encoded in different part of the genome. However, a blast search revealed that outside of *Eubacterium limosum* strain KIST 612, the nearest homolog of the carnitine-upregulated non-Pyl MttB was in *Acetobacterium dehalogenans* where it was in a gene cluster also encoding a highly homologous corrinoid protein and THF methylase.

Biochemical characterization of QA demethylation enzymes.

E. limosum, like many anaerobes from the gut, does not have an established heuristic genetic system, but much can be learned by biochemical experiments. Such techniques allowed first elucidation of the methylamine-dependent 3-component systems (16, 25, 26, 28).

MtgB, the glycine betaine MT I, methylates the corrinoid cofactor cob(I)alamin. No other methylamine was a substrate (41). Analogous activities in *E. limosum* cell extracts were tested using the same spectral assay. Carnitine-grown, but not lactate-grown, cell extracts had a robust carnitine:cob(I)alamin activity. No other QAs were substrates. This activity suggests that an MtgB-like enzyme is upregulated in carnitine cells, perhaps the non-Pyl MttB discussed above. A butyrobetaine-grown extract also was seen to have a similar activity, but specific for butyrobetaine.

The *Methanosarcina barkeri* Pyl-MttB can methylate cognate Co(I)-corrinoid protein MttC, but not free cofactor. Recombinant MttB was already in hand, but only the MttC apoprotein was produced in *E. coli*. This was solved by expression in the methanogen *Methanosarcina acetivorans*, which yielded corrinoid bound to MttC in unit stoichiometry. Unlike some corrinoid proteins, MttC cannot be reduced to the active Co(I) state by Ti(III)citrate but must be reduced in an ATP-dependent reaction by the oxygen sensitive iron-sulfur protein RamA. Expression in aerobically grown *E. coli* produced an apo-protein lacking the two tetranuclear iron-sulfur clusters, however, anaerobic growth in the presence of fumarate, iron chloride, and cysteine allowed anaerobic purification of RamA as an active protein.

The structure of MtgB, as a putative methyltransferase, was solved previously by the Joint Center for Structural Genomics (pdb id: 2QNE). The MtgB active site cavity was identified by

comparison to structurally homologous corrinoid dependent methyltransferases with known active sites. Q-site finder (69) was used to identify probable ligand binding residues highly conserved within the MtgB clade creating a putative glycine betaine motif. At the active site bottom, it was noted that the motif's R and H could interact with the betaine carboxyl, while Y and F placed above could enter into pi interactions with the cation. Substitution of these residues with dissimilar ones found at the same position in distant MttB relatives decreased activity by 99%.

Determining the relative abundance of QA degradation genes in the human gut microbiome.

A vast amount of uncultivated diversity exists in the gut (74, 75), a portion of this diversity involves a previously unsuspected ecology surrounding QAs and their derivatives. Recent studies have focused on taxonomic groups that might generate TMA from QAs, but not QA demethylation. Microbial taxa identified by 16S rRNA in mice whose diets are supplemented with carnitine and choline were correlated with increased TMA and TMAO concentrations (3, 6, 10). Choline diet-dependent atherosclerosis and TMAO levels in mice are transmittable via cecal microbiome transplantation (76) Most of the microbial taxa highly correlated to TMAO levels lack cultivated or genomically-sampled representatives, for example, uncultivated order RF39 (76), whose metabolism is unknown. The lack of knowledge about the metabolic potential of uncultivated microbes catalyzing QA transformations that are benign (demethylation) or detrimental (TMA generation) hinders the ability to discern a causal role between the presence or absence of different gut microbes and atherosclerosis susceptibility.

Metagenomics is cultivation independent and enables metabolic analyses, in a community context, of the 99% of microbes in nature that are uncultivated. One approach can reconstruct genomes of members comprising only 0.05% of community abundance in human stool (74, 77). The advancement of sequencing and informatics tools enables read assembly and accurate assignment of genome fragments to specific microbes from complex microbial communities like the human gut (74, 78, 79).

Several lines of evidence suggest that normal microbiota can use QA demethylation pathways. First, *E. limosum* demethylates multiple QAs and is an integral member of the microbiome (83). Second, the Human Microbiome Project (HMP) isolate database (PRJNA28331) was mined for genes encoding Pyl and non-Pyl MttBs using Blast searches with custom-built hidden Markov models. Non-pyl MttB homologs were found in *D. hafniense DP7*, *Clostridium sp D5* and *barletti*, *Bilophila wadsworthia*, *Blautia producta*, *Kineosphaera limosa*, *Butyricicoccus pullicaecorum*, *Pseudoflavonifractor capillosus*, and *Ruminicoccus gauvreauii*, while the methanogen *Methanomassiliicoccus* spp, and *B. wadsworthia* and *D. hafniense* also contained Pyl containing MttB that could likely convert TMA to DMA.

References Cited

1. Rebouche CJ, Seim H. Carnitine metabolism and its regulation in microorganisms and mammals. Annu Rev Nutr. 1998;18:39-61. doi: 10.1146/annurev.nutr.18.1.39. PubMed PMID: 9706218.

2. Heinzmann SS, Merrifield CA, Rezzi S, Kochhar S, Lindon JC, Holmes E, et al. Stability and robustness of human metabolic phenotypes in response to sequential food challenges. Journal of proteome research. 2012;11(2):643-55. doi: 10.1021/pr2005764. PubMed PMID: 21999107.

3. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472(7341):57-63. doi: 10.1038/nature09922. PubMed PMID: 21475195; PubMed Central PMCID: PMC3086762.

4. Zhang AQ, Mitchell SC, Smith RL. Dietary precursors of trimethylamine in man: a pilot study. Food Chem Toxicol. 1999;37(5):515-20. PubMed PMID: 10456680.

5. Craciun S, Balskus EP. Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. Proc Natl Acad Sci USA. 2012;109(52):21307-12. doi: 10.1073/pnas.1215689109. PubMed PMID: 23151509; PubMed Central PMCID: PMC3535645.

6. Zhu Y, Jameson E, Crosatti M, Schafer H, Rajakumar K, Bugg TD, et al. Carnitine metabolism to trimethylamine by an unusual Rieske-type oxygenase from human microbiota. Proc Natl Acad Sci USA. 2014;111(11):4268-73. doi: 10.1073/pnas.1316569111. PubMed PMID: 24591617; PubMed Central PMCID: PMC3964110.

7. Meyer M, Granderath K, Andreesen JR. Purification and characterization of protein PB of betaine reductase and its relationship to the corresponding proteins glycine reductase and sarcosine reductase from *Eubacterium acidaminophilum*. Eur. J. Biochem. 1995;234(1):184-91. PubMed PMID: 8529639.

8. Koeth RA, Levison BS, Culley MK, Buffa JA, Wang Z, Gregory JC, et al. gamma-Butyrobetaine is a proatherogenic intermediate in gut microbial metabolism of L-carnitine to TMAO. Cell Metab. 2014;20(5):799-812. doi: 10.1016/j.cmet.2014.10.006. PubMed PMID: 25440057; PubMed Central PMCID: PMC4255476.

9. Lang DH, Yeung CK, Peter RM, Ibarra C, Gasser R, Itagaki K, et al. Isoform specificity of trimethylamine N-oxygenation by human flavin-containing monooxygenase (FMO) and P450 enzymes: selective catalysis by FMO3. Biochem. Pharmacol. 1998;56(8):1005-12. PubMed PMID: 9776311.

10. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat. Med.

2013;19(5):576-85. doi: 10.1038/nm.3145. PubMed PMID: 23563705; PubMed Central PMCID: PMC3650111.

11. Wang Z, Tang WH, Buffa JA, Fu X, Britt EB, Koeth RA, et al. Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. Eur Heart J. 2014;35(14):904-10. doi: 10.1093/eurheartj/ehu002. PubMed PMID: 24497336; PubMed Central PMCID: PMC3977137.

12. Brown JM, Hazen SL. The gut microbial endocrine organ: bacterially derived signals driving cardiometabolic diseases. Annu Rev Med 2015. p. 343-59.

13. Martin FP, Wang Y, Sprenger N, Yap IK, Lundstedt T, Lek P, et al. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. Molecular systems biology. 2008;4:157. doi: 10.1038/msb4100190. PubMed PMID: 18197175; PubMed Central PMCID: PMC2238715.

14. Brown JM, Hazen SL. Metaorganismal nutrient metabolism as a basis of cardiovascular disease. Curr Opin Lipidol. 2014;25(1):48-53. doi: 10.1097/MOL.0000000000000036. PubMed PMID: 24362355; PubMed Central PMCID: PMC4018574.

15. Brugere JF, Borrel G, Gaci N, Tottey W, O'Toole PW, Malpuech-Brugere C. Archaebiotics: proposed therapeutic use of archaea to prevent trimethylaminuria and cardiovascular disease. Gut microbes. 2014;5(1):5-10. doi: 10.4161/gmic.26749. PubMed PMID: 24247281; PubMed Central PMCID: PMC4049937.

16. Ferguson DJ, Jr., Krzycki JA. Reconstitution of trimethylamine-dependent coenzyme M methylation with the trimethylamine corrinoid protein and the isozymes of methyltransferase II from *Methanosarcina barkeri*. J Bacteriol. 1997;179(3):846-52.

17. Paul L, Ferguson DJ, Krzycki JA. The trimethylamine methyltransferase gene and multiple dimethylamine methyltransferase genes of *Methanosarcina barkeri* contain in-frame and read-through amber codons. J Bacteriol. 2000;182(9):2520-9.

18. Soares JA, Zhang L, Pitsch RL, Kleinholz NM, Jones RB, Wolff JJ, et al. The residue mass of L-pyrrolysine in three distinct methylamine methyltransferases. J Biol Chem. 2005;280(44):36962-9. PubMed PMID: 16096277.

19. Krzycki JA. Function of genetically encoded pyrrolysine in corrinoid-dependent methylamine methyltransferases. Curr Opin Chem Biol. 2004;8(5):484-91. Epub 2004/09/29. doi: 10.1016/j.cbpa.2004.08.012 S1367-5931(04)00110-3 [pii]. PubMed PMID: 15450490.

20. Craig SA. Betaine in human nutrition. Am J Clin Nutr. 2004;80(3):539-49. PubMed PMID: 15321791.

21. Matsumoto M, Kibe R, Ooga T, Aiba Y, Kurihara S, Sawaki E, et al. Impact of intestinal microbiota on intestinal luminal metabolome. Sci Rep. 2012;2:233. doi: 10.1038/srep00233. PubMed PMID: 22724057; PubMed Central PMCID: PMC3380406.

22. Lustgarten MS, Price LL, Chale A, Fielding RA. Metabolites related to gut bacterial metabolism, peroxisome proliferator-activated receptor-alpha activation, and insulin sensitivity are associated with physical function in functionally-limited older adults. Aging cell. 2014;13(5):918-25. doi: 10.1111/acel.12251. PubMed PMID: 25041144; PubMed Central PMCID: PMC4331755.

23. Wargo MJ. Choline catabolism to glycine betaine contributes to Pseudomonas aeruginosa survival during murine lung infection. PloS one. 2013;8(2):e56850. doi: 10.1371/journal.pone.0056850. PubMed PMID: 23457628; PubMed Central PMCID: PMC3572970.

24. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, et al. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A. 2009;106(10):3698-703. doi: 10.1073/pnas.0812874106. PubMed PMID: 19234110; PubMed Central PMCID: PMC2656143.

25. Burke SA, Krzycki JA. Involvement of the "A" isozyme of methyltransferase II and the 29-kilodalton corrinoid protein in methanogenesis from monomethylamine. J Bacteriol. 1995;177:4410-6.

26. Burke SA, Krzycki JA. Reconstitution of monomethylamine:coenzyme M methyl transfer with a corrinoid protein and two methyltransferases purified from *Methanosarcina barkeri*. J Biol Chem. 1997;272(26):16570-7.

27. Burke SA, Lo SL, Krzycki JA. Clustered genes encoding the methyltransferases of methanogenesis from monomethylamine. JBacteriol. 1998;180(13):3432-40.

28. Ferguson DJ, Jr., Gorlatova N, Grahame DA, Krzycki JA. Reconstitution of dimethylamine:coenzyme M methyl transfer with a discrete corrinoid protein and two methyltransferases purified from *Methanosarcina barkeri*. J Biol Chem. 2000;275(37):29053-60.

29. Siebert A, Schubert T, Engelmann T, Studenik S, Diekert G. Veratrol-O-demethylase of *Acetobacterium dehalogenans*: ATP-dependent reduction of the corrinoid protein. Arch Microbiol. 2005;183(6):378-84. PubMed PMID: 15968525.

30. Studenik S, Vogel M, Diekert G. Characterization of an O-demethylase of *Desulfitobacterium hafniense* DCB-2. J Bacteriol. 2012;194(13):3317-26. doi: 10.1128/JB.00146-12. PubMed PMID: 22522902; PubMed Central PMCID: PMC3434752.

31. Sauer K, Harms U, Thauer RK. Methanol:coenzyme M methyltransferase from *Methanosarcina barkeri*. Purification, properties and encoding genes of the corrinoid protein MT1. Eur J Biochem. 1997;243(3):670-7. PubMed PMID: 9057830.

32. Naidu D, Ragsdale SW. Characterization of a three-component vanillate O-demethylase from *Moorella thermoacetica*. J Bacteriol. 2001;183(11):3276-32781. PubMed PMID: 11344134.

33. Krzycki JA. Function of genetically encoded pyrrolysine in corrinoid-dependent methylamine methyltransferases. Curr Opin Chem Biol. 2004;8:484-91.

34. Harms U, Thauer RK. Methylcobalmin:coenzyme M methyltransferase isoenzymes MtaA and MtbA from *Methanosarcina barkeri*. Eur J Biochem. 1996;235:653-9.

35. Paul L, Krzycki JA. Sequence and transcript analysis of a novel *Methanosarcina barkeri* methyltransferase II homolog and its associated corrinoid protein homologous to methionine synthase. J Bacteriol. 1996;178(22):6599-607.

36. Ferguson DJ, Jr., Krzycki JA, Grahame DA. Specific roles of methylcobamide:coenzyme M methyltransferase isozymes in metabolism of methanol and methylamines in *Methanosarcina barkeri*. J Biol Chem. 1996;271(9):5189-94.

37. Kreher S, Studenik S, Diekert G. Ether cleaving methyltransferases of the strict anaerobe *Acetobacterium dehalogenans*: controlling the substrate spectrum by genetic engineering of the N-terminus. Mol Microbiol. 2010;78(1):230-7. doi: 10.1111/j.1365-2958.2010.07333.x. PubMed PMID: 20923421.

38. Ferguson T, Soares JA, Lienard T, Gottschalk G, Krzycki JA. RamA, a Protein Required for Reductive Activation of Corrinoid-dependent Methylamine Methyltransferase Reactions in Methanogenic Archaea. J Biol Chem. 2009;284(4):2285-95. PubMed PMID: 19043046.

39. Hao B, Zhao G, Kang PT, Soares JA, Ferguson TK, Gallucci J, et al. Reactivity and chemical synthesis of L-pyrrolysine- the 22(nd) genetically encoded amino acid. Chem Biol. 2004;11(9):1317-24. Epub 2004/09/24. doi: 10.1016/j.chembiol.2004.07.011 S1074552104002182 [pii]. PubMed PMID: 15380192.

40. Hao B, Gong W, Ferguson TK, James CM, Krzycki JA, Chan MK. A new UAG-encoded residue in the structure of a methanogen methyltransferase. Science. 2002;296:1462-6.

41. Ticak T, Kountz DJ, Girosky KE, Krzycki JA, Ferguson DJ, Jr. A nonpyrrolysine member of the widely distributed trimethylamine methyltransferase family is a glycine betaine methyltransferase. Proc Natl Acad Sci USA. 2014;111(43):E4668-76. doi: 10.1073/pnas.1409642111. PubMed PMID: 25313086; PubMed Central PMCID: PMC4217433.

42. Eggerth AH. The Gram-positive non-spore-bearing anaerobic bacilli of human feces. Journal of bacteriology. 1935;30(3):277-99. PubMed PMID: 16559837; PubMed Central PMCID: PMC543656.

43. Wang RF, Cao WW, Cerniglia CE. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Applied and environmental microbiology. 1996;62(4):1242-7. PubMed PMID: 8919784; PubMed Central PMCID: PMC167889.

44. Song Y, Cho BK. Draft Genome Sequence of Chemolithoautotrophic Acetogenic Butanol-Producing *Eubacterium limosum* ATCC 8486. Genome Announc. 2015;3(1). doi: 10.1128/genomeA.01564-14. PubMed PMID: 25676768; PubMed Central PMCID: PMC4333668.

45. Muller E, Fahlbusch K, Walther R, Gottschalk G. Formation of N,N-Dimethylglycine, Acetic Acid, and Butyric Acid from Betaine by *Eubacterium limosum*. Applied and environmental microbiology. 1981;42(3):439-45. PubMed PMID: 16345842; PubMed Central PMCID: PMC244033.

46. Schippa S, Iebba V, Santangelo F, Gagliardi A, De Biase RV, Stamato A, et al. Cystic fibrosis transmembrane conductance regulator (CFTR) allelic variants relate to shifts in faecal microbiota of cystic fibrosis patients. PloS one. 2013;8(4):e61176. doi: 10.1371/journal.pone.0061176. PubMed PMID: 23613805; PubMed Central PMCID: PMC3629184.

47. Biagi E, Nylund L, Candela M, Ostan R, Bucci L, Pini E, et al. Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PloS one. 2010;5(5):e10667. doi: 10.1371/journal.pone.0010667. PubMed PMID: 20498852; PubMed Central PMCID: PMC2871786.

48. Kanauchi O, Fukuda M, Matsumoto Y, Ishii S, Ozawa T, Shimizu M, et al. *Eubacterium limosum* ameliorates experimental colitis and metabolite of microbe attenuates colonic inflammatory action with increase of mucosal integrity. World J Gastroenterol. 2006;12(7):1071-7. PubMed PMID: 16534848; PubMed Central PMCID: PMC4087899.

49. Karlsson FH, Fak F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, et al. Symptomatic atherosclerosis is associated with an altered gut metagenome. Nat Commun. 2012;3:1245. doi: 10.1038/ncomms2266. PubMed PMID: 23212374; PubMed Central PMCID: PMC3538954.

50. Watkins AJ, Roussel EG, Parkes RJ, Sass H. Glycine betaine as a direct substrate for methanogens (*Methanococcoides* spp.). Applied and environmental microbiology.

2014;80(1):289-93. doi: 10.1128/AEM.03076-13. PubMed PMID: 24162571; PubMed Central PMCID: PMC3911008.

51. Watkins AJ, Roussel EG, Webster G, Parkes RJ, Sass H. Choline and N,N-dimethylethanolamine as direct substrates for methanogens. Applied and environmental microbiology. 2012;78(23):8298-303. doi: 10.1128/AEM.01941-12. PubMed PMID: 23001649; PubMed Central PMCID: PMC3497383.

52. Ticak T, Hariraju D, Arcelay MB, Arivett BA, Fiester SE, Ferguson DJ, Jr. Isolation and characterization of a tetramethylammonium-degrading *Methanococcoides* strain and a novel glycine betaine-utilizing *Methanolobus* strain. Arch Microbiol. 2015;197(2):197-209. doi: 10.1007/s00203-014-1043-6. PubMed PMID: 25319587.

53. Tallant TC, Krzycki JA. Methylthiol:coenzyme M methyltransferase from *Methanosarcina barkeri*, an enzyme of methanogenesis from dimethylsulfide and methylmercaptopropionate. J Bacteriol. 1997;179(22):6902-11.

54. Poehlein A, Gottschalk G, Daniel R. First Insights into the Genome of the Gram-Negative, Endospore-Forming *Organism Sporomusa ovata* Strain H1 DSM 2662. Genome Announc. 2013;1(5). doi: 10.1128/genomeA.00734-13. PubMed PMID: 24029766; PubMed Central PMCID: PMC3772150.

55. Cornforth JW, Henry AJ. 108. L-stachydrine from the Fruit of *Capparis tomentosa*. J Chem Soc 1952;1952:601-2.

56. Blight SK, Larue RC, Mahapatra A, Longstaff DG, Chang E, Zhao G, et al. Direct charging of $tRNA_{CUA}$ with pyrrolysine *in vitro* and *in vivo*. Nature. 2004;431(7006):333-5.

57. Longstaff DG, Larue RC, Faust JE, Mahapatra A, Zhang L, Green-Church KB, et al. A natural genetic code expansion cassette enables transmissible biosynthesis and genetic encoding of pyrrolysine. Proc Natl Acad Sci USA. 2007;104(3):1021-6. PubMed PMID: 17204561.

58. Gaston MA, Zhang L, Green-Church KB, Krzycki JA. The complete biosynthesis of the genetically encoded amino acid pyrrolysine from lysine. Nature. 2011;471(7340):647-50. Epub 2011/04/02. doi: nature09918 [pii] 10.1038/nature09918. PubMed PMID: 21455182; PubMed Central PMCID: PMC3070376.

59. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics. 2005;4(9):1265-72. doi: 10.1074/mcp.M500061-MCP200. PubMed PMID: 15958392.

60. Merkley ED, Wrighton KC, Castelle CJ, Anderson BJ, Wilkins MJ, Shah V, et al. Changes in protein expression across laboratory and field experiments in *Geobacter bemidjiensis*. J Proteome Res. 2015;14(3):1361-75. doi: 10.1021/pr500983v. PubMed PMID: 25496566.

61. Guan Y, Ngugi DK, Blom J, Ali S, Ferry JG, Stingl U. Draft Genome Sequence of an Obligately Methylotrophic Methanogen, *Methanococcoides methylutens*, Isolated from Marine Sediment. Genome Announc. 2014;2(6). doi: 10.1128/genomeA.01184-14. PubMed PMID: 25414501; PubMed Central PMCID: PMC4239356.

62. Zhao S, Kumar R, Sakai A, Vetting MW, Wood BM, Brown S, et al. Discovery of new enzymes and metabolic pathways by using structure and genome context. Nature. 2013;502(7473):698-702. doi: 10.1038/nature12576. PubMed PMID: 24056934; PubMed Central PMCID: PMC3966649.

63. Ziegler C, Bremer E, Kramer R. The BCCT family of carriers: from physiology to crystal structure. Mol Microbiol. 2010;78(1):13-34. doi: 10.1111/j.1365-2958.2010.07332.x. PubMed PMID: 20923416.

64. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. The I-TASSER Suite: protein structure and function prediction. Nat Methods. 2015;12(1):7-8. doi: 10.1038/nmeth.3213. PubMed PMID: 25549265; PubMed Central PMCID: PMC4428668.

65. Nguyen HD, Studenik S, Diekert G. Corrinoid activation by a RACE protein: studies on the interaction of the proteins involved. FEMS microbiology letters. 2013;345(1):31-8. doi: 10.1111/1574-6968.12178. PubMed PMID: 23672517.

66. Hennig SE, Jeoung JH, Goetzl S, Dobbek H. Redox-dependent complex formation by an ATP-dependent activator of the corrinoid/iron-sulfur protein. Proc Natl Acad Sci USA. 2012;109(14):5235-40. doi: 10.1073/pnas.1117126109. PubMed PMID: 22431597; PubMed Central PMCID: PMC3325681.

67. Florindo C, Ferreira R, Borges V, Spellerberg B, Gomes JP, Borrego MJ. Selection of reference genes for real-time expression studies in *Streptococcus agalactiae*. J Microbiol Methods. 2012;90(3):220-7. doi: 10.1016/j.mimet.2012.05.011. PubMed PMID: 22634000.

68. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8. doi: 10.1006/meth.2001.1262. PubMed PMID: 11846609.

69. Laurie AT, Jackson RM. Q-SiteFinder: an energy-based method for the prediction of protein-ligand binding sites. Bioinformatics. 2005;21(9):1908-16. Epub 2005/02/11. doi: bti315 [pii] 10.1093/bioinformatics/bti315. PubMed PMID: 15701681.

70. Ferguson DJ, Jr., Longstaff DG, Krzycki JA. Assay of methylotrophic methyltransferases from methanogenic archaea. Methods Enzymol. 2011;494:139-58. Epub 2011/03/16. doi: B978-0-12-385112-3.00008-1 [pii] 10.1016/B978-0-12-385112-3.00008-1. PubMed PMID: 21402214.

71. Klock HE, Lesley SA. The Polymerase Incomplete Primer Extension (PIPE) method applied to high-throughput cloning and site-directed mutagenesis. Methods Mol Biol. 2009;498:91-103. Epub 2008/11/07. doi: 10.1007/978-1-59745-196-3_6. PubMed PMID: 18988020.

72. Pritchett MA, Metcalf WW. Genetic, physiological and biochemical characterization of multiple methanol methyltransferase isozymes in *Methanosarcina acetivorans* C2A. Mol Microbiol. 2005;56(5):1183-94. Epub 2005/05/11. doi: MMI4616 [pii] 10.1111/j.1365-2958.2005.04616.x. PubMed PMID: 15882413.

73. Tallant TC, Paul L, Krzycki JA. The MtsA subunit of the methylthiol:coenzyme M methyltransferase of *Methanosarcina barkeri* catalyses both half-reactions of corrinoid-dependent dimethylsulfide: coenzyme M methyl transfer. J Biol Chem. 2001;276(6):4485-93.

74. Sharon I, Morowitz MJ, Thomas BC, Costello EK, Relman DA, Banfield JF. Time series community genomics analysis reveals rapid shifts in bacterial species, strains, and phage during infant gut colonization. Genome Res. 2013;23(1):111-20. doi: 10.1101/gr.142315.112. PubMed PMID: 22936250; PubMed Central PMCID: PMC3530670.

75. He X, McLean JS, Edlund A, Yooseph S, Hall AP, Liu SY, et al. Cultivation of a human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. Proc Natl Acad Sci USA. 2015;112(1):244-9. doi: 10.1073/pnas.1419038112. PubMed PMID: 25535390; PubMed Central PMCID: PMC4291631.

76. Gregory JC, Buffa JA, Org E, Wang Z, Levison BS, Zhu W, et al. Transmission of atherosclerosis susceptibility with gut microbial transplantation. J Biol Chem. 2015;290(9):5647-60. doi: 10.1074/jbc.M114.618249. PubMed PMID: 25550161; PubMed Central PMCID: PMC4342477.

77. Castelle CJ, Hug LA, Wrighton KC, Thomas BC, Williams KH, Wu D, et al. Extraordinary phylogenetic diversity and metabolic versatility in aquifer sediment. Nat Commun. 2013;4:2120. doi: 10.1038/ncomms3120. PubMed PMID: 23979677; PubMed Central PMCID: PMC3903129.

78. Di Rienzi SC, Sharon I, Wrighton KC, Koren O, Hug LA, Thomas BC, et al. The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new candidate phylum sibling to Cyanobacteria. eLife. 2013;2:e01102. doi: 10.7554/eLife.01102. PubMed PMID: 24137540; PubMed Central PMCID: PMC3787301.

79. Sharon I, Banfield JF. Microbiology. Genomes from metagenomics. Science. 2013;342(6162):1057-8. doi: 10.1126/science.1247023. PubMed PMID: 24288324.

80. Wrighton KC, Thomas BC, Sharon I, Miller CS, Castelle CJ, VerBerkmoes NC, et al. Fermentation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial phyla. Science. 2012;337(6102):1661-5. doi: 10.1126/science.1224041. PubMed PMID: 23019650.

81. Greenblum S, Turnbaugh PJ, Borenstein E. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. Proc Natl Acad Sci USA. 2012;109(2):594-9. doi: 10.1073/pnas.1116053109. PubMed PMID: 22184244; PubMed Central PMCID: PMC3258644.

82. Tveit AT, Urich T, Frenzel P, Svenning MM. Metabolic and trophic interactions modulate methane production by Arctic peat microbiota in response to warming. Proc Natl Acad Sci USA. 2015;112(19):E2507-16. doi: 10.1073/pnas.1420797112. PubMed PMID: 25918393; PubMed Central PMCID: PMC4434766.

83. Schwiertz A, Le Blay G, Blaut M. Quantification of different Eubacterium spp. in human fecal samples with species-specific 16S rRNA-targeted oligonucleotide probes. Applied and environmental microbiology. 2000;66(1):375-82. PubMed PMID: 10618251; PubMed Central PMCID: PMC91833.

84. Franzosa EA, Hsu T, Sirota-Madi A, Shafquat A, Abu-Ali G, Morgan XC, et al. Sequencing and beyond: integrating molecular 'omics' for microbial community profiling. Nat Rev Microbiol. 2015;13(6):360-72. doi: 10.1038/nrmicro3451. PubMed PMID: 25915636.

85. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal. 2012;6(8):1621-4. doi: 10.1038/ismej.2012.8. PubMed PMID: 22402401; PubMed Central PMCID: PMC3400413.

86. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335-6. doi: 10.1038/nmeth.f.303. PubMed PMID: 20383131; PubMed Central PMCID: PMC3156573.

87. Wrighton KC, Virdis B, Clauwaert P, Read ST, Daly RA, Boon N, et al. Bacterial community structure corresponds to performance during cathodic nitrate reduction. The ISME journal. 2010;4(11):1443-55.

88. Handley KM, Wrighton KC, Miller CS, Wilkins MJ, Kantor RS, Thomas BC, et al. Disturbed subsurface microbial communities follow equivalent trajectories despite different structural starting points. Environmental microbiology. 2014. DOI: 10.1111/1462-2920.12467

89. Wrighton KC, Castelle CJ, Wilkins MJ, Hug La, Sharon I, Thomas BC, et al. Metabolic interdependencies between phylogenetically novel fermenters and respiratory organisms in an unconfined aquifer. The ISME journal. 2014;8(7):1452-63.

90. Sharon I, Kertesz M, Hug LA, Pushkarev D, Blauwkamp TA, Castelle CJ, et al. Accurate, multi-kb reads resolve complex populations and detect rare microorganisms. Genome Res. 2015;25(4):534-43. doi: 10.1101/gr.183012.114. PubMed PMID: 25665577; PubMed Central PMCID: PMC4381525.

91. Dridi B, Fardeau ML, Ollivier B, Raoult D, Drancourt M. *Methanomassiliicoccus luminyensis* gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. Int J Syst Evol Microbiol. 2012;62(Pt 8):1902-7. doi: 10.1099/ijs.0.033712-0. PubMed PMID: 22859731.

92. Dridi B. Laboratory tools for detection of archaea in humans. Clin Microbiol Infect. 2012;18:825-33.

93. Borrel G, Harris HM, Tottey W, Mihajlovski A, Parisot N, Peyretaillade E, et al. Genome sequence of "*Candidatus Methanomethylophilus alvus*" Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens. J Bacteriol. 2012;194(24):6944-5. doi: 10.1128/JB.01867-12. PubMed PMID: 23209209; PubMed Central PMCID: PMC3510639.

94. Wolfe RS. Techniques for cultivating methanogens. Methods in enzymology. 2011;494:1-22. doi: 10.1016/B978-0-12-385112-3.00001-9. PubMed PMID: 21402207.

95. Khelaifia S, Raoult D, Drancourt M. A versatile medium for cultivating methanogenic archaea. PloS one. 2013;8(4):e61563. doi: 10.1371/journal.pone.0061563. PubMed PMID: 23613876; PubMed Central PMCID: PMC3629087.

96. Paul K, Nonoh JO, Mikulski L, Brune A. "*Methanoplasmatales,*" *Thermoplasmatales*-related archaea in termite guts and other environments, are the seventh order of methanogens. Applied and environmental microbiology. 2012;78(23):8245-53. doi: 10.1128/AEM.02193-12. PubMed PMID: 23001661; PubMed Central PMCID: PMC3497382.

97. Metcalf WW, Zhang JK, Wolfe RS. An anaerobic, intrachamber incubator for growth of *Methanosarcina* spp. on methanol-containing solid media. Applied & Environmental Microbiology. 1998;64(2):768-70.

98. Cline JD. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol Oceanogr. 1969;14(3): 454–8.

99. Shand CA, Williams BL, Coutts G. Determination of N-species in soil extracts using microplate techniques. Talanta. 2008;74(4):648-54. doi: 10.1016/j.talanta.2007.06.039. PubMed PMID: 18371688.

100. Kremer JD, Cao X, Krzycki J. Isolation of two novel corrinoid proteins from acetate-grown *Methanosarcina barkeri*. J Bacteriol. 1993;175(15):4824-33.

101. Ingalls ST, Hoppel CL, Turkaly JS. Synthesis of radioactively methyl-labelled (L)-carnitine. J Labelled Comp Radiopharm. 1982;9(4):525-41.

Example 2. Proteomics reveals that *E. limosum* grown on quaternary amines upregulates genes encoding MttB family members and other proteins of three component methyltransferase systems.

E. limosum was cultivated on defined medium containing one of the indicated quaternary amines as the sole source of fixed carbon. Cells cultivated on lactate served as a control substrate, as these should not require MttB family proteins. Total protein was extracted from the cell pellets, digested with trypsin, and the peptide fragments analyzed by 2-dimensional mass spectrometry. Total abundance of the parent proteins identified with the peptide sequences were estimated using emPAI values calculated for each gene product. The mol % abundance for a particular protein was calculated using summed emPAI values for the circa 1500-1800 proteins detected in each sample. Values are the averages of four biological replicates.

Table 2. Abundance of mole percentage of each protein grown on each quaternary amine.

			Abundance (% mol total protein)					
Protein	**Genbank Acc. #**	**MW**	**Lactate**	**Carnitine**	**Gly Betaine**	**Pro Betaine**	**Butyrobetaine**	**Choline**
MtcB	WP_038351887.1	54 kDa	0.0000	2.1397	0.0000	0.0000	0.0000	0.0001
MtpB	WP_038353400.1	54 kDa	0.0000	0.0029	5.6496	3.7734	0.0128	0.0056
MtyB	WP_013382626.1	54 kDa	0.0000	0.0001	0.0000	0.0000	0.8848	0.0030
MtcC	WP_038352545.1	22 kDa	0.0085	0.5839	0.3438	0.2222	0.4631	0.2355
MtcA	WP_038351870.1	30 kDa	0.1000	3.5774	1.3168	1.6207	1.8328	1.0547
RamC	WP_038351871.1	64 kDa	0.0096	0.1364	0.0761	0.0218	0.0269	0.0329
MthB	WP_038350935.1	53 kDa	0.0004	0.0001	0.0000	0.0000	0.0000	2.6974
MthC	WP_038350932.1	22 kDa	0.0000	0.0001	0.0000	0.0000	0.0000	5.7573
MthA	WP_038350933.1	29 kDa	0.0003	0.0000	0.0000	0.0000	0.0000	0.7839

One of four different MttB family members was abundant in cells grown on each quaternary amine (MtcB with carnitine, MtpB with proline betaine and glycine betaine, MtyB with gamma butyrobetaine, or MthB with choline) but not in cells grown with lactic acid. Additionally,

corrinoid protein and methyl-corrinoid:THF methyltransferase homologs are also abundant in quaternary amine grown cells, but not cells grown on lactate. MtcC and MtcA are abundant in all quaternary amine grown cells, but MthC and MthA are abundant specifically in choline grown cells. Additionally, RamC, is upregulated and it is a homolog of proteins which activate corrinoid proteins for methyl transfer. These findings suggest that MttB homologs have evolved specificity for the different quaternary amines, and demethylate a specific quaternary amine as parts of three-component methyltransferase systems that underlie the ability of *E. limosum* and other organisms to demethylate proatherogenic quaternary amines.

During growth on carnitine, an MttB homolog ("MtcB"), two corrinoid proteins ("MtcC1" and "MtcC2") and a putative methylcorrinoid:THF methyltransferase ("MtcA") are upregulated when compared to growth on lactate.

Table 3. Genes upregulated by growth on carnitine

Protein	L-carnitine		Lactate		Fold upregulation on carnitine
	emPAI	Mole % protein	emPAI	Mole % protein	
MtcA	22	1.6	1.3	0.090	17
MtcB	0.51	0.036	Not detected		N.A.
MtcC1	10	0.71	1.7	0.12	6.1
MtcC2	0.31	0.022	Not detected		N.A.

Example 3. Analysis of products of QA metabolism in *E. limosum* cultures.

Previously, culture supernatants were analyzed by thin layer chromatography, and it was found that all cultures accumulated the product of a single demethylation of the QA added to the medium. That is, when growing on either glycine betaine, L-carnitine, γ-butyrobetaine, or choline, cultures respectively produced N,N-dimethylglycine, N,N-dimethylethanolamine, or norcarnitine, γ-dimethylaminobutyrate, or dimethylaminoethanol. Each demethylation product was identified by co-migration with authentic standards in TLC. In addition to TLC, the identitiy of norcarnitine and gamma-dimethylaminobutyrate have been confirmed by mass spectrometry. In addition, the identity of the choline demethylation product as dimethylethanolamine has also been identified by mass spectrometry. For each of the three demethylation products, the m/z values obtained for the demethylation product eluted from TLC plates was within 3 ppm of the theoretical value. In other words, it was a very good match that gave further proof of the identity.

Additionally, all cultures of *E. limosum* growing on glycine betaine, carnitine, choline, or g-butyrobetaine have been analyzed. It was shown that none produce trimethylamine during QA

metabolism. Analysis was done by gas chromatography and had an estimated lower limit of detection of 0.05 mM TMA.

Example 4. Strain adaptation of *E. limosum* cultures to growth on choline.

Unlike growth with other QAs, growth on choline is initially slower with *E. limosum*. However, as cultures are successively transferred to fresh medium, adaptation to growth by demethylation of choline occurs. Once fully adapted to growth on choline, the culture retains the ability to rapidly consume and grow on choline with successive transfers (Fig. 16).

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will appreciate that numerous changes and modifications can be made to the preferred embodiments of the invention and that such changes and modifications can be made without departing from the spirit of the invention. It is, therefore, intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

CLAIMS

We claim:

1. A probiotic composition comprising an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier.
2. The probiotic composition of claim 1, wherein the isolated *Eubacterium* spp. is selected from *Eubacterium limosum, Eubacterium aggregans, Eubacterium barkeri, Eubacterium callanderi.*
3. The probiotic composition of claim 2, wherein the isolated *Eubacterium* spp. is *Eubacterium limosum.*
4. The probiotic composition of claim 3, wherein the isolated *Eubacterium* spp. is *Eubacterium limosum* ATCC 8486.
5. The probiotic composition of any one of claims 1 to 4, wherein the isolated *Eubacterium* spp. is present at 10^5 to 10^{13} cfu *Eubacterium* per gram.
6. The probiotic composition of any one of claims 1 to 5, in the form of a suspension, capsule, tablets or a microencapsulated product.
7. The probiotic composition of any one of claims 1 to 6, further comprising an additional probiotic strain.
8. A method of treating or preventing heart disease, comprising administering to a subject in need thereof an effective amount of a probiotic composition, wherein the probiotic composition comprises an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier.
9. The method of claim 8, wherein the isolated *Eubacterium* spp. is selected from *Eubacterium limosum, Eubacterium aggregans, Eubacterium barkeri, Eubacterium callanderi.*
10. The method of claim 9, wherein the isolated *Eubacterium* spp. is *Eubacterium limosum.*
11. The method of claim 10, wherein the isolated *Eubacterium* spp. is *Eubacterium limosum* ATCC 8486.
12. The method of any one of claims 8 to 11, wherein the isolated *Eubacterium* spp. is present at 10^5 to 10^{13} cfu *Eubacterium* per gram.
13. The method of any one of claims 8 to 12, in the form of a suspension, capsule, tablets or a microencapsulated product.
14. The method of any one of claims 8 to 13, further comprising an additional probiotic strain.
15. The method of any one of claims 8 to 14, wherein the heart disease is selected from cardiac ischemia, myocardial infarction, angina, peripheral vascular disease, or stroke.
16. A method of reducing trimethylamine or trimethylamine-N-oxide levels in a subject, comprising administering to the subject an effective amount of a probiotic composition,

wherein the probiotic composition comprises an isolated *Eubacterium* spp. and a pharmaceutically acceptable carrier.

17. The method of claim 16, wherein the isolated *Eubacterium* spp. is selected from *Eubacterium limosum, Eubacterium aggregans, Eubacterium barkeri, Eubacterium callanderi.*
18. The method of claim 17, wherein the isolated *Eubacterium* spp. is *Eubacterium limosum.*
19. The method of claim 18, wherein the isolated *Eubacterium* spp. is *Eubacterium limosum* ATCC 8486.
20. The method of any one of claims 16 to 19, wherein the isolated *Eubacterium* spp. is present at 10^5 to 10^{13} cfu *Eubacterium* per gram.
21. The method of claims 16 to 20, in the form of a suspension, capsule, tablets or a microencapsulated product.
22. The method of claims 16 to 21, further comprising an additional probiotic strain.

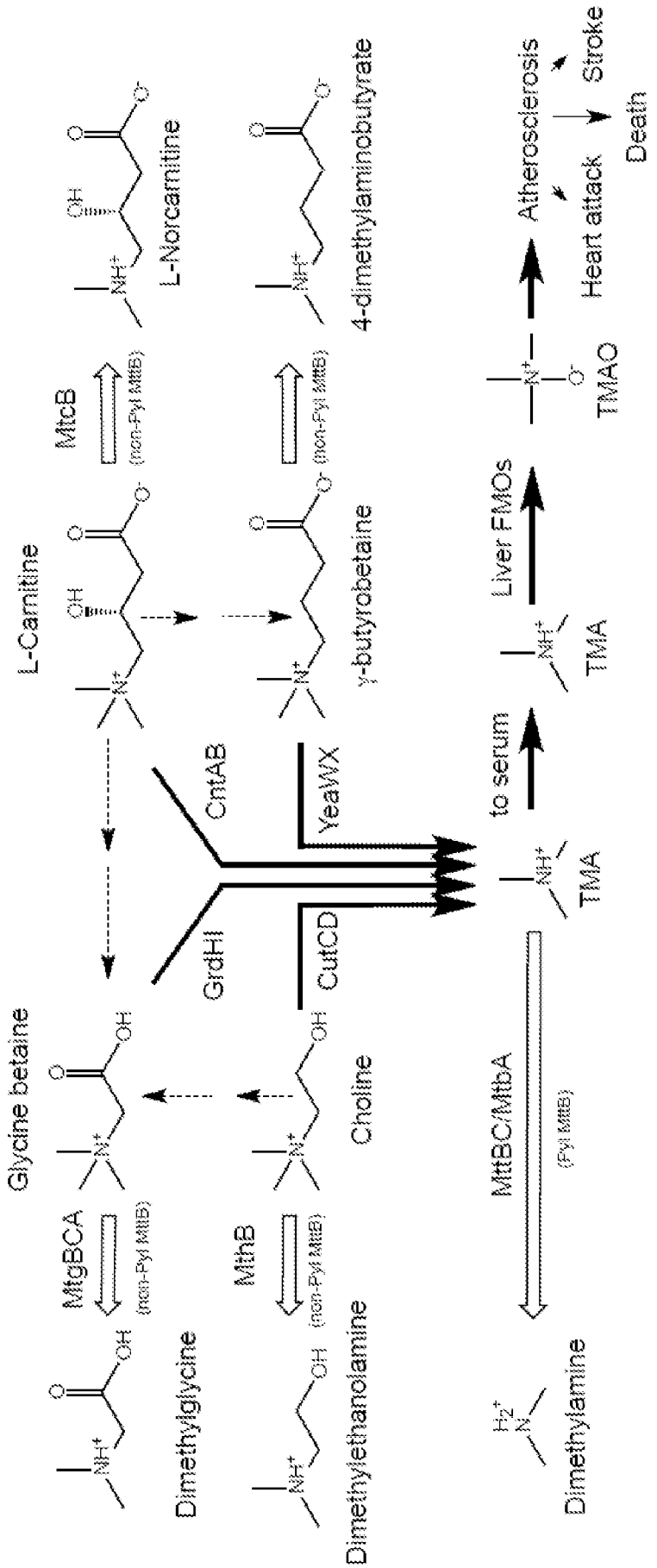

L-Carnitine
MtcB
(non-Pyl MttB)
L-Norcarnitine
γ-butyrobetaine
4-dimethylaminobutyrate
CntAB
YeaWX
GrdHI
CutCD
Glycine betaine
MtgBCA
(non-Pyl MttB)
Dimethylglycine
Choline
MttB
(non-Pyl MttB)
Dimethylethanolamine
MttBC/MttA
(Pyl MttB)
Dimethylamine
to serum
TMA
Liver FMOs
TMA
TMAO
Atherosclerosis
Heart attack
Stroke
Death

FIG. 1

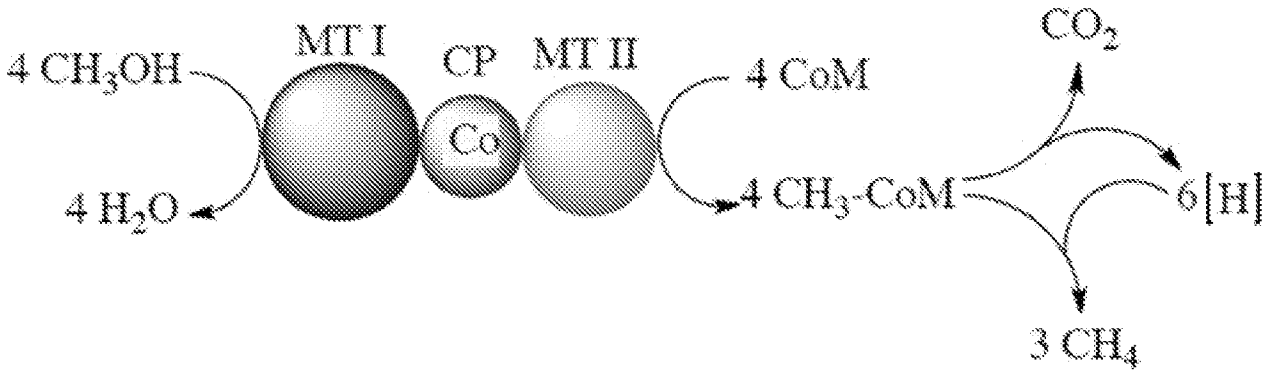

4 CH3OH
4 H2O
MT I
CP
Co
MT II
4 CoM
4 CH3-CoM
CO2
6[H]
3 CH4

FIG. 2A

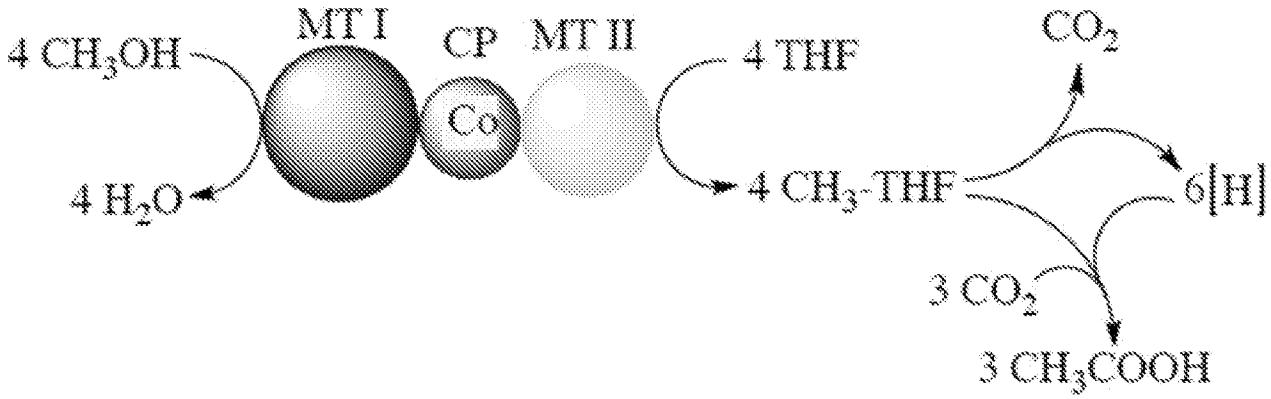

4 CH3OH
4 H2O
MT I
CP
Co
MT II
4 THF
4 CH3-THF
CO2
6[H]
3 CO2
3 CH3COOH

FIG. 2B

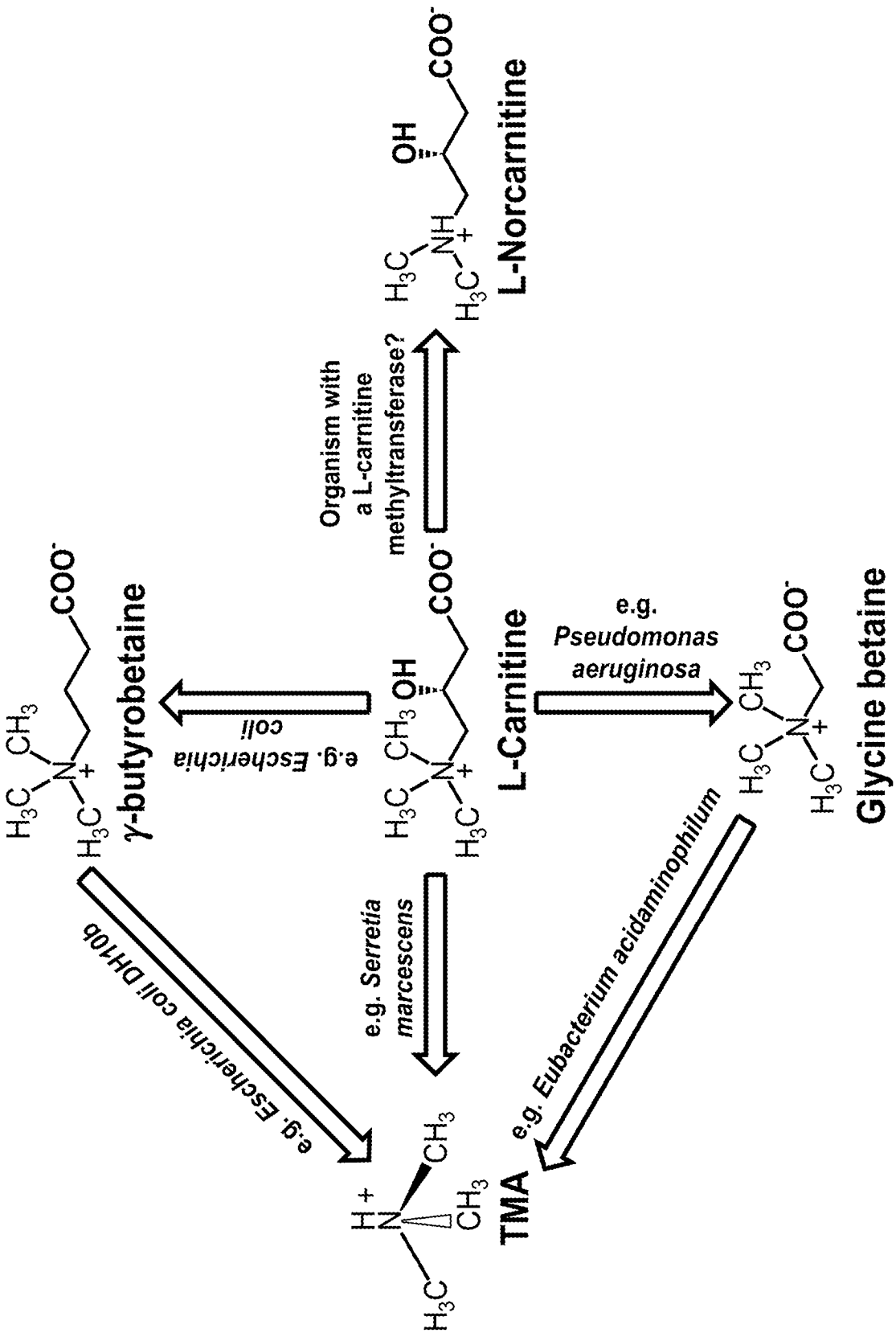

γ-butyrobetaine
e.g. Escherichia coli
L-Carnitine
Organism with a L-carnitine methyltransferase?
L-Norcarnitine
e.g. Pseudomonas aeruginosa
Glycine betaine
e.g. Escherichia coli DH10b
e.g. Serretia marcescens
e.g. Eubacterium acidaminophilum
TMA

FIG. 3

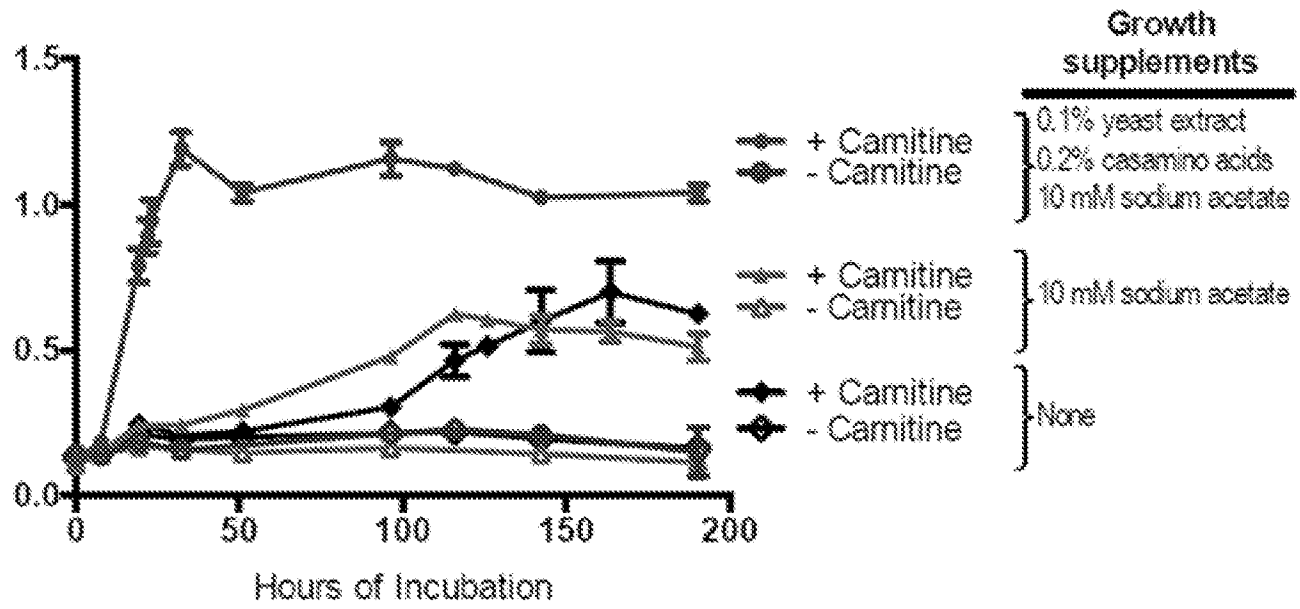

Growth supplements
1.5
1.0
0.5
0.0
0
50
100
150
200
Hours of Incubation
+ Carnitine
- Carnitine
0.1% yeast extract
0.2% casamino acids
10 mM sodium acetate
+ Carnitine
- Carnitine
10 mM sodium acetate
+ Carnitine
- Carnitine
None

FIG. 4

Substrate or product	L-carnitine consumed	CO_2 consumed	Norcarnitine produced	Acetate produced	Butyrate produced	Caproate produced
µmol change ± STD (n=5)	476 ± 51	102 ± 1	not determined	77 ± 18	78 ± 4	10 ± 2
% Carbon Recovery	90.8%					

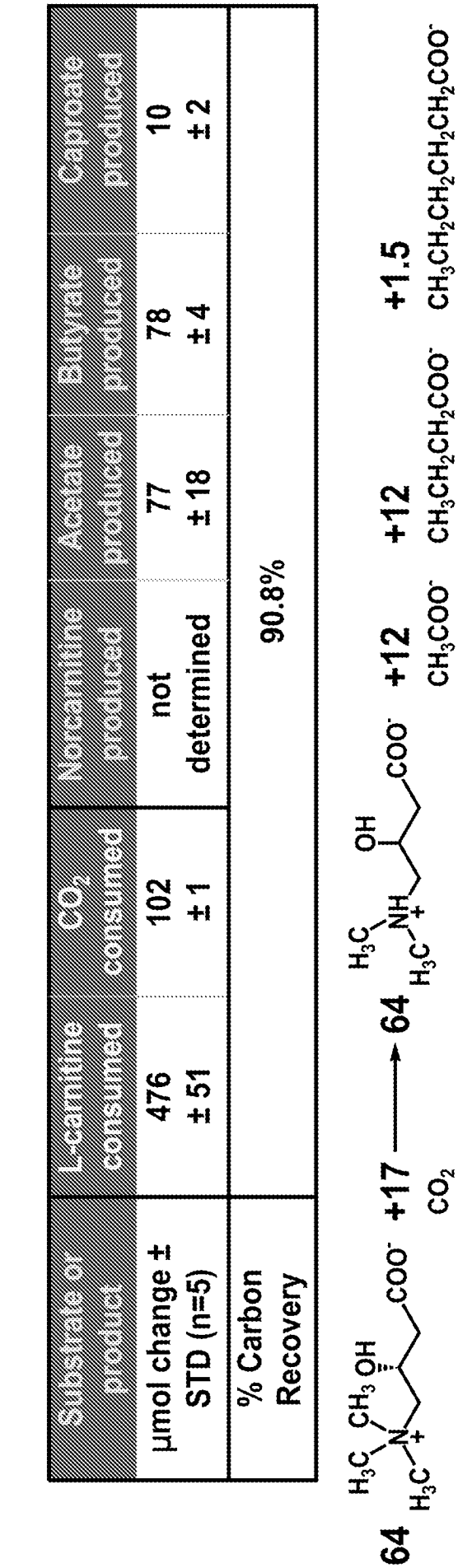


FIG. 5

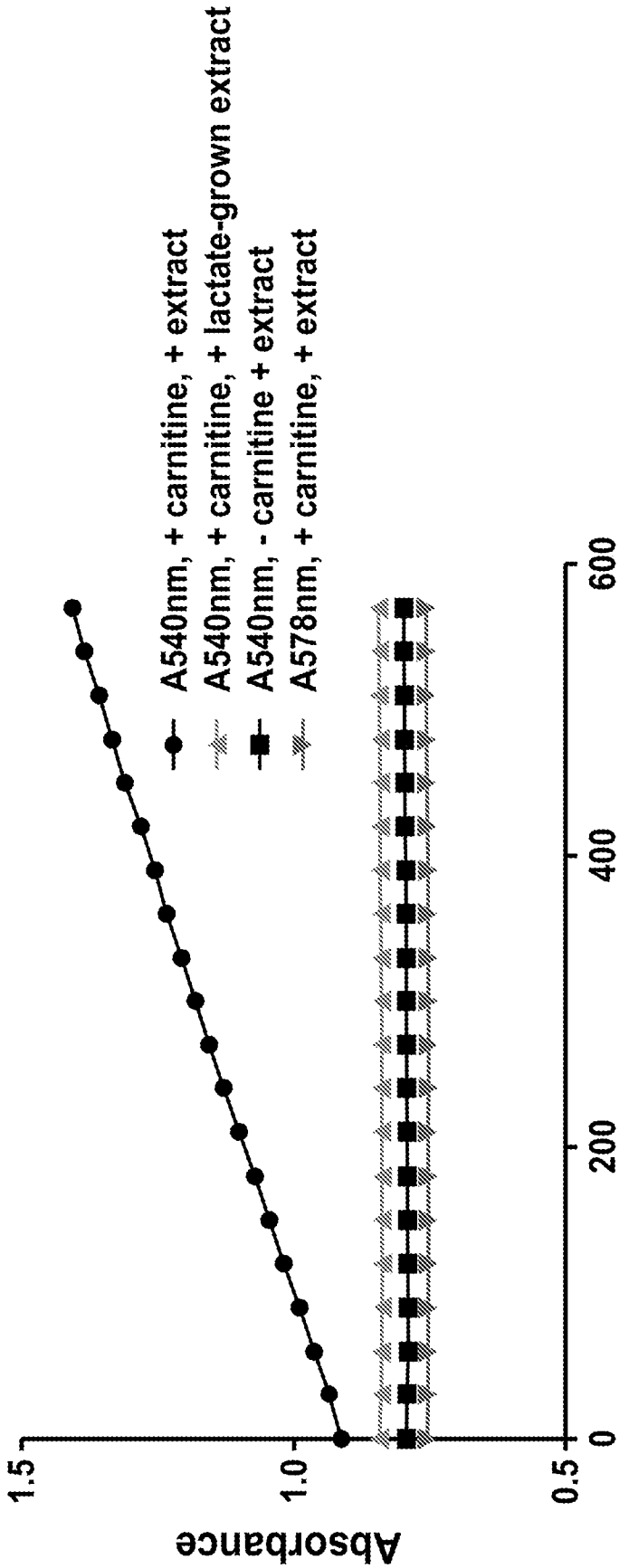

A540nm, + carnitine, + extract
A540nm, + carnitine, + lactate-grown extract
A540nm, - carnitine + extract
A578nm, + carnitine, + extract
Absorbance
1.5
1.0
0.5
0
200
400
600
Time (sec)

FIG. 6A

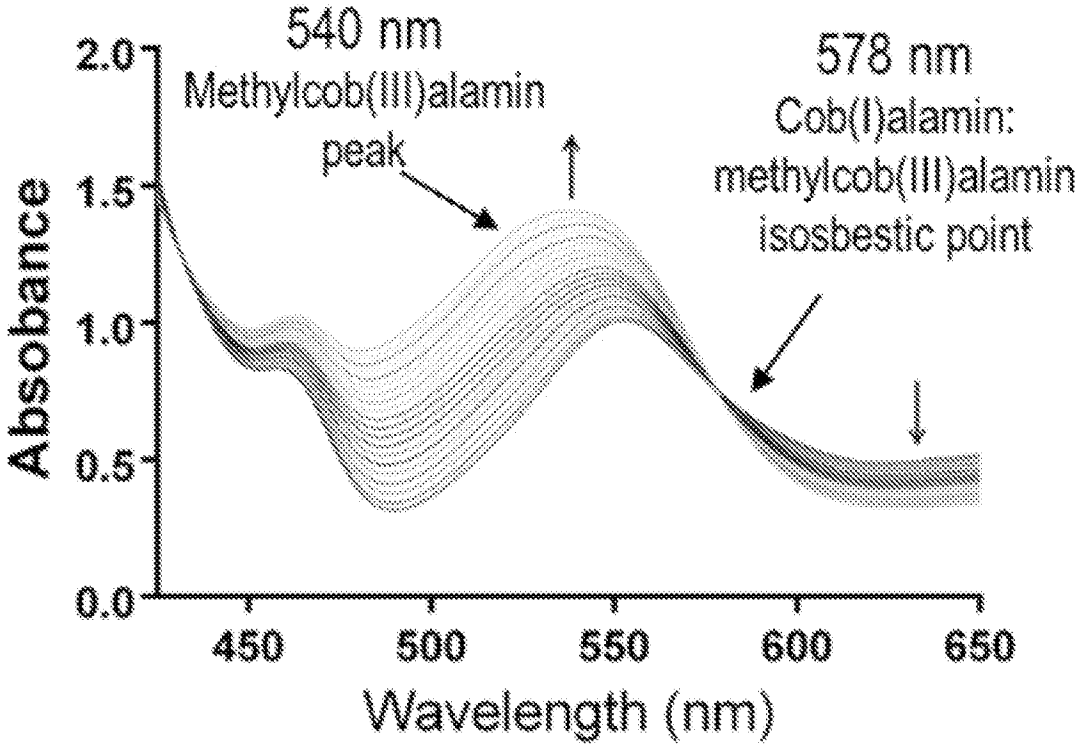

540 nm
Methylcob(III)alamin
peak
578 nm
Cob(I)alamin:
methylcob(III)alamin
isosbestic point
Absobance
2.0
1.5
1.0
0.5
0.0
450
500
550
600
650
Wavelength (nm)

FIG. 6B

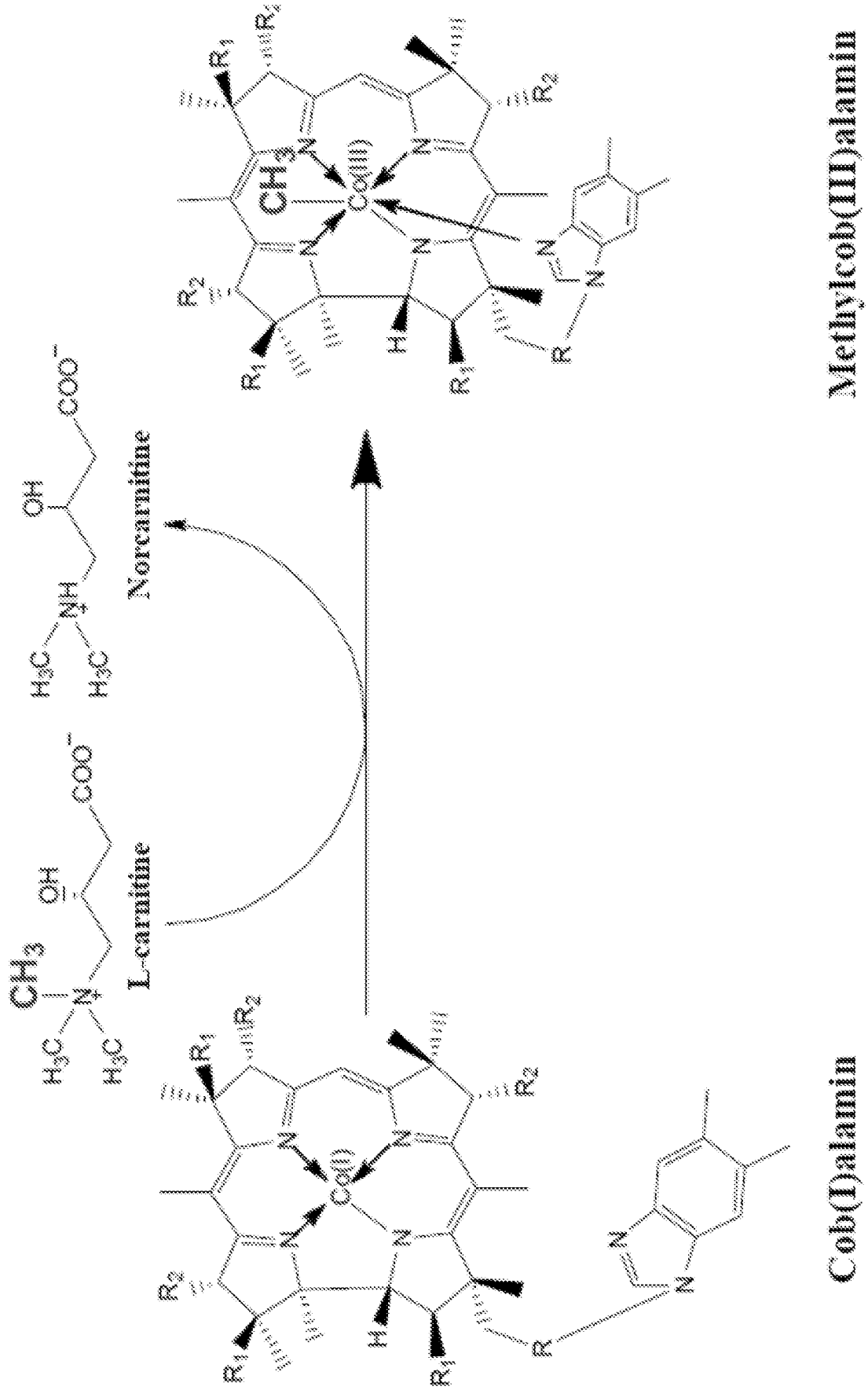

L-carnitine
Norcarnitine
Methylcob(III)alamin
Cob(I)alamin

FIG. 6C

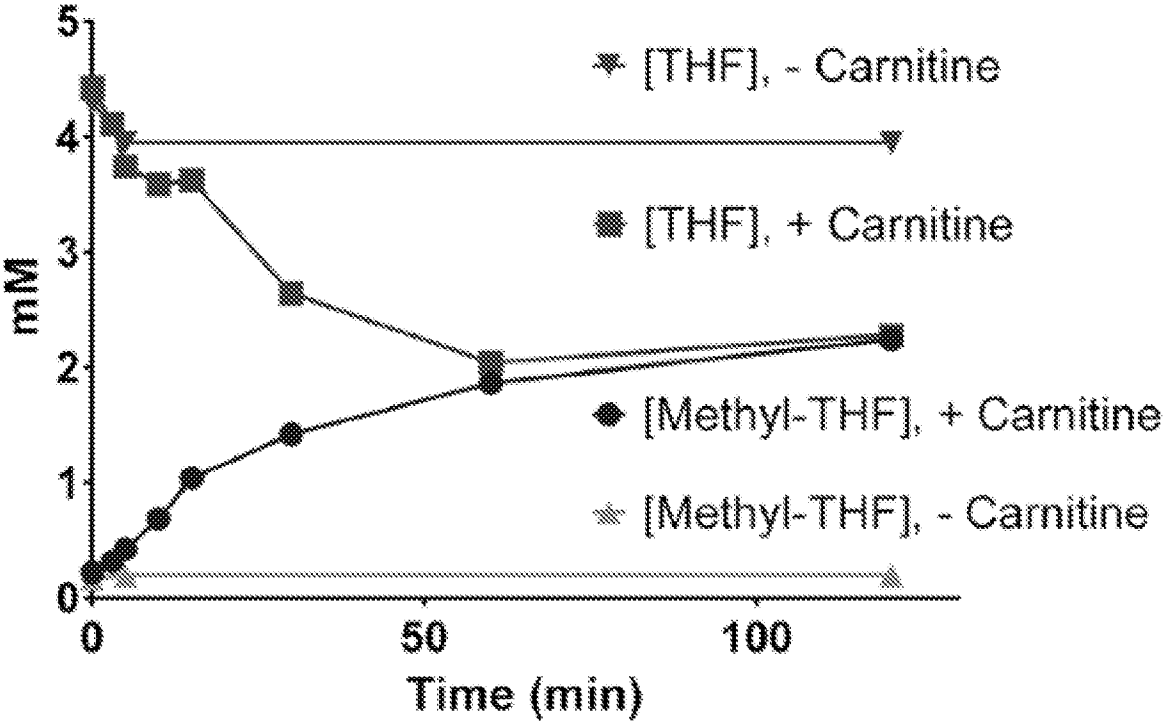

5
4
3
2
1
0
mM
0
50
100
Time (min)
[THF], - Carnitine
[THF], + Carnitine
[Methyl-THF], + Carnitine
[Methyl-THF], - Carnitine

FIG. 7A

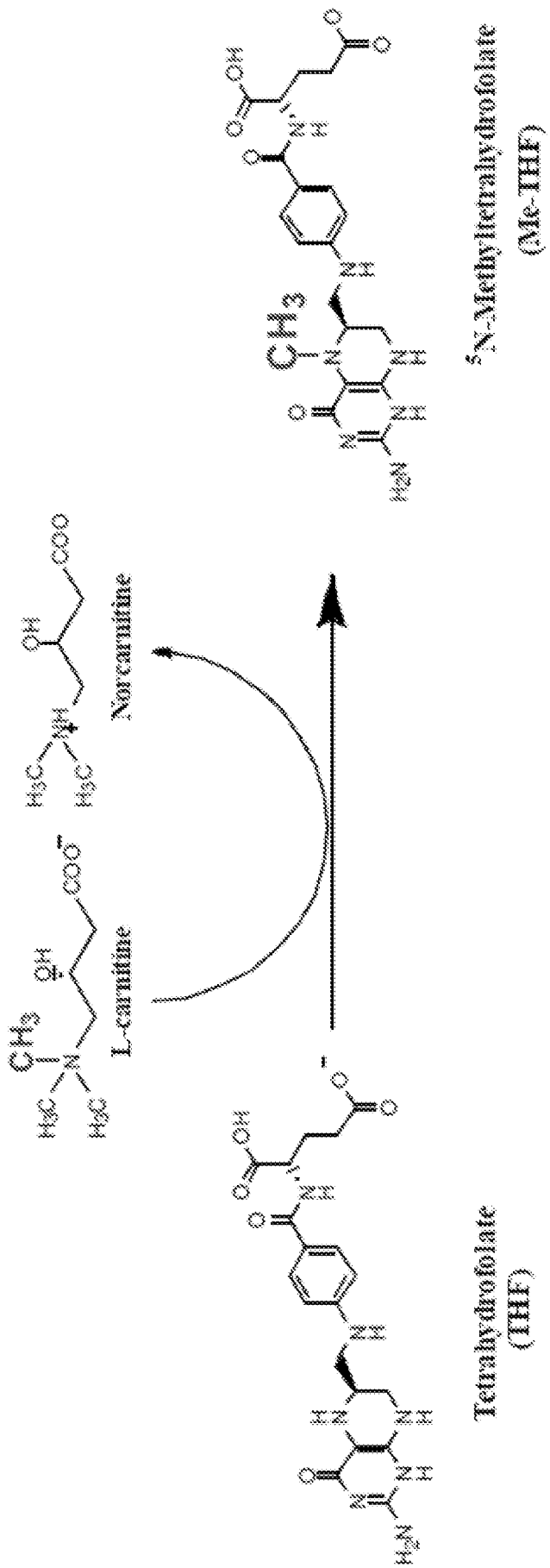

L-carnitine
Norcarnitine
Tetrahydrofolate (THF)
5-N-Methyltetrahydrofolate (Me-THF)

FIG. 7B

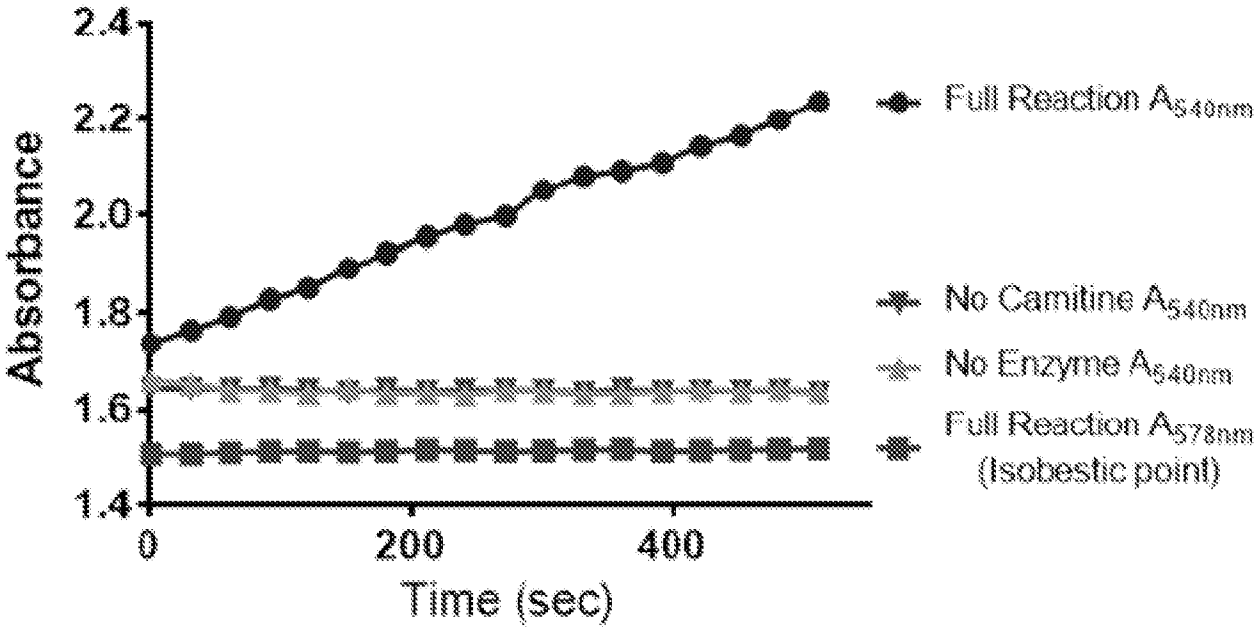

Absorbance
2.4
2.2
2.0
1.8
1.6
1.4
0
200
400
Time (sec)
Full Reaction A540nm
No Carnitine A540nm
No Enzyme A540nm
Full Reaction A578nm
(Isobestic point)

FIG. 8A

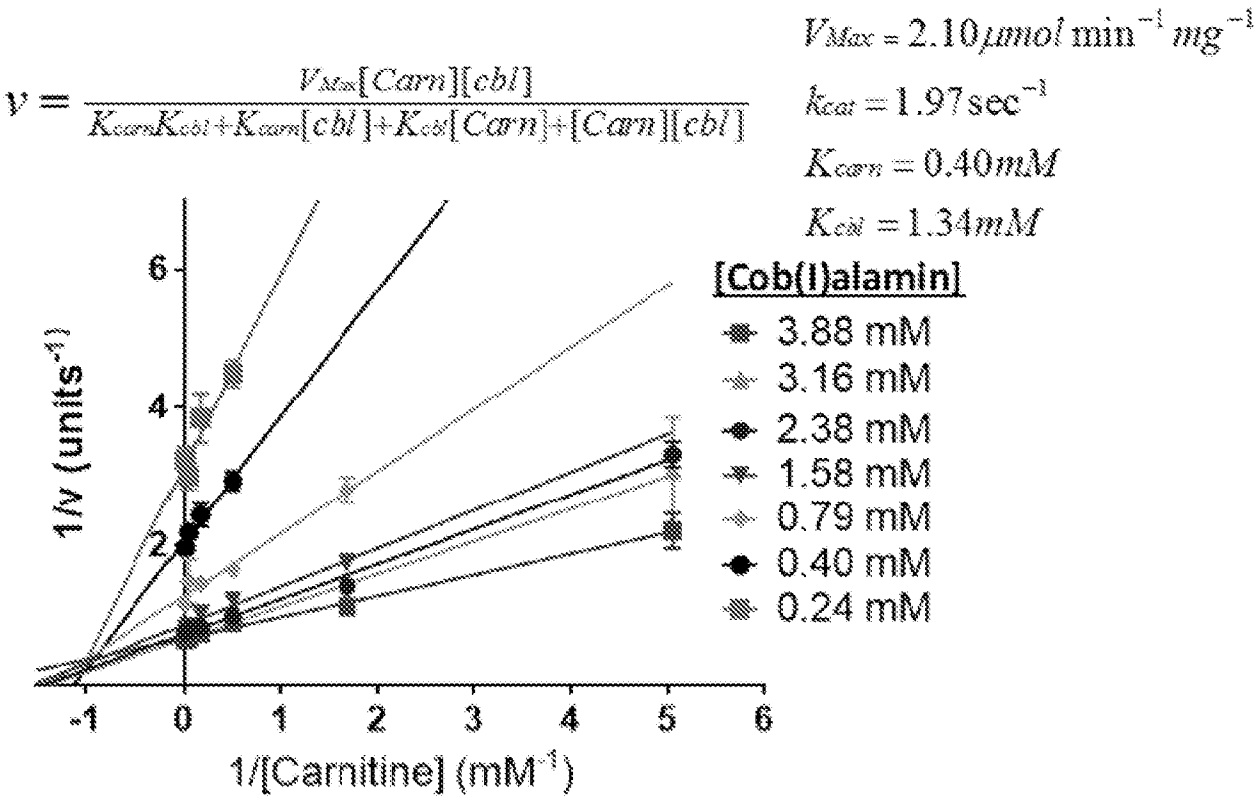

$v = \frac{V_{Max}[Carn][cbl]}{K_{carn}K_{cbl} + K_{carn}[cbl] + K_{cbl}[Carn] + [Carn][cbl]}$
$V_{Max} = 2.10 \mu mol\ \min^{-1} mg^{-1}$
$k_{cat} = 1.97 \sec^{-1}$
$K_{carn} = 0.40 mM$
$K_{cbl} = 1.34 mM$
[Cob(I)alamin]
3.88 mM
3.16 mM
2.38 mM
1.58 mM
0.79 mM
0.40 mM
0.24 mM
1/v (units^{-1})
6
4
2
-1
0
1
2
3
4
5
6
1/[Carnitine] (mM^{-1})

FIG. 8B

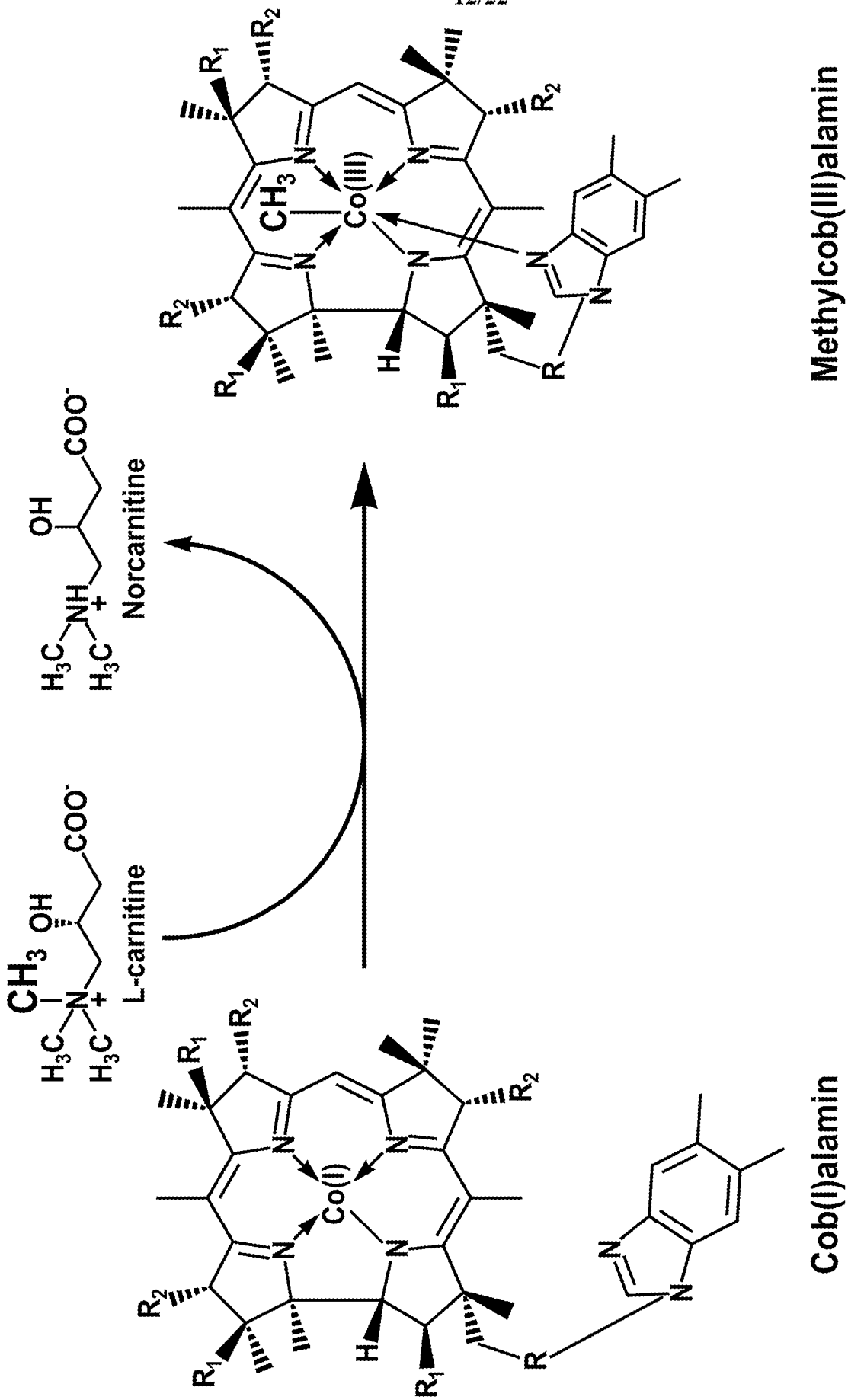

Methylcob(III)alamin
Norcarnitine
L-carnitine
Cob(I)alamin

FIG. 8C

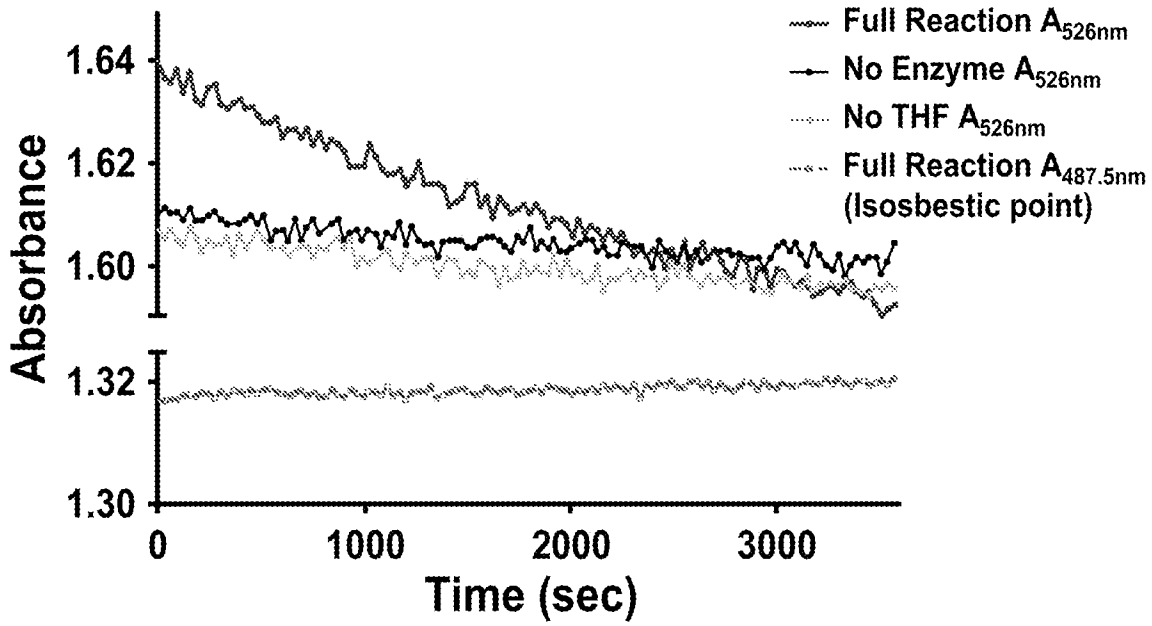

Full Reaction A_{526nm}
No Enzyme A_{526nm}
No THF A_{526nm}
Full Reaction $A_{487.5nm}$
(Isosbestic point)
Absorbance
1.64
1.62
1.60
1.32
1.30
0
1000
2000
3000
Time (sec)

FIG. 9A

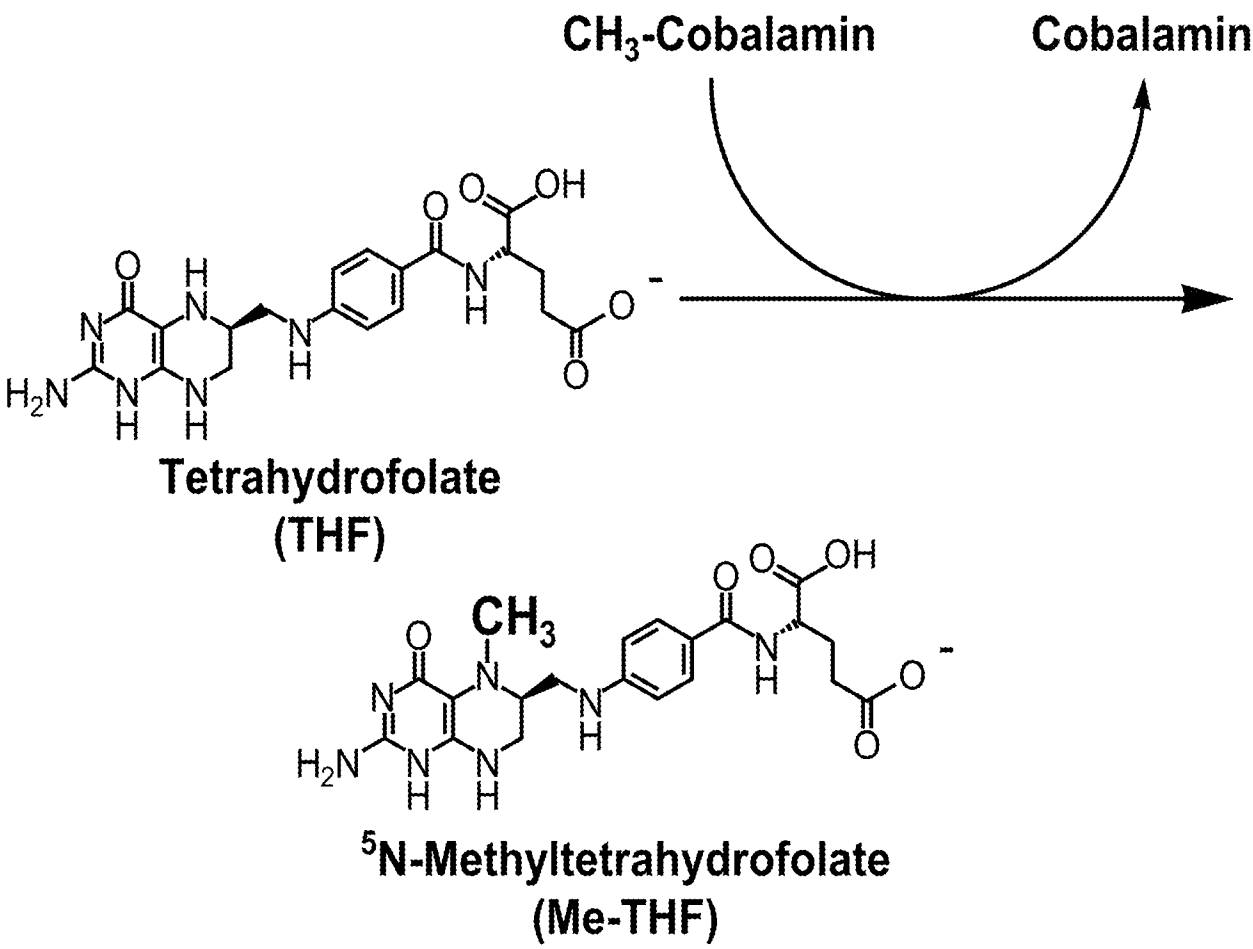

CH_3-Cobalamin
Cobalamin
Tetrahydrofolate
(THF)
^{5}N-Methyltetrahydrofolate
(Me-THF)

FIG. 9B

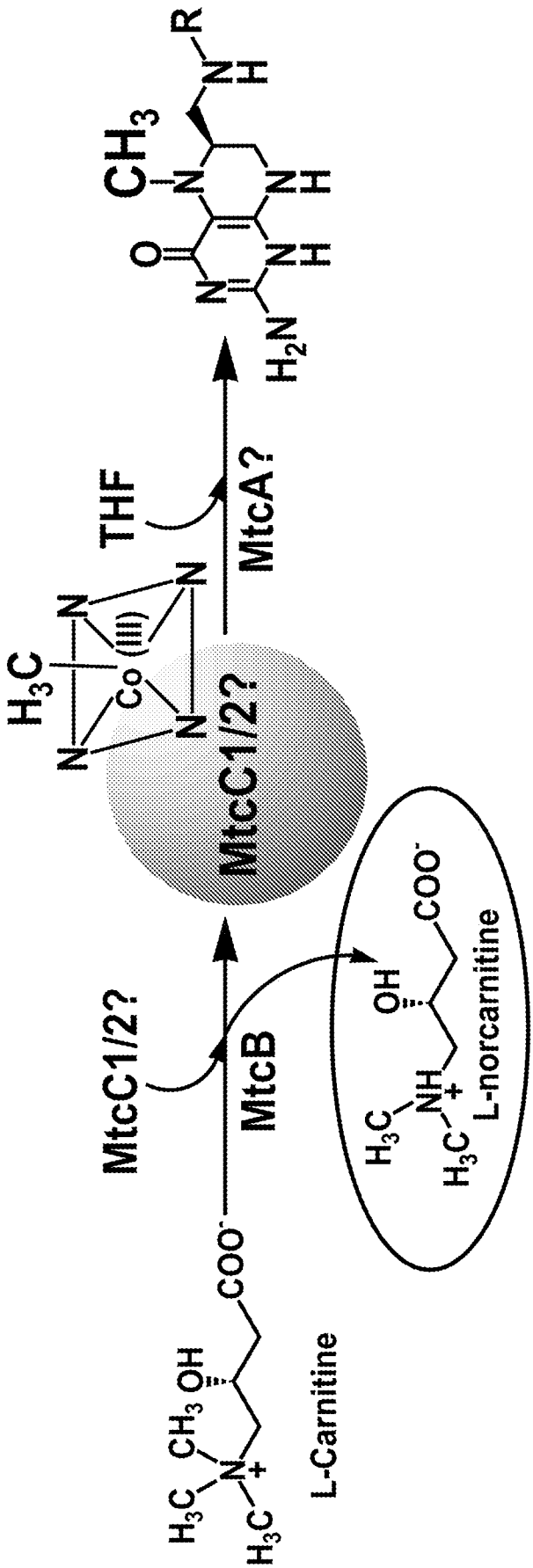


FIG. 10

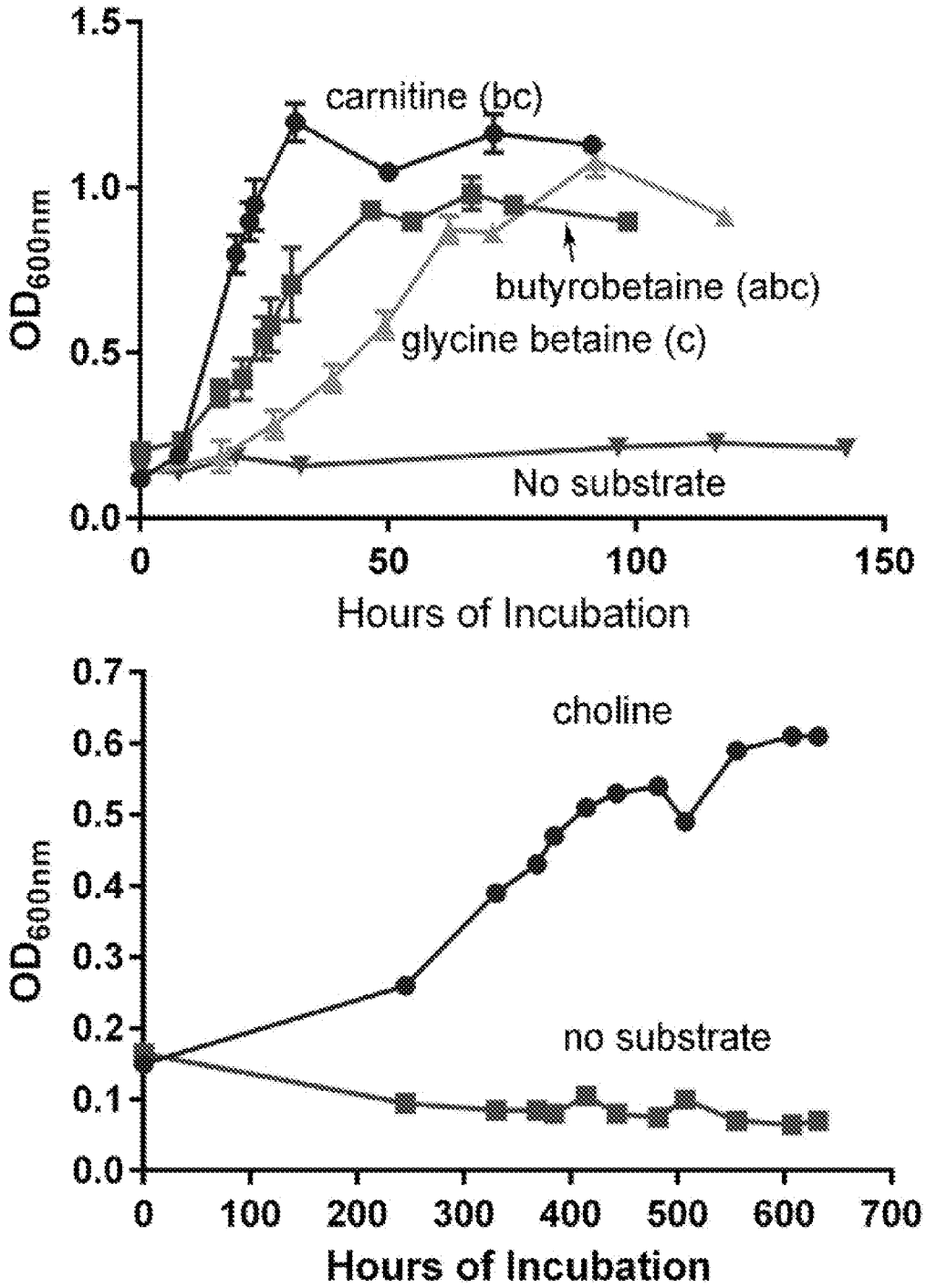

1.5
1.0
0.5
0.0
OD600nm
carnitine (bc)
butyrobetaine (abc)
glycine betaine (c)
No substrate
0
50
100
150
Hours of Incubation
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD600nm
choline
no substrate
0
100
200
300
400
500
600
700
Hours of Incubation

FIG. 11

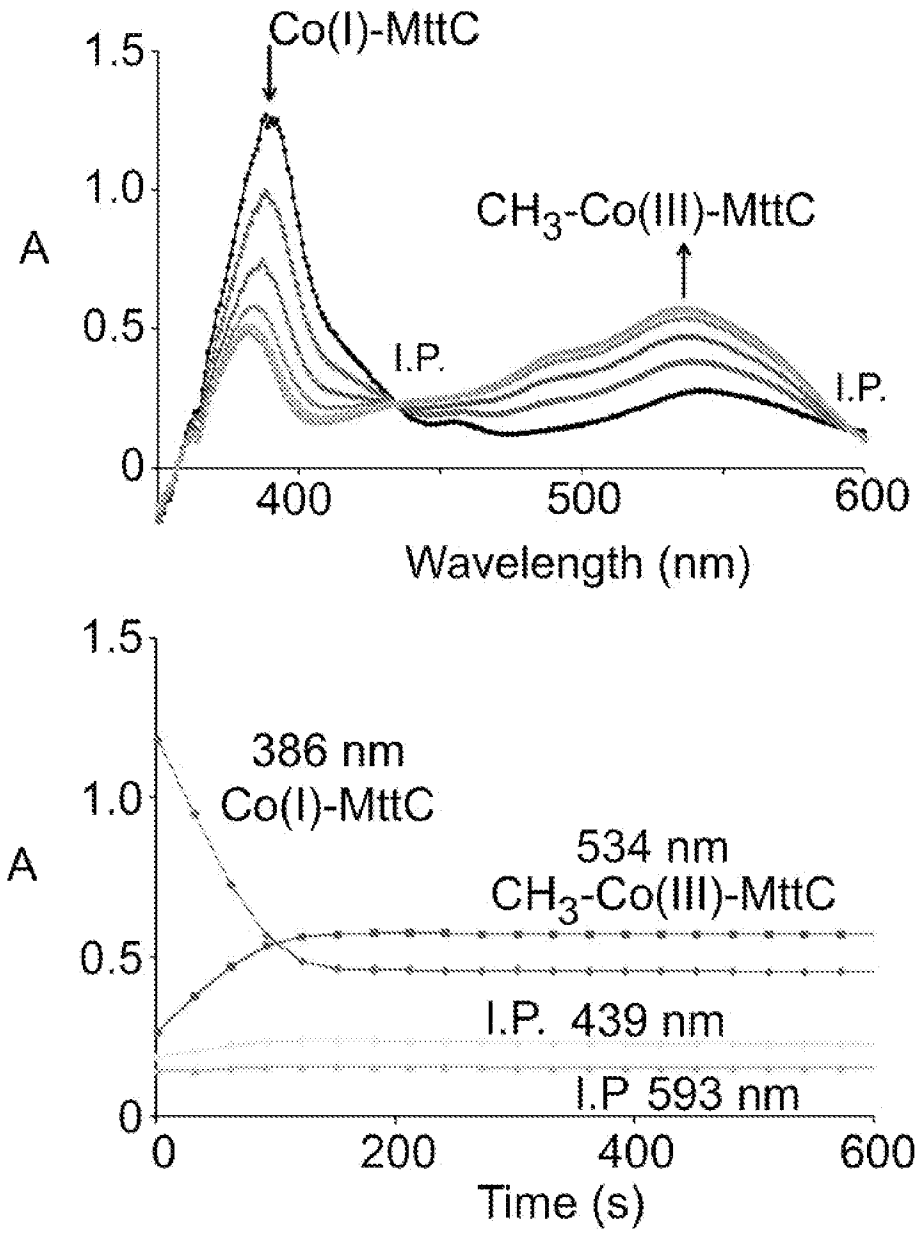

Co(I)-MttC
CH3-Co(III)-MttC
I.P.
I.P.
A
1.5
1.0
0.5
0
400
500
600
Wavelength (nm)
386 nm
Co(I)-MttC
534 nm
CH3-Co(III)-MttC
I.P. 439 nm
I.P 593 nm
A
1.5
1.0
0.5
0
0
200
400
600
Time (s)

FIG. 12

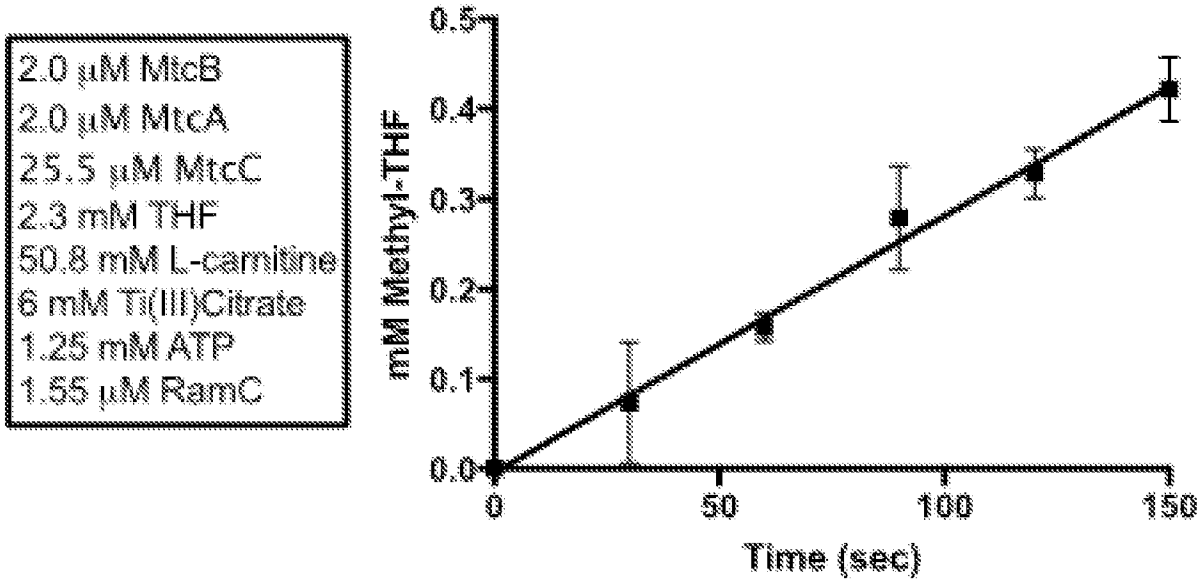

2.0 µM MtcB
2.0 µM MtcA
25.5 µM MtcC
2.3 mM THF
50.8 mM L-carnitine
6 mM Ti(III)Citrate
1.25 mM ATP
1.55 µM RamC
mM Methyl-THF
0.0
0.1
0.2
0.3
0.4
0.5
0
50
100
150
Time (sec)

FIG. 13A

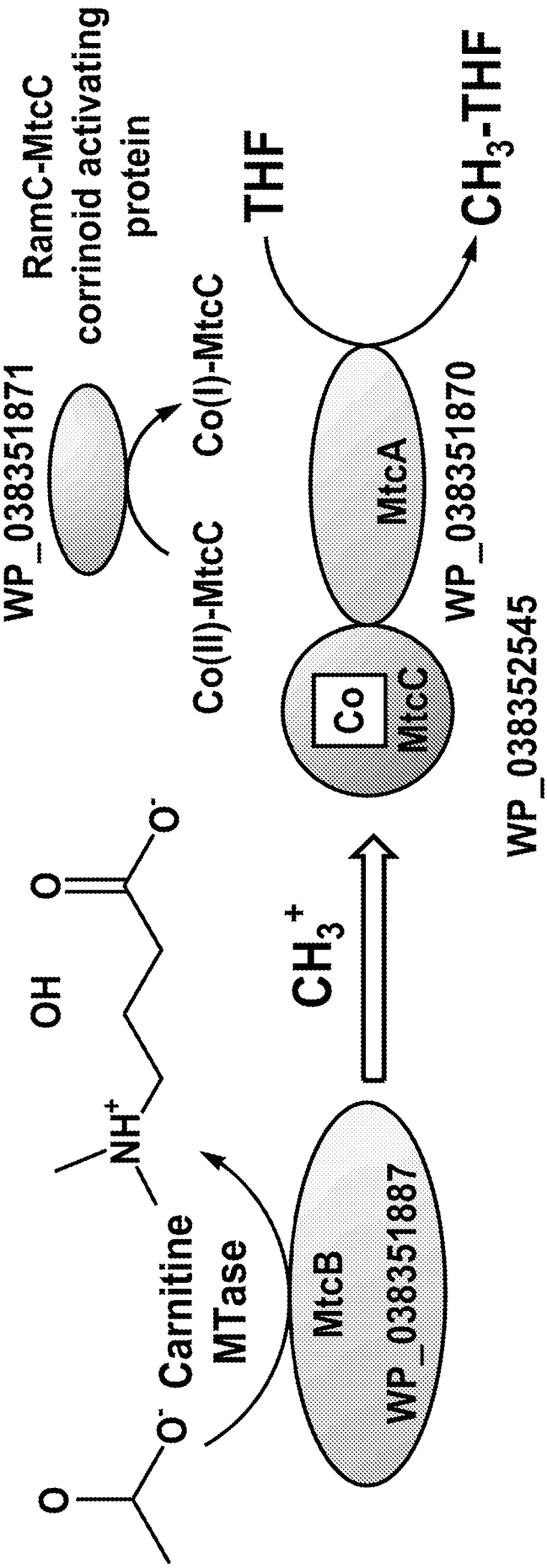

RamC-MtcC
corrinoid activating
protein
WP_038351871
Co(I)-MtcC
Co(II)-MtcC
THF
CH3-THF
MtcA
WP_038351870
Co
MtcC
WP_038352545
CH3+
OH
O
O-
NH+
O-
Carnitine
MTase
MtcB
WP_038351887

FIG. 13B

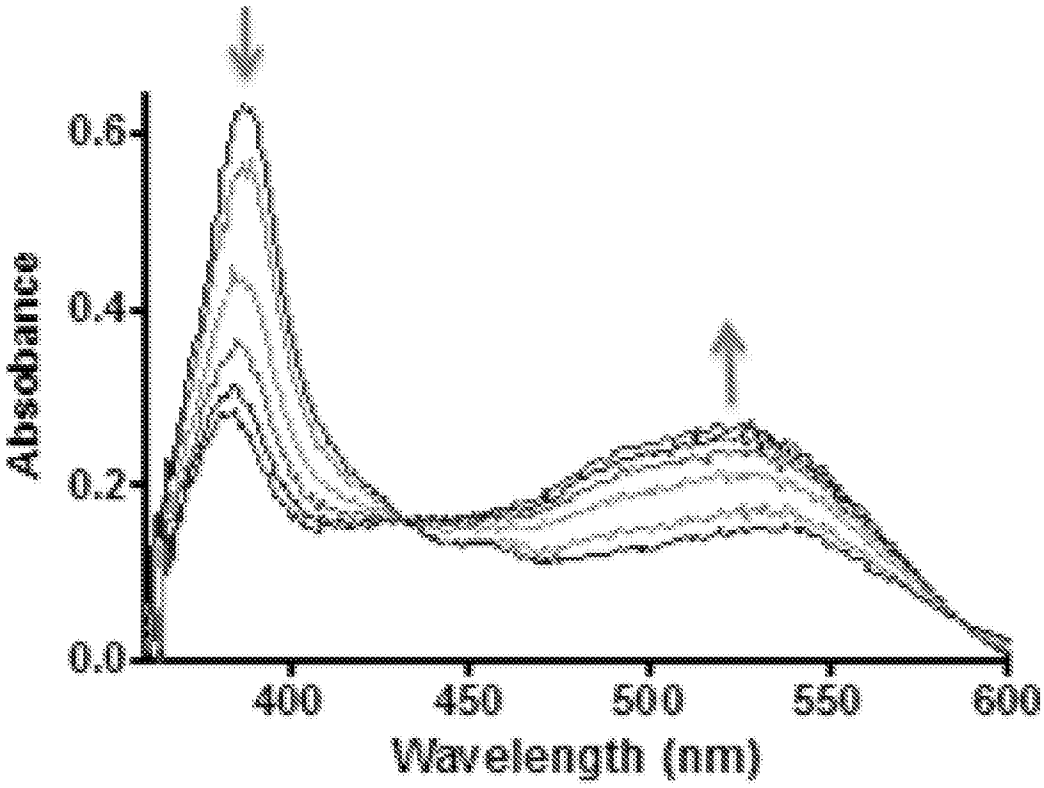


FIG. 14A

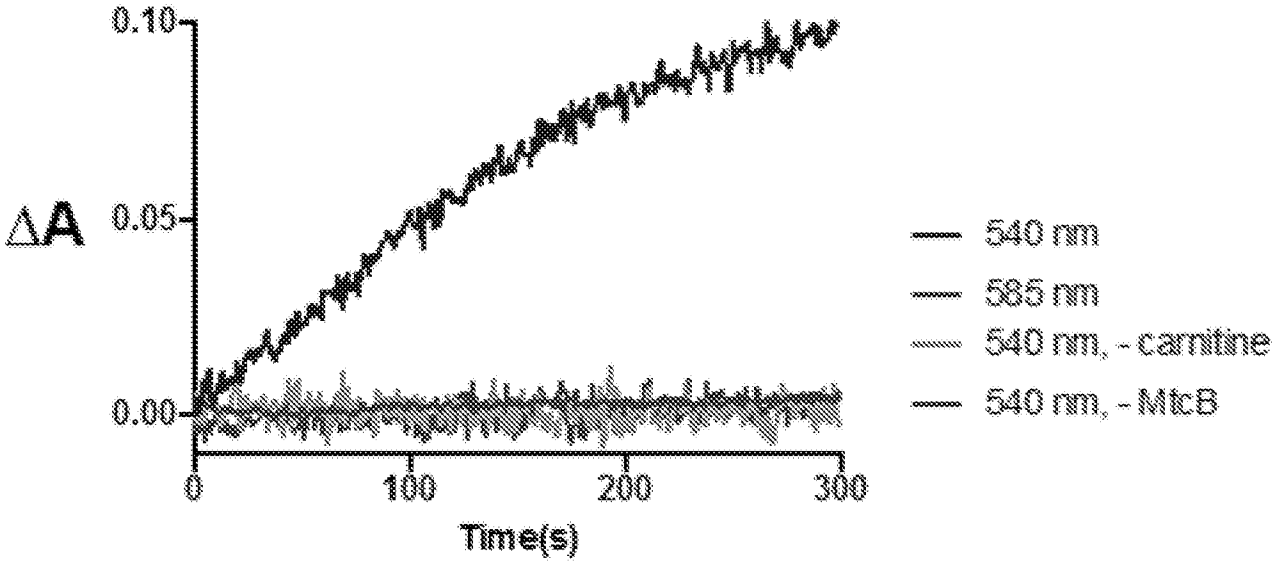


FIG. 14B

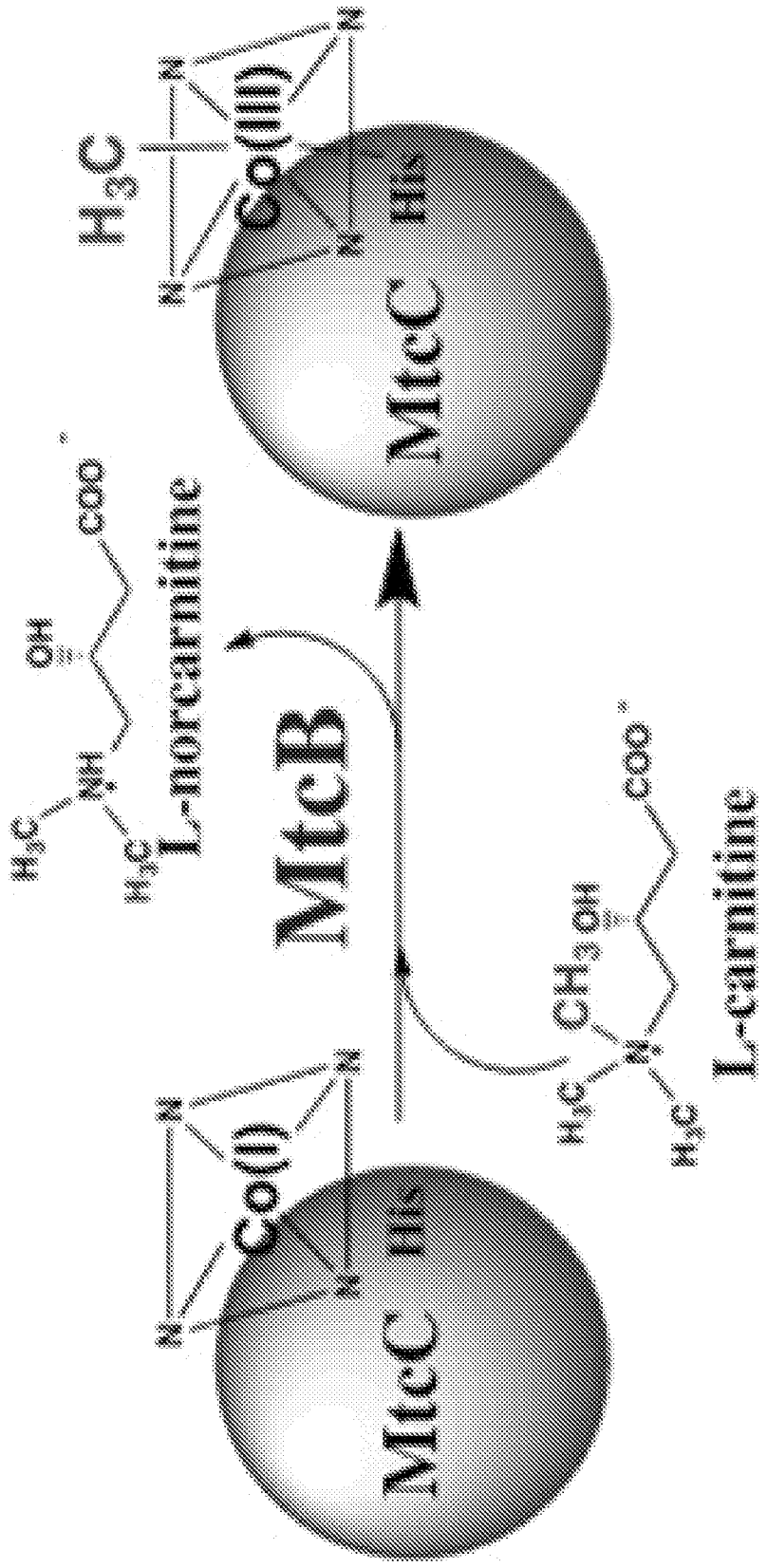


FIG. 14C

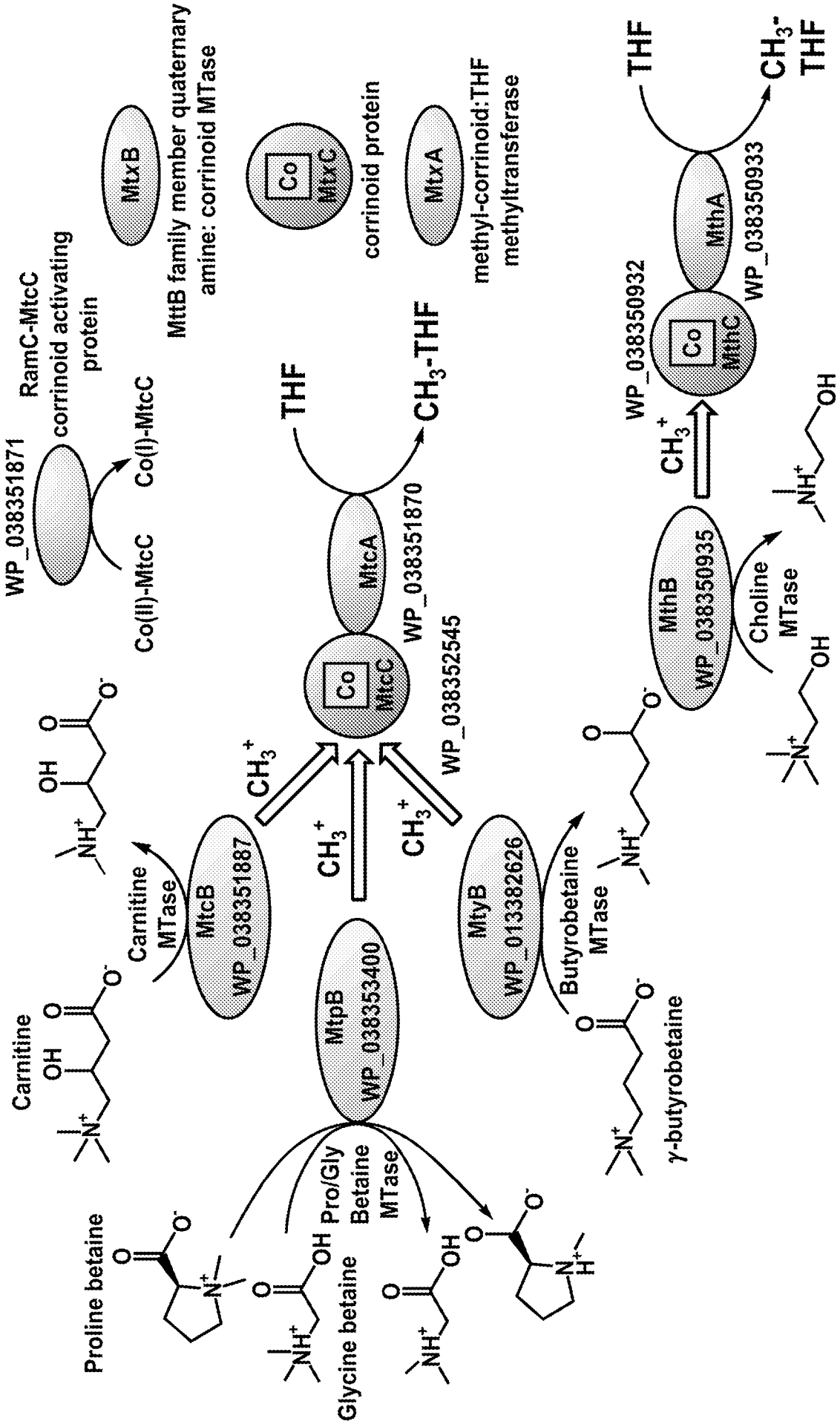

WP_038351871
RamC-MtcC
corrinoid activating
protein
Co(II)-MtcC
Co(I)-MtcC
MttB family member quaternary
amine: corrinoid MTase
MtxB
Co
MtxC
corrinoid protein
MtxA
methyl-corrinoid:THF
methyltransferase
THF
CH3-THF
MtcA
WP_038351870
Co
MtcC
WP_038352545
CH3+
CH3+
CH3+
Carnitine
MTase
MtcB
WP_038351887
MtpB
WP_038353400
MtyB
WP_013382626
Butyrobetaine
MTase
Carnitine
Proline betaine
Pro/Gly
Betaine
MTase
Glycine betaine
γ-butyrobetaine
MthB
WP_038350935
Choline
MTase
CH3+
WP_038350932
Co
MthC
MthA
WP_038350933
THF
CH3-
THF

FIG. 15

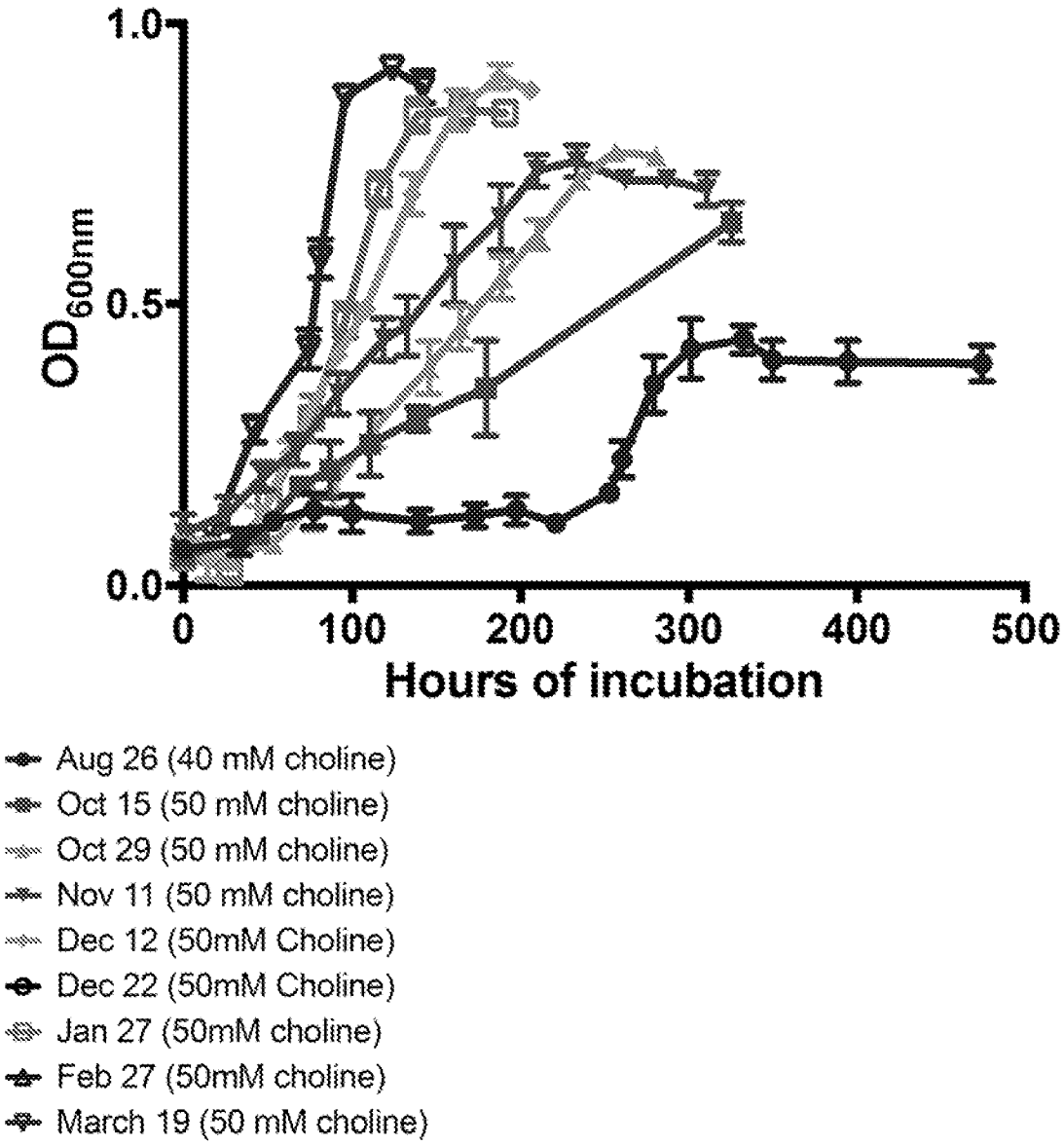

1.0
0.5
0.0
OD600nm
0
100
200
300
400
500
Hours of incubation
Aug 26 (40 mM choline)
Oct 15 (50 mM choline)
Oct 29 (50 mM choline)
Nov 11 (50 mM choline)
Dec 12 (50mM Choline)
Dec 22 (50mM Choline)
Jan 27 (50mM choline)
Feb 27 (50mM choline)
March 19 (50 mM choline)

FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37869

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 35/74 (2017.01)
CPC - A61K 36/28, A61Q 11/00, A61K 8/19, A61K 45/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 2008/0131401 A1 (Brown et al). 5 June 2008 (5.6.2008) abstract, para [0020], [0024]	1 ------- 2-5
X --- Y	US 2014/0328803 A1 (McKenzie et al.) 6 November 2014 (6.11.2014) para [0129], [0142], [0210], [0302]	8-12 ------ 2-5, 16-20
Y	Tang et al. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. The New England Journal of Medicine. vol. 368 no.17, April 25, 2013, pg1575, ln19-20	16-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 2017	15 SEP 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37869

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-7, 13-15, 21-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.