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Review

Carnitine in bacterial physiology and metabolism

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Carnitine is a quaternary amine compound found at high concentration in animal tissues, particularly muscle, and is most well studied for its contribution to fatty acid transport into mitochondria. In bacteria, carnitine is an important osmoprotectant, and can also enhance thermotolerance, cryotolerance and barotolerance. Carnitine can be transported into the cell or acquired from metabolic precursors, where it can serve directly as a compatible solute for stress protection or be metabolized through one of a few distinct pathways as a nutrient source. In this review, we summarize what is known about carnitine physiology and metabolism in bacteria. In particular, recent advances in the aerobic and anaerobic metabolic pathways as well as the use of carnitine as an electron acceptor have addressed some long-standing questions in the field.

Introduction

Carnitine (γ-trimethylamino-β-hydroxybutyric acid) (Fig. 1) is a quaternary amine compound that can be produced by all domains of life, and was discovered in muscle extract in 1905 by Gulewitsch & Krimberg (1905) and Kutscher (1905). It was shown to be essential for larval development of the mealworm *Tenebrio molitor* and was originally designated vitamin B_T based on this requirement. Later, it was discovered that carnitine can be synthesized in mammals and is now considered to be a quasi-nutrient or conditionally essential nutrient (Flanagan *et al.*, 2010), as neonates have reduced biosynthesis and rely on placental transfer of carnitine in utero and exogenous sources after birth (Combs, 2012). Fifty years after the discovery of carnitine, it was demonstrated that assorted Gram-positive and Gram-negative bacteria could use carnitine in either aerobic or anaerobic environments for a variety of cellular functions, including as an electron acceptor, as a compatible solute to survive environmental insults or as a sole carbon, nitrogen and energy source. Bacterial carnitine metabolism was most recently reviewed in 1998 (Bieber, 1988; Bremer, 1983; Kleber, 1997; Rebouche & Seim, 1998) and the field has seen important advances. This review summarizes what we knew at the time of the previous reviews and emphasizes what we have learned since, including: (i) how anaerobic bacteria synthesize and utilize crotonobetaine and carnitine as final electron acceptors, (ii) the impact of carnitine degradation by the intestinal microbiota and the genes responsible for this anaerobic conversion, (iii) the genes involved in aerobic degradation of carnitine, and (iv) how carnitine as a compatible solute impacts survival within and outside of the host.

Abbreviations: ABC, ATP-binding cassette; BKACE, β-keto acid cleavage enzyme; CDH, carnitine dehydrogenase; HTH, helix–turn–helix; TMAO, trimethylamine-*N*-oxide

Carnitine in the environment

Recent work makes it clear that while animals represent the most readily accessible source of carnitine, carnitine is often present and sometimes abundant in soil and natural waters. Quaternary ammonium compounds are abundant in a number of soil ecosystems, including comprising a quarter of the most abundant organic nitrogen compounds in the soil water of a subalpine grassland (Warren, 2013a). In this environment, carnitine was the most abundant quaternary ammonium compound (0.49 μM) and third most abundant soluble nitrogen compound overall, while acetylcarnitine was present at a slightly lower concentration (0.33 μM) (Warren, 2013a). It is apparent that carnitine concentration varies depending on sample location (Warren, 2013b), but there is need for a more thorough quantification of carnitine in other environments. The carnitine levels in soil and water may vary depending on the bacterial flora at the site and whether the bacteria inhabiting those environments are capable of carnitine metabolism. The presence and utility of carnitine in the environment is supported, in part, by the number of bacteria capable of carnitine metabolism, including a few newly identified species such as *Burkholderia caribensis* (Achouak *et al.*, 1999), *Bacillus decisifrondis* (Zhang *et al.*, 2007), *Pseudomonas kilonensis* (Sikorski *et al.*, 2001) from soil, and *Shewanella pacifica* from sea water (Ivanova *et al.*, 2004).

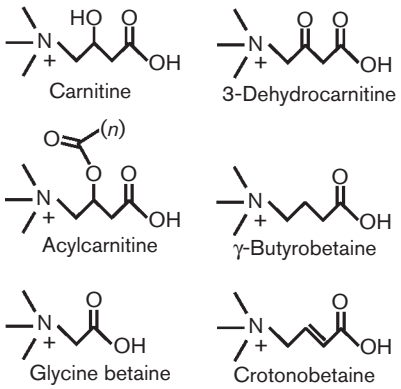

Fig. 1. Structures of carnitine and related compounds discussed in this review.

Importance of carnitine to animals (including humans)

Carnitine is most abundantly associated with animals and its physiology in animals provides an important backdrop to our review of microbial processes. Carnitine is a zwitterion and can exist as either D- or L-enantiomers, but the D stereoisomer is not utilized for normal physiology in animals and can inhibit acylcarnitine transferases, thereby resulting in tissue depletion of L-carnitine (Bieber, 1988). Therefore, unless specifically noted, we are discussing L-carnitine. Animals use the L-carnitine shuttle to transport long- to short-chain fatty acids in and out of the mitochondria by reversibly esterifying the β-carbon hydroxyl group with a fatty acid to form *O*-acylcarnitine (Fig. 1). β-Oxidation of very-long-chain fatty acids starts in the peroxisome, and once they have been converted to medium- or short-chain fatty acids, carnitine is employed to traffic them out of the peroxisome and into the mitochondria where β-oxidation is completed (Reddy & Hashimoto, 2001; Steiber *et al.*, 2004; Wanders & Waterham, 2006). Acetyl-CoA derived from β-oxidation can be used to generate ATP via the TCA cycle, while β-oxidation allows the cell to maintain the acetyl-CoA: CoA ratio and enables removal of specific harmful acylcarnitines derived from endogenous substances or from xenobiotics (Bieber, 1988).

In humans, ~95 % of total carnitine is found in skeletal and cardiac muscle, with the remaining 5 % circulating in the plasma (Cave *et al.*, 2008). Approximately 75 % of carnitine is obtained through diet, with foods from animal origin (e.g. meat and dairy products) having the highest carnitine content (Steiber *et al.*, 2004). The remaining 25 % of carnitine is synthesized endogenously from the essential amino acids L-methionine and L-lysine in the liver, kidney, testes, and brain (Bremer, 1983; Flanagan *et al.*, 2010; Rebouche, 2014). The body maintains homeostatic levels of carnitine by balancing carnitine absorption from the small intestine lumen, reabsorption by the kidneys, and modest endogenous synthesis (Rebouche & Seim, 1998). Carnitine and acylcarnitines are primarily absorbed from the lumen of the small intestine, where they are actively transported into enterocytes and diffuse past the serosal membrane into the circulatory system so they can then be transported into all other cells (Marciani *et al.*, 1991; Rebouche, 2004). Dietary carnitine that is not absorbed in the small intestines is metabolized by bacteria in the large intestine – there are no animal enzymes to break down carnitine (Rebouche & Chenard, 1991). Over the past decade, gut microbiome metabolism has become a topic receiving close review, and recently Koeth *et al.* (2013) associated the degradation of carnitine by intestinal microbiota with cardiovascular disease and the promotion of atherosclerosis. Some intestinal bacteria can convert carnitine to trimethylamine, which is subsequently oxidized in the liver to the proatherogenic species trimethylamine-*N*-oxide (TMAO) (Koeth *et al.*, 2013) – a metabolic pathway we discuss in more detail below. The role of bacterial metabolism of carnitine directly promoting cardiovascular disease has been shown in multiple studies (Hartiala *et al.*, 2014; Koeth *et al.*, 2013; Kuka *et al.*, 2014), but is still up for discussion in the scientific and medical communities (Johri *et al.*, 2014). However, there is no debate that bacteria, intestinal and otherwise, utilize carnitine in many different ways for their benefit (Ussher *et al.*, 2013).

Physiological benefits of carnitine for bacteria

Animals have been the focus of research on carnitine since its discovery in 1905 (Gulewitsch & Krimberg, 1905) and microbial carnitine metabolism was not described until more than 50 years later (Fraenkel & Friedman, 1957). In the intervening years, microbiologists have described the roles for carnitine in bacteria, where it can be used as a final electron acceptor, a compatible solute, or as a sole carbon, nitrogen, and energy source. Regardless of its eventual role, carnitine is transported into the bacterial cytosol by one of two different mechanisms. The first method is an ATP-dependent ATP-binding cassette (ABC) transport system utilizing the canonical three subunits – a transmembrane domain, an ATPase, and a periplasmic binding protein (also known as a substrate binding protein). The second method of import is by a betaine/choline/carnitine transporter (BCCT) that can be driven by the sodium or proton motive force, but in many cases where carnitine is the substrate often functions as a carnitine: γ-butyrobetaine antiporter (Ziegler *et al.*, 2010). Carnitine import or import of immediate precursors is critical for bacterial acquisition of carnitine, as Verheul *et al.* (1998) demonstrated that *de novo* synthesis of carnitine does not occur in *Escherichia coli* and, to the best of our knowledge, *de novo* synthesis of carnitine has not been demonstrated in any bacterial species.

Carnitine: an organic compatible solute

Outside of the cosy confines of the laboratory, bacteria are subject to constantly changing environments to which they must respond rapidly to survive and thrive. One way to accommodate stresses caused by changing water content, salt, temperature or pressure is by synthesis or import of compatible solutes. Compatible solutes can be accumulated at high concentrations in the cytoplasm while not interfering with cellular processes, and thus contribute to proper protein function and cellular homeostasis (Brown & Simpson, 1972). Compatible solutes are typically organized into categories: carbohydrates, amino acids, methylamines, methylsulfonium solutes, and specific inorganic ions. Organic solutes that are either non-charged or zwitterions with no net charge at physiological pH are preferred compatible solutes, the intracellular concentrations of which are carefully regulated to maintain protein stability and cell physiology (Fitzsimmons *et al.*, 2012; Hoffmann *et al.*, 2013). Carnitine is an ideal compatible solute that can be imported and/or generated from direct precursors by many bacteria, and its abundance for infectious microbes in the host and presence in many natural environments suggests it is readily accessible (Warren, 2013a, b).

Osmoprotection

Drastic changes in water content in the environment can result in osmotic stress to the cell. Low external solute concentration is hypo-osmotic, with resulting pressure to drive water into the cell causing an increase in cell turgor pressure. Conversely, increased external solute concentration due to added solutes or loss of water is hyper-osmotic, with resulting pressure leading to water efflux from the cell, reducing turgor pressure. In addition to the altered physiology imparted by these turgor changes, both conditions can lead to cell death due to irreversible plasmolysis (loss of water) or cytolysis (too much water). To overcome osmotic stress, bacteria can either acquire or synthesize osmoprotectants – a process that is universal for bacteria. One way bacteria can overcome osmotic stress is by utilizing carnitine as an osmoprotectant and/or osmolyte.

Gram-negative osmoprotection by carnitine. The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* uses carnitine as both an osmoprotectant and an osmolyte. Intracellular carnitine can be accumulated directly via transport from extracellular sources through a BCCT (Malek *et al.*, 2011) or ABC transporter (Chen *et al.*, 2010), or indirectly by degradation of *O*-acylcarnitines (Lucchesi *et al.*, 1995; Meadows & Wargo, 2013). *Pseudomonas aeruginosa*, as with many environmental proteobacteria, can also metabolize carnitine to the osmolyte glycine betaine (Fig. 2) (Aurich *et al.*, 1967; Bastard *et al.*, 2014; Wargo & Hogan, 2009). *E. coli* also uses carnitine as an osmolyte, where it functions in aerobic, anaerobic, and high salt conditions (Verheul *et al.*, 1998). Import is primarily mediated through the ProU transport system, a substrate binding protein-dependent ABC transporter, along with modest uptake from the major facilitator superfamily transporter ProP in anaerobic and osmostressed aerobic cells. Although CaiT functions as a carnitine antiporter, its activity is not sufficient to confer osmoprotection (Verheul *et al.*, 1998). *Yersinia enterocolitica* also employs carnitine as an osmolyte, where unlike most Gram-negative bacteria, NMR observations demonstrated that added carnitine was not metabolized to the more potent osmolyte glycine betaine (Park *et al.*, 1995).

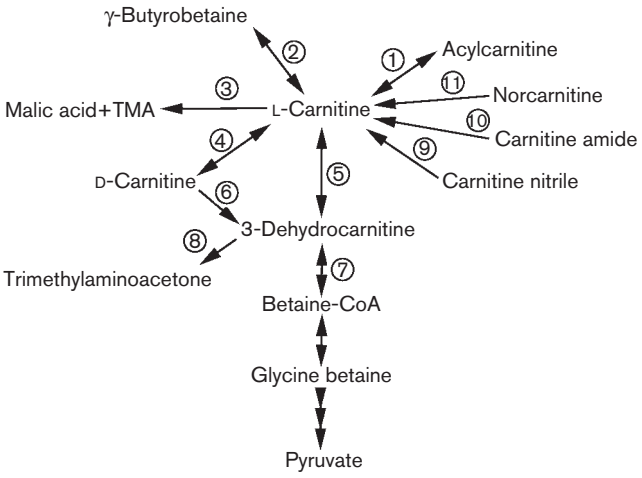

Fig. 2. Diagram of the multiple pathways for carnitine metabolism in bacteria based on Uanschou *et al.* (2005). Metabolic steps described in this review are noted with numbered circles: (1) acylcarnitine hydrolase, HocS, EC 3.1.1.28; (2) γ-butyrobetaine hydroxylase, EC 1.14.11.1; (3) oxidoreductase CntAB, EC 1.14.13.- (TMA, trimethylamine); (4) carnitine racemase, EC 5.1.-.-; (5) L-carnitine dehydrogenase, EC 1.1.1.108; (6) D-carnitine dehydrogenase, EC 1.1.1.108; (7) β-keto acid cleavage enzyme, EC 3.-.-.-; (8) no ATP, spontaneous decarboxylation; (9) nitrilase, EC 3.5.5.1; (10) carnitine amidase, EC 3.5.1.73; (11) carnitine methylase. Note that not all enzymic steps have full EC descriptors at this time.

Gram-positive osmoprotection by carnitine. The lactic acid bacteria *Tetragenococcus halophile* and *Lactobacillus plantarum* can import and use D- and L-carnitine as osmolytes (Kets *et al.*, 1994; Robert *et al.*, 2000). *Brevibacterium linens* can also use D- and L-carnitine as osmolytes, and while L-carnitine can be metabolized to glycine betaine, it is further metabolized as a sole carbon or nitrogen source (Jebbar *et al.*, 1998). *Bacillus subtilis* imports D- and L-carnitine, acetylcarnitine, crotonobetaine, γ-butyrobetaine and octanoylcarnitine via the ABC transporter OpuC. Both stereoisomers and the carnitine resulting from acetyl- and octanoylcarnitine degradation function as osmolytes to protect the cell from hyperosmotic stress (Kappes & Bremer, 1998). The carnitine generated or imported by *Bacillus subtilis* cannot be

metabolized further and therefore is thought to function primarily as an osmoprotectant. *Staphylococcus aureus*, a common member of the skin and nasal flora that is an important opportunistic pathogen, uses carnitine as a compatible solute in high salt (Vilhelmsson & Miller, 2002).

Carnitine appears to be particularly important for the common foodborne pathogen *Listeria monocytogenes*. *Listeria monocytogenes* primarily imports carnitine through the OpuC ABC transporter (Fraser & O'Byrne, 2002; Fraser *et al.*, 2000; Verheul *et al.*, 1995, 1997), where it functions as an osmolyte in high salt conditions (Beumer *et al.*, 1994). Mice infected with *Listeria monocytogenes* perorally show that OpuC is important for survival and infection in the small intestine, and for the proliferation and dissemination of the bacteria into other organs, including the liver and spleen (Sleator *et al.*, 2001, 2003; Wemekamp-Kamphuis *et al.*, 2002). One of these studies postulated that bacterial destruction of the intestinal epithelial layer allows carnitine release, which then alleviates the effects of the hyper-osmotic environment of the small intestine, where salt concentrations are two times higher than the blood (Sleator *et al.*, 2001). Additional mouse infections comparing mutants in the glycine betaine transport systems BetL and Gbu to mutants of OpuC led to the conclusion that carnitine transport is more important than glycine betaine for *Listeria* during infection (Sleator *et al.*, 2003; Wemekamp-Kamphuis *et al.*, 2002).

Beyond salt: role of carnitine in cryoprotection, bile tolerance and barotolerance

In addition to osmoprotection, compatible solutes can protect bacteria from additional sources of stress. *Listeria* and *Bacillus* have both been well studied in relation to carnitine uptake, and are good examples of bacteria that employ carnitine to protect against multiple stress conditions.

Listeria is a foodborne pathogen that has multiple lifestyles, thriving on decaying plant material and transiently living in the gastrointestinal tract of some animals, including humans. Any bacterium that lives or passes through the small intestine has to cope with bile salts, which aid in digestion of lipids, have antimicrobial activity, disrupt membranes and proteins, and induce oxidative stress and DNA damage (Begley *et al.*, 2005). The OpuC carnitine transport system in *Listeria monocytogenes* is important for protecting the bacteria against bile stress and its expression is co-regulated with the bile efflux system BilE (Watson *et al.*, 2009). Mice infected perorally with an *opuC* mutant strain had significantly reduced numbers of bacteria in the faeces and a decrease in systemic infection, measured as bacteria in the liver and spleen (Watson *et al.*, 2009). Carnitine enhances bile tolerance, and is important for dissemination and survival in the small intestine (Gahan & Hill, 2014; Sleator & Hill, 2010; Watson *et al.*, 2009).

Unlike many bacteria, *Listeria monocytogenes* is capable of growing at refrigeration temperatures (4 °C), making it a common contaminant of dairy, meats, fruits and vegetables (Ryser & Marth, 2007). Cold or chill stress affects protein structure (Jaenicke *et al.*, 1990), protein stability (Privalov & Gill, 1988), and membrane fluidity (Rudolph *et al.*, 1986), and compatible solutes, such as carnitine, can alleviate these negative effects due to cold (Bayles & Wilkinson, 2000). The expression of the carnitine uptake system *opuC* is increased at low temperatures, allowing the cell to acquire carnitine and maintain growth (Angelidis & Smith, 2003; Angelidis *et al.*, 2002; Sleator *et al.*, 2009; Wemekamp-Kamphuis *et al.*, 2004).

The ability to survive low-temperature stress is not the sole domain of bacteria in our refrigerators, but rather a required feature of bacteria that inhabit the environment, where mean soil surface temperatures range from 0 to 18 °C, mean freshwater ranges from 3 to 25 °C and while the mean ocean surface temperature is ~17 °C, the bulk of ocean water maintains a temperature of ~3 °C. Therefore, it is not surprising that successful environmental bacteria have also evolved pathways for cold tolerance that involve carnitine. In *Bacillus subtilis*, carnitine and its related metabolites crotonobetaine and γ-butyrobetaine have been shown to protect against both cold stress (15 °C) and heat stress (52 °C), via uptake through the OpuC transporter (Hoffmann & Bremer, 2011). It is likely that utilization of carnitine as a cryo- and thermoprotectant is not restricted to *Listeria* and *Bacillus*, and the taxonomic breadth and ecological impact of this process require further examination.

Pressure is another assault that *Listeria* has adapted to, as food processing uses high pressure for food preservation. *Listeria* exposed to elevated osmolarity and high pressure survive better and demonstrate substantial barotolerance when the compatible solute carnitine is present and imported into the cell (Smiddy *et al.*, 2004).

Carnitine metabolism

Carnitine can be utilized in different metabolic pathways to play a variety of physiological roles. Fig. 2 illustrates multiple described pathways for carnitine metabolism and highlights bacteria in which particular reactions have been demonstrated. For each of the pathways described below, it is important to note that strain-specific utilization of carnitine via particular pathways can be governed by alternative regulation or the presence/absence of metabolic genes. For example, *Pseudomonas syringae* B728a carries the genes for carnitine conversion to glycine betaine, while *Pseudomonas syringae* DC3000 has lost the entire carnitine catabolic operon (Chen & Beattie, 2007; Chen *et al.*, 2010).

Carnitine as a nutrient

Carnitine can be catabolized for use as a carbon source via two routes. The first pathway cleaves the backbone carbon–nitrogen bond to yield trimethylamine and malic

semialdehyde, in which the latter enters the TCA cycle. The second route begins with the metabolism of carnitine to glycine betaine, which is then subjected to three successive demethylations to yield glycine, which can enter central metabolism. Carnitine can also be used as a sole nitrogen source, most commonly via the glycine betaine route, where glycine conversion to serine is followed by deamination to form pyruvate and ammonia. Formation of trimethylamine from carnitine leaves the nitrogen unusable for most of the organisms covered in this review, but many bacteria can use trimethylamine as a nitrogen source, carbon source, osmoprotectant and, when oxidized to TMAO, as an electron acceptor (as reviewed by Barrett & Kwan, 1985; Strøm *et al.*, 1979; Yancey, 2005).

Carnitine to glycine betaine: a gateway reaction

Carnitine can be metabolized to glycine betaine via a multistep process, the first steps of which are encoded on genes located in the carnitine catabolism operon (Uanschou *et al.*, 2005; Wargo & Hogan, 2009). First, carnitine is converted to 3-dehydrocarnitine by the enzyme carnitine dehydrogenase (CDH, EC 1.1.1.108), an NAD^+-dependent oxidoreductase. *Pseudomonas aeruginosa* (Aurich *et al.*, 1967; Kleber & Aurich, 1967; Kleber *et al.*, 1967), *Xanthomonas translucens* (Arima *et al.*, 2010; Mori *et al.*, 1988), *Enterobacter* sp. (Hwang & Bang, 1997), *Pseudomonas putida* (Kleber *et al.*, 1978), *Pseudomonas fluorescens* (Hung & Kleber, 1985), *Burkholderia cepacia* (Dalmastri *et al.*, 2003), *Rhizobium* sp. (Arima *et al.*, 2010) and *Agrobacterium* sp. (Hanschmann *et al.*, 1996) all encode CDH enzymes that are specific for L-carnitine. 3-Dehydrocarnitine is relatively unstable, therefore if there is no ATP or CoA present, it will spontaneously decarboxylate to yield trimethylaminoacetone (*N*,*N*,*N*-trimethylaminopropanone) and carbon dioxide (Lindstedt *et al.*, 1967). If sufficient ATP and CoA are present, then 3-dehydrocarnitine is converted to acetylacetone and glycine betaine-CoA by the β-keto acid cleavage enzyme (BKACE). The gene encoding the carnitine-specific BKACE is located directly upstream of the CDH gene(s) in the carnitine catabolism operon (Uanschou *et al.*, 2005) and is designated *cdhC* in *Pseudomonas aeruginosa* (Bastard *et al.*, 2014; Wargo & Hogan, 2009). The identification of this gene was part of a massive biochemical screen of various bacterial BKACE members and proved very valuable for the identification of these CoA-dependent cleavage enzyme substrates (Bastard *et al.*, 2014). The glycine betaine-CoA derived from BKACE activity is then converted to glycine betaine and CoA by a CoA transferase, likely the DhcAB enzyme in *Pseudomonas aeruginosa* (Wargo & Hogan, 2009), which appears to function as a general amino acid CoA transferase (Palmer *et al.*, 2013). After carnitine is metabolized to glycine betaine, it can function as an osmolyte (as described above) or be utilized as a sole carbon, nitrogen, and energy source if the organism encodes the necessary enzymes, as in the case for *Pseudomonas aeruginosa* (Wargo *et al.*, 2008), *Xanthamonas translucens* (Arima *et al.*, 2010; Mori *et al.*, 1988), *Enterobacter* sp. (Hwang & Bang, 1997), *Rhizobium* sp. (Arima *et al.*, 2010; Goldmann *et al.*, 1991), *Sinorhizobium meliloti* (Goldmann *et al.*, 1991), *Burkholderia cepacia* (Dalmastri *et al.*, 2003) and *Agrobacterium* sp. (Hanschmann *et al.*, 1996; Nobile & Deshusses, 1986). The metabolism and homeostasis of glycine betaine has been reviewed recently (Wargo, 2013).

Cleaving the backbone carbon–nitrogen bond

An alternative pathway to use carnitine as a sole carbon source is to cleave the carbon–nitrogen bond of carnitine to form trimethylamine and malic acid. It has been known since the mid-1960s that *Serratia marcescens* aerobically splits the carbon–nitrogen bond of both D- and L-carnitine to form trimethylamine and malic acid, and that this reaction does not occur under anaerobic conditions as it requires uptake of oxygen (Unemoto *et al.*, 1966). Through the same reaction, *Acinetobacter calcoaceticus* can also use both D- and L-carnitine as sole carbon sources (Kleber *et al.*, 1977; Miura-Fraboin *et al.*, 1982), yet the responsible enzymes remained elusive until recently. The genes encoding the enzymes that are essential for the conversion of carnitine to trimethylamine and malic semialdehyde were recently identified as a two-subunit oxidoreductase in *Acinetobacter baumannii* (Zhu *et al.*, 2014), with CntA being a Rieske-family iron–sulfur cluster oxygenase and CntB being the reductase. The CntAB proteins likely function analogously to GbcAB, which comprise the oxidoreductase that demethylates glycine betaine to dimethylglycine (Wargo *et al.*, 2008). Both sets of proteins cleave one of the carbon–nitrogen bonds in a quaternary amine compound and these enzymes may represent a general evolutionary strategy for quaternary amine metabolism. Orthologues and homologues of the *cntAB* genes are found in a variety of gut microbiota: *Gammaproteobacteria* (*Klebsiella pneumoniae*, *E. coli*, *Citrobacter*, *Providencia* and *Shigella*), *Betaproteobacteria* (*Achromobacter*) and *Firmicutes* (*Sporosarcina*) (Zhu *et al.*, 2014). The malic semialdehyde produced during this reaction is converted to malic acid, which enters the TCA cycle. Trimethylamine formed from carnitine by gut bacteria is correlated with human cardiovascular health (Koeth *et al.*, 2013), where it is oxidized to TMAO by hepatic flavin monooxygenases in the liver (Bennett *et al.*, 2013). Therefore, the identification of CntAB provides a target for monitoring gut microbiota capacity for trimethylamine production and is a critical step forward in our understanding of carnitine metabolism by bacteria.

Carnitine as a final electron acceptor

Bacteria that live strictly or transiently in anaerobic environments, where oxygen cannot serve as the final electron acceptor, can use alternate electron acceptors, including sulfates, nitrates, ferric iron, carbon dioxide, and fumarate, amongst others. In cases where the common electron acceptors are not present, some *Enterobacteriaceae* (*E. coli*, *Salmonella typhimurium* and *Proteus*

spp.) can use carnitine and its catabolic product crotonobetaine as final electron acceptors in the absence of oxygen, and in the presence of additional carbon and nitrogen sources (Seim *et al.*, 1982a, b, c). Use of these compounds as electron acceptors is regulated by the transcriptional activator, CaiF, and the regulatory proteins CRP and FNR, which regulate expression of the divergently transcribed operons *caiTABCDE* and *fixABCX*, both required for carnitine metabolism in *E. coli* (Buchet *et al.*, 1998, 1999; Eichler *et al.*, 1995, 1996). For its role as an electron acceptor, carnitine is imported by the substrate: product antiporter CaiT, which exchanges carnitine for its metabolic product γ-butyrobetaine (Jung *et al.*, 1990, 2002; Kalayil *et al.*, 2013). Co-expression of these two operons is induced by carnitine and crotonobetaine, but repressed by oxygen, glucose, γ-butyrobetaine and more desirable final electron acceptors, such as nitrate and fumarate (Jung *et al.*, 1987; Seim *et al.*, 1982a, b). Expression of the carnitine metabolism genes in *E. coli* and *Proteus* spp. is also detectable in aerobic environments in the presence of inducing compounds, but to a much lesser extent than under anaerobic conditions (Elssner *et al.*, 1999; Engemann & Kleber, 2001; Obón *et al.*, 1999).

The *fix* operon is predicted to be involved in transferring electrons to carnitine (Eichler *et al.*, 1995, 1996; Walt & Kahn, 2002). The transformation of carnitine to crotonobetaine is reversible (Jung *et al.*, 1989) and was originally thought to occur via the single carnitine dehydratase CaiB with the co-substrates γ-butyrobetainyl-CoA or crotonobetainyl-CoA (Eichler *et al.*, 1994a; Elssner *et al.*, 2000; Jung *et al.*, 1989). However, Elssner *et al.* (2001) demonstrated that 'L-carnitine dehydratase does not exist' and two enzymes are responsible for the reversible conversion of carnitine to crotonobetaine. CaiB is a CoA transferase that transfers CoA from γ-butyrobetainyl-CoA, crotonobetainyl-CoA or carnitinyl-CoA to form γ-butryobetaine, crotonobetaine or carnitine. CaiD has enoyl-CoA hydratase activity and can dehydrate carnitinyl-CoA to crotonobetainyl-CoA, or can hydrate crotonobetainyl-CoA to carnitinyl-CoA; thus, CaiD has two potential names: crotonobetainyl-CoA hydratase or carnitinyl-CoA dehydratase. These enzymic processes also allow carnitine to be synthesized from crotonobetaine and vice versa (Fig. 3) (Elssner *et al.*, 2001; Engemann *et al.*, 2001, 2005). Crotonobetaine can be reduced to γ-butyrobetaine by two proteins: CaiB, with one of the co-substrates γ-butyrobetainyl-CoA or crotonobetainyl-CoA, and the unidirectional enzyme crotonobetaine reductase CaiA, which reduces crotonobetainyl-CoA to γ-butyrobetainyl-CoA (Engemann *et al.*, 2005; Preusser *et al.*, 1999; Roth *et al.*, 1994). Two other proteins necessary for anaerobic carnitine catabolism are CaiC and CaiE. CaiC is a betaine: -CoA ligase with CoA transferase activity *in vitro*, but is not sufficient *in vivo* to compensate for a *caiB* deletion. Therefore, CaiC is likely required for activation of trimethylammonium compounds (Bernal *et al.*, 2008; Eichler *et al.*, 1994b). The function of CaiE is still somewhat mysterious, but it is postulated to be required for activation or synthesis for an unknown cofactor necessary for carnitine metabolism (Eichler *et al.*, 1994b).

The enzymes for converting γ-butyrobetaine into carnitine, best described in the *Enterobacteriaceae*, are predicted to be present in other bacterial groups. The betaproteobacterium *Achromobacter cycloclast* and the gammaproteobacterium *Acinetobacter calcoaceticus* are predicted to have similar enzymes as no γ-butyrobetaine hydroxylase was detected during degradation of γ-butyrobetaine to carnitine. For *Acinetobacter calcoaceticus*, after γ-butyrobetaine is degraded to carnitine, the carnitine can then be broken down to trimethylamine and malic acid where it uses it as a carbon source (Miura-Fraboin *et al.*, 1982), whereas the fate of the newly synthesized carnitine was not investigated in *Acinetobacter cycloclast* (Naidu *et al.*, 2001).

Sensing and binding carnitine

For osmotic adaptation to, or metabolism of, carnitine, it is important for bacteria to regulate the expression of genes involved in these processes. To our knowledge, carnitine is sensed to regulate activity at the transcriptional level using so-called 'one-component' regulators, i.e. single polypeptides that sense ligand and regulate transcription. However, compared with our understanding of carnitine transport and metabolism, the detection of carnitine for transcriptional regulation is much more poorly understood. We know of only two transcription factors that sense carnitine to regulate transcription: CaiF in *E. coli* and CdhR in *Pseudomonas aeruginosa*. Both are transcriptional activators and induce transcription in response to carnitine (Buchet *et al.*, 1999; Wargo & Hogan, 2009), although importantly, direct interaction of either protein with carnitine has not been demonstrated.

In *E. coli*, the *cai* and *fix* operons (described above) are regulated by the global regulator CRP and the carnitine-responsive activator CaiF. The CaiF protein is small and is predicted to contain two AraC-like helix–turn–helix (HTH) domains and likely functions similar to the MarR 'HTH-only' activators (Buchet *et al.*, 1999). Interestingly, the lack of a canonical ligand-binding domain suggests that detection of the ligand occurs within what is typically thought of as the DNA-binding domain of the protein or is mediated by an independently encoded sensor protein. Using the Phyre2 protein prediction server (Kelley & Sternberg, 2009), CaiF has high structural homology to GrlA from enterohaemorrhagic *E. coli*, where the two-component response regulator GrlR has been shown to interact with GrlA, modulating transcriptional regulation (Creasey *et al.*, 2003; Russell *et al.*, 2007). While we have found no obvious GlrR homologues in the *fix* and *cai* operons, it remains a possibility that carnitine detection occurs via a GlrR orthologue or a cognate sensor kinase, such as GlrK (Yamamoto *et al.*, 2005).

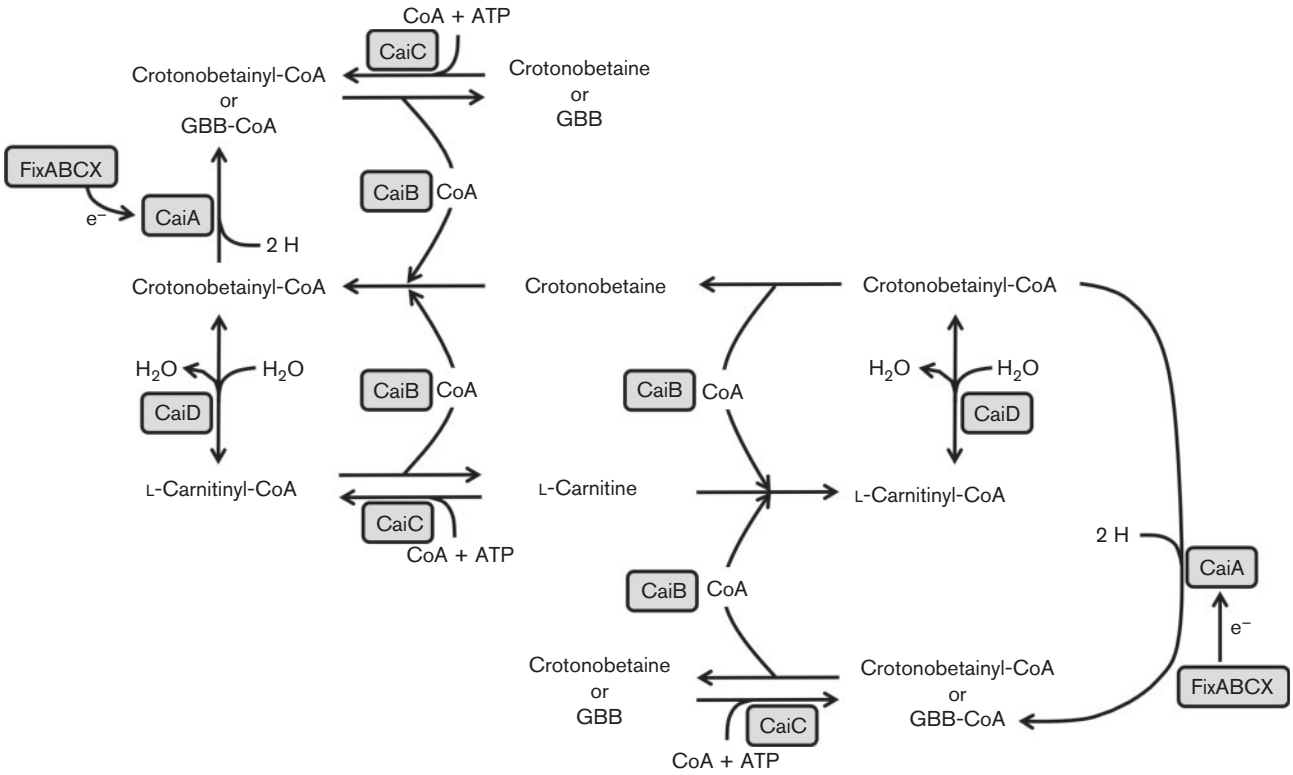

Fig. 3. Diagram of the γ-butyrobetaine–crotonobetaine–carnitine cycle primarily based on Elssner *et al.* (2001), with additions based on Bernal *et al.* (2008) and Canovas *et al.* (2003). GBB, γ-butyrobetaine; CaiA, crotonobetaine reductase; CaiB, CoA transferase; CaiC, betaine:CoA ligase; CaiD, enoyl-CoA hydratase; FixABCX, electron transfer flavoproteins. Electron movement is denoted by e^-.

In *Pseudomonas aeruginosa*, the transcription factor CdhR functions as the carnitine-responsive activator of the genes encoding the CDH enzymic pathway (Wargo & Hogan, 2009). CdhR is a member of the glutamine amidotransferase-1 subfamily of the AraC transcription factor family and has the canonical AraC family structure consisting of an N-terminal ligand sensing and dimerization domain, and a C-terminal DNA-binding domain composed of two HTH motifs. Orthologues of CdhR are found divergently transcribed from the aerobic CDH genes in a variety of organisms, including many species in the families *Pseudomonadaceae*, *Burkholderiaceae* and *Rhizobiaceae*, as well as in *Mesorhizobium loti* and *Silicibacter* sp. (Uanschou *et al.*, 2005). Given the conservation of the gene arrangement and predicted functions, we propose that further understanding of CdhR will yield insights into this regulation beyond *Pseudomonas*.

In Gram-positive bacteria, the CDH operons contain a TetR family transcription regulator that likely functions as the carnitine sensor for regulated expression of these operons. Compared with the Gram-negative bacteria, presence of the CDH and its cognate TetR-like regulator is much more restricted, occurring in *Brevibacterium linens*, *Staphylococcus epidermidis*, *Streptomyces coelicolor* and *Oceanobacillus* sp. (Uanschou *et al.*, 2005). For the first two organisms on this list, acquisition of this predicted horizontally acquired operon makes teleological sense given the prevalence of carnitine in milk and in the skin, respectively.

An integral part of transport, metabolism, and transcriptional activation is binding of the target molecule. In the case of carnitine, crystal structures have been solved in complex that include carnitine in complex with the periplasmic binding protein OpuCC (Du *et al.*, 2011), the carnitine:γ-butryobetaine antiporter CaiT (Schulze *et al.*, 2010; Tang *et al.*, 2010) and similar aromatic cage architecture of the quaternary amine-binding site with γ-butyrobetaine has been determined for γ-butyrobetaine hydroxylase (Tars *et al.*, 2014). Therefore, as in the situation for glycine betaine (Schiefner *et al.*, 2004), high-affinity binding of carnitine and its metabolites seems to occur via an aromatic cage enabling the cation–π interaction with the trimethylamine moiety coupled with properly spaced hydrogen-bonding residues to coordinate the carboxylic acid. While this binding arrangement has been demonstrated for transport and metabolic proteins, neither carnitine nor the related compounds choline or glycine betaine have been

crystallized with their cognate transcriptional regulators. Given the specificity and affinity provided by the cation–π interaction, it has been hypothesized that glycine betaine and carnitine-sensitive transcription factors utilize a functionally similar binding site (Bremer, 2011).

Multiple paths for carnitine acquisition

De novo carnitine synthesis

While bacteria can synthesize the quaternary amine compounds choline and glycine betaine from single unrelated carbon sources during growth on minimal media, such *de novo* synthesis of carnitine has not been demonstrated. Rather, as discussed below, carnitine is typically generated by metabolizing appropriate trimethylated precursors. While evidence for true *de novo* synthesis is lacking, N^{ε}-trimethyllysine is the starting point for mammalian carnitine synthesis (Vaz & Wanders, 2002) and bacteria are known to synthesize N^{ε}-trimethyllysine (Barbier *et al.*, 2013; Klagsbrun & Furano, 1975). Therefore, it remains a formal possibility that bacteria can use this N^{ε}-trimethyllysine to synthesize carnitine in a manner similar to mammals.

D-Carnitine ... not a dead end after all

Animals only synthesize and respond physiologically to L-carnitine, thus the presence of the D stereoisomer arises from bacterial processes on carnitine and its derivatives or from ingested food based on abiotic racemization. Despite the relative abundance of L-carnitine, some bacteria are capable of utilizing D-carnitine as a sole carbon and nitrogen source, including *Agrobacterium* sp., *Agrobacterium radiobacter* and *Enterobacter* (Hanschmann & Kleber, 1997; Hwang & Bang, 1997; Klüttermann *et al.*, 2002). *Agrobacterium* expresses both an L-CDH and a D-CDH, and its utilization of D-carnitine is dependent on the loss of chirality upon conversion of D-carnitine to the achiral 3-dehydrocarnitine (Hanschmann & Kleber, 1997). *Enterobacter* sp. KC-006 is also able to use D-carnitine as a sole carbon and nitrogen source. Mutations that significantly impair L-CDH activity permitted growth on D-carnitine as well as wild type, suggesting that a carnitine racemase was likely not responsible (Hwang & Bang, 1997). However, the D-carnitine metabolic pathway in *Enterobacter* has not been described fully. While *Enterobacter* likely does not have a carnitine racemase, there are bacteria that employ such an enzyme for D-carnitine metabolism. In *Pseudomonas* sp. AK1, a cytoplasmic carnitine racemase converts D-carnitine to L-carnitine, which is subsequently metabolized to glycine via glycine betaine to supply the cell with carbon and nitrogen (Monnich *et al.*, 1995). *E. coli* 044 K74 also expresses carnitine racemase activity, which is induced in the presence of L-carnitine or crotonobetaine, and repressed by glucose, oxygen and fumarate (Canovas *et al.*, 2003; Castellar *et al.*, 1998; Jung & Kleber, 1991). CaiD was initially suggested to function as the racemase, as the *caiD* gene is required for racemase activity (Eichler *et al.*, 1994b; Jung & Kleber, 1991; Jung *et al.*, 1987); however, this has not been tested directly. CaiD is still postulated to be involved in racemization of D-carnitine, as CaiC was shown to activate D-carnitine by adding a CoA group to produce D-carnitinyl-CoA, which is then theorized to be converted to L-carnitinyl-CoA by CaiD (Bernal *et al.*, 2008).

Carnitine synthesis by γ-butyrobetaine hydroxylase

The direct route to the formation of carnitine from γ-butyrobetaine occurs through the enzyme γ-butyrobetaine hydroxylase (EC 1.14.11.1). It has been identified in *Pseudomonas* sp. AK1 (Lindstedt *et al.*, 1970a, b; Rüetschi *et al.*, 1993) and *Pseudomonas* sp. L1 (Lu *et al.*, 2012), and both enzymes are homologous to the animal γ-butyrobetaine hydroxylase in their requirement for oxygen and the cofactors iron, ascorbate, and α-ketogluterate (Lindstedt & Lindstedt, 1970; Lindstedt *et al.*, 1968).

Acylcarnitine

A fatty acid moiety can be conjugated to the third carbon of carnitine resulting in *O*-acylcarnitines (Fig. 1), which can serve as sources of carnitine, but also can alter bacterial physiology directly (Nguyen *et al.*, 2012). *Pseudomonas aeruginosa* can utilize acylcarnitines with 2–16 fatty acid chain lengths as sole carbon, nitrogen and energy sources, with the exception of octanoylcarnitine, although the reason for this utilization gap is unknown (Meadows & Wargo, 2013). Short-chain acylcarnitines (acetyl- and butyrylcarnitine) are hydrolysed to L-carnitine and a short-chain fatty acid by the esterase HocS (Meadows & Wargo, 2013), while the medium- and long-chain acylcarnitine hydrolase(s) has not yet been identified. *Pseudomonas putida* can utilize L-acylcarnitines with 10–16 fatty acid chain lengths as sole carbon and nitrogen sources (Kleber *et al.*, 1978). The L-enantiomers of short-chain acylcarnitines acetyl-, propionyl-, butyryl-, and iso-butyrylcarnitine, the medium-chain lauroylcarnitine, and the long-chain palmitoylcarnitine can be hydrolysed to carnitine in *Acinetobacter calcoaceticus* (Kleber *et al.*, 1977). Hydrolysis of D- and L-octanoylcarnitine has been assessed for a consortium of yeast, bacteria, and fungi, resulting in the findings that *Bacillus subtilis* ATCC 6633, *Bacillus subtilis* sp. IMAM and *Penicillium notatum* IMAM were capable of hydrolysing D- and L-octanoylcarnitine, whilst *Pseudomonas fluorescens* IMAM, *Rhodotorula gracilis* IMAM and *Fusarium oxysporum* sp. *lini* IMAM had specificity for the L-enantiomer only. However, in this study, the biological function of the resulting carnitine or fatty acid in these strains was not assessed (Aragozzini *et al.*, 1986). An acylcarnitine hydrolase has also been purified from an *Alcaligenes* species, and was capable of hydrolysing acetyl-, propinoyl-, hexanoyl-, octanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl-, and stearoylcarnitine

(Takahashi & Ueda, 1995); however, the gene encoding this enzyme was not determined.

Other carnitine derivatives

Carnitine is used in nutritional supplements, energy drinks, to replace carnitine lost during dialysis, and in treatments for carnitine uptake disorders. As D-carnitine is inhibitory to uptake and metabolism in mammals, these industries have searched for cost-effective methods to synthesize the L-carnitine enantiomer. One strategy for L-carnitine synthesis is to start with a more easily synthesized enantiomeric precursor, such as L-carnitine nitrile or L-carnitine amide, in order to decrease the cost and streamline the production of L-carnitine. A bacterium isolated from the soil, DSM 6230 (no taxonomic classification has been published), metabolizes L-carnitine amide into L-carnitine and ammonia by a novel enzyme L-carnitine amidase (Kula & Joeres, 1993; Joeres & Kula, 1994). The identified carnitine amidase is highly specific for L-carnitine amide, and the D-enantiomer inhibits the enzyme and cannot be used as a substrate (Kula *et al.*, 1996). Another compound that can be metabolized to form carnitine is carnitine nitrile. A nitrilase from *Corynebacterium*, carnitine nitrile hydrolase, converts D- or L-carnitine nitrile into its corresponding D- or L-carnitine and ammonia (Kakayama *et al.*, 1991). Norcarnitine is a derivative of carnitine that has a diethylamino group instead of trimethylamino group, and can be used as a sole carbon and nitrogen source in *Pseudomonas putida* (Kleber *et al.*, 1978). The biological importance of these carnitine-related enzymes has not yet been examined, but the findings suggest that there is either enzymic flexibility in some of the carnitine metabolic enzymes or that there are additional, naturally occurring carnitine-like compounds in the environment.

Conclusions and future directions

Bacteria import, synthesize, and metabolize carnitine through various pathways that have different physiological effects. Our understanding of carnitine transport and metabolism is derived from studying extracellular or facultative intracellular bacteria and examining how carnitine is obtained from either the environment or within an animal host. However, there are a number of important questions that remain to be addressed related to carnitine-dependent transcriptional regulation, the ecological roles of carnitine, the role of carnitine in obligate intracellular bacteria and its importance during non-pathogenic interactions, such as symbioses.

In relation to obligate intracellular pathogens, we know surprisingly little. For instance, to date, no spirochaetes have been identified to use carnitine, but the causative agent of syphilis, *Treponema pallidum*, is predicted to harbour a carnitine transporter (Saier & Paulsen, 2000; Smajs *et al.*, 2005). With *Treponema pallidum* only having 1000 genes (Fraser *et al.*, 1998), carnitine transport may be important for survival in certain environments and for establishing infection. Furthermore, it is tempting to speculate that intracellular pathogens and symbionts can likely use carnitine based on the role of carnitine:fatty acid transport systems in the mitochondria, particularly in light of the endosymbiotic source of these organelles. As such, one might expect a role for carnitine import in the *Rickettsiales*.

Beyond pathogens and symbionts, the impact of environmental metabolism of the osmoprotectants glycine betaine and dimethylsulfionylproprionate has been reasonably well studied (reviewed by Curson *et al.*, 2011; Welsh, 2000), but carnitine has not received much attention in relation to its contributions outside of animal infection and likely deserves additional scrutiny. For instance, the source of carnitine detected in the environment (Warren, 2013a, b) is unknown and we know very little about its half-life as a soluble compound in the environment or the flux rate of carnitine in any environment. A priori, one would assume an important role for carnitine metabolism, both aerobic and anaerobic, during animal decomposition on land or in marine environments. In the deep oceans in particular, carnitine utilization from fish and marine mammal carcasses might represent an important pathway to scavenge all available nutrients in this harsh environment.

Finally, we know almost nothing about the mechanisms by which carnitine is bound and detected to mediate transcriptional regulation. Further investigations into CdhR-like, CaiF-like and the predicted carnitine-sensing TetR family regulators in Gram-positives will be needed to understand how ligand detection is accomplished and converted into regulation of gene expression. Of particular import will be understanding the binding pocket of these regulators to determine if they maintain the cation–π binding aromatic cage that typifies the known quaternary amine-binding proteins crystallized to date. From an evolutionary perspective, CdhR likely arose after gene duplication from a GbdR-like ancestor, while the Gram-positive TetR family proteins are reasonably similar to the choline-binding transcription factor BetI. Thus, characterization of any of these quaternary amine-binding transcription factors will provide crucial understanding of ligand binding and, by homology, present likely binding residues in their respective paralogues. Beyond direct ligand-sensing transcription regulators, we do not know of any two-component systems or chemotaxis regulators that sense and respond to carnitine. The GlrA/GlrK system may represent the actual carnitine-sensing input that impinges on CaiF to establish carnitine-sensitive gene induction. Additionally, many bacteria have been shown to chemotax towards the quaternary amines dimethylsulfionylproprionate and glycine betaine (Miller *et al.*, 2004; Seymour *et al.*, 2010; Stocker & Seymour, 2012), and therefore any discovery of chemotaxis towards carnitine will further our understanding of its role in bacterial biology.

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