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CARNITINE AND *O*-ACYLCARNITINES IN *PSEUDOMONAS AERUINGOSA:* METABOLISM, TRANSPORT, AND REGULATION

A Dissertation Presented

by

Jamie A. Meadows

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Microbiology and Molecular Genetics

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Dissertation Examination Committee:

Matthew J. Wargo, Ph.D., Advisor
Mary J. Dunlop, Ph.D., Chairperson
Christopher D. Huston, M.D.
Keith P. Mintz, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

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ABSTRACT

Pseudomonas aeruginosa is found in numerous environments and is an opportunistic pathogen affecting those who are immunocompromised. Its large genome encodes tremendous metabolic and regulatory diversity that enables *P. aeruginosa* to adapt to various environments. We are interested in how *P. aeruginosa* senses and responds to the host-derived compounds, carnitine and acylcarnitines. Acylcarnitines can be hydrolyzed to carnitine, where the liberated carnitine and its catabolic product glycine betaine can be used as osmoprotectants, for induction of the virulence factor phospholipase C, and as sole carbon, nitrogen, and energy sources. *P. aeruginosa* is incapable of *de novo* synthesis of carnitine and acylcarnitines and therefore imports these compounds from exogenous source. Short-chain acylcarnitines are imported by the ABC transporter CaiX-CbcWV. Medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically and the liberated carnitine is transported through CaiX-CbcWV. Once in the cytoplasm, short-chain acylcarnitines are hydrolyzed by the L-enantiomer specific hydrolase, HocS. The transcriptional regulator CdhR is divergently transcribed from the carnitine catabolism operon and we have identified the upstream activating region, the binding site sequence, and essential residues required for CdhR binding and induction of the carnitine operon. Carnitine catabolism is repressed by glucose and glycine betaine at the transcriptional level. Furthermore, using two different *cdhR* translational fusions we show that CdhR enhances its own expression and that GbdR, a related transcription factor, contributes to *cdhR* expression by enhancing the level of basal expression. These studies are the first to determine the mechanism of *O*-acylcarnitine transport, metabolism, and the regulation of these processes, which contribute to utilization of these compounds for *P. aeruginosa* survival in diverse environments.

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DEDICATION

I would like to dedicate this dissertation to David V. Pollack, Ph.D. You are my inspiration and you gave me the courage to take on this endeavor, and for that I am forever grateful.

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CHAPTER 1:

INTRODUCTION

Jamie A. Meadows [1] and Matthew J. Wargo [1,2]

[1] Department of Microbiology and Molecular Genetics, University of Vermont College of Medicine, Burlington, VT, USA

[2] The Vermont Lung Center, University of Vermont College of Medicine, Burlington, VT, USA

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1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium that is ubiquitous in nature. *P. aeruginosa* is capable of living in many different environments due to its large genome that encodes diverse metabolic pathways. The genome is over 6.2 megabases and has over 5,570 predicted open reading frames that enable tremendous functional diversity [1]. With over 10 % of the genome predicted to be involved in metabolism and 8.4 % to regulation of gene expression, it's no wonder *P. aeruginosa* readily adapts to changing environmental conditions and nutrient sources [1]. Even though its respiration is primarily aerobic, *P. aeruginosa* can respire anaerobically using nitrate as an electron acceptor in the absence of oxygen. *P. aeruginosa* has a polar monoflagellum and Type IV motile pili making it highly motile, allowing the bacterium to actively find nutrients, attach to surfaces, and seek desirable environments [2]. Furthermore, it is this versatility that makes *P. aeruginosa* such a widespread pathogen [3], causing disease in plants [4, 5], nematodes [6], insects [7], and most notably, as an opportunistic pathogen in immunocompromised people.

To successfully cause infection, *P. aeruginosa* must incorporate and metabolize molecules derived from the host. General maintenance and expression of virulence factors are minimal requirements, while efficient catabolism and resultant growth can lead to successful colonization and improved evasion of immune-mediated clearance. The host-derived compound carnitine is abundant in host tissues and is readily used by *P. aeruginosa* [8]. Carnitine can be transported into the cell or acquired from metabolic

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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 [9].

1.1.1. Infections caused by *P. aeruginosa*: acute and chronic

P. aeruginosa is one of the leading causes of nosocomial infections in the world [10] and often spreads from contaminated hospital materials (bedding, floors, sinks, etc.) and hospital personnel [11], although the actual routes of transmission from the contaminated sources to the patients have not been definitively shown. When *P. aeruginosa* establishes an infection it can develop into either an acute or chronic infection. This process is regulated via internal and external signals, often related to metabolism and nutrient acquisition, that alter motility and virulence expression to promote either an acute or chronic infection [12, 13].

Acute infections are severe, progressive infections that can result in septicemia and death if not treated properly. Virulence factors and toxins are often associated with acute infections. The exoenzyme type 3 secretion system (T3SS) of *P. aeruginosa* significantly enhances the pathogens ability to cause acute lung disease [14]. The Exotoxin (Exo) T and ExoS are GTPase-activating proteins that alter host actin cytoskeleton [15-17], while ExoU has patatin-like phospholipase activity affecting lipid metabolism and membrane integrity [18]. Together these exotoxins decrease host wound healing, cell motility and phagocytic capabilities, leading to septicemia and death [19]. People with severe burns are highly susceptible to infection due to breach in the natural skin barrier [11]. *P. aeruginosa* is the leading cause of infection in burns because

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elements such as elastase, exotoxin A, phospholipases, quorum sensing, and the ability to be motile, enable *P. aeruginosa* to persist in the wound and disseminate [20, 21]. Other common acute infections are folliculitis [22], ulcerative keratitis [11], urinary tract infections [23], green nail syndrome [24], and episodes of pulmonary exacerbation in the lung diseases cystic fibrosis and chronic obstructive pulmonary disorder [25].

Chronic infections are those that can last months to a lifetime and are slow progressing. Cystic fibrosis (CF) is an autosomal recessive disorder caused from a mutation in the CF transmembrane conductance regulator (CFTR) gene, an ABC transporter that moves chloride ions across epithelial cell membranes. CF affects many organ systems but the altered lung physiology is currently the leading cause of death. The dysfunction of water balance across the respiratory epithelium and resultant impact on mucociliary clearance leads to a thick viscous mucus layer, a perfect environment for bacterial colonization [26]. After childhood, *P. aeruginosa* is the most common pathogen in the lungs and generally persists as a chronic infection despite extensive eradication regimes [27]. Infection of the lungs and the resultant lung function decline is the leading cause of mortality and morbidity in CF patients and more than 95% of CF patients die from respiratory failure, although only 70% of these are now from *P. aeruginosa* [28, 29]. In chronic infections, *P. aeruginosa* cells form biofilms, repress flagella expression, change their metabolic profile, and can become phenotypically mucoid by producing alginate, enhancing antibiotic resistance and impairing clearance by the host [25, 30].

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1.1.2. Pathogenicity

The ability of *P. aeruginosa* to infect a wide range of hosts and cause significant disease and mortality is mediated by numerous factors that can be categorized as either virulence factors or virulence determinants [31]. Virulence factors, in the strictest definition, are secreted from the cell and damage the host. The most important group of secreted virulence factors are those secreted from the type three secretion systems (T3SS). The T3SS apparatus is a syringe-like complex that injects effector proteins directly into host cells to manipulate them [14]. In addition to T3SS, proteins can be secreted into the environment. Exotoxin A inhibits protein biosynthesis by inactivating elongation factor 2, resulting in cell death [32]. Phospholipase C, encoded by *plcH*, is secreted and cleaves phosphatidylcholine and sphingomyelin, components of eukaryotic cell membranes and lung surfactant. PlcH activity has been found to negatively impact lung function during infection, and can function to induce a proinflammatory response, suppress oxidative burst in neutrophils, degrade pulmonary surfactant, and increase endothelial cell death [33-38]. Elastases and proteases are other secreted virulence factors used by *P. aeruginosa* [11]. One of the most visually recognized virulence factors is pyocyanin because of its blue color. Pyocyanin is a redox-active secondary metabolite that not only kills eukaryotic host cells but also has the capacity to kill other bacteria through a myriad of mechanisms [31, 39].

Virulence determinants are factors that contribute to infection and disease, but not those that are for housekeeping maintenance [40]. Motility by flagella and pili are virulence determinants that contribute to pathogenicity through adherence, persistence in

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wound sites, and dissemination throughout the host [41]. Quorum sensing is a virulence determinant used by bacteria to communicate and determine cell population density, and adjusts gene expression accordingly, impacting virulence [42, 43]. It was recently demonstrated that quorum sensing activates dispersal of biofilms [44], a community-based lifestyle. Biofilms are a developmental process leading to long duration attachment to a surface, where the cells are highly resistant to antimicrobials making it difficult to eradicate the bacteria [42]. A virulence determinant often overlooked is catabolism of host compounds. For a pathogen to colonize the host, the bacterium must acquire and catabolize host compounds to serve as nutrients, which are discussed in greater detail later in the chapter.

P. aeruginosa is a successful opportunistic pathogen because of metabolic diversity, secreted virulence factors, and resistance to antimicrobials and drug therapies. Virulence and antibiotic resistance are not mutually exclusive; alginate production, quorum sensing, and biofilm formation directly contribute to drug resistance. Resistance is largely attributed to intrinsic features in the microbe. For starters, the *P. aeruginosa* outer membrane is more restrictive than many other Gram-negative bacteria, with a permeability 12-100 times lower than in *E. coli* [45], excluding passage of many antibiotic compounds. Efflux pumps contribute to antibiotic resistance by pumping those antibiotics that passed the limited permeability barrier of the outer membrane back out of the cell. Overexpression of the MexAB-OprM, MexXY-OprM, and MexEF-OprN efflux systems removes β-lactams, quinolones, aminoglycosides, carbapenems, and disinfectants, respectively [46]. Another intrinsic resistance method that *P. aeruginosa*

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possess is a chromosomal *ampC* gene, encoding a periplasmic β-lactamase that can inactivate β-lactam antibiotics [47]. Horizontal gene transfer and mutations can be acquired, providing additional mechanisms to escape the death blows from antibiotics and drugs.

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1.1.3. References

1. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S. & Olson, M. V. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen, *Nature.* **406**, 959-64.
2. O'Toole, G. A. & Kolter, R. (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development, *Molecular microbiology.* **30**, 295-304.
3. Silby, M. W., Winstanley, C., Godfrey, S. A., Levy, S. B. & Jackson, R. W. (2011) *Pseudomonas* genomes: diverse and adaptable, *FEMS microbiology reviews.* **35**, 652-80.
4. Silo-Suh, L., Suh, S. J., Sokol, P. A. & Ohman, D. E. (2002) A simple alfalfa seedling infection model for *Pseudomonas aeruginosa* strains associated with cystic fibrosis shows AlgT (sigma-22) and RhlR contribute to pathogenesis, *Proceedings of the National Academy of Sciences of the United States of America.* **99**, 15699-704.
5. Walker, T. S., Bais, H. P., Deziel, E., Schweizer, H. P., Rahme, L. G., Fall, R. & Vivanco, J. M. (2004) *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation, *Plant physiology.* **134**, 320-31.
6. Mahajan-Miklos, S., Tan, M. W., Rahme, L. G. & Ausubel, F. M. (1999) Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model, *Cell.* **96**, 47-56.
7. Jander, G., Rahme, L. G. & Ausubel, F. M. (2000) Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects, *Journal of bacteriology.* **182**, 3843-5.
8. Lindstedt, G., Lindstedt, S., Midtvedt, T., Tofft M. (1967) The Formation and Degradation of Carnitine in *Pseudomonas, Biochemistry.* **6**, 1262-1270.
9. Meadows, J. A. & Wargo, M. J. (2015) Carnitine in bacterial physiology and metabolism, *Microbiology.*
10. Koulenti, D., Lisboa, T., Brun-Buisson, C., Krueger, W., Macor, A., Sole-Violan, J., Diaz, E., Topeli, A., DeWaele, J., Carneiro, A., Martin-Loeches, I., Armaganidis, A., Rello, J. & Group, E.-V. C. S. (2009) Spectrum of practice in the diagnosis of nosocomial pneumonia in patients requiring mechanical ventilation in European intensive care units, *Critical care medicine.* **37**, 2360-8.
11. Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist, *Microbes and infection / Institut Pasteur.* **2**, 1051-60.
12. Furukawa, S., Kuchma, S. L. & O'Toole, G. A. (2006) Keeping their options open: acute versus persistent infections, *Journal of bacteriology.* **188**, 1211-7.

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13. Yahr, T. L. & Greenberg, E. P. (2004) The genetic basis for the commitment to chronic versus acute infection in *Pseudomonas aeruginosa*, *Molecular cell.* **16**, 497-8.
14. Hauser, A. R. (2009) The type III secretion system of *Pseudomonas aeruginosa*: infection by injection, *Nature reviews Microbiology.* **7**, 654-65.
15. Krall, R., Sun, J., Pederson, K. J. & Barbieri, J. T. (2002) In vivo rho GTPase-activating protein activity of *Pseudomonas aeruginosa* cytotoxin ExoS, *Infection and immunity.* **70**, 360-7.
16. Cowell, B. A., Chen, D. Y., Frank, D. W., Vallis, A. J. & Fleiszig, S. M. (2000) ExoT of cytotoxic *Pseudomonas aeruginosa* prevents uptake by corneal epithelial cells, *Infection and immunity.* **68**, 403-6.
17. Geiser, T. K., Kazmierczak, B. I., Garrity-Ryan, L. K., Matthay, M. A. & Engel, J. N. (2001) *Pseudomonas aeruginosa* ExoT inhibits in vitro lung epithelial wound repair, *Cellular microbiology.* **3**, 223-36.
18. Hauser, A. R., Kang, P. J. & Engel, J. N. (1998) PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence, *Molecular microbiology.* **27**, 807-18.
19. Sawa, T. (2014) The molecular mechanism of acute lung injury caused by *Pseudomonas aeruginosa*: from bacterial pathogenesis to host response, *Journal of intensive care.* **2**, 10.
20. Church, D., Elsayed, S., Reid, O., Winston, B. & Lindsay, R. (2006) Burn wound infections, *Clinical microbiology reviews.* **19**, 403-34.
21. Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995) Common virulence factors for bacterial pathogenicity in plants and animals, *Science.* **268**, 1899-902.
22. Yu, Y., Cheng, A. S., Wang, L., Dunne, W. M. & Bayliss, S. J. (2007) Hot tub folliculitis or hot hand-foot syndrome caused by *Pseudomonas aeruginosa*, *Journal of the American Academy of Dermatology.* **57**, 596-600.
23. Moore, K. N., Day, R. A. & Albers, M. (2002) Pathogenesis of urinary tract infections: a review, *Journal of clinical nursing.* **11**, 568-74.
24. Nenoff, P., Paasch, U. & Handrick, W. (2014) [Infections of finger and toe nails due to fungi and bacteria], *Der Hautarzt; Zeitschrift fur Dermatologie, Venerologie, und verwandte Gebiete.* **65**, 337-48.
25. Doring, G., Parameswaran, I. G. & Murphy, T. F. (2011) Differential adaptation of microbial pathogens to airways of patients with cystic fibrosis and chronic obstructive pulmonary disease, *FEMS microbiology reviews.* **35**, 124-46.
26. Matsui, H., Grubb, B. R., Tarran, R., Randell, S. H., Gatzy, J. T., Davis, C. W. & Boucher, R. C. (1998) Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease, *Cell.* **95**, 1005-15.
27. Heijerman, H. (2005) Infection and inflammation in cystic fibrosis: a short review, *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society.* **4 Suppl 2**, 3-5.
28. Dodge, J. A., Lewis, P. A., Stanton, M. & Wilsher, J. (2007) Cystic fibrosis mortality and survival in the UK: 1947-2003, *The European respiratory journal.* **29**, 522-6.

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29. Folkesson, A., Jelsbak, L., Yang, L., Johansen, H. K., Ciofu, O., Hoiby, N. & Molin, S. (2012) Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective, *Nature reviews Microbiology.* **10**, 841-51.
30. Murray, T. S., Egan, M. & Kazmierczak, B. I. (2007) *Pseudomonas aeruginosa* chronic colonization in cystic fibrosis patients, *Current opinion in pediatrics.* **19**, 83-8.
31. Ran, H., Hassett, D. J. & Lau, G. W. (2003) Human targets of *Pseudomonas aeruginosa* pyocyanin, *Proceedings of the National Academy of Sciences of the United States of America.* **100**, 14315-20.
32. Pollack, M. (1983) The role of exotoxin A in *Pseudomonas* disease and immunity, *Reviews of infectious diseases.* **5 Suppl 5**, S979-84.
33. Berk, R. S., Brown, D., Coutinho, I. & Meyers, D. (1987) In vivo studies with two phospholipase C fractions from *Pseudomonas aeruginosa*, *Infect Immun.* **55**, 1728-30.
34. Meyers, D. J. & Berk, R. S. (1990) Characterization of phospholipase C from *Pseudomonas aeruginosa* as a potent inflammatory agent, *Infect Immun.* **58**, 659-66.
35. Terada, L. S., Johansen, K. A., Nowbar, S., Vasil, A. I. & Vasil, M. L. (1999) *Pseudomonas aeruginosa* hemolytic phospholipase C suppresses neutrophil respiratory burst activity, *Infect Immun.* **67**, 2371-6.
36. Ostroff, R. M., Vasil, A. I. & Vasil, M. L. (1990) Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa*, *J Bacteriol.* **172**, 5915-23.
37. Wiener-Kronish, J. P., Sakuma, T., Kudoh, I., Pittet, J. F., Frank, D., Dobbs, L., Vasil, M. L. & Matthay, M. A. (1993) Alveolar epithelial injury and pleural empyema in acute *P. aeruginosa* pneumonia in anesthetized rabbits, *J Appl Physiol.* **75**, 1661-9.
38. Wargo, M. J., Gross, M. J., Rajamani, S., Allard, J. L., Lundblad, L. K., Allen, G. B., Vasil, M. L., Leclair, L. W. & Hogan, D. A. (2011) Hemolytic phospholipase C inhibition protects lung function during *Pseudomonas aeruginosa* infection, *American journal of respiratory and critical care medicine.* **184**, 345-54.
39. Lau, G. W., Hassett, D. J., Ran, H. & Kong, F. (2004) The role of pyocyanin in *Pseudomonas aeruginosa* infection, *Trends in molecular medicine.* **10**, 599-606.
40. Mekalanos, J. J. (1992) Environmental signals controlling expression of virulence determinants in bacteria, *Journal of bacteriology.* **174**, 1-7.
41. Sato, H., Okinaga, K. & Saito, H. (1988) Role of pili in the pathogenesis of *Pseudomonas aeruginosa* burn infection, *Microbiology and immunology.* **32**, 131-9.
42. Veesenmeyer, J. L., Hauser, A. R., Lisboa, T. & Rello, J. (2009) *Pseudomonas aeruginosa* virulence and therapy: evolving translational strategies, *Critical care medicine.* **37**, 1777-86.
43. Bjarnsholt, T. & Givskov, M. (2007) The role of quorum sensing in the pathogenicity of the cunning aggressor *Pseudomonas aeruginosa*, *Analytical and bioanalytical chemistry.* **387**, 409-14.
44. Solano, C., Echeverz, M. & Lasa, I. (2014) Biofilm dispersion and quorum sensing, *Current opinion in microbiology.* **18**, 96-104.

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45. Hancock, R. E. (1998) Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria, *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* **27 Suppl 1**, S93-9.
46. Lambert, P. A. (2002) Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*, *Journal of the Royal Society of Medicine.* **95 Suppl 41**, 22-6.
47. Lister, P. D., Wolter, D. J. & Hanson, N. D. (2009) Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms, *Clinical microbiology reviews.* **22**, 582-610.

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1.2 Carnitine in bacterial physiology and metabolism

1.2.1. Summary

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.

1.2.2. Introduction

Carnitine (gamma-trimethylamino-beta-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 and Krimberg (Gulewitsch & Krimberg, 1905), and Kutscher (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

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transfer of carnitine in utero and exogenous sources after birth (Combs Jr., 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 fifteen years ago (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.

1.2.3. 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 sub-alpine grassland (Warren, 2013a). In this environment, carnitine was the most abundant quaternary ammonium compound (0.49

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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 dependent 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).

1.2.4. The importance of carnitine to animals (like us)

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 beta carbon hydroxyl group with a fatty acid to form *O*-acylcarnitine (Fig. 1). Beta oxidation of very long- chain fatty acids starts in the peroxisome, and once they

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have been converted to medium- or short fatty acid chain lengths, carnitine is employed to traffic them out of the peroxisome and into the mitochondria where beta oxidation is completed (Reddy & Hashimoto, 2001; Steiber *et al.*, 2004; Wanders & Waterham, 2006). Acetyl-CoA derived from beta oxidation can be used to generate ATP via the TCA cycle, while beta 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, about 95% of total carnitine is found in skeletal and cardiac muscle, with the remaining 5% circulates in the plasma (Cave *et al.*, 2008). Approximately 75% of carnitine is obtained through diet; with foods from animal origin like meat and dairy 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 where 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 and colleagues

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associated the degradation of carnitine by intestinal microbiota with cardiovascular disease (CVD) and the promotion of atherosclerosis (Koeth *et al.*, 2013). Some intestinal bacteria can convert carnitine to trimethylamine, which is subsequently oxidized in the liver to the proatherogenic species trimehtylamine-*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 CVD 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).

1.2.5. The physiologic 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 wasn't described until almost fifty 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 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 BCCT (betaine-choline-carnitine) transporters that

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can be driven by the sodium or proton motive force, but in many cases where carnitine is the substrate, often functions as a carnitine:gamma butyrobetaine antiporter (Ziegler *et al.*, 2010). Carnitine import or import of immediate precursors is critical for bacterial acquisition of carnitine, as Verheul et al. demonstrated that de novo synthesis of carnitine does not occur in *Escherichia coli* (Verheul *et al.*, 1998), 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 cozy 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, 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 concentration 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

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bacteria and its abundance for infectious microbes in the host and presence in many natural environments suggests it is readily accessible (Warren, 2013a; Warren, 2013b).

Osmoprotection

Drastic changes in water content in the environment can result in osmotic stress to the cell. Low external solute concentration is hypoosmotic, 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 hyperosmotic, 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). *P. aeruginosa*, like many environmental proteobacteria, can also metabolize carnitine to the osmolyte glycine

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betaine (Fig. 2) (Aurich *et al.*, 1967; Bastard *et al.*, 2014; Wargo & Hogan, 2009). *Escherichia coli* also use 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 MFS-family 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, nuclear magnetic resonance observations demonstrated that added carnitine was not metabolized to the more potent osmolyte glycine betaine (Park *et al.*, 1995).

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, gamma-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 *B. subtilis* cannot be metabolized further and therefore is thought to function primarily as an

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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*. *L. monocytogenes* primarily imports carnitine through the OpuC ABC transporter (Fraser & O'Byrne, 2002; Fraser *et al.*, 2000; Verheul *et al.*, 1995; Verheul *et al.*, 1997), where it functions as an osmolyte in high salt conditions (Beumer *et al.*, 1994). Mice infected with *L. 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; Sleator *et al.*, 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 hyperosmotic 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: the 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

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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 *L. monocytogenes* is important for protecting the bacteria against bile stress and its expression is coregulated 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 feces 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, *L. 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, 1990), protein stability (Privalov & Gill, 1988), and membrane fluidity (Rudolph *et al.*, 1986), and compatible solutes, like 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

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(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 average soil surface temperatures range from 0 – 18 °C, average freshwater ranges from 3 – 25 °C, and while the average ocean surface temperature is ~17 °C the bulk of ocean water maintains a temperature of about 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 gamma butyrobetaine have been shown to protect against both cold stress (15 °C) and heat stress (52 °C), via uptake through the OpuC transporter (Hoffmann and Bremer, 2011). It is likely that utilization of carnitine as a cryo- and thermo-protectant is not restricted to *Listeria* and *Bacillus* and the taxonomic breadth and ecological impact of this process requires 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 survives better and demonstrates substantial barotolerance when the compatible solute carnitine is present and imported into the cell (Smiddy *et al.*, 2004).

1.2.6. Carnitine metabolism

Carnitine can be utilized in different metabolic pathways to play a variety of physiologic roles. Figure 2 illustrates multiple described pathways for carnitine

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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 *P. 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, functions as an electron acceptor (as reviewed in (Barrett & Kwan, 1985; Strom *et al.*, 1979; Yancey, 2005)).

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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. *P. 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), *P. putida* (Kleber *et al.*, 1978), *P. 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 Coenzyme A (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-dehydrocaritine is converted to acetylacetone and glycine betaine-CoA by the beta-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 *P. 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

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is then converted to glycine betaine and CoA by a CoA transferase, likely the DhcAB enzyme in *P. 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 *P. 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), and metabolism and homeostasis of glycine betaine has recently been reviewed (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 1960's 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 in anaerobic conditions since 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, 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

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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 C-N bonds in a quaternary amine compound, and these enzymes may represent a general evolutionary strategy for quaternary amine metabolism. Orthologs and homologs 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 to human cardiovascular health (Koeth *et al.*, 2013), where it is oxidized to trimethylamine-*N*-oxide (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, among others. In cases where

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the common electron acceptors are not present, some Enterobacteriaceae (*E. coli, S. 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; Seim *et al.*, 1982b; Seim *et al*., 1982c). 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; Buchet *et al.*, 1999; Eichler *et al.*, 1995; Eichler *et al.*, 1996). For its role as an electron acceptor, carnitine is imported by the substrate:product antiporter, CaiT, which exchanges carnitine for its metabolic product gamma-butyrobetaine (Jung *et al.*, 1990; Jung *et al.*, 2002; Kalayil *et al.*, 2013). Co-expression of these two operons is induced by carnitine and crotonobetaine, but repressed by oxygen, glucose, gamma-butyrobetaine, and more desirable final electron acceptors such as nitrate and fumarate (Jung *et al.*, 1987; Seim *et al.*, 1982a; Seim *et al.*, 1982b). 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; Obon *et al.*, 1999).

The *fix* operon is predicted to be involved in transferring electrons to carnitine (Eichler *et al.*, 1995; Eichler *et al.*, 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 cosubstrates gamma-

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butyrobetainyl-CoA or crotonobetainyl-CoA (Eichler *et al.*, 1994a; Elssner *et al.*, 2000; Jung *et al.*, 1989). However Elssner *et al.* demonstrated that "L-carnitine dehydratase does not exist" and two enzymes are responsible for the reversible conversion of carnitine to crotonobetaine (Elssner *et al.*, 2001). CaiB is a CoA transferase that transfers CoA from gamma-butyrobetainyl-CoA, crotonobetainyl-CoA, or carnitinyl-CoA to form gamma-butryobetaine, crotonobetaine or carnitine. CaiD has enoyl-CoA hydratase activity and can dehydrate carnitinyl-CoA to crotonobetainyl-CoA, or can hydrate crotonobetainyl-CoA to carntinyl-CoA, thus CaiD has two potential names: crotonobetainyl-CoA hydratase or carnitinyl-CoA dehydratase. These enzymatic processes also allow carnitine to be synthesized from crotonobetaine and vice versa, (Figure 3) (Elssner *et al.*, 2001; Engemann *et al.*, 2001; Engemann *et al.*, 2005). Crotonobetaine can be reduced to gamma-butyrobetaine by two proteins, CaiB, with one of the cosubstrates gamma-butyrobetainyl-CoA or crotonobetainyl-CoA, and the unidirectional enzyme crotonobetaine reductase, CaiA, which reduces crotonobetainyl-CoA to gamma-butryobetainyl-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).

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The enzymes for converting gamma-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 gamma-butyrobetaine hydroxylase was detected during degradation of gamma butyrobetaine to carnitine. For *A. calcoaceticus,* after gamma 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 *A. cycloclast* (Naidu *et al.*, 2001).

1.2.7. 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 to 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.

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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 Enterohemorrhagic *E. coli* (EHEC), 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 homologs in the *fix* and *cai* operons, it remains a possibility that carnitine detection occurs via a GlrR ortholog or a cognate sensor kinase, like GlrK (Yamamoto *et al.*, 2005).

In *P. aeruginosa*, the transcription factor CdhR functions as the carnitine-responsive activator of the genes encoding the carnitine dehydrogenase enzymatic pathway (Wargo & Hogan, 2009). CdhR is a member of the glutamine amidotransferase-1 (GATase-1) sub-family 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 comprised of two HTH motifs. Orthologs of CdhR are found divergently transcribed from the aerobic carnitine dehydrogenase genes in a variety of organisms including many species in the families

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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 carnitine dehydrogenase operons contain a TetR-family transcription regulator that likely functions as the carnitine sensor for regulated expression of these operons. Compared to the Gram negative bacteria, presence of the carnitine dehydrogenase 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:gamma butryobetaine antiporter CaiT (Schulze *et al.*, 2010; Tang *et al.*, 2010), and similar aromatic cage architecture of the quaternary amine binding site with gamma butyrobetaine has been determined for gamma butyrobetaine hydroxylase (Tars *et al.*, 2014). Therefore, like 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-pi interaction with the trimethylamine moiety coupled with properly spaced hydrogen bonding residues to

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

1.2.8. 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

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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; Kluttermann *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, 1997). However, the D-carnitine metabolic pathway in *Enterobacter* has not been fully described. 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 Iet 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 directly tested. CaiD is still postulated to be involved in racemization of D-carnitine, since CaiC was shown to activate D-carnitine by adding a

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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 gamma-butyrobetaine hydroxylase

The direct route to the formation of carnitine from gamma-butryobetaine occurs through the enzyme gamma-butryobetaine hydroxylase (EC 1.14.11.1). It has been identified in *Pseudomonas* sp. AK1 (Lindstedt *et al.*, 1970a; Lindstedt *et al.*, 1970b; Ruetschi *et al.*, 1993), and *Pseudomonas* sp. L1 (Lu *et al.*, 2012), and both enzymes are homologous to the animal gamma-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). *P. 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 hydrolyzed 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. *P. putida* can utilize L-acylcarnitines with 10-16

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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 hydrolyzed 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 *B. subtilis* ATCC 6633, *B. subtilis* sp. IMAM, and *Penicillium notatum* IMAM were capable of hydrolyzing D- and L-octanoylcarnitine, while *P. 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 hydrolyzing 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. Because 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, like

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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; Ulrich & 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 carnitinenitrile. A nitrilase from *Corynebacterium*, carnitinenitrile hydrolase, converts D- or L-carnitinenitrile into its corresponding D- or L-carnitine and ammonia (Kakayama, August 1991). Norcarnitine is a deriviative of carnitine that has a diethylamino group instead of trimethylamino group, can be used as a sole carbon and nitrogen source in *P. 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 enzymatic flexibility in some of the carnitine metabolic enzymes, or that there are additional, naturally-occurring carnitine-like compounds in the environment.

1.2.9. 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

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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 like symbioses.

In relation to obligate intracellular pathogens, we know surprisingly little. For instance, to date, no spirochetes have been identified to use carnitine, but the causative agent of syphilis, *Treponema pallidum,* is predicted to harbor a carnitine transporter (Saier & Paulsen, 2000; Smajs *et al.*, 2005). With *T. pallidum* only having a thousand genes (Fraser *et al.*, 1998), carnitine transport may be important 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 (DMSP) has been reasonably well studied (reviewed in (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; Warren, 2013b) 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

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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-pi binding aromatic cage that typifies the known quaternary amine binding proteins crystalized 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 paralogs. Beyond direct ligand-sensing transcription regulators, we do not know of any two-component systems or chemotaxis regulators that sense and response 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 DMSP and glycine betaine (Miller *et al.*, 2004; Seymour *et al.*, 2010; Stocker & Seymour, 2012), and therefore any

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discovery of chemotaxis towards carnitine will further our understanding of its role in bacterial biology.

1.2.10. Acknowledgements

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1.2.11. Figures

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

Figure 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, ACH, EC 3.1.1.28; (2) gamma butyrobetaine hydroxylase, GBBH, EC 1.14.11.1; (3) oxidoreductase CntAB, EC 1.14.13.-; (4) carnitine racemase, EC 5.1.-.-; (5) L-carnitine dehydrogenase, L-CDH, EC 1.1.1.108; (6) D-carnitine dehydrogenase, D-CDH, EC 1.1.1.108; (7) beta-keto acid cleavage enzyme, BKACE, 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 enzymatic steps have full EC descriptors at this time.

Figure 3. Diagram of the Gamma butyrobetaine-Crotonobetaine-Carnitine cycle. Primarily based on Elssner et al. (2001), with additions based on Bernal et al. (2008) and Cánovas et al. (2003). GBB – gamma-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^-.

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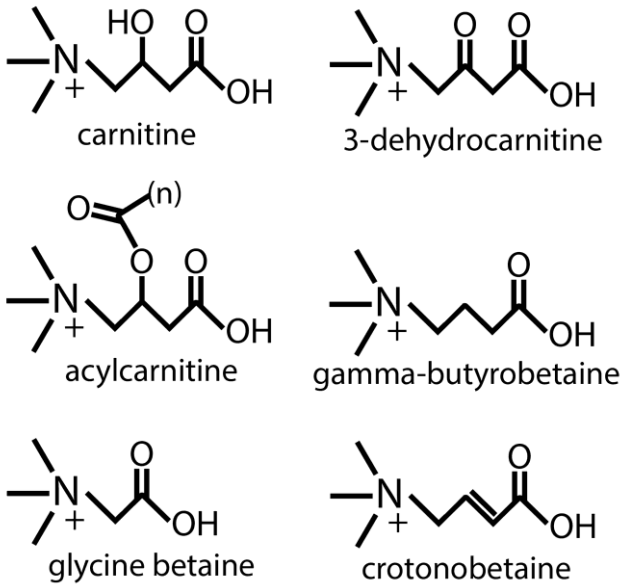

Figure 1. Structures of carnitine and related compounds

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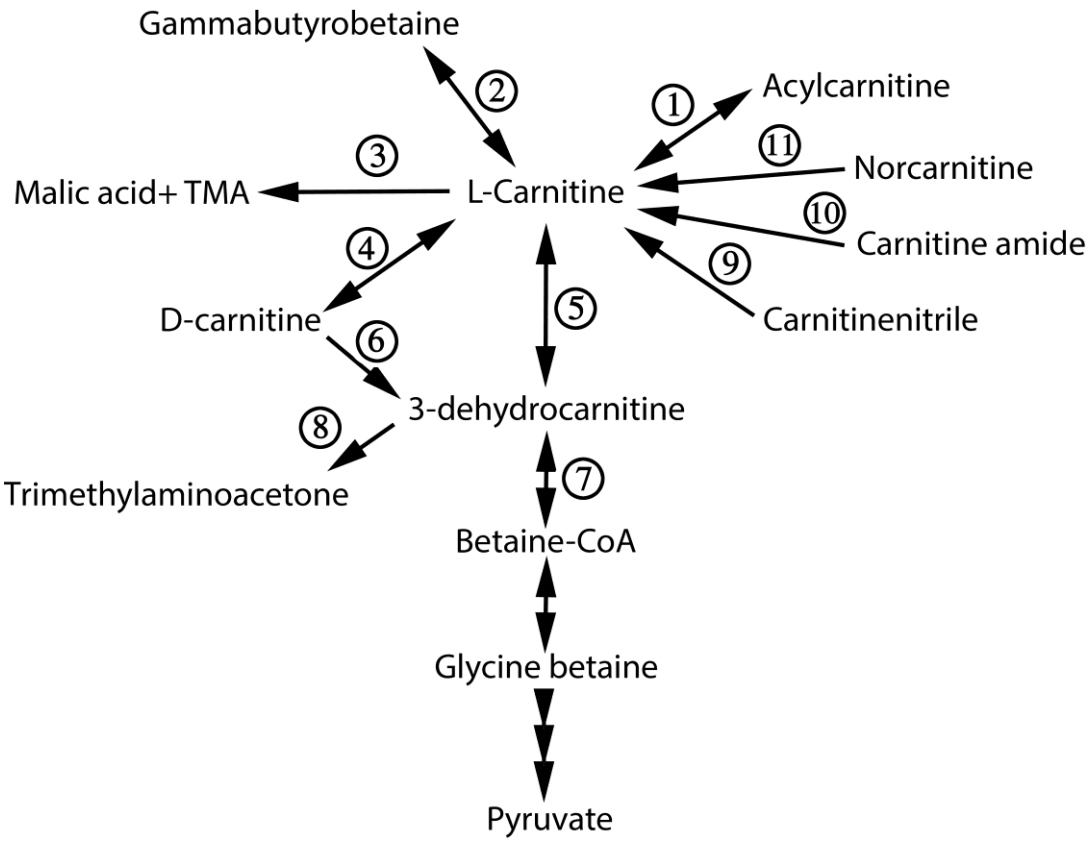

Figure 2. Diagram of the multiple pathways for carnitine metabolism in bacteria

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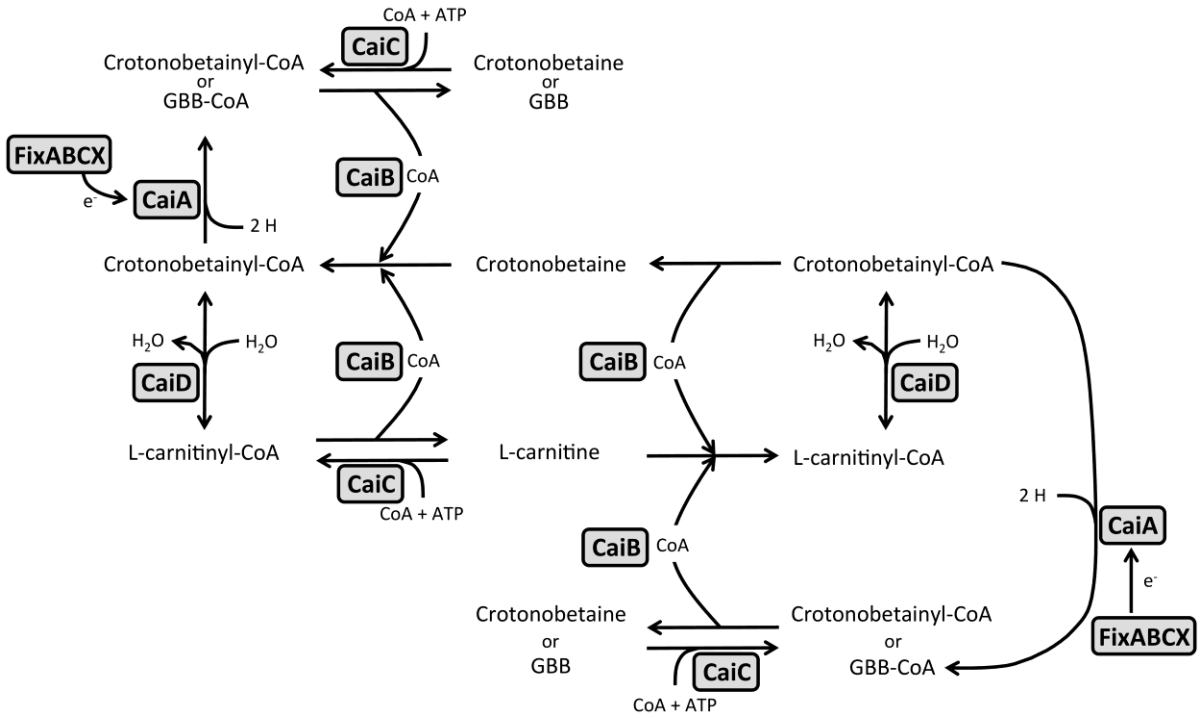

Figure 3. Diagram of the Gamma butyrobetaine-Crotonobetaine-Carnitine cycle

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1.2.12. References

Achouak, W., Christen, R., Barakat, M., Martel, M. H. & Heulin, T. (1999). *Burkholderia caribensis* sp. nov., an exopolysaccharide-producing bacterium isolated from vertisol microaggregates in Martinique. *Int J syst Bacteriol* **Pt 2**, 787-794.

Angelidis, A. S. & Smith, G. M. (2003). Role of the glycine betaine and carnitine transporters in adaptation of *Listeria monocytogenes* to chill stress in defined medium. *Appl Environ Microbiol* **69**, 7492-7498.

Angelidis, A. S., Smith, L. T., Hoffman, L. M. & Smith, G. M. (2002). Identification of *opuC* as a chill-activated and osmotically activated carnitine transporter in *Listeria monocytogenes*. *Appl Environ Microbiol* **68**, 2644-2650.

Aragozzini, F., Manzoni, M., Cavazzoni, V. & Craveri, R. (1986). D,L-carnitine resolution by *Fusarium oxysporum*. *Biotechnol Lett* **8**, 95-98.

Arima, J., Uesumi, A., Mitsuzumi, H. & Mori, N. (2010). Biochemical characterization of L-carnitine dehydrogenases from *Rhizobium* sp. and *Xanthomonas translucens*. *Biosci Biotech Bioch* **74**, 1237-1242.

Aurich, H., Kleber, H. P. & Schopp, W. D. (1967). An inducible carnitine dehydrogenase from *Pseudomonas aeruginosa*. *Biochim Biophys Acta* **139**, 505-507.

Barbier, M., Owings, J. P., Martinez-Ramos, I., Damron, F. H., Gomila, R., Blazquez, J., Goldberg, J. B. & Alberti, S. (2013). Lysine trimethylation of EF-Tu mimics platelet-activating factor to initiate *Pseudomonas aeruginosa* pneumonia. *mBio* **4**, e00207-00213.

Barrett, E. L. & Kwan, H. S. (1985). Bacterial reduction of trimethylamine oxide. *Annu Rev Microbiol* **39**, 131-149.

Bastard, K., Smith, A. A., Vergne-Vaxelaire, C., Perret, A., Zaparucha, A., De Melo-Minardi, R., Mariage, A., Boutard, M., Debard, A. & other authors (2014). Revealing the hidden functional diversity of an enzyme family. *Nat Chem Biol* **10**, 42-49.

Bayles, D. O. & Wilkinson, B. J. (2000). Osmoprotectants and cryoprotectants for *Listeria monocytogenes*. *Lett Appl Microbiol* **30**, 23-27.

Begley, M., Gahan, C. G. & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiol Rev* **29**, 625-651.

Bennett, B. J., de Aguiar Vallim, T. Q., Wang, Z., Shih, D. M., Meng, Y., Gregory, J., Allayee, H., Lee, R., Graham, M. & other authors (2013). Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab* **17**, 49-60.

Bernal, V., Arense, P., Blatz, V., Mandrand-Berthelot, M. A., Canovas, M. & Iborra, J. L. (2008). Role of betaine:CoA ligase (CaiC) in the activation of betaines and the transfer of coenzyme A in *Escherichia coli*. *J Appl Microbiol* **105**, 42-50.

www.manaraa.com

Beumer, R. R., Te Giffel, M. C., Cox, L. J., Rombouts, F. M. & Abee, T. (1994). Effect of exogenous proline, betaine, and carnitine on growth of *Listeria monocytogenes* in a minimal medium. *Appl Environ Microbiol* **60**, 1359-1363.

Bieber, L. L. (1988). Carnitine. *Annu Rev Biochem* **57**, 261-283.

Bremer, E. (2011). Crystal ball - 2011. *Environ Microbiol Rep* **3**, 1-26.

Bremer, J. (1983). Carnitine-metabolism and functions. *Physiol Rev* **63**, 1420-1480.

Brown, A. D. & Simpson, J. R. (1972). Water relations of sugar-tolerant yeasts: the role of intracellular polyols. *J Gen Microbiol* **72**, 589-591.

Buchet, A., Eichler, K. & Mandrand-Berthelot, M. A. (1998). Regulation of the carnitine pathway in *Escherichia coli:* investigation of the *cai-fix* divergent promoter region. *J Bacteriol* **180**, 2599-2608.

Buchet, A., Nasser, W., Eichler, K. & Mandrand-Berthelot, M. A. (1999). Positive co-regulation of the *Escherichia coli* carnitine pathway cai and fix operons by CRP and the CaiF activator. *Mol Microbiol* **34**, 562-575.

Canovas, D., Castellar, M. R., Obon, T., Torroglosa, C., Olivares, J. L. & Iborra, J. L. (2003). Racemisation of D(+)-carnitine into L(-)-carnitine by *Escherichia coli* strains. *Process Biochem* **39**, 287-293.

Castellar, M. R., Canovas, M., Kleber, H. P. & Iborra, J. L. (1998). Biotransformation of D(+)-carnitine into L(-)-carnitine by resting cells of *Escherichia coli* O44 K74. *J Appl Microbiol* **85**, 883-890.

Cave, M. C., Hurt, R. T., Frazier, T. H., Matheson, P. J., Garrison, R. N., McClain, C. J. & McClave, S. A. (2008). Obesity, inflammation, and the potential application of pharmaconutrition. *J Parenter Enteral Nutr* **23**, 16-34.

Chen, C. & Beattie, G. A. (2007). Characterization of the osmoprotectant transporter OpuC from *Pseudomonas syringae* and demonstration that cystathionine-beta-synthase domains are required for its osmoregulatory function. *J Bacteriol* **189**, 6901-6912.

Chen, C., Malek, A. A., Wargo, M. J., Hogan, D. A. & Beattie, G. A. (2010). The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds. *Mol Microbiol* **75**, 29-45.

Combs Jr., G. F. (2012). *Vitamins Fundamental Aspects in Health and Nutrtion*, Fourth edn.: Academic Press, Elsevier Inc. .

Creasey, E. A., Delahay, R. M., Daniell, S. J. & Frankel, G. (2003). Yeast two-hybrid system survey of interactions between LEE-encoded proteins of enteropathogenic *Escherichia coli*. *Microbiol* **149**, 2093-2106.

Curson, A. R., Todd, J. D., Sullivan, M. J. & Johnston, A. W. (2011). Catabolism of dimethylsulphoniopropionate: microorganisms, enzymes and genes. *Nat Rev Microbiol* **9**, 849-859.

Dalmastri, C., Fiore, A., Alisi, C., Bevivino, A., Tabacchioni, S., Giuliano, G., Sprocati, A. R., Segre, L., Mahenthiralingam, E. & other authors (2003). A rhizospheric *Burkholderia cepacia* complex population: genotypic and phenotypic diversity of *Burkholderia cenocepacia* and *Burkholderia ambifaria*. *FEMS Microbiol Ecol* **46**, 179-187.

www.manaraa.com

Du, Y., Shi, W. W., He, Y. X., Yang, Y. H., Zhou, C. Z. & Chen, Y. (2011). Structures of the substrate-binding protein provide insights into the multiple compatible solute binding specificities of the *Bacillus subtilis* ABC transporter OpuC. *Biochem J* **436**, 283-289.

Eichler, K., Schunck, W. H., Kleber, H. P. & Mandrand-Berthelot, M. A. (1994a). Cloning, nucleotide sequence, and expression of the *Escherichia coli* gene encoding carnitine dehydratase. *J Bacteriol* **176**, 2970-2975.

Eichler, K., Bourgis, F., Buchet, A., Kleber, H. P. & Mandrand-Berthelot, M. A. (1994b). Molecular characterization of the *cai* operon necessary for carnitine metabolism in *Escherichia coli*. *Mol Microbiol* **13**, 775-786.

Eichler, K., Buchet, A., Bourgis, F., Kleber, H. P. & Mandrand-Berthelot, M. A. (1995). The fix *Escherichia coli* region contains four genes related to carnitine metabolism. *J Basic Microbiol* **35**, 217-227.

Eichler, K., Buchet, A., Lemke, R., Kleber, H. P. & Mandrand-Berthelot, M. A. (1996). Identification and characterization of the *caiF* gene encoding a potential transcriptional activator of carnitine metabolism in *Escherichia coli*. *J Bacteriol* **178**, 1248-1257.

Elssner, T., Preusser, A., Wagner, U. & Kleber, H. P. (1999). Metabolism of L(-)-carnitine by Enterobacteriaceae under aerobic conditions. *FEMS Microbiol Lett* **174**, 295-301.

Elssner, T., Engemann, C., Baumgart, K. & Kleber, H. P. (2001). Involvement of coenzyme A esters and two new enzymes, an enoyl-CoA hydratase and a CoA-transferase, in the hydration of crotonobetaine to L-carnitine by *Escherichia coli*. *Biochemistry* **40**, 11140-11148.

Elssner, T., Hennig, L., Frauendorf, H., Haferburg, D. & Kleber, H. P. (2000). Isolation, identification, and synthesis of gamma-butyrobetainyl-CoA and crotonobetainyl-CoA, compounds involved in carnitine metabolism of *E. coli*. *Biochemistry* **39**, 10761-10769.

Engemann, C. & Kleber, H. P. (2001). Epigenetic regulation of carnitine metabolising enzymes in *Proteus* sp. under aerobic conditions. *FEMS Microbiol Lett* **196**, 1-6.

Engemann, C., Elssner, T. & Kleber, H. P. (2001). Biotransformation of crotonobetaine to L(-)-carnitine in *Proteus* sp. *Arch Microbiol* **175**, 353-359.

Engemann, C., Elssner, T., Pfeifer, S., Krumbholz, C., Maier, T. & Kleber, H. P. (2005). Identification and functional characterisation of genes and corresponding enzymes involved in carnitine metabolism of *Proteus* sp. *Arch Microbiol* **183**, 176-189.

Fitzsimmons, L. F., Hampel, K. J. & Wargo, M. J. (2012). Cellular choline and glycine betaine pools impact osmoprotection and phospholipase C production in *Pseudomonas aeruginosa*. *J Bacteriol* **194**, 4718-4726.

Flanagan, J. L., Simmons, P. A., Vehige, J., Willcox, M. D. & Garrett, Q. (2010). Role of carnitine in disease. *Nutr Metab* **7**, 30.

Fraenkel, G. & Friedman, S. (1957). Carnitine. *Vitam Horm* **15**, 73-118.

Fraser, C. M., Norris, S. J., Weinstock, G. M., White, O., Sutton, G. G., Dodson, R., Gwinn, M., Hickey, E. K., Clayton, R. & other authors (1998). Complete

www.manaraa.com

genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science* **281**, 375-388.

Fraser, K. R. & O'Byrne, C. P. (2002). Osmoprotection by carnitine in a *Listeria monocytogenes* mutant lacking the OpuC transporter: evidence for a low affinity carnitine uptake system. *FEMS Microbiol Lett* **211**, 189-194.

Fraser, K. R., Harvie, D., Coote, P. J. & O'Byrne, C. P. (2000). Identification and characterization of an ATP binding cassette L-carnitine transporter in *Listeria monocytogenes*. *Appl Environ Microbiol* **66**, 4696-4704.

Gahan, C. G. & Hill, C. (2014). *Listeria monocytogenes*: survival and adaptation in the gastrointestinal tract. C*ell infect Microbiol* **4**, 9.

Goldmann, A., Boivin, C., Fleury, V., Message, B., Lecoeur, L., Maille, M. & Tepfer, D. (1991). Betaine use by rhizosphere bacteria: genes essential for trigonelline, stachydrine, and carnitine catabolism in *Rhizobium meliloti* are located on pSym in the symbiotic region. *Mol Plant Microbe In* **4**, 571-578.

Gulewitsch, W. & Krimberg, R. (1905). Uber das Carnitin. *Hoppe-Seyler's Z Physiol Chem* **45**, 326-330.

Hanschmann, H. & Kleber, H. P. (1997). Purification and characterization of D(+)-carnitine dehydrogenase from *Agrobacterium* sp.-a new enzyme of carnitine metabolism. *Biochim Biophys Acta* **1337**, 133-142.

Hanschmann, H., Ehricht, R. & Kleber, H. P. (1996). Purification and properties of L(-)-carnitine dehydrogenase from *Agrobacterium* sp. *Biochim Biophys Acta* **1290**, 177-183.

Hartiala, J., Bennett, B. J., Tang, W. H., Wang, Z., Stewart, A. F., Roberts, R., McPherson, R., Lusis, A. J., Hazen, S. L. & other authors (2014). Comparative genome-wide association studies in mice and humans for trimethylamine N-oxide, a proatherogenic metabolite of choline and L-carnitine. *Arterioscler Thromb Vasc Biol* **34**, 1307-1313.

Hoffmann, T., Wensing, A., Brosius, M., Steil, L., Volker, U. & Bremer, E. (2013). Osmotic control of *opuA* expression in *Bacillus subtilis* and its modulation in response to intracellular glycine betaine and proline pools. *J Bacteriol* **195**, 510-522.

Hoffmann, T. & Bremer, E. (2011). Protection of *Bacillus subtilis* against cold stress via compatible-solute acquistion. *J. Bacteriol* **193,** 1552-1562.

Hung, K. & Kleber, H. P. (1985). Vorkommen und Regulation der carnitindehydrogenase *Pseudomonas*-species. *Wiss Z Karl-Marx-Univ Leipzig, Math-Nat R* **34**, 293-296.

Hwang, K. C. & Bang, W. (1997). Optimal resolution of L-carnitine from racemic DL-carnitine by *Enterobacter* sp. assimilating D-carnitine. *J Microbiol Biotechn* **7**, 318-322.

Ivanova, E. P., Gorshkova, N. M., Bowman, J. P., Lysenko, A. M., Zhukova, N. V., Sergeev, A. F., Mikhailov, V. V. & Nicolau, D. V. (2004). *Shewanella pacifica* sp. nov., a polyunsaturated fatty acid-producing bacterium isolated from sea water. *Int J Syst Evol Microbiol* **54**, 1083-1087.

www.manaraa.com

Jaenicke, R. (1990). Protein structure and function at low temperatures. *Philos t roy soc B* **326**, 535-551; discussion 551-533.

Jebbar, M., Champion, C., Blanco, C. & Bonnassie, S. (1998). Carnitine acts as a compatible solute in *Brevibacterium linens*. *Res Microbiol* **149**, 211-219.

Johri, A. M., Heyland, D. K., Hetu, M. F., Crawford, B. & Spence, J. D. (2014). Carnitine therapy for the treatment of metabolic syndrome and cardiovascular disease: Evidence and controversies. *Nutr Metab Cardiovasc Dis*.

Jung, H., Jung, K. & Kleber, H. P. (1989). Purification and properties of carnitine dehydratase from *Escherichia coli*-a new enzyme of carnitine metabolization. *Biochim Biophys Acta* **1003**, 270-276.

Jung, H., Jung, K. & Kleber, H. P. (1990). L-carnitine uptake by *Escherichia coli*. *J Basic Microbiol* **30**, 507-514.

Jung, H., Buchholz, M., Clausen, J., Nietschke, M., Revermann, A., Schmid, R. & Jung, K. (2002). CaiT of *Escherichia coli*, a new transporter catalyzing L-carnitine/gamma -butyrobetaine exchange. *J Biol Chem* **277**, 39251-39258.

Jung, H. & Kleber, H. P. (1991). Metabolism of D(+)carnitine by *Escherichia coli*. *Appl Microbiol Biotechnol* **35**, 191-395.

Jung, K., Jung, H. & Kleber, H. P. (1987). Regulation of L-carnitine metabolism in *Escherichia coli*. *J Basic Microbiol* **27**, 131-137.

Kakayama, K., Honda, H., Ogawa, Y., Ohta, T. & Ozawa, T. (1991). Method of producing carnitine. *US Patent* **5,041,375**.

Kalayil, S., Schulze, S. & Kuhlbrandt, W. (2013). Arginine oscillation explains Na+ independence in the substrate/product antiporter CaiT. *Proc Natl Acad Sci U S A* **110**, 17296-17301.

Kappes, R. M. & Bremer, E. (1998). Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and y-butyrobetaine via the ABC transport system OpuC. *Microbiol* **144**, 83-90.

Kelley, L. A. & Sternberg, M. J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* **4**, 363-371.

Kets, E. P. W., Galinski, E. A. & de Bont, J. A. M. (1994). Carnitine: a novel compatible in *Lactobacillus plantarum*. *Arch Microbiol* **162**, 243-248.

Klagsbrun, M. & Furano, A. V. (1975). Methylated amino acids in the proteins of bacterial and mammalian cells. *Arch Biochem Biophys* **169**, 529-539.

Kleber, H. P. (1997). Bacterial carnitine metabolism. *FEMS Microbiol Lett* **147**, 1-9.

Kleber, H. P. & Aurich, H. (1967). [Damped oscillations in the synthesis of carnitine dehydrogenase by *Pseudomonas aeruginosa*]. *Hoppe Seylers Z Physiol Chem* **348**, 1727-1729.

Kleber, H. P., Seim, H., Aurich, H. & Strack, E. (1978). [Interrelationships between carnitine metabolism and fatty acid assimilation in *Pseudomonas putida* (author's transl)]. *Arch Microbiol* **116**, 213-220.

Kleber, H. P., Schopp, W., Sorger, H., Tauchert, H. & Aurich, H. (1967). [Formation of 3-dehydrocarnitine from L-carnitine through the action of a *Pseudomonas aeruginosa* enzyme]. *Acta Biol Med Ger* **19**, 659-667.

www.manaraa.com

Kleber, H. P., Seim H., Aurich, H. & Strack, E. (1977). Verwertung von Trimethylammoniumverbindungen durch *Acinetobacter calcoaceticus*. *Arch Microbiol* **112**, 201-206.

Kluttermann, K., Tauchert, H. & Kleber, H. P. (2002). Synthesis of Poly-beta-hydroxybutyrate by *Agrobacterium radiobacter* after Growth on D-carnitine. *Acta biotechnol* **22**, 261-269.

Koeth, R. A., Wang, Z., Levison, B. S., Buffa, J. A., Org, E., Sheehy, B. T., Britt, E. B., Fu, X., Wu, Y. & other authors (2013). Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* **19**, 576-585.

Kuka, J., Liepinsh, E., Makrecka-Kuka, M., Liepins, J., Cirule, H., Gustina, D., Loza, E., Zharkova-Malkova, O., Grinberga, S. & other authors (2014). Suppression of intestinal microbiota-dependent production of pro-atherogenic trimethylamine N-oxide by shifting L-carnitine microbial degradation. *Life Sci* **117**, 84-92.

Kula, M. R., Joeres, U. & Stelkes-Ritter, U. (1996). New Microbial Amidases. *Ann N Y Acad Sci* **799**, 725-728.

Kula, M. R. & Joeres, U. (1993). L-carnitine amidase produced by a microorganism. *US Patent* **5,238,838**.

Kutscher, F. (1905). Zur Kenntnis des Novains. *Z Physiol Chem* **4947-49**.

Lindstedt, G. & Lindstedt, S. (1970). Cofactor requirements of gamma-butyrobetaine hydroxylase from rat liver. *J Biol Chem* **245**, 4178-4186.

Lindstedt, G., Lindstedt, S. & Tofft, M. (1970a). Gamma-butyrobetaine hydroxylase from *Pseudomonas* sp AK 1. *Biochemistry* **9**, 4336-4342.

Lindstedt, G., Lindstedt, S., Olander, B. & Tofft, M. (1968). Alpha-ketoglutarate and hydroxylation of gamma-butyrobetaine. *Biochim Biophys Acta* **158**, 503-505.

Lindstedt, G., Lindstedt, S., Midtvedt, T. & Tofft, M. (1970b). Inducible gamma-butyrobetaine-degrading enzymes in *Pseudomonas* species AK 1. *J Bacteriol* **101**, 1094-1095.

Lindstedt, G., Lindstedt, S., Midtvedt, T. & Tofft, M. (1967). The formation and degradation of carnitine in *Pseudomonas*. *Biochemistry* **6**, 1262-1270.

Lu, X., Zhang, P., Li, Q., Liu, H., Lin, X. & Ma, X. (2012). [Cloning, expression and characterization of a gamma-butyrobetaine hydroxylase gene *bbh* from *Pseudomonas* sp. L-1]. *Wei Sheng Wu Xue Bao* **52**, 602-610.

Lucchesi, G. I., Lisa, T. A., Casale, C. H. & Domenech, C. E. (1995). Carnitine resembles choline in the induction of cholinesterase, acid phosphatase, and phospholipase C and in its action as an osmoprotectant in *Pseudomonas aeruginosa*. *Curr Microbiol* **30**, 55-60.

Malek, A. A., Chen, C., Wargo, M. J., Beattie, G. A. & Hogan, D. A. (2011). Roles of three transporters, CbcXWV, BetT1, and BetT3, in *Pseudomonas aeruginosa* choline uptake for catabolism. *J Bacteriol* **193**, 3033-3041.

Marciani, P., Lindi, C., Marzo, A., Arrigoni Martelli, E., Cardace, G. & Esposito, G. (1991). L-carnitine and carnitine ester transport in the rat small intestine. *Pharmacol Res* **23**, 157-162.

www.manaraa.com

Meadows, J. A. & Wargo, M. J. (2013). Characterization of *Pseudomonas aeruginosa* growth on O-acylcarnitines and identification of a short-chain acylcarnitine hydrolase. *Appl Environ Microbiol* **79**, 3355-3363.

Miller, T. R., Hnilicka, K., Dziedzic, A., Desplats, P. & Belas, R. (2004). Chemotaxis of *Silicibacter* sp. strain TM1040 toward dinoflagellate products. *Appl Environ Microbiol* **70**, 4692-4701.

Miura-Fraboin, J., Kleber, H.P. & Englard, S. (1982). Assimilation of gamma-butyrobetaine, and D- and L-carnitine by resting cell suspensions of *Acinetobacter calcoaceticu*s and *Pseudomonas putida*. *Arch Microbiol* **133**, 217-221.

Monnich, K., Hanschmann, H. & Kleber, H. P. (1995). Utilization of D-carnitine by *Pseudomonas* sp. AK 1. *FEMS Microbiol Lett* **132**, 51-55.

Mori, N., Kasugai, T., Kitamoto, Y. & Ichikawa, Y. (1988). Purification and some properties ofcarnitine dehydrogenase from *Xanthomonas translucens*. *Agric Biol Chem* **52**, 249-250.

Naidu, G., Lee, I. Y., Cho, O. K. & Park, Y. H. (2001). Conversion of gamma-butyrobetaine to L-carnitine by *Achromobacter cycloclast*. *J Ind Microbiol Biotechnol* **26**, 309-315.

Nguyen, U. T., Wenderska, I. B., Chong, M. A., Koteva, K., Wright, G. D. & Burrows, L. L. (2012). Small-molecule modulators of *Listeria monocytogenes* biofilm development. *Appl Environ Microbiol* **78**, 1454-1465.

Nobile, S. & Deshusses, J. (1986). Transport of gamma-butyrobetaine in an *Agrobacterium* species isolated from soil. *J Bacteriol* **168**, 780-784.

Obon, J. M., Maiquez, J. R., Canovas, M., Kleber, H. P. & Iborra, J. L. (1999). High-density *Escherichia coli* cultures for continuous L(-)-carnitine production. *Appl Microbiol Biotechnol* **51**, 760-764.

Palmer, G. C., Jorth, P. A. & Whiteley, M. (2013). The role of two *Pseudomonas aeruginosa* anthranilate synthases in tryptophan and quorum signal production. *Microbiol* **159**, 959-969.

Park, S., Smith, L. T. & Smith, G. M. (1995). Role of glycine betaine and related osmolytes in osmotic stress adaptation in *Yersinia enterocolitica* ATCC 9610. *Appl Environ Microbiol* **61**, 4378-4381.

Preusser, A., Wagner, U., Elssner, T. & Kleber, H. P. (1999). Crotonobetaine reductase from *Escherichia coli* consists of two proteins. *Biochim Biophys Acta* **1431**, 166-178.

Privalov, P. L. & Gill, S. J. (1988). Stability of protein structure and hydrophobic interaction. *Adv Protein Chem* **39**, 191-234.

Rebouche, C. J. (2004). Kinetics, pharmacokinetics, and regulation of L-carnitine and acetyl-L-carnitine metabolism. *Ann N Y Acad Sci* **1033**, 30-41.

Rebouche, C. J. (2014). *Modern Nutrition in Health and Disease*, Eleventh edn.: Woldters Kluwer, Lippincott Williams and Wilkins.

Rebouche, C. J. & Chenard, C. A. (1991). Metabolic fate of dietary carnitine in human adults: identification and quantification of urinary and fecal metabolites. *J Nutr* **121**, 539-546.

www.manaraa.com

Rebouche, C. J. & Seim, H. (1998). Carnitine metabolism and its regulation in microorganisms and mammals. *Annu Rev Nutr* **18**, 39-61.

Reddy, J. K. & Hashimoto, T. (2001). Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annu Rev Nutr* **21**, 193-230.

Robert, H., Le Marrec, C., Blanco, C. & Jebbar, M. (2000). Glycine betaine, carnitine, and choline enhance salinity tolerance and prevent the accumulation of sodium to a level inhibiting growth of *Tetragenococcus halophila*. *Appl Environ Microbiol* **66**, 509-517.

Roth, S., Jung, K., Jung, H., Hommel, R. K. & Kleber, H. P. (1994). Crotonobetaine reductase from *Escherichia coli*--a new inducible enzyme of anaerobic metabolization of L(-)-carnitine. *Antonie van Leeuwenhoek* **65**, 63-69.

Rudolph, A. S., Crowe, J. H. & Crowe, L. M. (1986). Effects of three stabilizing agents-proline, betaine, and trehalose-on membrane phospholipids. *Arch Biochem Biophys* **245**, 134-143.

Ruetschi, U., Nordin, I., Odelhog, B., Jornvall, H. & Lindstedt, S. (1993). gamma-Butyrobetaine hydroxylase. Structural characterization of the *Pseudomonas* enzyme. *Eur J Biochem* **213**, 1075-1080.

Russell, R. M., Sharp, F. C., Rasko, D. A. & Sperandio, V. (2007). QseA and GrlR/GrlA regulation of the locus of enterocyte effacement genes in enterohemorrhagic *Escherichia coli*. *J Bacteriol* **189**, 5387-5392.

Ryser, E. T. & Marth, E. H. (2007). *Listeria*, listeriosis, and food safety, Third edn.: CRC Press, Taylor and Francis Group, LLC.

Saier Jr, M. H. & Paulsen, I. T. (2000). Whole genome analyses of transporters in spirochetes: *Borrelia burgdorferi* and *Treponema pallidum*. *J Mol Microb Biotech* **2**, 393-399.

Schiefner, A., Breed, J., Bosser, L., Kneip, S., Gade, J., Holtmann, G., Diederichs, K., Welte, W. & Bremer, E. (2004). Cation-pi interactions as determinants for binding of the compatible solutes glycine betaine and proline betaine by the periplasmic ligand-binding protein ProX from *Escherichia coli*. *J Biol Chem* **279**, 5588-5596.

Schulze, S., Koster, S., Geldmacher, U., Terwisscha van Scheltinga, A. C. & Kuhlbrandt, W. (2010). Structural basis of Na(+)-independent and cooperative substrate/product antiport in CaiT. *Nature* **467**, 233-236.

Seim, H., Loster, H., Claus, R., Kleber, H. P. & Strack, E. (1982a). Stimulation of the anaerobic growth of *Salmonella typhimurium* by reduction of L-carnitine, carnitine derivatives and structure-related trimethylammonium compounds. *Arch Microbiol* **132**, 91-95.

Seim, H., Loster, H., Claus, R., Kleber, H. P. & Strack, E. (1982b). Formation of gamma-butryobetaine and trimethylamine from quaternary ammonium compounds structure-related to L-carnitine and choline by *Proteus vularis*. *FEMS Microbiol Lett* **13**, 201-205.

www.manaraa.com

Seim H., Loster, H. & Kleber, H. P. (1982c). Reduktiver stoffwechsel des L-carnitins and strukturverwandter trimethylammoniumverbindugen in *Escherichia coli*. *Acta Biol Med Ger* **41**, 1009-1019.

Seymour, J. R., Simo, R., Ahmed, T. & Stocker, R. (2010). Chemoattraction to dimethylsulfoniopropionate throughout the marine microbial food web. *Science* **329**, 342-345.

Sikorski, J., Stackebrandt, E. & Wackernagel, W. (2001). *Pseudomonas kilonensis* sp. nov., a bacterium isolated from agricultural soil. *Int J Syst Evol Microbiol* **51**, 1549-1555.

Sleator, R. D. & Hill, C. (2010). Compatible solutes: the key to *Listeria*'s success as a versatile gastrointestinal pathogen? *Gut Pathog* **2**, 20.

Sleator, R. D., Banville, N. & Hill, C. (2009). Carnitine enhances the growth of *Listeria monocytogenes* in infant formula at 7 degrees C. *J Food Prot* **72**, 1293-1295.

Sleator, R. D., Wouters, J., Gahan, C. G., Abee, T. & Hill, C. (2001). Analysis of the role of OpuC, an osmolyte transport system, in salt tolerance and virulence potential of *Listeria monocytogenes*. *Appl Environ Microbiol* **67**, 2692-2698.

Sleator, R. D., Francis, G. A., O'Beirne, D., Gahan, C. G. & Hill, C. (2003). Betaine and carnitine uptake systems in *Listeria monocytogenes* affect growth and survival in foods and during infection. *J Appl Microbiol* **95**, 839-846.

Smajs, D., McKevitt, M., Howell, J. K., Norris, S. J., Cai, W. W., Palzkill, T. & Weinstock, G. M. (2005). Transcriptome of *Treponema pallidum*: gene expression profile during experimental rabbit infection. *J Bacteriol* **187**, 1866-1874.

Smiddy, M., Sleator, R. D., Patterson, M. F., Hill, C. & Kelly, A. L. (2004). Role for compatible solutes glycine betaine and L-carnitine in listerial barotolerance. *Appl Environ Microbiol* **70**, 7555-7557.

Steiber, A., Kerner, J. & Hoppel, C. L. (2004). Carnitine: a nutritional, biosynthetic, and functional perspective. *Mol Aspects Med* **25**, 455-473.

Stocker, R. & Seymour, J. R. (2012). Ecology and physics of bacterial chemotaxis in the ocean. *Microbiol Mol Biol R* **76**, 792-812.

Strom, A. R., Olafsen, J. A. & Larsen, H. (1979). Trimethylamine oxide: a terminal electron acceptor in anaerobic respiration of bacteria. *J Gen Microbiol* **112**, 315-320.

Takahashi, M. & Ueda, S. (1995). Method of assaying for acyl-L- carnitine and short-chain acyl-carnitine. *US Patent* **5,385,829**.

Tang, L., Bai, L., Wang, W. H. & Jiang, T. (2010). Crystal structure of the carnitine transporter and insights into the antiport mechanism. *Nat Struct Mol Biol* **17**, 492-496.

Tars, K., Leitans, J., Kazaks, A., Zelencova, D., Liepinsh, E., Kuka, J., Makrecka, M., Lola, D., Andrianovs, V. & other authors (2014). Targeting carnitine biosynthesis: discovery of new inhibitors against gamma-butyrobetaine hydroxylase. *J Med Chem* **57**, 2213-2236.

www.manaraa.com

Uanschou, C., Frieht, R. & Pittner, F. (2005). What to learn from comparative genomic sequence analysis of L-carnitine dehydrogenase. *Monatshefte fur Chemie* **136**, 1365-1381.

Ulrich, J. & Kula, M. R. (1994). Purification and characterisation of a microbial L-carnitine amidase. *Appl Microbiol Biotechnol* **40**, 606-610.

Unemoto, T., Hayashi, M., Miyaki, K. & Hayashi, M. (1966). Formation of trimethylamine from DL-carnitine by *Serratia marcescens*. *Biochim Biophys Acta* **121**, 220-222.

Ussher, J. R., Lopaschuk, G. D. & Arduini, A. (2013). Gut microbiota metabolism of L-carnitine and cardiovascular risk. *Atherosclerosis* **231**, 456-461.

Vaz, F. M. & Wanders, R. J. (2002). Carnitine biosynthesis in mammals. *Biochem J* **361**, 417-429.

Verheul, A., Rombouts, F. M., Beumer, R. R. & Abee, T. (1995). An ATP-dependent L-carnitine transporter in *Listeria monocytogenes* Scott A is involved in osmoprotection. *J Bacteriol* **177**, 3205-3212.

Verheul, A., Glaasker, E., Poolman, B. & Abee, T. (1997). Betaine and L-carnitine transport by *Listeria monocytogenes* Scott A in response to osmotic signals. *J Bacteriol* **179**, 6979-6985.

Verheul, A., Wouters, J. A., Rombouts, F. M. & Abee, T. (1998). A possible role of ProP, ProU and CaiT in osmoprotection of *Escherichia coli* by carnitine. *J Appl Microbiol* **85**, 1036-1046.

Vilhelmsson, O. & Miller, K. J. (2002). Humectant permeability influences growth and compatible solute uptake by *Staphylococcus aureus* subjected to osmotic stress. *J Food Prot* **65**, 1008-1015.

Walt, A. & Kahn, M. L. (2002). The *fixA* and *fixB* genes are necessary for anaerobic carnitine reduction in *Escherichia coli*. *J Bacteriol* **184**, 4044-4047.

Wanders, R. J. & Waterham, H. R. (2006). Biochemistry of mammalian peroxisomes revisited. *Annu Rev Biochem* **75**, 295-332.

Wargo, M. J. (2013). Homeostasis and catabolism of choline and glycine betaine: lessons from *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **79**, 2112-2120.

Wargo, M. J. & Hogan, D. A. (2009). Identification of genes required for *Pseudomonas aeruginosa* carnitine catabolism. *Microbiol* **155**, 2411-2419.

Wargo, M. J., Szwergold, B. S. & Hogan, D. A. (2008). Identification of two gene clusters and a transcriptional regulator required for *Pseudomonas aeruginosa* glycine betaine catabolism. *J Bacteriol* **190**, 2690-2699.

Warren, C. R. (2013a). High diversity of small organic N observed in soil water. *Soil Biol Biochem* **57**, 444-450.

Warren, C. R. (2013b). Quaternary ammonium compounds can be abundant in some soils and are taken up as intact molecules by plants. *New Phytol* **198**, 476-485.

Watson, D., Sleator, R. D., Casey, P. G., Hill, C. & Gahan, C. G. (2009). Specific osmolyte transporters mediate bile tolerance in *Listeria monocytogenes*. *Infect Immun* **77**, 4895-4904.

www.manaraa.com

Welsh, D. T. (2000). Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. *FEMS Microbiol Rev* **24**, 263-290.

Wemekamp-Kamphuis, H. H., Sleator, R. D., Wouters, J. A., Hill, C. & Abee, T. (2004). Molecular and physiological analysis of the role of osmolyte transporters BetL, Gbu, and OpuC in growth of *Listeria monocytogenes* at low temperatures. *Appl Environ Microbiol* **70**, 2912-2918.

Wemekamp-Kamphuis, H. H., Wouters, J. A., Sleator, R. D., Gahan, C. G., Hill, C. & Abee, T. (2002). Multiple deletions of the osmolyte transporters BetL, Gbu, and OpuC of *Listeria monocytogenes* affect virulence and growth at high osmolarity. *Appl Environ Microbiol* **68**, 4710-4716.

Yamamoto, K., Hirao, K., Oshima, T., Aiba, H., Utsumi, R. & Ishihama, A. (2005). Functional characterization in vitro of all two-component signal transduction systems from *Escherichia coli*. *J Biol Chem* **280**, 1448-1456.

Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *The Journal of experimental biology* **208**, 2819-2830.

Zhang, L., Xu, Z. & Patel, B. K. (2007). *Bacillus decisifrondis* sp. nov., isolated from soil underlying decaying leaf foliage. *Int J Syst Evol Microbiol* **57**, 974-978.

Zhu, Y., Jameson, E., Crosatti, M., Schafer, H., Rajakumar, K., Bugg, T. D. & Chen, Y. (2014). Carnitine metabolism to trimethylamine by an unusual Rieske-type oxygenase from human microbiota. *Proc Natl Acad Sci U S A* **111**, 4268-4273.

Ziegler, C., Bremer, E. & Kramer, R. (2010). The BCCT family of carriers: from physiology to crystal structure. *Mol Microbiol* 78, 13-34.

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1.3 Catabolism of host-compounds during extracellular bacterial infections

1.3.1 Abstract

Efficient catabolism of host-derived compounds is essential for bacterial survival and virulence. While these links in intracellular bacteria are well-studied, such studies in extracellular bacteria lag behind, mostly for technical reasons. The field has identified important metabolic pathways, but the mechanisms by which they impact infection and in particular, establishing the importance of a compound's catabolism versus alternate metabolic roles has been difficult. In this review we will examine evidence for catabolism during extracellular bacterial infections in animals and known or potential roles in virulence. In the process, we point out key gaps in the field that will require new or newly adapted techniques.

1.3.2 Introduction

To successfully cause infection, a bacterium must incorporate and metabolize molecules derived from the host. General maintenance and expression of virulence factors are minimal requirements, while efficient catabolism and resultant growth can lead to successful colonization and improved evasion of immune-mediated clearance. Here we will use the strict definition of catabolism: degradation of a compound yielding energy [Russell and Cook, 1995]. When we view bacterial catabolism in this way, the most straightforward analogy is to view the host as the growth medium [Brown et al., 2008]. This paradigm, while it's been around for 135 years and has seen intermittent resurgence [Garber, 1960; Pasteur, 1878], has seen many contributions within the last

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two decades. Armed with the powerful techniques of modern bacteriology, solving the problems of in vivo bacterial catabolism, growth, and resultant virulence should be easy, right?

In one sense the answer is, undoubtedly, yes. In the case of intracellular pathogens, the eukaryotic host cells generate the correct 'growth medium' in the infected compartment. Experiments in tissue culture, presuming the correct cell type and growth condition, can very closely mimic the experience of the bacteria in a similar cell within the whole organism. These in vitro systems benefit tremendously from a few handy simplifications: (i) one or a few host cell types, (ii) high bacteria to host cell ratio, and (iii) facile monitoring and manipulation of both sides of the interaction. For many intracellular pathogens, their tractability has led to a reasonably good understanding of in vivo catabolism and its connection to virulence, particularly for *Legionella*, *Listeria*, *Salmonella*, and *Mycobacterium*, which have been well reviewed previously [Eisenreich et al., 2010] and will not be repeated here. Instead, this review focuses on extracellular bacterial pathogens; the little that is known, the vast areas that are not, and some thoughts about how to address the current unknowns. Our principle goal is to discuss what is known about the catabolic pathways governing in vivo growth and survival.

For extracellular bacteria, the primary technical issue can be boiled down to one thing: not knowing the growth medium. What looks to be straightforward turns out to be fraught with problems. We have certainly made important headway by using overly-simplified models, but we are left with a wide gap between these models and reality. We lose the dynamic, homeostatic nature of the extracellular niche when we remove this

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niche from the organism or reconstitute it from parts [Smith, 2000]. Such in vitro characterizations of the extracellular milieu lack dynamics, while in vivo experiments are plagued with the problems readily overcome when studying intracellular pathogens: (i) numerous host cell types, tissues, and organs compared to a specific microenvironment within a specific cell type; (ii) overwhelming number of host cells to bacterial invaders; and (iii) poor spatial and temporal resolution of goings-on within the infection ([Smith, 2000] provides a thought provoking analysis of conceptual and technical problems associated with the study of catabolism in vivo). These issues do not make identifying important metabolic pathways overly difficult, rather they make dissecting the specific mechanism by which an implicated pathway alters in vivo growth, catabolism, and virulence incredibly hard. To bridge this gap in knowledge we need new techniques, greater incorporation of techniques from other fields, and a push towards mechanistic understanding of the importance of in vivo catabolism and specific catabolic pathways.

1.3.3. Assessing catabolism vs. alternate metabolic usage

Catabolism has been extensively studied in vitro, where chemostats, microcalorimeters, and oxygen utilization measures have helped biochemists and microbiologists decipher the multiple roles of metabolized compounds during growth. Even within these in vitro systems, determining a compound's actual utilization requires both growth measures and flux analyses to extract information about the role of a compound in energy production versus growth yield [Russell and Cook, 1995]. Overlaid onto the challenges of studying catabolism in vivo is the issue that many important

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compounds have multiple roles within the cell. Amino acids can be catabolized as an energy source, but they also participate in protein synthesis, are interconverted to other amino acids, and can have other functions: proline is a useful osmoprotectant [Csonka and Hanson, 1991]; serine is used to synthesize phosphatidylserine and as part of the C1 acquisition cycle [Anthony, 2011; Geiger et al., 2010]; tryptophan feeds into formation of many secondary metabolites including virulence-related molecules like PQS produced by *P. aeruginosa* [Farrow and Pesci, 2007], etc. Amino acids are not alone in having multiple functions: host-derived carbohydrates can often be used to decorate pathogen surfaces and incorporated into exopolysaccharides, while lipids can impact efflux mechanisms and envelope stress. In this review, we have tried to catalogue the strength of evidence for catabolism itself versus alternate usage of host-derived compounds. We will examine major classes of compounds in the following subsections, which are summarized in Table 1.

1.3.4. Carbohydrates

Colonization of mucosal surfaces is a key step in pathogenesis for many opportunistic pathogens. The mucosal surface is dominated by mucins, large glycoproteins that coat mucosal surfaces to trap particles and infectious agents [Thornton et al., 2008]. Mucins are enriched in sialic acid, an anionic nine-carbon carbohydrate [Vimr et al., 2004], thus it is not surprising that many mucosal pathogens have acquired pathways for sialic acid utilization. Some of the best evidence for catabolism of carbohydrates as a major source of energy is utilization of sialic acid by intestinal

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pathogens. Pathogenic species of *Vibrio* can be transmitted by the ingestion of contaminated food or water and subsequently colonize the host intestine. *V. cholerae* causes acute diarrheal disease and *V. vulnificus* results in gastroenteritis that can lead to septicemia in immunocompromised individuals. *V. vulnificus* carries the nan genes for sialic acid degradation on its core chromosome, whereas *V. cholerae* strains have the nan genes on the Vibrio Pathogenicity Island 2 (VPI-2) [Almagro-Moreno and Boyd, 2009; Jeong et al., 2009]. In vitro, deletion of the nan genes in both species results in the inability to utilize sialic acid as a sole carbon and energy source and in vivo studies demonstrate colonization and survival defects in the intestine of infected mice [Almagro-Moreno and Boyd, 2009; Jeong et al., 2009]. In vitro growth assays for both species reveal no toxic effects from sialic acid accumulation intracellularly or in the growth medium due to this defective catabolism, suggesting that the observed growth defects in vitro correlate with the colonization defect in the intestine. Jeong et al. postulate that sialic acid catabolism contributes to pathogenesis by contributing directly to multiplication (energy production and growth yield) and Almagro-Moreno et al. point out that it may serve as a carbon source in times of high competition when colonizing the intestine. Both groups concur that sialic acid catabolism as an energy source ensures survival during infection [Almagro-Moreno and Boyd, 2009; Jeong et al., 2009].

As with many potential catabolic substrates, sialic acid has other uses for some pathogens that can cloud the interpretation of in vivo studies. Sialic acid can be used to sialate lipids or exopolysaccharides as a means of immune evasion by bacterial pathogens [Vimr et al., 2004]. Deletions in genes encoding sialidases or sialic acid transporters have

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been shown to cause colonization and survival defects in non-typeable *Haemophilus influenzae*, the major causative agents of middle ear infections [Jurcisek et al., 2005], as well as respiratory tract infections by *Streptococcus pneumoniae* [Marion et al., 2011]. In these studies, while sialic acid utilization as a catabolic substrate is possible, alterations in sialylation are also a potential mechanism. In addition, it is critical to separate catabolism from incorporation. It is possible that while sialic acid is broken down as an energy source by some of these pathogens, its importance may be for supplying carbohydrate substrates for sugar-intensive biosynthetic processes like capsule synthesis or biofilm formation. Degradation of sialic acid requires an initial investment of ATP and produces acetate and ammonia during partial metabolism even prior to the catabolic steps that would lead to energy production [Vimr et al., 2004]. The resultant ammonia could function as a nitrogen source, while the acetate may be important in a more global sense because acetate, and its co-regulated acetyl-CoA and acetate phosphate pools, impact lysine acetylation [Verdin and Ott, 2013]. Lysine acetylation has been implicated in global regulation of central metabolism and multiple catabolic pathways [Wang et al., 2010]. Consequently, such metabolic byproducts can impact catabolism of other substrates indirectly.

The carbohydrates deoxyribose and fucose have been implicated as catabolic substrates for pathogenic strains of *Escherichia coli*. *E. coli* is a Gram negative bacterium that is a normal inhabitant of the human intestinal flora. Pathogenic strains can be transmitted through fecal-oral or fecal-ureter route where they can colonize the intestine or the urinary tract resulting in extreme diarrhea or urinary tract infections, respectively.

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For deoxyribose, many pathogenic *E. coli* contain the *deoK* operon encoding proteins for deoxyribose catabolism, allowing these organisms to use deoxyribose as a carbon source [Bernier-Febreau et al., 2004; Jonsen et al., 1959]. Strains incapable of deoxyribose catabolism have defects in intestinal colonization and are outcompeted by those strains capable of deoxyribose catabolism [Martinez-Jehanne et al., 2009]. It is known that deoxyribose is present in the intestinal mucus, the colonization defect of the *deoK* mutant is clear, and the presence of the *deoK* gene on one of the pathogenicity islands underlines its contribution to virulence, however, the evidence for direct catabolism is not strong.

It is well documented that the gut is populated with a variety of bacteria and that metabolite sharing is a common phenomenon. Freter's nutrient theory states that in order for invading bacteria to compete and thrive in the intestine it must be able to more efficiently utilize a limiting nutrient better than resident bacteria [Freter et al., 1983]. Although *E. coli* can utilize fucose as a carbon source [Hacking and Lin, 1976], they do not contain genes encoding fucosidases [Hoskins et al., 1985], which cleave fucose from glycans. But by being in close proximity with the gut commensal *Bacteroidetes thetaiotamicron* and other strains, *E. coli* can capitalize on *B. thetaiotamicron*'s fucose cleavage by importing and catabolizing released fucose [Salyers et al., 1977; Xu et al., 2003]. In a bovine rectal colonization model, wild type *E. coli* O157:H7 out-competed a fucose catabolic mutant, *fucAO* (fucose aldolse and oxido-reductase respectively) exhibiting the importance of fucose utilization for *E. coli* colonization and maintenance of the population [Snider et al., 2009].

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E. coli in vitro gene expression studies showed that genes involved in catabolism of gluconate and other sugars were highly induced when cells were grown in intestine-like conditions, 50% mouse cecal mucus. Gluconate was shown to be the preferred nutrient in vitro through mutation in edd, which encodes a 6-phosphogluconate dehydratase, which catalyzes a key step in gluconate entry into the Entner-Doudoroff pathway. In a mouse cocolonization model, the edd mutant had a defect in initiation and maintenance of intestinal colonization [Chang et al., 2004]. The gluconate catabolism was also shown to be important for intestinal colonization of *V. cholerae*, as the *edd* mutant failed to colonize the intestine in an infant mouse model [Patra et al., 2012].

1.3.5. Amino Acids

We previously remarked on the pleiotropic roles of amino acids within the cell. This integration with the whole metabolome often makes concrete conclusions about amino acid catabolism difficult. The best evidence for catabolic utilization comes from *Campylobacter jejuni*, for which serine is a preferred carbon and energy source [Leach et al., 1997]. *C. jejuni* is commensal to avian species but is spread to humans through the consumption of contaminated food and causes acute gastroenteritis. Mutations in L-serine deaminase (*sdaA*), which converts serine to pyruvate, lead to major *C. jejuni* colonization and survival defects in both the avian and mouse intestines, and the mouse liver [Hofreuter et al., 2012; Velayudhan et al., 2004]. Likewise, in uropathogenic *E. coli* (UPEC), L-serine can also be catabolized as a sole carbon and energy source [Su and Newman, 1991]. For UPEC, L-serine utilization is important for colonization of the

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bladder, as an sdaAsdaB double mutant was defective in colonization of the mouse bladder [Anfora et al., 2007]. The reaction catalyzed by serine deaminase directly produces pyruvate, strongly suggesting serine as a catabolic substrate. However, pyruvate is also a central player in anabolic reactions and while separable in vitro, in vivo attribution of mechanism is difficult. Also, given the additional product of the reaction, ammonia, the role of L-serine as a nitrogen source in these infection models cannot be wholly discounted.

As mentioned above, *C. jejuni* has a propensity to utilize amino acids within the intestines of mice and birds, rather than the dietary and mucin-derived carbohydrates that are often preferred by other intestinal pathogens [Velayudhan and Kelly, 2002]. Therefore, it is not surprising that *C. jejuni* genes responsible for amino acid catabolism, including aspartate and proline, have also been shown to be important in intestinal colonization [Guccione et al., 2008; Hofreuter et al., 2012]. Proline is also important during Staphylococcus aureus burn infections and abscess formation [Schwan et al., 1998]. In these studies, it is not easy to ascertain the role being played by proline, as the gene disruptions were made in the proline transporter. Proline can be transported into the cell and accumulated in the cytosol, where it can function as an osmoprotectant. Accumulation of various host-derived osmoprotectants is important for a variety of infectious agents and infection sites [Sleator et al., 2001], therefore it is likely proline plays this role for S. aureus, though catabolism has not been ruled out.

Mekalanos' group took a top down approach when it came to identifying factors that contribute to the virulence of *V. cholerae*. Using a suckling mouse intestine model,

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they paired individual LysR-type transcriptional regulator mutants with the parental strain and tested for in vivo fitness of the mutant compared to the wild type [Bogard et al., 2012]. Of the 38 LysR-family mutants tested, two mutants were impaired in colonizing the intestine, one of which was *metR*, the methionine biosynthesis regulator that also has a role in catabolism [Bogard et al., 2012]. Two MetR controlled genes that are particularly important for mouse intestine colonization are metJ which encodes a methionine repressor and *glyA1* which encodes serine hydroxymethyltransferase [Bogard et al., 2012]. GlyA1 is potentially interesting, as it controls the flux of glycine to pyruvate via serine, but also glycine biosynthesis via serine. Based on the evidence from *C. jejuni* infections mentioned previously, serine may be abundant in the intestine and could play a role in either the catabolic or anabolic reactions; particularly the later if glycine or other glycine precursors are limiting.

The concentrations of host-derived metabolites are substantially altered during bacterial infection, particularly near the site of infection [Beisel, 1975; Smith, 2000]. A dramatic case of such metabolic change occurs during chronic lung infections of people with the genetic disorder cystic fibrosis by the opportunistic pathogen *Pseudomonas aeruginosa* [Burns et al., 1998]. During these chronic infections, tissue damage, immune response, and bacterial activities sculpt the mileu, resulting in thick mucus plugs in airways that can be expectorated as sputum [Boucher, 2004]. *P. aeruginosa* catabolizes many amino acids within sputum from cystic fibrosis patients in vitro, but displays a preference for alanine [Palmer et al., 2007]. The importance of alanine catabolism was demonstrated in a chronic rat lung infection model, showing that *P. aeruginosa* mutants

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incapable of converting alanine to pyruvate could not compete well against wild-type *P. aeruginosa* [Boulette et al., 2009]. Similar to the cases of serine catabolism in *C. jejuni* and UPEC, this is a deamination in *P. aeruginosa*, resulting in the formation of ammonia along with pyruvate. Therefore, while loss of alanine catabolism is among the likely mechanisms driving the mutant phenotype, the impact of pyruvate-dependent anabolic pathways and the role of the released ammonia need to be considered.

1.3.6. Other compounds

Glycerol can be phosphorylated and incorporated into glycolysis and is also a critical contributor to phospholipid synthesis. In *Borrelia burgdorferi*, the causative agent of Lyme Disease, deletion of the glycerol catabolism gene *glpD*, which converts glycerol to dihdroxyacetone phosphate (DHAP), results in clear replication defects within the adult tick vector and nymph stage, while the deletion did not have any effect on growth or survival within the mouse model of infection [He et al., 2011; Pappas et al., 2011]. Deletion of *glpD* with the rest of the *glpDFK* operon resulted in a robust growth defect within the tick [He et al., 2011], likely because deletion of the entire operon limits glycerol incorporation into phospholipids in addition to elimination of a shunt of glycerol into glycolysis [Pappas et al., 2011].

L-lactate can be the sole energy source and is the preferred carbon source for the opportunistic dental and endocardial pathogen *Aggregatibacter actinomycetemcomitans* (Aa), even in the presence of high-yield compounds such as glucose or fructose [Brown and Whiteley, 2009]. Like many bacteria, *Aa* exists in multispecies communities and is

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often found in conjunction with oral streptococci, which produce L-lactate as a catabolic product. *Streptococcus gordonii* and an *Aa lctD* (NAD independent L-lactate dehydrogenase) mutant co-inoculated into a mouse thigh abscess model was used to show that lactate catabolism in *Aa* is important in vivo for establishing polymicrobial infections [Ramsey et al., 2011].

The conversion of pyruvate to lactate by lactate dehydrogenase (ldh) is also an important part of NAD+ regeneration in fermentative systems, which allows the continued operation of glycolysis. An example can be found in *Enterococcus faecalis*, a pathogen that can cause endocarditis, bacteremia, urinary tract infections and meningitis. *E. faecalis* has two *ldh* genes and when both are deleted there is a defect in colonization and persistence of the bacterium in an intravenous mouse model infection in the liver and kidney [Rana et al., 2013]. While NAD+ also functions in other metabolic processes, the flux required by glycolysis suggests that the *ldh* activity in *E. faecalis* plays its main role in enabling robust glycolysis.

1.3.7. The importance of catabolism and its link to virulence

Catabolism of host-derived compounds is necessary for successful infection. These compounds may be derived directly from the host or by scavenging compounds released by other pathogenic or commensal bacteria [Freter et al., 1983]. The studies described above have determined metabolic pathways important for bacterial survival and growth within the host, but in most cases, direct evidence for catabolism contributing to the in vivo phenotype is limited (well described in [Vimr, 2013]). From the most human-

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centric standpoint, the exact mechanism is not critical to drive therapeutic development – efficacy and safety trump mechanism. However, we think that understanding the direct mechanism may lead to better future therapies by suggesting synergistic targets to inhibit with the appropriate drug cocktail. For example, if serine was truly a major energy source for a pathogen, combining the serine pathway inhibitor with an inhibitor of the next most important energy source would likely boost efficacy. However, if the importance of serine is to provide a ready supply of glycine or contribution to C1 or C2 biosynthetic units, a better strategy would be to target alternate pathways that produce the limiting intermediate. Therefore, a more complete understanding of the nature of the metabolic deficiency during infection will benefit future antimicrobial therapeutic development.

Determining the link between catabolism and virulence can be difficult. It is obvious that eliminating catabolism of host-derived compounds decreases the pathogenicity of the bacterial population. On the other hand, microbiologists often look at virulence in terms of production of anti-host products termed virulence factors, while catabolic pathways important for survival would fall under the term virulence determinant.

Under the heading of virulence determinant, we can certainly include all of the metabolic pathways discussed in this review. Each genetic mutation reduced the survival or growth of the bacteria and thus contributed to virulence in the model discussed. While virulence determinants can work indirectly, all of the bacterial species mentioned here also produce secreted virulence factors. Most of these are transported through the general or type III secretion systems and target host cells or specific pathways to boost

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pathogenesis by increasing host damage and decreasing immune clearance. In the simplest sense, producing virulence factors requires energy and therefore are dependent on catabolic pathways. However, it is interesting to note two things. First, secreted proteins are generally less energy intensive than membrane or cytosolic proteins [Smith and Chapman, 2010] and consequently metabolically cheaper. Second, secreted virulence factors are a good investment during nutrient stress, as many described virulence factors, particularly extracellular enzymes transported by the general secretion system, can also be thought of as nutrient requisition systems [Rohmer et al., 2011], providing amino acids (proteases), sugars (glycosidases), nucleic acids (nucleases), and lipids, glycerol, and polar headgroups (lipases). The direct regulatory links between specific catabolism and virulence factor production are less readily apparent. However, it is very clear that, globally, the cell's nutrient and energy status strongly impacts virulence factor production, reviewed in [Poncet et al., 2009].

1.3.8. Methods: current and a call for renewed and newly-adapted techniques

Most studies of bacterial catabolism have not started as such. With some exceptions described here, catabolic pathways have often been identified during medium or high-throughput screens to identify mutants with altered in vivo survival. Moderate throughput has generally relied on direct counts of surviving colonies, often in competition with wild type cells. High-throughput techniques that have been particularly successful are in vivo expression technology (IVET) [Slauch et al., 1994], signature-tagged mutagenesis (STM) [Lehoux and Levesque, 2000], and transposon insertion

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sequencing (TnSeq) [van Opijnen et al., 2009], all of which offer ways to assess large numbers of mutants simultaneously during infection. While generally useful, there are caveats associated with these methods of identification of catabolic pathways. One complication is that there are a variety of available carbon sources in a host – many at very high concentrations – so the contribution of any one carbon source may be relatively small, particularly for catabolically versatile bacteria.

Direct labeling: In the very simplest sense, we want to identify what compounds bacteria are eating in the host to provide them with energy, carbon, and nitrogen. There are direct and indirect ways to do this. A classic and still very useful method is to track the fate of radiolabeled substrates. While simple in vitro, many technical issues crop up in vivo. In general, a substrate is fed or injected into the animal and conversion of the substrate into radiolabeled CO2, acetate, or some other catabolic product is monitored. By comparing to an uninfected animal one can determine the proportion of compound utilized during infection. However, practically this is made very difficult for two reasons. First, host metabolism changes during infection, alterations due to bacterial processes are difficult to separate from changes due to the way the host changes its metabolism to fight the pathogen [Beisel, 1975]. Second, there is the gut microbiota, which by sheer numbers, provide a huge catabolic reservoir to convert substrates of interest. This leaves direct labeling useful mainly in two cases: gnotobiotic mice and compartmentalized substrates. While the former eliminates or reduces issues with gut metabolism, the later takes advantage of the fact that many compartments and organs maintain stable pools of compounds that are not rapidly converted or moved from the compartment. Examples

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include particular proteoglycans in the joints, certain glycosphingolipids in the brain, and choline in the lung and brain. Other compounds that can be studied with this technique are those that are not catabolized by the host, such as L-carnitine, which while utilized in the carnitine/acylcarnitine shuttle system, is not catabolized by host cells [Peluso et al., 2000], although its anaerobic breakdown by the gut flora shows important links to cardiovascular health [Koeth et al., 2013].

Stable isotope probing: Stable isotope probing has been exceptionally useful for the study of catabolic processes in microbial ecology [Neufeld et al., 2007]. Briefly, an organism catabolizing a heavy-isotope substrate will incorporate the isotope into their DNA and the DNA of an otherwise 'light' organism will shift towards 'heavy'. This shift is assessed with cesium gradient ultracentrifugation followed by fractionation. This technique falls victim to the same issues as direct radiolabeling, but the cesium gradient method allows discrimination of the source of the DNA by specific nucleic acid hybridization techniques. This technique, however, directly assesses the incorporation of label into DNA, an anabolic process. While these building blocks can be derived from catabolic end products and siphoned from catabolic intermediates, it is not a direct marker of catabolism.

Using transcriptional evidence to understand catabolism: One of the best ways to understand the range of possible food choices for the bacteria (but not their relative importance) is to look at the genes whose expression is induced in response to the host. As with the rest of bacterial catabolism in the host, the intracellular pathogens have yielded much more compelling transcriptional stories than their extracellular counterparts

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at the site of infection. For extracellular pathogens, the staggeringly abundant host RNA has generally made sensitive measurement of the bacterial transcriptome difficult. There are some exceptions, where bacterial abundance or localized infections have allowed ex vivo transcriptomics [Bielecki et al., 2008; Chaffin et al., 2012; Larocque et al., 2005], although this field is changing rapidly with many host RNA subtraction techniques and massively parallel sequencing. Therefore, many host-pathogen systems are now sufficiently sensitive to determine the transcriptome of the bacterial population during infection.

A particularly useful application of these techniques would be to couple transcriptomics and metabolomics during infection in order to determine what compounds the bacteria senses and simultaneously measure the fate of the related metabolites. In most infection sites, the bacteria may not be able to exceed host homeostasis for many metabolites, therefore simultaneous analysis of the local transcriptome of the host may help determine compounds that the host senses it must make more of, essentially responding to a small decrease by making or transporting more of a given compound.

While global transcriptomics has its place, often we are asking questions about specific pathways or genes. In these cases, sensitive and quantifiable reporters are critical tools for analysis. The current generation of reporter genes works well for microscopic evaluation (fluorescent proteins) and high levels of expression (lux and lacZ). While these are both quantifiable under optimal conditions, a better proposition might be to use the power of reporter genes in combination with the sensitivity of radiodetection. One

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potential method we have been discussing is to use the SNAP and CLIP proteins (NEB) as reporters, with increasing the sensitivity and specificity by labeling with radioactive substrates instead of fluorescent or biotin-linked substrates. A strain carrying one of the reporters as a translational fusion to the gene of interest and the other to a control gene would allow relative expression values to be sensitively determined by SDS-PAGE of the labeled tissue homogenate followed by autoradiography.

1.3.9. Conclusions

Bacterial nutrition is a critical component of understanding infection and developing effective treatments. Part of this nutrition is utilization of compounds as energy sources, i.e. catabolism. Here we have provided some glimpses of specific compounds used by pathogens, but we're confident that the current work only scratches the surface of important host-derived compounds. The challenge for the field is to generate methods or new systems to determine the metabolic role of individual compounds for the infecting bacteria.

1.3.10. Acknowledgements

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1.3.11. Tables

Table 1. Summary of studies on extracellular pathogens implicating catabolism of host-derived compounds during animal infection

Bacteria	Disease	Catabolite	Reference
Gram positive			
Enterococcus faecalis	Endocarditis, UTI, bacteremia, meningitis	Lactate	Rana et al. (2013)
Staphylococcus aureus	Skin infections, endocarditis, pneumonia, meningitis, bacteremia	Proline	Schwan et al. (1998)
Steptococcus pneumonia	Pneumoina, meningitis, endocarditis, pericarditis, bacteremia	Sialic acid	Marion et al. (2011)
Gram negative			
Aggregatibacter actinomycetemcomitans	Perionditis, infective endocarditis	Lactate	Ramsey et al. (2011)
Borrelia burgdorferi	Arthritis, Lymes	Glycerol	Pappas et al. (2011), He et al. (2011)
Campylobacter jejuni	Gastroenteritis, Guillain–Barré syndrome	Serine	Velayudhan et al. (2004)
		Proline	Hofreuter et al. (2012)
		Aspertate	Guccione et al. (2008)
Escherichia coli	Gastroenteritis, UTI, neonatal meningitis	Serine	Anfora et al. (2007)
		Deoxyribose	Martinez-Jehanne et al. (2009)
		Fucose	Snider et al. (2009)
		Gluconate	Chang et al. (2004)
Haemophilus influenzae	Otitis media, pneumonia, meningitis, bacteremia	Sialic acid	Jurcisek et al. (2005)
Pseudomonas aeruginosa	Lung infection, keratitis, UTI, bacteremia, folliculitis, endocarditis	Alanine	Boulette et al. (2009)
Vibrio cholerae	Acute diarrhea	Methionine	Bogard et al. (2012)
		Sialic acid	Almagro-Moreno et al. (2009)
		Gluconate	Patra et al. (2012)
Vibrio vulnificus	Gastroenteritis, septicemia	Sialic acid	Jeong et al. (2009)

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1.3.12. References

Almagro-Moreno S, Boyd EF. 2009. Sialic acid catabolism confers a competitive advantage to pathogenic *Vibrio cholerae* in the mouse intestine. Infect Immun 77:3807-16.

Anfora AT, Haugen BJ, Roesch P, Redford P, Welch RA. 2007. Roles of serine accumulation and catabolism in the colonization of the murine urinary tract by *Escherichia coli* CFT073. Infect Immun 75:5298-304.

Anthony C. 2011. How half a century of research was required to understand bacterial growth on C1 and C2 compounds; the story of the serine cycle and the ethylmalonyl-CoA pathway. Sci Prog 94:109-37.

Beisel WR. 1975. Metabolic response to infection. Annu Rev Med 26:9-20.

Bernier-Febreau C, du Merle L, Turlin E, Labas V, Ordonez J, Gilles AM, Le Bouguenec C. 2004. Use of deoxyribose by intestinal and extraintestinal pathogenic *Escherichia coli* strains: a metabolic adaptation involved in competitiveness. Infect Immun 72:6151-6.

Bielecki P, Glik J, Kawecki M, Martins dos Santos VA. 2008. Towards understanding *Pseudomonas aeruginosa* burn wound infections by profiling gene expression. Biotechnol Lett 30:777-90.

Bogard RW, Davies BW, Mekalanos JJ. 2012. MetR-regulated *Vibrio cholerae* metabolism is required for virulence. MBio 3.

Boucher RC. 2004. New concepts of the pathogenesis of cystic fibrosis lung disease. Eur Respir J 23:146-58.

Boulette ML, Baynham PJ, Jorth PA, Kukavica-Ibrulj I, Longoria A, Barrera K, Levesque RC, Whiteley M. 2009. Characterization of alanine catabolism in *Pseudomonas aeruginosa* and its importance for proliferation in vivo. J Bacteriol 191:6329-34.

Brown SA, Palmer KL, Whiteley M. 2008. Revisiting the host as a growth medium. Nat Rev Microbiol 6:657-66.

Brown SA, Whiteley M. 2009. Characterization of the L-lactate dehydrogenase from *Aggregatibacter actinomycetemcomitans*. PLoS One 4:e7864.

Burns JL, Emerson J, Stapp JR, Yim DL, Krzewinski J, Louden L, Ramsey BW, Clausen CR. 1998. Microbiology of sputum from patients at cystic fibrosis centers in the United States. Clin Infect Dis 27:158-63.

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Chaffin DO, Taylor D, Skerrett SJ, Rubens CE. 2012. Changes in the *Staphylococcus aureus* transcriptome during early adaptation to the lung. PLoS One 7:e41329.

Chang DE, Smalley DJ, Tucker DL, Leatham MP, Norris WE, Stevenson SJ, Anderson AB, Grissom JE, Laux DC, Cohen PS, Conway T. 2004. Carbon nutrition of *Escherichia coli* in the mouse intestine. Proc Natl Acad Sci U S A 101:7427-32.

Csonka LN, Hanson AD. 1991. Prokaryotic osmoregulation: genetics and physiology. Annu Rev Microbiol 45:569-606.

Eisenreich W, Dandekar T, Heesemann J, Goebel W. 2010. Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. Nat Rev Microbiol 8:401-12.

Farrow JM, 3rd, Pesci EC. 2007. Two distinct pathways supply anthranilate as a precursor of the *Pseudomonas* quinolone signal. J Bacteriol 189:3425-33.

Freter R, Brickner H, Fekete J, Vickerman MM, Carey KE. 1983. Survival and implantation of *Escherichia coli* in the intestinal tract. Infect Immun 39:686-703.

Garber ED. 1960. The host as a growth medium. Ann N Y Acad Sci 88:1187-94.
Geiger O, Gonzalez-Silva N, Lopez-Lara IM, Sohlenkamp C. 2010. Amino acid-containing membrane lipids in bacteria. Prog Lipid Res 49:46-60.

Guccione E, Leon-Kempis Mdel R, Pearson BM, Hitchin E, Mulholland F, van Diemen PM, Stevens MP, Kelly DJ. 2008. Amino acid-dependent growth *of Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. Mol Microbiol 69:77-93.

Hacking AJ, Lin EC. 1976. Disruption of the fucose pathway as a consequence of genetic adaptation to propanediol as a carbon source in *Escherichia coli*. J Bacteriol 126:1166-72.

He M, Ouyang Z, Troxell B, Xu H, Moh A, Piesman J, Norgard MV, Gomelsky M, Yang XF. 2011. Cyclic di-GMP is essential for the survival of the lyme disease spirochete in ticks. PLoS Pathog 7:e1002133.

Hofreuter D, Mohr J, Wensel O, Rademacher S, Schreiber K, Schomburg D, Gao B, Galan JE. 2012. Contribution of amino acid catabolism to the tissue specific persistence of *Campylobacter jejuni* in a murine colonization model. PLoS One 7:e50699.

Hoskins LC, Agustines M, McKee WB, Boulding ET, Kriaris M, Niedermeyer G. 1985. Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains

www.manaraa.com

that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. J Clin Invest 75:944-53.

Jeong HG, Oh MH, Kim BS, Lee MY, Han HJ, Choi SH. 2009. The capability of catabolic utilization of N-acetylneuraminic acid, a sialic acid, is essential for *Vibrio vulnificus* pathogenesis. Infect Immun 77:3209-17.

Jonsen J, Laland S, Strand A. 1959. Adaptation of *E. coli* to 2-deoxy D-ribose. Acta Pathol Microbiol Scand 47:75-9.

Jurcisek J, Greiner L, Watanabe H, Zaleski A, Apicella MA, Bakaletz LO. 2005. Role of sialic acid and complex carbohydrate biosynthesis in biofilm formation by nontypeable *Haemophilus influenzae* in the chinchilla middle ear. Infect Immun 73:3210-8.

Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, Smith JD, DiDonato JA, Chen J, Li H, Wu GD, Lewis JD, Warrier M, Brown JM, Krauss RM, Tang WH, Bushman FD, Lusis AJ, Hazen SL. 2013. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 19:576-85.

Larocque RC, Harris JB, Dziejman M, Li X, Khan AI, Faruque AS, Faruque SM, Nair GB, Ryan ET, Qadri F, Mekalanos JJ, Calderwood SB. 2005. Transcriptional profiling of *Vibrio cholerae* recovered directly from patient specimens during early and late stages of human infection. Infect Immun 73:4488-93.

Leach S, Harvey P, Wali R. 1997. Changes with growth rate in the membrane lipid composition of and amino acid utilization by continuous cultures of *Campylobacter jejuni*. J Appl Microbiol 82:631-40.

Lehoux DE, Levesque RC. 2000. Detection of genes essential in specific niches by signature-tagged mutagenesis. Curr Opin Biotechnol 11:434-9.

Marion C, Burnaugh AM, Woodiga SA, King SJ. 2011. Sialic acid transport contributes to pneumococcal colonization. Infect Immun 79:1262-9.

Martinez-Jehanne V, du Merle L, Bernier-Febreau C, Usein C, Gassama-Sow A, Wane AA, Gouali M, Damian M, Aidara-Kane A, Germani Y, Fontanet A, Coddeville B, Guerardel Y, Le Bouguenec C. 2009. Role of deoxyribose catabolism in colonization of the murine intestine by pathogenic *Escherichia co*li strains. Infect Immun 77:1442-50.

Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW, Murrell JC. 2007. DNA stable-isotope probing. Nat Protoc 2:860-6.

www.manaraa.com

Palmer KL, Aye LM, Whiteley M. 2007. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. J Bacteriol 189:8079-87.

Pappas CJ, Iyer R, Petzke MM, Caimano MJ, Radolf JD, Schwartz I. 2011. *Borrelia burgdorferi* requires glycerol for maximum fitness during the tick phase of the enzootic cycle. PLoS Pathog 7:e1002102.

Pasteur L. 1878. La Theorie des Germes. Comptes Rendus l'Academie des Science 86:1037-1043.

Patra T, Koley H, Ramamurthy T, Ghose AC, Nandy RK. 2012. The Entner-Doudoroff pathway is obligatory for gluconate utilization and contributes to the pathogenicity of *Vibrio cholerae*. J Bacteriol 194:3377-85.

Peluso G, Barbarisi A, Savica V, Reda E, Nicolai R, Benatti P, Calvani M. 2000. Carnitine: an osmolyte that plays a metabolic role. J Cell Biochem 80:1-10.

Poncet S, Milohanic E, Maze A, Nait Abdallah J, Ake F, Larribe M, Deghmane AE, Taha MK, Dozot M, De Bolle X, Letesson JJ, Deutscher J. 2009. Correlations between carbon metabolism and virulence in bacteria. Contrib Microbiol 16:88-102.

Ramsey MM, Rumbaugh KP, Whiteley M. 2011. Metabolite cross-feeding enhances virulence in a model polymicrobial infection. PLoS Pathog 7:e1002012.

Rana NF, Sauvageot N, Laplace JM, Bao Y, Nes I, Rince A, Posteraro B, Sanguinetti M, Hartke A. 2013. Redox Balance via Lactate Dehydrogenase Is Important for Multiple Stress Resistance and Virulence in *Enterococcus faecalis*. Infect Immun 81:2662-8.

Rohmer L, Hocquet D, Miller SI. 2011. Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. Trends Microbiol 19:341-8.

Russell JB, Cook GM. 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiol Rev 59:48-62.

Salyers AA, Vercellotti JR, West SE, Wilkins TD. 1977. Fermentation of mucin and plant polysaccharides by strains of *Bacteroides* from the human colon. Appl Environ Microbiol 33:319-22.

Schwan WR, Coulter SN, Ng EY, Langhorne MH, Ritchie HD, Brody LL, Westbrock-Wadman S, Bayer AS, Folger KR, Stover CK. 1998. Identification and characterization of the PutP proline permease that contributes to in vivo survival of *Staphylococcus aureus* in animal models. Infect Immun 66:567-72.

Slauch JM, Mahan MJ, Mekalanos JJ. 1994. In vivo expression technology for selection of bacterial genes specifically induced in host tissues. Methods Enzymol 235:481-92.

www.manaraa.com

Sleator RD, Gahan CG, Hill C. 2001. Identification and disruption of the *proBA* locus in *Listeria monocytogenes*: role of proline biosynthesis in salt tolerance and murine infection. Appl Environ Microbiol 67:2571-7.

Smith DR, Chapman MR. 2010. Economical evolution: microbes reduce the synthetic cost of extracellular proteins. MBio 1.

Smith H. 2000. Questions about the behaviour of bacterial pathogens in vivo. Philos Trans R Soc Lond B Biol Sci 355:551-64.

Snider TA, Fabich AJ, Conway T, Clinkenbeard KD. 2009. *E. coli* O157:H7 catabolism of intestinal mucin-derived carbohydrates and colonization. Vet Microbiol 136:150-4.
Su H, Newman EB. 1991. A novel L-serine deaminase activity in *Escherichia coli* K-12. J Bacteriol 173:2473-80.

Thornton DJ, Rousseau K, McGuckin MA. 2008. Structure and function of the polymeric mucins in airways mucus. Annu Rev Physiol 70:459-86.

van Opijnen T, Bodi KL, Camilli A. 2009. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. Nat Methods 6:767-72.

Velayudhan J, Jones MA, Barrow PA, Kelly DJ. 2004. L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by *Campylobacter jejuni*. Infect Immun 72:260-8.

Velayudhan J, Kelly DJ. 2002. Analysis of gluconeogenic and anaplerotic enzymes in *Campylobacter jejuni*: an essential role for phosphoenolpyruvate carboxykinase. Microbiology 148:685-94.

Verdin E, Ott M. 2013. Acetylphosphate: A Novel Link between Lysine Acetylation and Intermediary Metabolism in Bacteria. Mol Cell 51:132-4.

Vimr ER. 2013. Unified theory of bacterial sialometabolism: how and why bacteria metabolize host sialic acids. ISRN Microbiol 2013:816713.

Vimr ER, Kalivoda KA, Deszo EL, Steenbergen SM. 2004. Diversity of microbial sialic acid metabolism. Microbiol Mol Biol Rev 68:132-53.

Wang Q, Zhang Y, Yang C, Xiong H, Lin Y, Yao J, Li H, Xie L, Zhao W, Yao Y, Ning ZB, Zeng R, Xiong Y, Guan KL, Zhao S, Zhao GP. 2010. Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. Science 327:1004-7.

www.manaraa.com

Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chiang HC, Hooper LV, Gordon JI. 2003. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. Science 299:2074-6.

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1.4. Carnitine transport

1.4.1. Background

In order for nutrients to get into the cell and for waste products to exit they must cross cytosolic membrane. Gram-negative bacteria have an additional membrane, the outer membrane that is semi-permeable and slows the diffusion of small hydrophilic molecules while inhibiting the passage of large and charged molecules [1, 2]. The outer membrane has transmembrane protein channels called porins that can allow passage of specific or nonspecific cargo and have many functions, one of which is to allow entry of nutrients and solutes [3, 4]. The cytoplasmic membrane acts as a semi-permeable barrier that prevents passive leakage of the cytosolic components but does not completely inhibit small or nonpolar molecules from diffusion, like oxygen or carbon dioxide. Large, hydrophilic, or charged molecules must be transported in and out of the cell.

Transport of nutrients into the cytoplasm occurs via simple diffusion, facilitated diffusion, or active transport. Simple diffusion is the movement of small, hydrophobic as nonpolar molecules directly across the membrane. Facilitated diffusion moves larger, polar, charged, and hydrophilic molecules into the cell using membrane proteins that span the cytosolic membrane. Both simple and facilitated diffusion are typically put in the same category of passive transport because neither requires energy and the molecules move from a higher concentration to a lower concentration or are driven by the electrochemical gradient of the cell [5]. However, active transport requires energy in order to translocate molecules and is often coupled with ATP hydrolysis or actively maintained ion gradients [5].

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Carnitine is a polar zwitteronic compound that cannot pass through cell membrane and is actively transported using either ATP hydrolysis, ion motive force, or driven by a substrate:product antiporter. Many bacteria utilize and benefit from carnitine transport by using it as osmoprotectants, inducers of virulence factors, or as sole carbon, nitrogen, and energy sources [6], as discussed earlier in this section.

1.4.2. ATP-Binding Cassette transporter

ATP-binding cassette transporters, ABC transporters for short, are conserved through all domains of life [7] and have been present in every species studied as of 2006 [8]. The majority of characterized eukaryotic ABC transporters export substrates out of organelles or the cell, whereas most bacterial ABC transporters import substrates [9]. ABC transporters are composed of four core domains that span the lipid membrane and drive translocation of solutes by ATP hydrolysis. The core transporter forms a pore which ligand is transported into the cell, and is composed of two transmembrane domains (TMD) that bind ligand and two nucleotide binding domains (NBD) that bind and hydrolyze ATP. The core forms inward and outward facing configurations: the inward conformation has the TMD open to the cytoplasm, and the outward conformation has the TMD open to the periplasm [10]. ABC transporters also have substrate binding proteins; periplasmic in Gram-negative bacteria, or tethered to the cell membrane in Gram-positive, which binds ligand and delivers it to the TMD [7, 9].

ABC importers are divided into three classes or types. Type I and Type II transporters differ in that- Type I have an inward facing TMD and fewer transmembrane domains,

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whereas Type II have more transmembrane domains, an outward facing TMD. Additionally, the interaction between the substrate binding protein (SBP) and the core transporter is weakened upon binding of substrate in Type II transporters [11, 12]. A recently categorized ABC transporter is Type III or ECF for energy-coupling factor. Type III transporters consist of the TMD-NBD and an S component instead of the SBP [11]. The S component binds substrates that are typically metal ions and vitamins [13].

1.4.3. Components of ABC transporters

Bacterial import of molecules via an ABC transporter requires a substrate binding protein that binds the substrate for delivery to the core transporter TMD-NBD. The SBP is either tethered to the cell membrane by an N-terminal lipid anchor or is covalently attached the TMD, as in Gram-positive bacteria. In Gram-negative bacteria, the untethered SBP is periplasmic [14, 15] and is in stoichiometric excess compared to the core components to increase the probability of substrate import. A good example of this is the *E. coli* maltose import system, which has 30 fold more SBP than TMD-NBD [16]. To date, all SBPs have the same tertiary structure with two lobes joined by a hinge. The SBP is in an open conformation until substrate binding when the lobes close around the substrate, described by the "venus fly trap" model [17]. Each SBP binds one substrate molecule at a time and delivers it to the TMD [18]. Ligand specificity of a SBP is not necessarily limited to one substrate, and some SBPs have multiple ligands; notable examples are OpuC in *B. subtilis* (carnitine, crotonobetaine, and γ-butyrobetaine) [19,

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20], LivJ in *E. coli* (leucine, isoleucine, and valine) [21], and CbcX in *P. aeruginosa* (choline and glycine betaine) [22].

The transmembrane domain of the ABC transporter spans the cytoplasmic membrane and is composed of two homodimers or heterodimers. Although each dimer typically has 6 alpha helices, 10-20 alpha helices have been reported [11]. TMD's have one or two substrate binding sites, although the two binding site configuration is more common [23]. A crystal structure of the *E. coli* BtuCD TMD revealed a 16 amino acid helix-turn-helix EAA motif, also known as the L loop, is conserved and located in the cytoplasm [24, 25]. This EAA motif that interfaces with the NBD and this interaction is likely how NBD conformational changes driven by ATP hydrolysis are transduced to the TMD [14]. Similar to the SBP, TMDs can bind and interact with multiple SBPs, which is observed in *P. aeruginosa* CbcWV [22] and *Salmonella typhimurium* HisQMP [26].

Energy required to drive import and export is generated from ATP hydrolysis. NBDs are highly conserved and are homologous throughout the family with seven domains: aromatic, Walker A, Walker B, ABC signature domain (LSGGQ), and Q, D, and H loops [7]. Two NBDs are required for each transporter complex and both must be functional for ATP hydrolysis and transport of substrates [9].

1.4.4. Mechanism of action

Import by ABC transporters begins with the SBP binding to its ligand and interacting with the TMD causing a conformation change, which is thought to initiate binding of ATP to the NBD [7]. The two NBDs each bind a magnesium-ATP, which

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catalyzes dimerization, wherein the TMDs switch between the inward and outward conformation (or outward to inward depending on type of transporter). This interaction, ATP binding, and switching of the TMDs, is thought to transmit a signal to the SBP to release the substrate into the middle of the TMDs [9]. ATP hydrolysis takes places, releasing ADP, inorganic phosphate, and substrate resulting in what is called the "power stroke", all of which resets the NBD and TMD for the next cycle of import [9, 12].

1.4.5. Carnitine and ABC transporters

Gram-positive and Gram-negative bacteria use ABC transporters to import carnitine for multiple uses: osmoprotection, cryoprotection, barotolerance, bile tolerance, and as a carbon, nitrogen, and energy source [6]. The components of the *P. aeruginosa* carnitine ABC transporter are located in two separate operons: the TMD and NBD are in the *cbcXWV* operon, and the SBP, *caiX*, is encoded in the carnitine catabolism operon. *cbcV* encodes the NBD and the TMD encoded by *cbcW* is predicted to have six transmembrane alpha helices, resulting in a total of 12 helices in the membrane spanning component upon homodimerization [27]. The SBP *caiX* is in the first gene in the carnitine catabolism operon and imports carnitine, acetylcarnitine, and butyrylcarnitine. The molecular details of how CaiX binds its ligands are not known but may be similar to the *B. subtilis* OpuCC protein, which binds carnitine, glycine betaine, and choline [20]. The cleft of OpuCC has an aromatic girdle composed of four tyrosine residues that interact with the trimethylammonium through cation-π and hydrophobic interactions and

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the carboxyl group is bound by hydrogen bonding to the amino acids glutamine, serine and asparagine within the binding pocket [20].

1.4.6. Betaine/choline/carnitine transporters (BCCT)

BCCT transporters, as the name would suggest, transport the compatible solutes choline, carnitine, and glycine betaine, all of which are quaternary amine compounds. More recently the substrate specificity has been broadened to include other compatible solutes: proline betaine, acetylcholine, and dimethylsulphoniopropionate [28]. Structurally, BCCTs typically have 12 transmembrane domains with the N- and C-termini extending into the cytosol. The driving force behind transport of substrate is either proton motive force, sodium motive force, or less commonly by substrate: product antiport, including a quaternary amine carnitine:γ-butyrobetaine antiporter. The most widely studied carnitine BCCTs are the CaiT orthologs from *E. coli* and *Proteus mirabilis.* CaiT is a substrate:product antiporter that imports carnitine during anaerobic conditions when glucose and a final electron acceptor are absent [29]. Under those conditions, carnitine and its metabolite crotonobetaine work as final electron acceptors and are both converted to gamma butyrobetaine, which is exported as the product in the antiporter system [29]. Recent studies demonstrate that CaiT binds carnitine or γ-butyrobetaine, which is similar to the substrate binding protein OpuCC (mentioned above). CaiT binds the positively charge amine head group using cation-π and van der Waals interactions and the carboxyl tail by hydrogen bonds to amino acids in the pocket [30].

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1.4.7. References

1. Nikaido, H. & Vaara, M. (1985) Molecular basis of bacterial outer membrane permeability, *Microbiological reviews.* **49**, 1-32.
2. Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited, *Microbiology and molecular biology reviews : MMBR.* **67**, 593-656.
3. Cantisani, M., Vitiello, M., Falanga, A., Finamore, E., Galdiero, M. & Galdiero, S. (2012) Peptides complementary to the active loop of porin P2 from Haemophilus influenzae modulate its activity, *International journal of nanomedicine.* **7**, 2361-71.
4. Schirmer, T. (1998) General and specific porins from bacterial outer membranes, *Journal of structural biology.* **121**, 101-9.
5. Cooper, G. (2000) *The Cell: A molecular approach*, 6th edn, Sinauer Associates, Inc, Sunderland, MA.
6. Meadows, J. A. & Wargo, M. J. (2015) Carnitine in bacterial physiology and metabolism, *Microbiology*.
7. Linton, K. J. (2007) Structure and function of ABC transporters, *Physiology.* **22**, 122-30.
8. Linton, K. J. & Higgins, C. F. (2007) Structure and function of ABC transporters: the ATP switch provides flexible control, *Pflugers Archiv : European journal of physiology.* **453**, 555-67.
9. Higgins, C. F. & Linton, K. J. (2004) The ATP switch model for ABC transporters, *Nature structural & molecular biology.* **11**, 918-26.
10. Rees, D. C., Johnson, E. & Lewinson, O. (2009) ABC transporters: the power to change, *Nature reviews Molecular cell biology.* **10**, 218-27.
11. Rice, A. J., Park, A. & Pinkett, H. W. (2014) Diversity in ABC transporters: type I, II and III importers, *Critical reviews in biochemistry and molecular biology.* **49**, 426-37.
12. Wilkens, S. (2015) Structure and mechanism of ABC transporters, *F1000prime reports.* **7**, 14.
13. Berntsson, R. P., ter Beek, J., Majsnerowska, M., Duurkens, R. H., Puri, P., Poolman, B. & Slotboom, D. J. (2012) Structural divergence of paralogous S components from ECF-type ABC transporters, *Proceedings of the National Academy of Sciences of the United States of America.* **109**, 13990-5.
14. Jumpertz, T., Holland, B., Schmitt, L. (2009) *ABC tansporter in microorganisms*, Caister Academic Press, Norfolk, UK.
15. van der Heide, T. & Poolman, B. (2002) ABC transporters: one, two or four extracytoplasmic substrate-binding sites?, *EMBO reports.* **3**, 938-43.
16. Treptow, N. A. & Shuman, H. A. (1985) Genetic evidence for substrate and periplasmic-binding-protein recognition by the MalF and MalG proteins, cytoplasmic membrane components of the *Escherichia coli* maltose transport system, *Journal of bacteriology.* **163**, 654-60.
17. Mao, B., Pear, M. R., McCammon, J. A. & Quiocho, F. A. (1982) Hinge-bending in L-arabinose-binding protein. The "Venus's-flytrap" model, *The Journal of biological chemistry.* **257**, 1131-3.

www.manaraa.com

18. Borths, E. L., Locher, K. P., Lee, A. T. & Rees, D. C. (2002) The structure of *Escherichia coli* BtuF and binding to its cognate ATP binding cassette transporter, *Proceedings of the National Academy of Sciences of the United States of America.* **99**, 16642-7.
19. Kappes, R. M., Bremer E. (1998) Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and y-butyrobetaine via the ABC transport system OpuC *Microbiology.* **144**, 83-90.
20. Du, Y., Shi, W. W., He, Y. X., Yang, Y. H., Zhou, C. Z. & Chen, Y. (2011) Structures of the substrate-binding protein provide insights into the multiple compatible solute binding specificities of the *Bacillus subtilis* ABC transporter OpuC, *The Biochemical journal.* **436**, 283-9.
21. Trakhanov, S., Vyas, N. K., Luecke, H., Kristensen, D. M., Ma, J. & Quiocho, F. A. (2005) Ligand-free and -bound structures of the binding protein (LivJ) of the *Escherichia coli* ABC leucine/isoleucine/valine transport system: trajectory and dynamics of the interdomain rotation and ligand specificity, *Biochemistry.* **44**, 6597-608.
22. Chen, C., Malek, A. A., Wargo, M. J., Hogan, D. A. & Beattie, G. A. (2010) The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds, *Molecular microbiology.* **75**, 29-45.
23. van Veen, H. W., Callaghan, R., Soceneantu, L., Sardini, A., Konings, W. N. & Higgins, C. F. (1998) A bacterial antibiotic-resistance gene that complements the human multidrug-resistance P-glycoprotein gene, *Nature.* **391**, 291-5.
24. Schmitt, L., Benabdelhak, H., Blight, M. A., Holland, I. B. & Stubbs, M. T. (2003) Crystal structure of the nucleotide-binding domain of the ABC-transporter haemolysin B: identification of a variable region within ABC helical domains, *Journal of molecular biology.* **330**, 333-42.
25. Mourez, M., Hofnung, M. & Dassa, E. (1997) Subunit interactions in ABC transporters: a conserved sequence in hydrophobic membrane proteins of periplasmic permeases defines an important site of interaction with the ATPase subunits, *The EMBO journal.* **16**, 3066-77.
26. Nikaido, K. & Ames, G. F. (1999) One intact ATP-binding subunit is sufficient to support ATP hydrolysis and translocation in an ABC transporter, the histidine permease, *The Journal of biological chemistry.* **274**, 26727-35.
27. Winsor, G. L., Lam, D. K., Fleming, L., Lo, R., Whiteside, M. D., Yu, N. Y., Hancock, R. E. & Brinkman, F. S. (2011) *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes, *Nucleic acids research.* **39**, D596-600.
28. Ziegler, C., Bremer, E. & Kramer, R. (2010) The BCCT family of carriers: from physiology to crystal structure, *Molecular microbiology.* **78**, 13-34.
29. Jung, H., Buchholz, M., Clausen, J., Nietschke, M., Revermann, A., Schmid, R. & Jung, K. (2002) CaiT of *Escherichia coli*, a new transporter catalyzing L-carnitine/gamma -butyrobetaine exchange, *The Journal of biological chemistry.* **277**, 39251-8.

www.manaraa.com

30. Schulze, S., Koster, S., Geldmacher, U., Terwisscha van Scheltinga, A. C. & Kuhlbrandt, W. (2010) Structural basis of Na(+)-independent and cooperative substrate/product antiport in CaiT, *Nature*. **467**, 233-6.

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1.5. Regulation of bacterial gene expression

In order for bacteria to be able to prosper and survive in their environment, they need to be able to precisely control the expression level and timing of critical genes. *P. aeruginosa* PAO1 has a large genome that consists of 5,570 predicted open reading frames that encode systems for exceptional metabolic diversity [1]. It would not be advantageous for bacteria to transcribe and translate unnecessary genes, as it uses the energy and resources of the cell. Therefore, the cell tightly regulates gene expression and protein function in many ways. So, as part of this dissertation centers on two different transcription factors and how they contribute to carnitine regulation, the focus of this section will be on initiation of transcription, one of the steps for gene regulation.

1.5.1. Initiation of transcription

Transcription is a concerted effort between multiple proteins to make a RNA transcript. Chromosome size, compactness, DNA binding proteins, and epigenetics all play a role in initiation of transcription, but the required protein for transcription is the multisubunit RNA polymerase (RNAP). In order for RNAP to initiate transcription at a promoter, it must first interact with a sigma factor subunit to form the RNAP holoenzyme. *P. aeruginosa* PAO1 has 24 sigma factors that recognize and bind to the -10 and -35 elements (or functionally equivalent elements) of the promoter DNA, which are located 10 and 35 bases upstream from the transcriptional start site [2, 3]. The -10 site interacts with domain 2 and the -35 site interacts with domain 4 of the RNAP sigma 70 subunit, the housekeeping or primary sigma factor. Once the RNAP is bound, the DNA is

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denatured and forms an open promoter complex. The sigma factor dissociates and allows for elongation of the transcript to occur [4].

1.5.2. Transcription factors: inhibiting or promoting initiation

Transcription factors (TF) are proteins that couple environmental or internal signals and to gene expression. By binding specific DNA sequences TF activate or inhibit transcription by recruiting or blocking RNAP from binding promoter regions [5]. TF are divided into families based on protein structure but typically have a sensing domain and an effector domain. Transcription factors generally are two-domain proteins (ligand and DNA binding domains), meanwhile two component regulatory systems consist of a membrane bound kinase and a cytoplasmic response regulation that interacts with the DNA to regulate gene expression [6]. In order to be able to respond to a wide array of environmental signals, bacteria need many different regulatory proteins and in *P. aeruginosa* PAO1 there are over 400 transcriptional regulators [7]. TFs can act as activators or repressors, with some having dual activity.

Activators bind DNA sequences called enhancers and are classified by the mechanism they use to recruit RNAP. Class I activators bind upstream of the promoter region and bind to the alpha subunit C-terminal domain (αCTD) of RNAP. Class II activators have an enhancer site that overlaps the promoter -35 element. Lastly, class III activators bind enhancers near or overlapping the promoter, resulting in a conformational change of the promoter DNA to an orientation that is favorable to RNAP binding [5].

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Repressors inhibit transcription by impeding RNAP from binding the promoter. There are three basic mechanisms of repression: (i) repression by steric hindrance, typically by binding the promoter region and blocking RNAP from binding the promoter, (ii) looping the DNA making the promoter site inaccessible, and (iii) by modulating an activator [5].

Many promoters are controlled by two or more TFs that work in concert to tune gene expression to match environmental signals. Multiple activators can work together to recruit RNAP, or an activator can inhibit a repressor allowing for a second activator to initiate transcription. The regulatory circuit combinations are vast [5].

1.5.3. The AraC transcription factor family

Proteins of the AraC transcription factor family, named after the arabinose responsive regulator in *E. coli*, are present in Gram-positive and Gram-negative bacteria and function as activators or repressors [8]. The family is defined by a conserved 99 amino acid C-terminal domain [8], which consists of two helix-turn-helix motifs that bind to the major grooves of the DNA. Each monomer contacts one of the two half sites in the DNA binding sequence, typically oriented in direct repeats or inverted repeats [9]. While the N-terminal domain functions in ligand binding and dimerization, the sequence and structure of the N-terminus is not conserved, such that there are a number of subfamilies based on the identities of the N-termini. [10, 11]. The first 18 amino acids of the N-terminal domain is the arm, which interacts with the DNA binding domain in the absence of ligand and holds the protein in a conformation not amenable to dimerization. On the

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hand, in the presence of ligand the arm undergoes a conformational change to fold over the ligand [12, 13]. Both domains are linked together by a flexible linker that tethers the two domains together. The length, flexibility, and structure of the linker play a role in the regulation between the two domains [14, 15].

The mechanism for AraC regulation of the arabinose operon *araBAD* in *E. coli* has been established and is termed the "light switch" mechanism (figure 1). In the absence of arabinose, the N-terminal arm binds the C-terminal DNA-binding domain allowing for a conformation that promotes the DNA-binding domains to bind the half sites I_1 and O_2, looping the DNA and inhibiting transcription [16]. When arabinose binds, it is more favorable for the arm to dissociate from the DNA-binding domain and interacts with the dimerization domain and ligand [17, 18]. It is then energetically favorable for the DNA-binding domains to bind I_1 and I_2 instead of looping the DNA and this leads to transcriptional induction [16, 17]. This mechanism has been studied extensively with AraC but is not ubiquitous for all AraC TFs.

Schleif and colleagues constructed a chimeric protein consisting of the AraC ligand binding domain and LexA DNA binding domain that showed the LexA DNA binding domain bound its operator in an arabinose dependent manner, suggesting that the two domains can function independently from one another, at least in the case of AraC [19]. The N-terminal domain provides the ligand and sensory specificity for the regulator. The variability in the N-termini among the AraC-family TFs allows for control and regulation of carbon metabolism, stress responses, and virulence [8]. Rob, MarA, and SoxS are a few AraC TFs that respond to stresses such as antibiotic, oxidative stress, or organic

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solvents, respectively [20]. Carbon metabolism of arabinose in *E. coli* [21] is the most studied AraC-family TF and is described in detail above [22]. Lastly, AraC-family TFs can regulate virulence. ToxT regulates the toxin-coregulated pilus and cholera toxin, two virulence factors controlled by environmental stimuli. Bicarbonate increases the affinity of ToxT to the promoters while unsaturated fatty acids decrease DNA binding affinity [23, 24].

1.5.4. The GATase-1 AraC transcription factor subfamily

The varied AraC N-terminal domains are divided into subfamilies, one of which is a focus in our laboratory: the Type 1 glutamine amidotranferase- like domain (GATase-1, pfam00117). Glutamine amidotransferases are enzymes that have a catalytic triad of cysteine, glutamine, and histidine that remove an ammonia group from glutamate and transfer it to a substrate. The GATase-1 domain is conserved in some AraC TFs in *P. aeruginosa* and the members do not have a catalytic triad, and instead have a conserved cysteine residue at the nucleophilic elbow. The TFs CdhR and GbdR presented in this thesis belong to the GATase-1 AraC TF subfamily and together allow detection of the charged amine compounds carnitine, glycine betaine (GB), and dimethylglycine (DMG).

P. aeruginosa GbdR, the glycine betaine-dimethylglcyine regulator, has been studied to a much greater extent and regulates carbon metabolism and virulence [25]. Eukaryotic plasma membranes and lung surfactants contain significant amounts of phosphatidylcholine (PC) and sphingomyelin (SM). Phospholipase C (PlcH) enzymatically cleaves PC or SM to yield phosphorylcholine, which is dephosphorylated

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by PchP [26, 27]. *In vitro* experiments show *plcH* and *pchP* transcripts were induced in the presence of surfactant- and are dependent on GbdR [25], and *in vivo* mouse lung pneumonia models show that PlcH reduces lung function [28]. Choline liberated from PC/SM is catabolized to GB and subsequently to pyruvate. This pathway is primarily controlled by GbdR [29, 30] and serves multiple purposes for *P. aeruginosa* by generating an osmoprotectant [31], inducing *plcH* [25], and producing carbon, nitrogen, and energy sources [32]. Import of choline and GB is also partially regulated by GbdR [29, 30, 33].

The lesser known of the two GATase-1 TFs is CdhR, the carnitine dehydrogenase regulator. CdhR is divergently transcribed from the carnitine catabolism operon. Prior to the work presented here, our lab demonstrated that CdhR is required for growth on carnitine and is necessary for induction of the carnitine catabolism operon [22].

1.5.5. Post-transcriptional regulation

Regulation is complex and there can be many layers of control. As soon as a ribosome binding site (RBS), also known as the Shine-Delgarno sequence, of an mRNA exits the RNAP, translation can start, and in bacteria this can be concomitant with transcription. However, it is now appreciated that most translation occurs on free mRNAs [34]. With that being said, there is post-transcriptional regulation that often modulates stress responses and metabolic pathways [35].

RNA secondary structure can influence translation. Riboswitches are hairpin loop structures in the RNA 5' UTR that respond to environmental signals (temperature,

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uncharged tRNAs, or metabolites) in a protein independent manner to modulate gene expression [36]. Typically genes that control metabolite synthesis or import are next to the riboswitch [36]. Riboswitches can activate but more often repress gene expression by forming a termination stem loop blocking or allowing RNAP access to the ribosome binding site [37]. Small RNAs (sRNA), usually small noncoding snRNA, are *cis*- or *trans*-encoded regulatory elements that bind RNA resulting in negative gene expression by inhibiting translation, or by promoting RNA degradation or cleavage. However, stimulation of gene expression can occur by sRNAs but is not as common. The sRNAs act by relieving secondary RNA structures that would normally inhibit ribosome binding but upon binding causes a conformational change stimulating translation [35]. Post-transcriptional regulation can also be controlled by proteins interacting with 5' UTR, affecting RNA stability and translation. In times of decreased nutrient availability or entry into stationary phase, the *E. coli* CsrA protein will bind RNA to modify gene expression of proteins involved in carbon metabolism and motility [38, 39].

1.5.6. Other forms of regulation beyond RNA

These other forms of regulation are vast and expansive subjects as well, but will not be discussed in detail. The experiments in this dissertation examine transcriptional regulation, with limited analysis of post-transcriptional regulation, and how they impact growth. Post-translational modification (PTM) occurs after protein translation, often in response to environmental changes or factors with more than 300 types known as of 2010 [40]. Enzymes chemically modify proteins allowing for quick and specific changes that

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alter the proteins function, localization, and activity. One of the most common forms of PTM is the reversible process of phosphorylation. Specific proteins will have a phosphate group(s) added or removed to change their activity. Protein degradation is a very useful way to regulate processes. Bacterial pathogens *S. typhimurium* and *Listeria* have even found a way to hijack the host ubiquitin protein degradation system to degrade their effector proteins [40]. Some other common PTMs are glycosylation, acetylation, deamination, and many more, as reviewed in Ribet and Cossart [40].

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1.5.7. Figures

Figure 1. The regulatory region of the *araCBAD* genes. O1, I1, and I2 are half-sites that a single subunit of AraC binds. When there is no arabinose, the AraC monomers bind to half sites O1 and I1 and cause the DNA to loop and inhibit transcription. In the presence of arabinose an AraC monomer switches from the O1 to the I2 half site resulting in a formation that allows transcription of the *araBAD* operon.

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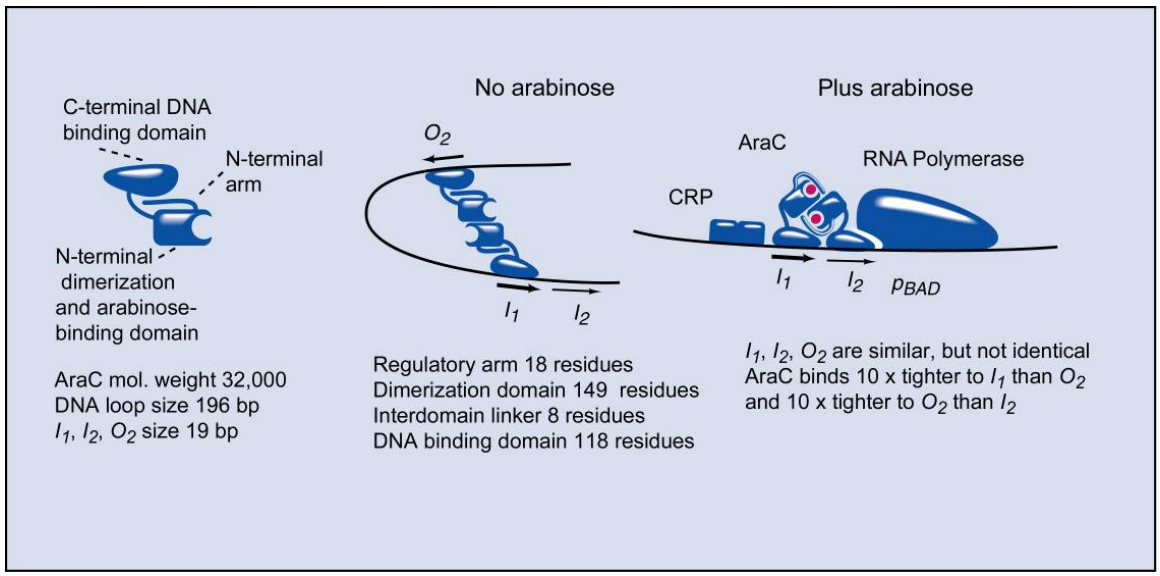

Figure 1. AraC light switch mechanism http://gene.bio.jhu.edu/

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1.5.8. References

1. Winsor, G. L., Lam, D. K., Fleming, L., Lo, R., Whiteside, M. D., Yu, N. Y., Hancock, R. E. & Brinkman, F. S. (2011) *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes, *Nucleic acids research.* **39**, D596-600.
2. Potvin, E., Sanschagrin, F. & Levesque, R. C. (2008) Sigma factors in *Pseudomonas aeruginosa*, *FEMS microbiology reviews.* **32**, 38-55.
3. Bielecki, P., Jensen, V., Schulze, W., Godeke, J., Strehmel, J., Eckweiler, D., Nicolai, T., Bielecka, A., Wille, T., Gerlach, R. G. & Haussler, S. (2015) Cross talk between the response regulators PhoB and TctD allows for the integration of diverse environmental signals in *Pseudomonas aeruginosa*, *Nucleic acids research*.
4. Murakami, K. S. & Darst, S. A. (2003) Bacterial RNA polymerases: the wholo story, *Current opinion in structural biology.* **13**, 31-9.
5. Browning, D. F. & Busby, S. J. (2004) The regulation of bacterial transcription initiation, *Nature reviews Microbiology.* **2**, 57-65.
6. Stock, A. M., Robinson, V. L. & Goudreau, P. N. (2000) Two-component signal transduction, *Annual review of biochemistry.* **69**, 183-215.
7. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S. & Olson, M. V. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen, *Nature.* **406**, 959-64.
8. Gallegos, M. T., Schleif, R., Bairoch, A., Hofmann, K. & Ramos, J. L. (1997) Arac/XylS family of transcriptional regulators, *Microbiology and molecular biology reviews : MMBR.* **61**, 393-410.
9. Carra, J. H. & Schleif, R. F. (1993) Variation of half-site organization and DNA looping by AraC protein, *The EMBO journal.* **12**, 35-44.
10. Martin, R. G. & Rosner, J. L. (2001) The AraC transcriptional activators, *Current opinion in microbiology.* **4**, 132-7.
11. Wu, M. & Schleif, R. (2001) Mapping arm-DNA-binding domain interactions in AraC, *Journal of molecular biology.* **307**, 1001-9.
12. Rodgers, M. E., Holder, N. D., Dirla, S. & Schleif, R. (2009) Functional modes of the regulatory arm of AraC, *Proteins.* **74**, 81-91.
13. Saviola, B., Seabold, R. & Schleif, R. F. (1998) Arm-domain interactions in AraC, *Journal of molecular biology.* **278**, 539-48.
14. Harmer, T., Wu, M. & Schleif, R. (2001) The role of rigidity in DNA looping-unlooping by AraC, *Proceedings of the National Academy of Sciences of the United States of America.* **98**, 427-31.
15. Seedorff, J. & Schleif, R. (2011) Active role of the interdomain linker of AraC, *Journal of bacteriology.* **193**, 5737-46.

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16. Schleif, R. (2003) AraC protein: a love-hate relationship, *BioEssays : news and reviews in molecular, cellular and developmental biology.* **25**, 274-82.
17. Cole, S. D. & Schleif, R. (2012) A new and unexpected domain-domain interaction in the AraC protein, *Proteins.* **80**, 1465-75.
18. Schleif, R. (2010) AraC protein, regulation of the l-arabinose operon in *Escherichia coli*, and the light switch mechanism of AraC action, *FEMS microbiology reviews.* **34**, 779-96.
19. Bustos, S. A. & Schleif, R. F. (1993) Functional domains of the AraC protein, *Proceedings of the National Academy of Sciences of the United States of America.* **90**, 5638-42.
20. Alekshun, M. N. & Levy, S. B. (1997) Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon, *Antimicrobial agents and chemotherapy.* **41**, 2067-75.
21. Sheppard, D. E. & Englesberg, E. (1967) Further evidence for positive control of the L-arabinose system by gene *araC*, *Journal of molecular biology.* **25**, 443-54.
22. Wargo, M. J. & Hogan, D. A. (2009) Identification of genes required for *Pseudomonas aeruginosa* carnitine catabolism, *Microbiology.* **155**, 2411-9.
23. Abuaita, B. H. & Withey, J. H. (2009) Bicarbonate Induces Vibrio cholerae virulence gene expression by enhancing ToxT activity, *Infection and immunity.* **77**, 4111-20.
24. Lowden, M. J., Skorupski, K., Pellegrini, M., Chiorazzo, M. G., Taylor, R. K. & Kull, F. J. (2010) Structure of *Vibrio cholerae* ToxT reveals a mechanism for fatty acid regulation of virulence genes, *Proceedings of the National Academy of Sciences of the United States of America.* **107**, 2860-5.
25. Wargo, M. J., Ho, T. C., Gross, M. J., Whittaker, L. A. & Hogan, D. A. (2009) GbdR regulates *Pseudomonas aeruginosa plcH* and *pchP* transcription in response to choline catabolites, *Infection and immunity.* **77**, 1103-11.
26. Berka, R. M., Gray, G. L. & Vasil, M. L. (1981) Studies of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*, *Infection and immunity.* **34**, 1071-4.
27. Massimelli, M. J., Beassoni, P. R., Forrellad, M. A., Barra, J. L., Garrido, M. N., Domenech, C. E. & Lisa, A. T. (2005) Identification, cloning, and expression of *Pseudomonas aeruginosa* phosphorylcholine phosphatase gene, *Current microbiology.* **50**, 251-6.
28. Wargo, M. J., Gross, M. J., Rajamani, S., Allard, J. L., Lundblad, L. K., Allen, G. B., Vasil, M. L., Leclair, L. W. & Hogan, D. A. (2011) Hemolytic phospholipase C inhibition protects lung function during *Pseudomonas aeruginosa* infection, *American journal of respiratory and critical care medicine.* **184**, 345-54.
29. Malek, A. A., Chen, C., Wargo, M. J., Beattie, G. A. & Hogan, D. A. (2011) Roles of three transporters, CbcXWV, BetT1, and BetT3, in *Pseudomonas aeruginosa* choline uptake for catabolism, *Journal of bacteriology.* **193**, 3033-41.
30. Hampel, K. J., LaBauve, A. E., Meadows, J. A., Fitzsimmons, L. F., Nock, A. M. & Wargo, M. J. (2014) Characterization of the GbdR regulon in *Pseudomonas aeruginosa*, *Journal of bacteriology.* **196**, 7-15.

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31. D'Souza-Ault, M. R., Smith, L. T. & Smith, G. M. (1993) Roles of N-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress, *Applied and environmental microbiology.* **59**, 473-8.
32. Wargo, M. J., Szwergold, B. S. & Hogan, D. A. (2008) Identification of two gene clusters and a transcriptional regulator required for *Pseudomonas aeruginosa* glycine betaine catabolism, *Journal of bacteriology.* **190**, 2690-9.
33. Chen, C., Malek, A. A., Wargo, M. J., Hogan, D. A. & Beattie, G. A. (2010) The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds, *Molecular microbiology.* **75**, 29-45.
34. Miller, O. L., Jr., Hamkalo, B. A. & Thomas, C. A., Jr. (1970) Visualization of bacterial genes in action, *Science.* **169**, 392-5.
35. Waters, L. S. & Storz, G. (2009) Regulatory RNAs in bacteria, *Cell.* **136**, 615-28.
36. Winkler, W. C. & Breaker, R. R. (2005) Regulation of bacterial gene expression by riboswitches, *Annual review of microbiology.* **59**, 487-517.
37. Nudler, E. & Mironov, A. S. (2004) The riboswitch control of bacterial metabolism, *Trends in biochemical sciences.* **29**, 11-7.
38. Dubey, A. K., Baker, C. S., Suzuki, K., Jones, A. D., Pandit, P., Romeo, T. & Babitzke, P. (2003) CsrA regulates translation of the *Escherichia coli* carbon starvation gene, *cstA*, by blocking ribosome access to the *cstA* transcript, *Journal of bacteriology.* **185**, 4450-60.
39. Wei, B. L., Brun-Zinkernagel, A. M., Simecka, J. W., Pruss, B. M., Babitzke, P. & Romeo, T. (2001) Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*, *Molecular microbiology.* **40**, 245-56.
40. Ribet, D. & Cossart, P. (2010) Post-translational modifications in host cells during bacterial infection, *FEBS letters.* **584**, 2748-58.

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CHAPTER 2:

CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* GROWTH ON *O*-ACYLCARNITINES AND IDENTIFICATION OF A SHORT-CHAIN ACYLCARNITINE HYDROLASE

Jamie A. Meadows [1] and Matthew J. Wargo[1,2]

[1] Department of Microbiology and Molecular Genetics, University of Vermont College of Medicine, Burlington, VT, USA

[2] The Vermont Lung Center, University of Vermont College of Medicine, Burlington, VT, USA

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2.1. Abstract

To survive in varied environments, from host tissue to soil, opportunistic bacterial pathogens must be metabolically flexible and able to use a variety of nutrient sources. We are interested in *Pseudomonas aeruginosa*'s catabolism of quaternary amine compounds that are prevalent in association with eukaryotes. Carnitine and acylcarnitines are abundant in animal tissues, particularly skeletal muscle, and are used to shuttle fatty acids in and out of the mitochondria where they undergo β-oxidation. We previously identified the genes required for carnitine catabolism as the first four genes in the carnitine operon (*caiXcdhCAB, PA5388-PA5384*). However, the last gene in the operon, *PA5384*, was not required for carnitine catabolism. We were interested in determining the function of PA5384. Bioinformatic analyses along with the genomic location of *PA5384* led us to hypothesize a role for PA5384 in acylcarnitine catabolism. Here we have characterized PA5384 as an L-enantiomer specific short-chain acylcarnitine hydrolase that is required for growth and hydrolysis of acetyl- and butyrylcarnitine to carnitine and the respective short-chain fatty acid. The liberated carnitine and its downstream catabolic product glycine betaine are subsequently available to function as osmoprotectants in hyperosmotic environments and induce transcription of the virulence factor phospholipase C, *plcH*. Furthermore, we confirmed that acylcarnitines from 2 to 16 carbon chain lengths, except for octanoylcarnitine (8-carbon), can be utilized by *P. aeruginosa* as sole carbon and nitrogen sources. These findings expand our knowledge of short-chain acylcarnitine catabolism and also point to remaining questions related to acylcarnitine transport and hydrolysis of medium- and long-chain acylcarnitines.

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2.2. Introduction

Carnitine and acylcarnitines are quaternary amine compounds that are abundant in mammalian tissues and play important roles in eukaryotic metabolism (1). Carnitine facilitates the translocation of fatty acids into the mitochondrial matrix where they undergo β-oxidation to produce energy for the cell (2, 3). This translocation is dependent on the formation of an ester linkage between the fatty acid and the hydroxyl oxygen on carbon three; this esterified carnitine is referred to as *O*-acylcarnitine or, as we will use throughout this study, acylcarnitine. While there are no eukaryotic enzymes that catabolize carnitine (2), free carnitine can be utilized by some bacteria, including *Pseudomonas aeruginosa*, as a sole carbon, nitrogen and energy source (4, 5). The concentration of carnitine and acylcarnitines in humans range from 50 μM in plasma to the low mM range in tissues, thus these compounds are potential sources of energy in colonization and infection (2, 6, 7). In *P. aeruginosa*, carnitine and its downstream catabolic product, glycine betaine, can function as osmoprotectants (8-10) and may be important for enhanced growth in hyperosmotic conditions, such as the environment of the lungs of people with cystic fibrosis and the urinary tract (11, 12). The generation of glycine betaine via carnitine catabolism also allows carnitine to induce expression of the virulence factor hemolytic phospholipase C, PlcH (8). During infection, PlcH can function to induce a proinflammatory response, suppress oxidative burst in neutrophils, degrade pulmonary surfactant, and increase endothelial cell death (13-18).

We previously identified the metabolic pathway responsible for carnitine catabolism in *P. aeruginosa* and determined that all but the last gene in the carnitine

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catabolism operon are required for carnitine degradation (19). The last gene in the operon, *PA5384*, is immediately downstream of the carnitine dehydrogenase genes (CDH), but is not required for growth on carnitine (19). However, the genomic location of *PA5384* provided insight into its potential function. Uanschou *et al*. compared the genomic sequences of predicted CDH genes in Gram positive and Gram negative bacteria and determined that many of the CDH-containing operons had an unidentified esterase/lipase downstream of the CDH gene(s) (7). *PA5384* fits this pattern, as it is immediately downstream of *cdhB*, and is bioinformatically predicted to be a lipolytic enzyme (19, 20). The prediction made by Uanschou *et al.* that PA5384 and homologs encode an acylcarnitine hydrolase, has not been directly tested. Previous work has briefly biochemically characterized an acylcarnitine hydrolase from an *Alcaligenes sp.* (21) that is used as a diagnostic tool for measuring acetylcarnitine in serum. However, this study provided no genetic identification associated with the purified hydrolase (22). Acylcarnitine catabolism has been studied in *Pseudomonas putida* (23) but it was not known if *P. aeruginosa* can hydrolyze acylcarnitines or employ them as a sole carbon and nitrogen source.

In this study we demonstrate that *P. aeruginosa* can utilize acylcarnitines as sole carbon, nitrogen, and energy sources. We also demonstrate that hydrolysis of acylcarnitines enables osmoprotection via production of carnitine and the downstream catabolite glycine betaine; improving cell survival in hyperosmotic environments and inducing the expression of the virulence factor PlcH. In addition, we characterize the function of the PA5384 enzyme and determine its role in acylcarnitine metabolism.

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2.3. Materials and Methods

2.3.1. Strains and growth conditions

P. aeruginosa PA14 (24), PAO1 (25), and derivative strains were maintained on Pseudomonas Isolation Agar (PIA, Difco) plates or in LB broth with gentamicin added when necessary at 50 µg ml^{-1} or 40 µg ml^{-1}, respectively. *E. coli* NEB5α was maintained on LB plates or in LB broth supplemented when necessary, with gentamicin at 10 µg ml^{-1} or 7 µg ml^{-1}, respectively, or in LB broth with 100 µg ml^{-1} kanamycin when needed.

To assess growth on acylcarnitines, *P. aeruginosa* cultures were pre-grown overnight at 37°C on a rotary wheel in 3 ml of MOPS minimal medium with 20 mM pyruvate and 5 mM glucose added as carbon sources (26). When necessary, gentamicin was added to these minimal media cultures at 20 µg ml^{-1}. Cells from these overnight cultures were added to MOPS media without a nitrogen source or MOPS media with 20 mM of the specified carbon source in 48 well plastic culture dishes at a final OD_{600} of 0.05. These plates were shaken at 170 rpm for 24 hours at 37°C for carnitine and acylcarnitines with 2-14 carbon chain lengths (designated 2C, 4C, …16C) or at room temperature for palmitoylcarnitine due to micelle formation of palmitoylcarnitine at 37°C. Growth was assessed by OD_{600} on a Synergy 2 BioTek plate reader. Measurement of growth at OD_{600} was not possible for palmitoylcarnitine due to the optical interference from the micelles that formed; therefore, triplicate serial dilutions were plated onto PIA and incubated for 24 hours to determine CFU ml^{-1}. The suppliers for the compounds are as follows: L-carnitine, L-acetylcarnitine, and D,L-palmitoylcarnitine are from Sigma, L-butyrylcarnitine and D-acetylcarnitine are from Crystal Chem., D,L-hexanoylcarnitine,

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D,L-decanoylcarnitine, D,L-lauryolcarnitine and D,L-myristoylcarnitine are from Biotrend Chemicals and D,L-acetylcarnitine is from Tocris Bioscience. The D,L-octanoylcarnitine was purchased from two different suppliers, Tocris Bioscience and Biotrend Chemicals. Biotrend Chemicals synthesize their compounds onsite in Germany, whereas Tocris Bioscience purchased the D,L-octanoylcarnitine from a producer in the United Kingdom (ascertained through personal communication).

2.3.2. Octanoylcarnitine competition assay

To determine if octanoylcarnitine inhibits growth on other acylcarnitines, *P. aeruginosa* PA14 was pre-grown overnight at 37°C on a rotary wheel in 3 ml of MOPS media with 20 mM pyruvate and 5 mM glucose added as carbon sources. Cells from the overnight culture were added at a final OD_{600} 0.05 to MOPS media with 10 mM of either acetylcarnitine or decanoylcarnitine as the carbon source. Octanoylcarnitine (Tocris Biosciences or Biotrend Chemicals) was added at 5 mM and the cultures incubated at 37°C overnight. Growth was assessed by OD_{600} on a Synergy 2 BioTek plate reader.

2.3.3. Expression and deletion constructs

The *PA5384* deletion construct was generated in the pMQ30 plasmid (27) and the deletion in *P. aeruginosa* PA14 was made by recombination as described previously (28, 29). Briefly, the upstream and downstream regions of the *PA5384* gene were amplified by PCR from pMW79 using the primers: PA5384-GOI-A 5'-aagcttGCCTGACCTTCCAGGACAT-3', PA5384-SOE-A 5'-

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aagtacgaaggcgactcgaccatggGGCGAGGCAGGGTTCTAT-3', PA5384-SOE-B 5'-ccatggtcgagtcgccttcgtacttCCGGAGACAGCGGATACTT-3', and PA5384-GOI-B 5'-gaattcATTGCCCTGGACCTACCTG-3'. Lower case letter sequences in the above primers represent complementary regions for splice overlap extension or engineered restriction sites. The splice overlap extension PCR product was cloned into the pCR-Blunt vector (Invitrogen), excised with HindIII and EcoRI, and cloned into similarly cut pMQ30 by *E. coli*-based cloning as described previously to generate pMW86. Donor *E. coli* S17/λpir carrying the *PA5384* deletion construct (pMW86) were mated with *P. aeruginosa* PA14 and single crossover mutants selected for growth on PIA plates with gentamicin. Recombinants were verified by PCR. Double crossover events were selected by growth on 5% sucrose LB plates with no NaCl (28, 30).

The *PA5384* expression vector (pJAM8) was made by amplifying *PA5384* from the plasmid pMW79 (19) using primers 5384ET30a F 5'-ggatccGCTGCGAAGTATCCGCTGTCT-3' and 5384ET30a R 5'-aagcttCTATTCGCCTGGCTGGTG-3'. This product was cloned into the pCR-Blunt vector using the Zero Blunt cloning kit (Invitrogen), with subsequent transformation and plasmid preparation as described above. The resulting plasmid was digested with HindIII and BamHI-HF (NEB) and the ~1kb fragment was extracted from an agarose gel and ligated into the similarly digested pET-30a expression vector (Novagen). The newly assembled plasmid, pJAM8, was transformed into chemically competent T7 Express Competent *E. coli* (NEB C2566) and transformants selected on LB with kanamycin.

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2.3.4. Trans complementation of *PA5384::tn* mutant strains

To complement the PAO1 *PA5384*::*Tn* mutant strains (31) (MJ206 and MJ207), we placed the *PA5384* gene at the *attTn7* site under control of the promoter regulating the native operon. The promoter of the carnitine catabolism operon (*PA5388*) was amplified from plasmid pMW79 using primers 5388promfor-5384F 5'-TAGCggtaccGGTTGAGGTTGCGCAGCC-3' and 5388promfor5384R 5'-ATCAggatccCATCGGTCTCCCCTCGTG-3'. The 213 bp product was ligated into the pCR-zero blunt vector using the Zero Blunt cloning kit (Invitrogen), with subsequent transformation and plasmid preparation as described above. The resulting plasmid was digested with KpnI-HF and BamHI-HF (NEB) and the 213 bp fragment was extracted from an agarose gel and ligated into the similarly digested pJAM8 to generate pJAM61. The pJAM61 plasmid was digested with KpnI-HF and HindIII-HF and the ~1.2 kbp fragment was ligated into the similarly cut plasmid pUC18-mini-Tn7T-Gm (32) and transformed into chemically competent *E. coli* NEB5α. Transformants harboring the newly generated plasmid, pJAM62, were selected in the presence of 7 μg ml^{-1} gentamicin. The *PA5388* promoter fused to *PA5384* was integrated onto the chromosome of the PAO1 *PA5384::tn* mutant strains as previously described (18, 32). Briefly, the plasmids pJAM62 and pTNS2 (33) were co-electroporated into each of the PAO1 *PA5384::tn* mutant strains and transformants were selected on 50 μg ml^{-1} gentamicin.

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2.3.5. Expression and purification of PA5384

Overnight cultures of JM17, a T7 Express Competent *E. coli* (NEB) carrying pJAM8, in LB with 100 µg ml^{-1} of kanamycin were inoculated into flasks containing 300 mL LB supplemented with 100 µg ml^{-1} of kanamycin and grown to an OD_{600} of 0.3-0.4. The cultures were then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 3 hours at 37°C. Cells were collected by centrifugation and the pellet was resuspended in lysis buffer (B-PER, Thermo Scientific) at 4 ml per 1 g of cells. Halt™ protease inhibitor cocktail, EDTA-free 100X (Thermo) at 1X concentration, 150 mM NaCl, 2 µl ml^{-1} lysozyme (50 units ml^{-1}, Thermo Scientific), and 2 µl ml^{-1} DNase (2500 units ml^{-1}, Thermo Scientific), were added to the culture and incubated with gentle shaking at 37°C for 20 minutes. The lysate was clarified by centrifugation at 8,000 x g for 20 minutes at 4°C. The clarified lysate was applied to a HisPur cobalt-NTA column (Thermo), washed thoroughly with wash buffer #1 (10 mM imidazole, 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4), and wash buffer #2 (wash buffer #1 with 33 mM imidazole), and protein eluted off the column with elution buffer (70 mM imidazole, 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4). The elution fractions containing N-terminal hexahistidine PA5384, determined by SDS-PAGE gel electrophoresis, were pooled and dialyzed in a 20,000 MWCO Slide-A-Lyzer (Thermo Scientific) in 0.1 M potassium phosphate buffer pH 7.2 and stored at 4°C. The protein concentration was determined to be 37 µg ml^{-1} by using bovine serum albumin standards in a Coomassie Plus – The Better Bradford™ Assay Kit according to Thermo instructions.

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2.3.6. Acetylcarnitine hydrolysis assay

An assay developed to detect acetylcarnitine for a diagnostic application previously described by Tomita *et al.* 2001 (22) was modified for the purpose of detecting enzymatic activity of the purified protein encoded by *P. aeruginosa PA5384.* The coupled assay of Tomita *et al.* was modified as follows. Purified 6xHis-PA5384 in 0.1 M potassium phosphate buffer pH 7.2 was added to the reaction mixture (0.1 M potassium phosphate buffer pH 7.2, 7 mM $MgSO_4$, 50 mM KCl, 2.5 mM ATP, 0.5 M phosphoenolpyruvate, 0.3 mM NADH, 20 units ml^{-1} acetate kinase (Sigma), 9 units ml^{-1} pyruvate kinase (Sigma), and 4 units ml^{-1} L-lactate dehydrogenase (Sigma)) in a 96 well-plate and incubated with shaking at 30°C (Synergy 2 BioTek) for 15 minutes. After this equilibration period, substrate (L-acetylcarnitine, D-acetylcarnitine, D,L-acetylcarnitine, or acetylcholine) was added to each well at 250 µM or 125 µM and incubated for an additional 3 hours at 30°C. During this incubation the absorbance was read at 340 nm every 3 minutes with each read preceded by 15 seconds of shaking.

2.3.7. Hydrolysis reactions for other acylcarnitines and detection of enzymatic activity

The substrate specificity of PA5384 was determined by detecting free L-carnitine using a colorimetric L-carnitine assay (BioVision). The assay was performed as described by the manufacturer with the modifications described here. First, in a 96 well plate, 15 µl of 0.1 M potassium phosphate buffer pH 7.2 was added to each well of L-carnitine standard and brought to a final volume of 50 µl with the provided assay buffer. In a 96

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well plate L-acylcarnitines 2C to 4C were added to a final concentration of 0.5 mM and D,L-acylcarnitines 6C to 16C were added to a final concentration of 1 mM in 0.1 M potassium phosphate buffer pH 7.2. The assay had technical triplicates and had more than three biological replicates for each carbon chain length. 0.5 µg of 6xHis-PA5384 was added to each well and incubated at 30°C while shaking for 15 seconds every 3 minutes for 3.5 hours. As a control, in triplicate, 0.1 M potassium phosphate buffer pH 7.2 and 0.5 mM acylcarnitine 2C to 4C or 1 mM 6C to 16C were added to the assay to detect signal from free carnitine present in these compounds. Assay controls according to BioVision's protocols were also performed with the modification of adding 0.5 µg 6xHis-PA5384 to the background control, which has no converting enzyme.

2.3.8. PA5384-FLAG-tag and localization by western blot

For western blot analysis, PA5384 was C-terminally FLAG-tagged via chromosomal integration by PCR amplifying the C terminus of *PA5384* with primers 5384Flag F 5'-ggtaccGAGCAGCCCCTGGAGTTC-3' and 5384Flag R 5'-aagcttCTACTTATCATCATCATCCTTGTAATCCGGCTTCAGGTTCATCCTG-3' which contains the FLAG-tag. The 585 base pair product was cloned into the pCR-Blunt vector (Invitrogen) and excised with KpnI and HindIII. The excised product was cloned into similarly cut pMQ30 (27). Donor *E. coli* S17/λpir carrying the *PA5384*-FLAG construct, pJAM34, underwent conjugation with *P. aeruginosa* PA14 and single crossover mutants selected for growth on PIA plates with gentamicin. Recombinants were verified by growth in MOPS with 20 mM acetylcarnitine as a sole carbon source

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supplemented with 20 µg ml^{-1} gentamicin and by whole lysate western blots using anti-FLAG monoclonal antibodies (Sigma). The western blots were performed as follows: electrophoresis in 8% or 12% SDS-PAGE gel followed by protein transfer to a PVDF membrane (Immobilon-P Millipore). The membrane was blocked overnight in 5% nonfat dried milk in Tris buffered saline – Tween 20 (TBS-T: 150 mM NaCl, 50 mM Tris HCl, 0.1% Tween 20, 0.01% sodium azide, pH 7.6). The membrane was washed in TBS-T, then incubated for 1 hour with the anti-FLAG M2 monoclonal antibody (Sigma) or anti-SadB antibody (34) and washed in TBS-T before detection with anti-mouse IgG HRP secondary antibody (GE Healthcare) or anti-rabbit IgG IRDye 800CW (Odyssey). After washing with TBS-T, HRP was detected via enhanced chemiluminescence western blotting detection reagent (GE Healthcare) prior to exposing membrane to film.

PA5384-FLAG strains JM116 and JM117, and control JM141 were grown overnight to mid log phase in MOPS with 5 mM glucose, 20 mM carnitine and 15 µg ml^{-1} gentamicin. JM141 is a control to account for nonspecific binding of the anti-FLAG monoclonal antibody to a random pMQ30 integrant (JM141 is with a single crossover integrant in *PA4921*, a gene irrelevant to the CDH pathway). Cells were collected by centrifugation at 8,000 x g for 10 minutes, and the supernatant was saved as the extracellular fraction. The pellets were resuspended in 20 mM Tris HCl pH 7.3 and lysed by repeated passage through a French Press at 9,000 PSI. The lysates were centrifuged at 8,000 x g for 10 minutes to remove non-lysed cells and large cellular debris. Separation of membrane from soluble fraction (cytoplasmic and periplasmic) was performed by ultracentrifugation at 100,000 x g for 30 minutes. The whole membrane fraction (outer

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and inner membrane) was resuspended in 20 mM Tris HCl pH 7.3 and washed three times. The soluble fraction centrifuged at 100,000 x g three times to ensure no membrane contamination. The protein from the extracellular fraction was concentrated using a 10,000 MWCO Centriprep Centrifugal Filter (Millipore). The extracellular, soluble, and membrane fractions were then analyzed by western blot following the protocol above.

2.3.9. Osmoprotection assay

Osmoprotection was determined by first growing *P. aeruginosa* PA14 WT and Δ*PA5384* overnight at 37°C on a rotary wheel in 3 mL of MOPS medium with 20 mM pyruvate and 5 mM glucose. Cells were collected by centrifugation, washed, and resuspended to a final OD_{600} of 0.05 in MOPS (which contains 50 mM NaCl) with an additional 800 mM NaCl, 20 mM glucose, and 0.2 mM carnitine or acetylcarnitine in 48 well plates. Cultures were grown at 37°C for 48 hours, due to the slow growth rate in a high salt medium, and growth was determined by OD_{600}.

2.3.10. *plcH* promoter activity by means of *lacZYA* fusion

Transcriptional induction from the *plcH* promoter was measured as previously described (35). Briefly, the *plcH-lacZYA* reporter construct, pMW22 (35), was transformed by electroporation into *P. aeruginosa* PA14 WT and Δ*PA5384* and transformants selected for growth on gentamicin. Cells were grown overnight in MOPS with 20 mM pyruvate, 5 mM glucose, and 20 µg ml^{-1} gentamicin. Cells were collected by centrifugation, washed, and resuspended in MOPS with 20 mM pyruvate and 20 µg ml^{-1}

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gentamicin with the addition of 1 mM carnitine or acetylcarnitine for induction samples. Cells were induced for 5.5 hours at 37°C and β-galactosidase assays were performed according to the method of Miller (36).

2.3.11. NPPC assay

Phospholipase C (PLC) activity was measured by ρ-nitrophenylphosphorylcholine (NPPC) hydrolysis according to the method of Kurioka and Matsuda (37), as previously modified in our laboratory (18, 35). Briefly, cells were grown overnight in MOPS, 20 mM pyruvate and 5 mM glucose. Cells were collected by centrifugation, washed, and resuspended in MOPS with 20 mM pyruvate and 1 mM carnitine or acetylcarnitine and grown overnight at 37°C in a 48 well plate. Supernatants from these cultures were obtained and added to 2X reaction buffer (200 mM Tris-HCl pH 7.2, 50% glycerol, 20 mM NPPC) in a 1:1 ratio. Hydrolysis of NPPC was quantified by measuring absorbance at 410 nm using the extinction coefficient 17,700 $M^{-1}cm^{-1}$.

2.4 Results

2.4.1. *PA5384* is required for growth on acetylcarnitine.

We previously demonstrated that the first four genes in the *caiXcdhCAB-PA5384* (*PA5388-PA5384)* operon (Fig. 1A) were critical for aerobic carnitine transport and metabolism (19, 38). *PA5384*, the last gene in the operon, was not required for carnitine catabolism (19). We hypothesized that *PA5384* may be associated with acylcarnitine catabolism based on its presence in the carnitine catabolism operon and bioinformatic

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prediction of lipase/esterase function. To determine the function of *PA5384*, we made an unmarked deletion of *PA5384* (Δ*PA5384*) in *P. aeruginosa* PA14 and measured growth on acetylcarnitine. The *PA5384* deletion mutant was unable to grow on acetylcarnitine as a sole carbon source (Fig. 1B). To test whether *P. aeruginosa* could use either acetylcarnitine stereoisomer, we used both the L- and D-enantiomers of acetylcarnitine in a growth assay, and found that *P. aeruginosa* can only grow on the L-enantiomer of acetylcarnitine (Fig. 1 B). It has been shown that carnitine can be used as a sole nitrogen source (23) and we tested if acetylcarnitine can be used as a sole nitrogen source. *P. aeruginosa* PA14 can grow on acetylcarnitine as the sole nitrogen source whereas the Δ*PA5384* deletion strain cannot (Fig. 1C).

We were unable to complement the acetylcarnitine growth defect of *PA5384* deletion strain using a high-copy constitutive plasmid (*PA5384* on the pUCP22 backbone), or using an arabinose-inducible high-copy system (*PA5384* under P_{BAD} control on the pMQ80 backbone). Therefore, to test if the growth phenotype in the *PA5384* mutant strain was due to mutation of *PA5384*, we constructed a *PA5388* (*caiX)* promoter fusion with *PA5384* (P_{caiX}-*PA5384*) and integrated it onto the chromosome at the *attTn7* site in two independent PAO1 transposon mutant strains. The two PAO1 transposon mutants, MJ207 and MJ206, carry a Tn5 transposon that confers resistance to tetracycline inserted into the *PA5384* coding sequence at base pairs 236 and 570, respectively (Fig. 1A) (31). Both strains carrying P_{caiX}-*PA5384* at the *attTn7* site (JM118 and JM119) were able to grow on acetylcarnitine as a sole carbon source (Fig. 1D). These data confirm the necessity of *PA5384* for growth on acetylcarnitine.

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2.4.2. PA5384 exhibits acetylcarnitine hydrolase activity.

P. aeruginosa requires *PA5384* for growth on acetylcarnitine (Fig. 1). To determine if *PA5384* encoded an acetylcarnitine hydrolase, we expressed recombinant PA5384 protein with an N-terminal hexahistidine tag (6xHis) and purified it by cobalt affinity chromatography and dialysis as described in the methods section. To measure acetylcarnitine hydrolase activity of PA5384, we adapted an enzymatic method designed to measure acetylcarnitine in serum (22), which uses four coupled enzymatic reactions to convert acetate abundance to an increase in NAD+ concentration, resulting in decreased UV absorbance at 340 nm dependent upon acetate. We predicted that PA5384 was an acetylcarnitine hydrolase that would hydrolyze acetylcarnitine to carnitine and acetate, and allow detection of acetate using the coupled reaction system. Using this assay we showed that PA5384 has L-enantiomer specific acetylcarnitine hydrolase activity, as it hydrolyzes L-acetylcarnitine and not D-acetylcarnitine (Fig. 2). The specificity of PA5384 was examined further by determining if the presence of the carnitine backbone of acetylcarnitine was necessary for hydrolysis. The inability of PA5384 to hydrolyze acetylcholine, which is another quaternary amine containing ester, to choline and acetate suggests that the carnitine backbone is necessary for acetylcarnitine hydrolysis and that PA5384 is not a general hydrolase (Fig. 2).

2.4.3. PA5384 has specificity for short-chain acylcarnitines.

As demonstrated above, *PA5384* is required for growth on acetylcarnitine and purified 6xHis-PA5384 hydrolyzes acetylcarnitine in vitro. In the infection environment,

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P. aeruginosa encounters a variety of acylcarnitines with different *O*-acyl chain lengths. To determine whether *PA5384* was required for growth on *O*-acyl chain lengths longer than acetylcarnitine we compared PA14 wild type to a Δ*PA5384* mutant strain for growth on acylcarnitines of 2 to 16 carbon (2C to 16C) lengths. Here we show that *PA5384* is required for growth on short-chain acylcarnitines (2C and 4C), but not for growth on medium or long-chain acylcarnitines (Fig. 3A). Interestingly, *P. aeruginosa* cannot use octanoylcarnitine as a sole carbon source (Fig. 3A), nor does octanoylcarnitine inhibit growth on acetylcarnitine or decanoylcarnitine as the sole carbon source (data not shown). This observation will be addressed in the discussion section.

We sought to determine if the chain length specificity for the observed growth phenotype was due to enzymatic specificity of PA5384. To test in vitro hydrolysis of different acylcarnitines by PA5384, we measured L-carnitine release using a colorimetric assay for L-carnitine detection (BioVision). Each acylcarnitine from 2C to 16C chain length was assayed for free L-carnitine either in the presence or absence of purified 6xHis-PA5384. The samples with no PA5384 enzyme added allowed for subtraction of contaminating free L-carnitine in the starting materials. The results show that PA5384 can hydrolyze the short-chain acylcarnitines, acetyl and butyryl, but it has no activity on medium-chain and long-chain acylcarnitines (Fig. 3B). Based on the above L-carnitine assay and our genetic evidence, PA5384 will now be referred to as HocS (Hydrolase of *O*-acylcarnitine, short-chains), with *hocS* being the corresponding gene name for *PA5384*.

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2.4.4. HocS is located in the cytoplasm.

HocS was bioinformatically predicted to be in the cytoplasm using the PSORTb database v3.0 with a cytoplasmic score of 9.97, well above the accepted cut-off of 7.5 (39, 40). To confirm this prediction, we tracked the localization of a chromosomally incorporated C-terminally FLAG-tagged HocS. Cells with the *hocS-FLAG* allele were grown in carnitine to induce expression (19). Western blot analysis was performed on the extracellular, soluble, and insoluble fractions and HocS-FLAG was detected by use of an anti-FLAG monoclonal antibody (Fig. 4) compared to the untagged HocS control. A known cytoplasmic protein, SadB, was used as a control for cytoplasmic localization as well as cytoplasmic contamination in the extracellular and insoluble fractions using the anti-SadB antibody (34). Based on this data, HocS is most likely cytoplasmic, although this data does not conclusively exclude periplasmic localization.

2.4.5. HocS is important for osmoprotection and induction of the virulence factor PlcH.

Carnitine is known to be an osmoprotectant (8) and to induce phospholipase C activity (PLC) (8). The ability of HocS to liberate carnitine from short-chain acylcarnitines led us to predict its involvement in osmoprotection and PlcH activity under conditions where short-chain acylcarnitines were present. To determine if *hocS* has a role in osmoprotection we compared PA14 wild type to the *hocS* deletion strain for the ability to grow in the presence of 850 mM NaCl. Under these conditions carnitine functions as an osmoprotectant allowing for growth in wild type and Δ*hocS* mutant strain (Fig. 5A).

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Osmoprotection was also observed with acetylcarnitine as the osmoprotectant source in PA14 wild type but not in the Δ*hocS* mutant strain, which had a three-fold decrease in growth compared to wild type (Fig. 5A). Choline is oxidized to glycine betaine, a potent osmoprotectant (9), and was used as control to show that deleting *hocS* did not compromise the choline catabolic pathway or general osmoprotection. These results support two conclusions. First, acetylcarnitine is not an effective osmoprotectant prior to hydrolysis, and second, that hydrolysis of acetylcarnitine by HocS can provide osmoprotection in hyperosmotic environments.

Release of carnitine by HocS activity would also be predicted to impact *plcH* transcription and PLC enzyme production by supplying glycine betaine for GbdR dependent induction of *plcH*. To test this prediction, we measured *plcH* transcription using a plasmid based *plcH-lacZYA* reporter (pMW22) (35), and measured PLC enzymatic activity using the NPPC assay (37). Carnitine induced transcription of *plcH* as previously reported (35), in both the PA14 wild type and Δ*hocS* mutant strains. However in the Δ*hocS* mutant strain acetylcarnitine showed no *plcH* transcriptional induction whereas PA14 wild type showed robust induction (Fig. 5B). Activity of secreted PLC was determined for both PA14 wild type and Δ*hocS* using the NPPC assay. A seven fold increase of PLC activity induced by acetylcarnitine was observed in PA14 wild type compared to Δ*hocS* (Fig. 5C). These results demonstrate that *hocS* hydrolysis of acetylcarnitine is required for acetylcarnitine-dependent induction of *plcH* transcription and PlcH activity.

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2.5. Discussion

In this study, we identified HocS (PA5384) as a short-chain acylcarnitine hydrolase that is required for *P. aeruginosa* to utilize acetyl- and butyrylcarnitine as sole carbon, nitrogen, and energy sources. Using a coupled enzymatic assay we show that HocS is specific for acylcarnitines and in conjunction with the L-carnitine assay we demonstrate that HocS specifically hydrolyzes short-chain acylcarnitines. The free carnitine generated can induce *plcH* transcription and result in induced PLC activity. *P. aeruginosa* can also utilize the rendered carnitine as an osmoprotectant to promote growth in hyperosmotic conditions. Through trans complementation we were able to show that *hocS* is required for *P. aeruginosa* grow on acetylcarnitine. We speculate that plasmid complementation was not successful due to the concentration of HocS compared to the other carnitine dehydrogenase proteins.

Acylcarnitine degradation has only been evaluated in *Pseudomonas putida* and *Pseudomonas fluorescens* (23, 41), which led us to inquire if *P. aeruginosa* can utilize acylcarnitines as sole carbon and nitrogen sources. In humans, acetylcarnitine is the most prevalent acylcarnitine intracellularly and in circulation but medium- and long-chain acylcarnitines are also present throughout the body, with long-chain acylcarnitines being the dominate acylcarnitine in bile (1). *P. putida* cannot utilize acylcarnitines from 2 to 8 carbon chain lengths but it is capable of using acylcarnitines 10 carbon chain lengths and longer as a sole carbon source (23). This led us to hypothesize that *P. aeruginosa* can utilize longer chain fatty acid length acylcarnitines as well. To the best of our knowledge, we are the first to show that *P. aeruginosa* can utilize acylcarnitines from 2 to 16 carbon

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chain lengths as sole carbon and nitrogen sources (Fig. 1C and Fig. 3A). Interestingly, our data illustrate that *P. aeruginosa* cannot use octanoylcarnitine as a sole carbon source and octanoylcarnitine does not inhibit growth on either acetyl- or decanoylcarnitine. The failure to grow on octanoylcarnitine may be due to an inability to transport or hydrolyze octanoylcarnitine, either or both of which suggest that *P. aeruginosa* is specifically restricted in its use of octanoylcarnitine (Fig. 3A). We used two different sources of octanoylcarnitine that were manufactured in two different sites (United Kingdom and Germany) to rule out toxic contaminants and defective synthesis, so we think that this restriction is a biological one, although we do not know the mechanism governing this restriction. *P. fluorescens* strain IMAM on the other hand can hydrolyze L-octanoylcarnitine (41) but no study to date has shown growth on octanoylcarnitine as the sole carbon source in the pseudomonads.

Takahashi demonstrated, through crude purification, that an acylcarnitine hydrolase from *Alcaligenes* could effectively be employed to identify acetylcarnitine in biological samples (22, 42, 43). However, the gene responsible for the acetylcarnitine hydrolase activity in *Alcaligenes* was not determined. We successfully identified the gene and expressed the protein responsible for hydrolysis of short-chain acylcarnitines in *P. aeruginosa*, PA5384 (HocS), and determined that it is likely localized in the cytoplasm (Fig. 4). Data from the L-carnitine assay further illustrate that *hocS* only hydrolyzes the short-chain acylcarnitines acetyl- and butyrylcarnitine (Fig. 3B). Since *hocS* hydrolyzes only substrates with a carnitine backbone, it suggests that an accessible fatty acid alone is not sufficient to achieve hydrolase activity and that the hydrolase activity is specific to

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the bond position on carnitine, which is further supported by the enantiomeric selectivity of the enzyme. These data and the ability of Δ*hocS* mutant strain to grow on medium- or long-chain acylcarnitines as the sole carbon source suggest that *P. aeruginosa* possesses an alternate hydrolase(s) that can liberate carnitine from medium- and long-chain acylcarnitines.

An additional goal of this study was to determine if short-chain acylcarnitines have a physiological role in *P. aeruginosa* in addition to use as nutrient sources. The virulence factor PlcH is induced by phosphate deprivation and by the products of choline and carnitine catabolism; glycine betaine and dimethylglycine (8, 44, 45). As access to carnitine enables induction of *plcH*, we hypothesized that the free carnitine generated by HocS hydrolysis of short-chain acylcarnitines would provide a source of carnitine to induce *plcH*. The lack of induction in either *plcH* transcription or PLC activity in the Δ*hocS* mutant in the presence of acetylcarnitine lead us to conclude that the carnitine generated from hydrolysis of short-chain acylcarnitines by HocS is sufficient to induce *plcH* expression (Fig. 5B-C).

The ability of *P. aeruginosa* to survive in hyperosmotic conditions by employing quaternary amine osmoprotectants has long been established (1, 8), such as choline-O-sulfate, carnitine, and glycine betaine. Even though acylcarnitines had not been evaluated in *P. aeruginosa* as osmoprotectants, our data shows that free carnitine generated by HocS hydrolysis acts as an osmoprotectant for *P. aeruginosa* in hyperosmotic conditions but that acetylcarnitine itself is not an osmoprotectant (Fig. 5A). In *Bacillus subtilis*, acetylcarnitine is a precursor to carnitine which is then used as an osmoprotectant (46). In

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Listeria monocytogenes, acetylcarnitine functions as an osmoprotectant (47, 48), but these studies did not address if acetylcarnitine is catabolized to carnitine. *P. aeruginosa* has been shown to catabolize carnitine to glycine betaine in hyperosmotic environments (46). Short-chain acylcarnitines are a source of carnitine that can ultimately be converted to glycine betaine.

This study has expanded our knowledge on *P. aeruginosa* acylcarnitine catabolism. Similar to *P. putida*, *P. aeruginosa* can grow on acylcarnitines from 10 to 16 carbon chain lengths, but *P. aeruginosa* is additionally capable of utilizing acylcarnitines from 2 to 6 carbon chain lengths. *P. aeruginosa* hydrolyzes short-chain acylcarnitines using an L-enantiomer specific hydrolase we have described here, HocS. The liberated carnitine from short-chain acylcarnitines hydrolysis has multiple physiological impacts, including induction of virulence factors and providing access to osmoprotectants.

2.6. Acknowledgements

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2.7. Figures

Figure 1. *PA5384* is required for *P. aeruginosa* growth on L-acetylcarnitine.

(A) Diagram of the carnitine catabolism operon in *P. aeruginosa* PAO1. ORFs are designated as arrows and are scaled relative to other ORFs. Black triangles represent transposon insertion sites in two separate PAO1 *PA5384* mutant strains with position of insertion noted by base pair number above the triangle. Gene designations are given below the arrows (19, 38) along with our proposed gene name for *PA5384*. (B) PA14 WT and Δ*PA5384* mutant cells were grown in MOPS minimal media supplemented with 20mM of carnitine, L-acetylcarnitine (LAC), D-acetylcarnitine (DAC), or racemic mix of D- and L-acetylcarnitine (DLAC) as sole sources of carbon. (C) PA14 WT and Δ*PA5384* mutant cells were grown in MOPS minimal media or MOPS minimal media without nitrogen supplemented with 20mM of carnitine (Carn) or L-acetylcarnitine (LAC) as the sole carbon and nitrogen source. (D) Trans complementation of two independent PAO1 *PA5384* transposon mutants. Cells were incubated in MOPS minimal media with 20 mM carnitine or LAC. MJ206 is the strain designation for the *PA5384*::Tn with transposon insertion at bp 570 and the derivative *attTn7::PA5384* strain is designated JM118. MJ207 is the strain designation for *PA5384*::Tn with transposon insertion at bp 236 and the derived *attTn7::PA5384* strain is designated JM119. Errors bars represent standard deviations of three replicates and results are representative of three independent experiments.

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Figure 2. PA5384 can hydrolyze L-acetylcarnitine. Coupled enzymatic assay with purified 6xHis-PA5384 and substrates (Δ) 250 μM L-acetylcarnitine (LAC), (○) 125 μM (LAC), (♦) 250 μM D-acetylcarnitine (DAC), (●) 250 μM D,L-acetylcarnitine (DLAC), (■) 250 μM acetylcholine (AcCho), (□) no substrate, or (▲) 250 μM LAC with no added PA5384. Lines have been added from the key to the absorbance curves to clarify the line identities. Errors bars represent standard deviations of triplicate samples and results are representative of three separate experiments.

Figure 3. *PA5384* encodes a short-chain acylcarnitine hydrolase. (A) PA14 WT to 16C acylcarnitine chain lengths. Number on x-axis indicates chain length. The y-axis shows relative growth of the mutant compared to WT, expressed as a percent of WT and the Δ*PA5384* mutant were grown in MOPS minimal media with 20 mM of carnitine (0) or 2C growth (i.e. WT growth = 100%). Growth for carnitine to acylcarnitines with 12C chain lengths was measured by OD_{600} and growth on acylcarnitines with chain lengths of 14C and 16C was determined by serial dilution and plating. (*) Neither PA14 WT nor ΔPA*5384* mutant strain grew on octanoylcarnitine as the sole carbon source. (B) Free L-carnitine generated from hydrolysis of acylcarnitines by 6xHis-PA5384 was detected using a colorimetric L-carnitine assay. The dotted line represents the assay sensitivity limit, below which measurements of carnitine are not reliable. Errors bars represent standard deviations of triplicate samples and results are representative of three separate experiments.

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Figure 4. HocS is likely localized to the cytoplasm. Cells from two independent strains, HocS-FLAG-1 (JM116) and HocS-FLAG-2 (JM117) that integrate a C-terminal FLAG-tag on HocS, and control strain JM141 were grown in carnitine. The extracellular (E), soluble (S), and insoluble fractions (I) were prepared as described in the methods section. After SDS-PAGE and blotting, the FLAG-tag was detected with a monoclonal anti-FLAG antibody and the cytoplasmic control, SadB, was detected with an anti-SadB antibody. The western blot shown is a representative of four comparable blots.

Figure 5. HocS has a role in osmoprotection and induction of the virulence factor *plcH*. (A) Osmoprotection was determined by growing PA14 WT and Δ*hocS* mutant cells for 48 hours in MOPS minimal media (50 mM NaCl) supplemented with 800 mM NaCl, 20 mM glucose as the carbon source and with the compatible solutes carnitine or L-acetylcarnitine (LAC) at a final concentration of 0.2 mM. (B) Induction of *plcH-lacZYA* (pMW22) by carnitine (Carn) or LAC was measured by inducing PA14 WT and Δ*hocS* mutant strain cells for 4 hours prior to the β-galactosidase assay. (C) To measure PLC activity, PA14 WT and Δ*hocS* mutant cells were grown overnight in MOPS supplemented with 20 mM pyruvate and either 1 mM carnitine or LAC. The resultant culture supernatants were used in an NPPC assay to determine PLC activity. Error bars represent standard deviations of triplicate samples and results are representative of three separate experiments.

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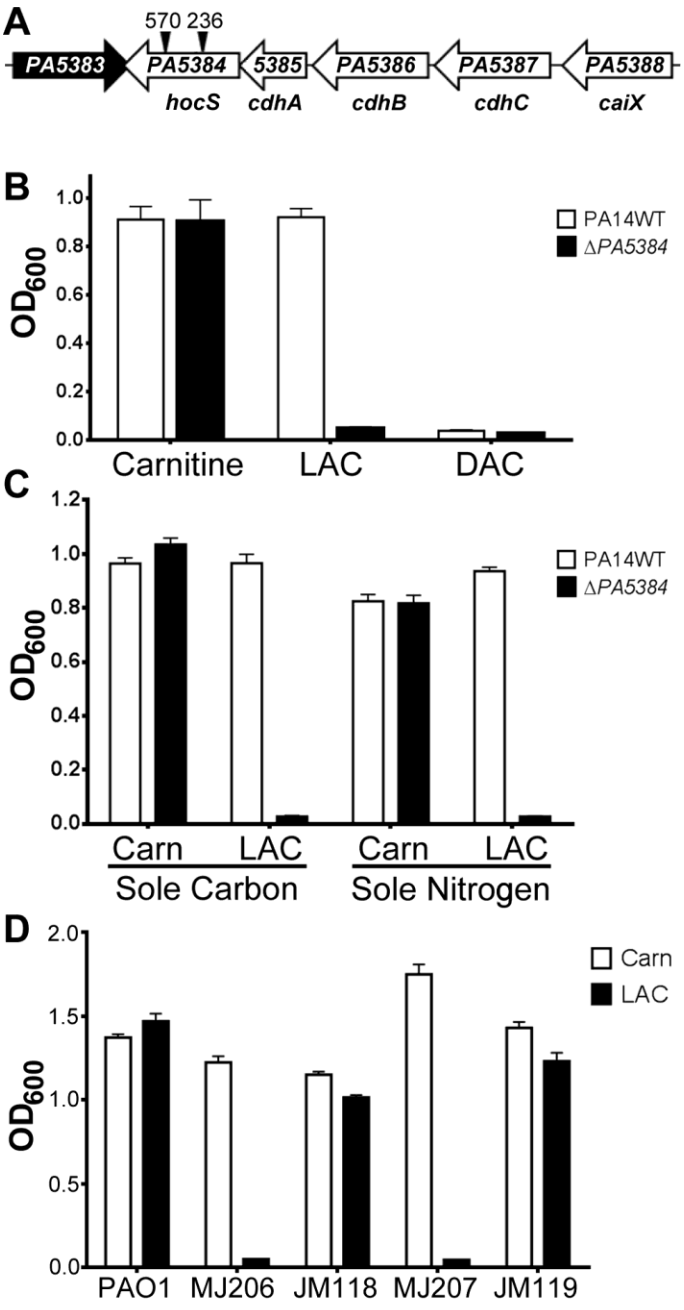

Figure 1. *PA5384* is required for *P. aeruginosa* growth on L-acetylcarnitine.

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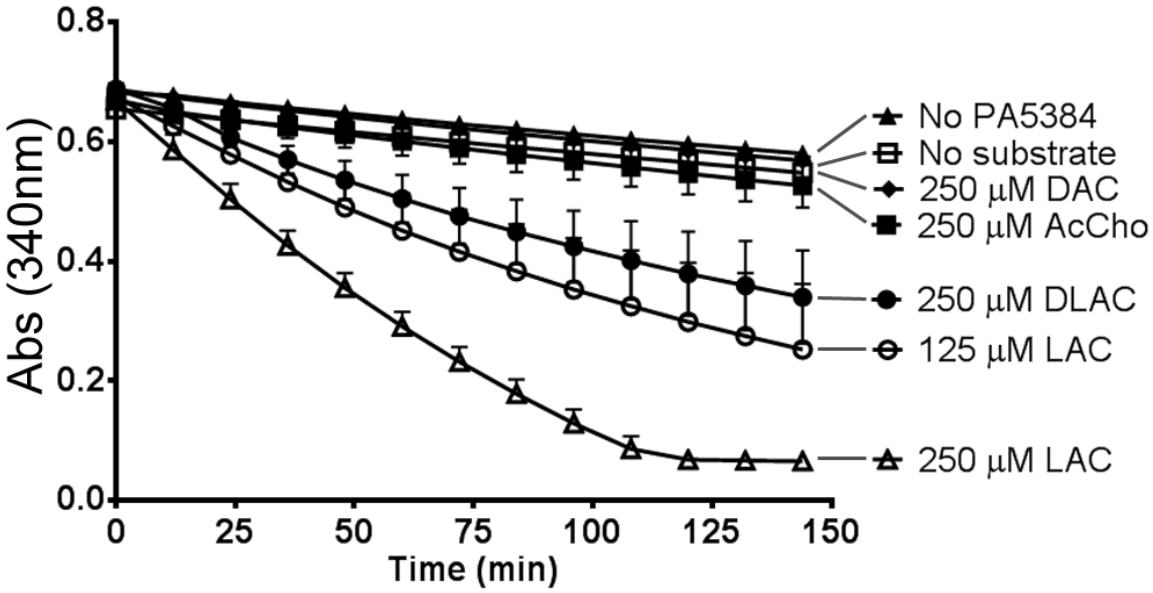

Figure 2. PA5384 can hydrolyze L-acetylcarnitine.

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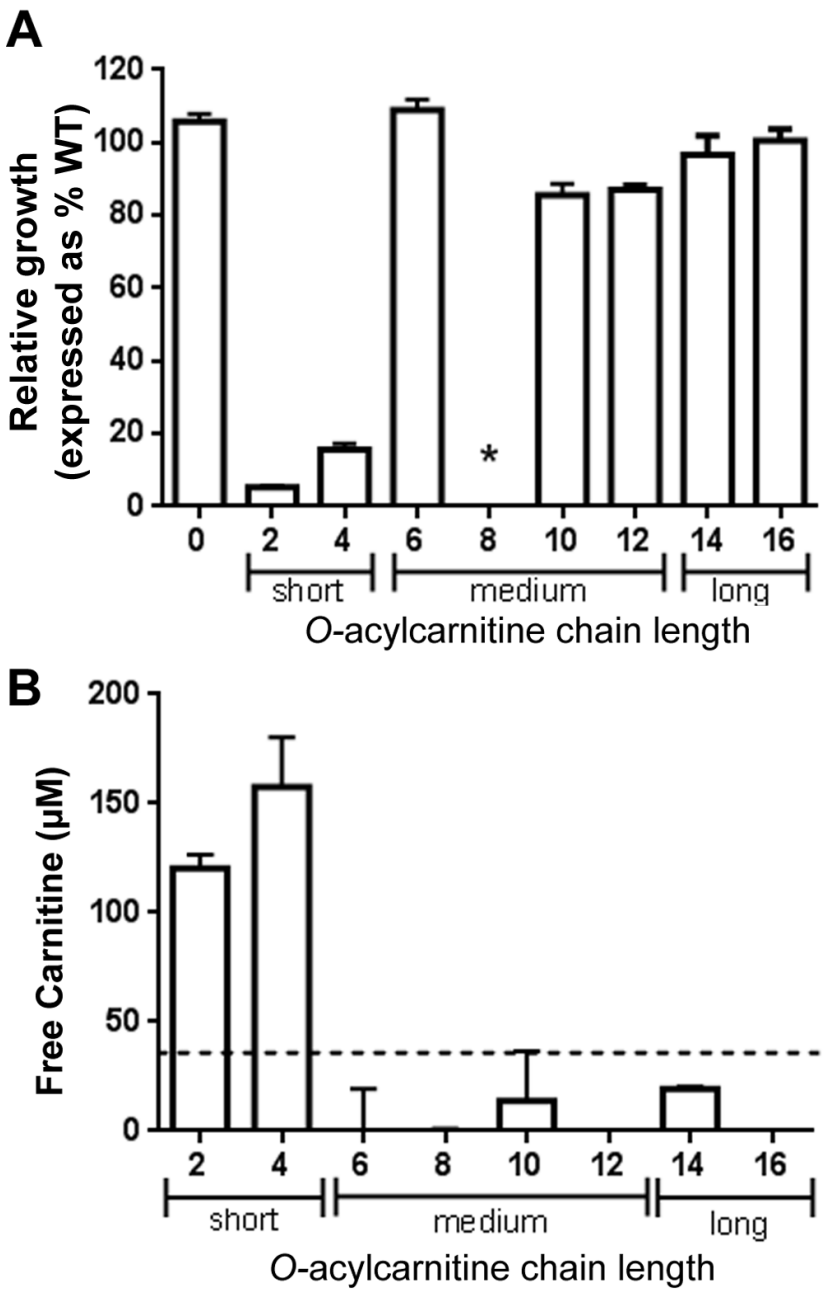

Figure 3. *PA5384* encodes a short-chain acylcarnitine hydrolase.

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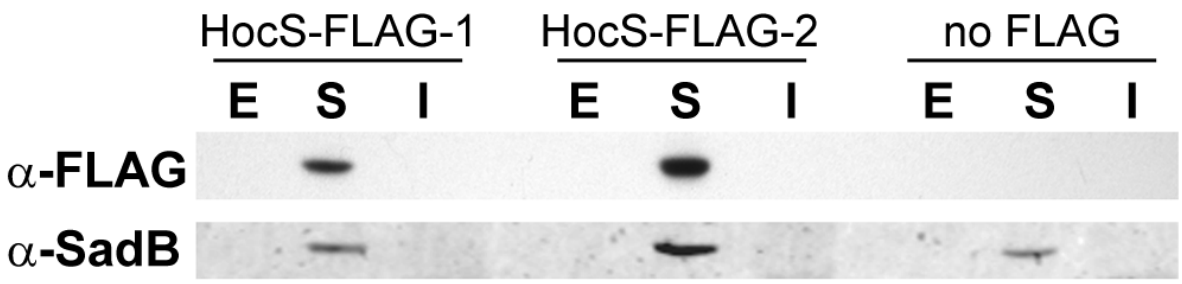

Figure 4. HocS is likely localized to the cytoplasm.

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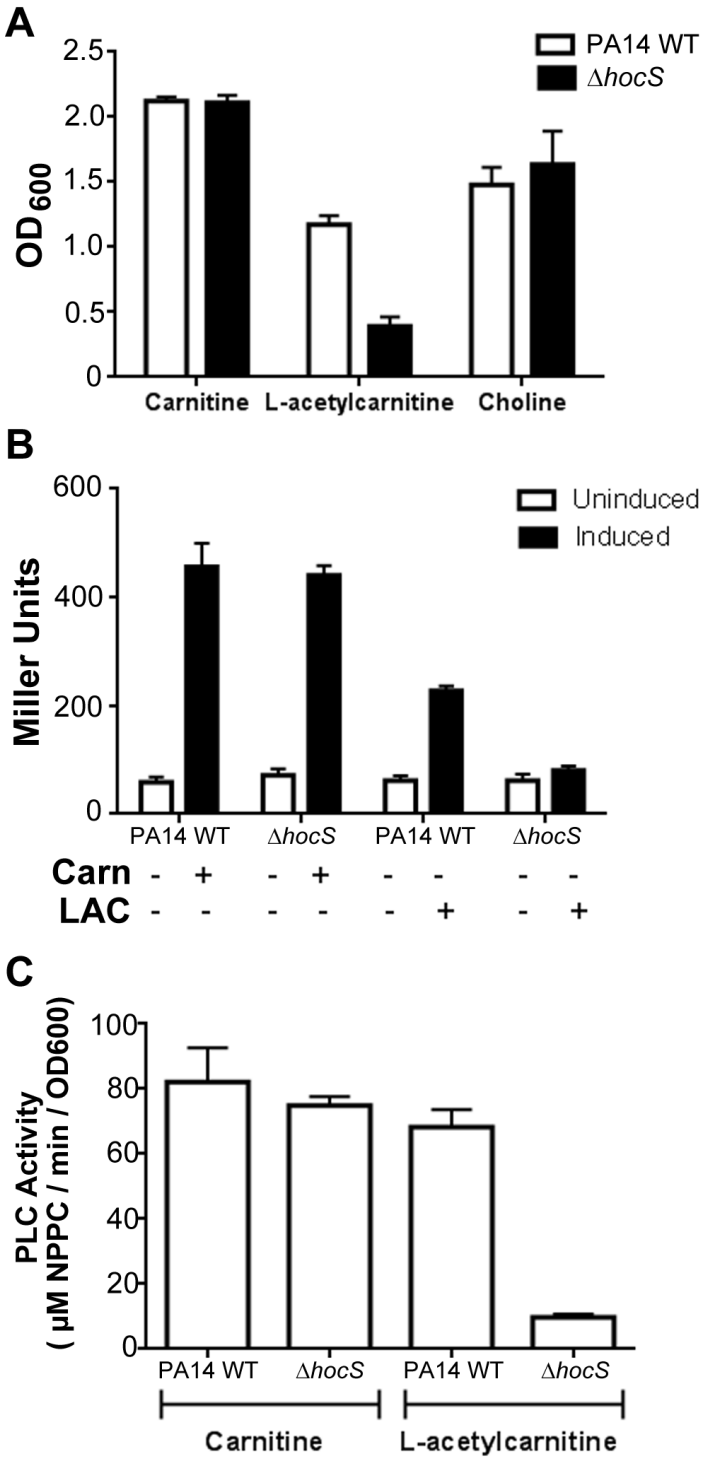

Figure 5. HocS has a role in osmoprotection and induction of the virulence factor *plcH*.

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2.8. Tables

Table 1. Strains and plasmids used in this study

Strain or plasmid	Database no.	Description (reference or source)[a]
P. aeruginosa PAO1		
PAO1 wild type	MJ79	*P. aeruginosa* WT (25)
PA5384::Tn*5*	MJ206	PA01 transposon mutant ID 47947, position in ORF 570 (31)
PA5384::Tn*5*	MJ207	PA01 transposon mutant ID 52172, position in ORF 236 (31)
PA5384::Tn*5 att*Tn*7*::*PA5384*	JM118	*trans*-complementation of *PA5384*::Tn ID 47947 (this study)
PA5384::Tn*5 att*Tn*7*::*PA5384*	JM119	*trans*-complementation of *PA5384*::Tn ID 52172 (this study)
P. aeruginosa PA14		
PA14 wild type	MJ101	*P. aeruginosa* WT (24)
Δ*PA5384*	MJ264	Clean *PA5384* deletion (this study)
PA5384-FLAG	JM116	PA14 with *PA5384* C-terminal FLAG-tag clone 1 (this study)
PA5384-FLAG	JM117	PA14 with *PA5384* C-terminal FLAG-tag clone 2 (this study)
PA14 pMQ30 integrant	JM141	PA14 with *PA4921*-pMQ30 genome integrant (this study)
E. coli		
E. coli wild type	MJ340	S17/λpir
E. coli T7 Express		NEB C2566
E. coli NEB5α		NEB C2987
E. coli T7 Express with pJAM8	JM17	T7 Express with pJAM8 (this study)
Plasmids		
pMQ30		Suicide vector, Gm^r (27)
pMQ80		High-copy-number *Pseudomonas* vector, Gm^r (27)
pET-30a		T7 expression vector, 6× His N-terminal tag, Kan^r (Novagen)
pUCP22		High-copy-number *Pseudomonas* stabilization vector, Gm^r (49)
pUC18-mini-Tn*7*T-Gm		Gm^r on mini-Tn*7*T (32)
pTNS2		Plasmid carrying the *att*Tn*7* transposase (33)
pMW22		Promoter *plcH-lacZYA* transcriptional fusion (35)
pMW79		PA14 genomic clone with *PA5380–PA5389* in pMQ8 (19)
pMW86		Causes deletion of *PA5384* in pMQ30 (this study)
pJAM6		*PA5384-PA5388* in pUCP22, Gm^r (derived from pMW79) (this study)
pJAM8		*PA5384* in pET-30a expression vector (this study)
pJAM10		PA14 *PA5384* in pMQ30 (this study)
pJAM34		PA14 *PA5384*FLAG, C terminal (this study)
pJAM61		PA14 *PA5388* promoter in pJAM8 (this study)
pJAM62		PA14 *PA5388* promoter fused to *PA5384* in pUC18-mini-Tn*7*T-Gm (32 and this study)

[a] ORF, open reading frame.

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2.9. References

1. **Rebouche CJ, Seim H.** 1998. Carnitine metabolism and its regulation in microorganisms and mammals. Annu. Rev. Nutr. **18:**39-61.
2. **Bremer J.** 1983. Carnitine-metabolism and functions. Physiol. Rev. **63:**1420-1480.
3. **Fritz IB.** 1963. Carnitine and Its Role in Fatty Acid Metabolism. Adv. Lipid Res. **1:**285-334.
4. **Aurich H, Kleber HP, Schopp WD.** 1967. An inducible carnitine dehydrogenase from *Pseudomonas aeruginosa*. Biochim. Biophys. Acta. **139:**505-507.
5. **Aurich H, Lorenz I.** 1959. [On the catabolism of carnitine by *Pseudomonas pyocyanea*]. Acta. Biol. Med. Ger. **3:**272-275.
6. **Rebouche CJ.** 1992. Carnitine function and requirements during the life cycle. FASEB J **6:**3379-3386.
7. **Uanschou C, Frieht R, Pittner F.** 2005. What to learn from a comparative genomic sequence analysis of L-carnitine dehydrogenase. Monatsh. Chem. **136:**1365-1381.
8. **Lucchesi GI, Lisa TA, Casale CH, Domenech CE.** 1995. Carnitine resembles choline in the induction of cholinesterase, acid phosphatase, and phospholipase C and in its action as an osmoprotectant in *Pseudomonas aeruginosa*. Curr. Microbiol. **30:**55-60.
9. **D'Souza-Ault MR, Smith LT, Smith GM.** 1993. Roles of N-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress. Appl. Environ. Microbiol. **59:**473-478.
10. **Vasil ML, A. I. Vasil, and V. D. Shortridge.** 1994. Phosphate in Microorganisms: Cellular and Molecular Biology. *In* E. Torriani-Gorrini EY, and S. Silver (ed.), Phosphate and osmoprotectants in the pathogenesis of *Pseudomonas aeruginosa*. ASM Press, Washington D.C.
11. **Kunin CM, Hua TH, Van Arsdale White L, Villarejo M.** 1992. Growth of *Escherichia coli* in human urine: role of salt tolerance and accumulation of glycine betaine. J. Infect. Dis. **166:**1311-1315.
12. **Gilljam H, Ellin A, Strandvik B.** 1989. Increased bronchial chloride concentration in cystic fibrosis. Scandinavian journal of clinical and laboratory investigation **49:**121-124.
13. **Berk RS, Brown D, Coutinho I, Meyers D.** 1987. In vivo studies with two phospholipase C fractions from *Pseudomonas aeruginosa*. Infect. Immun. **55:**1728-1730.
14. **Meyers DJ, Berk RS.** 1990. Characterization of phospholipase C from *Pseudomonas aeruginosa* as a potent inflammatory agent. Infect. Immun. **58:**659-666.
15. **Terada LS, Johansen KA, Nowbar S, Vasil AI, Vasil ML.** 1999. *Pseudomonas aeruginosa* hemolytic phospholipase C suppresses neutrophil respiratory burst activity. Infect. Immun. **67:**2371-2376.

www.manaraa.com

16. **Ostroff RM, Vasil AI, Vasil ML.** 1990. Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa*. J. Bacteriol. **172:**5915-5923.
17. **Wiener-Kronish JP, Sakuma T, Kudoh I, Pittet JF, Frank D, Dobbs L, Vasil ML, Matthay MA.** 1993. Alveolar epithelial injury and pleural empyema in acute *P. aeruginosa* pneumonia in anesthetized rabbits. J. Appl. Physiol. **75:**1661-1669.
18. **Wargo MJ, Gross MJ, Rajamani S, Allard JL, Lundblad LK, Allen GB, Vasil ML, Leclair LW, Hogan DA.** 2011. Hemolytic phospholipase C inhibition protects lung function during *Pseudomonas aeruginosa* infection. Am. J. Respir. Crit. Care Med. **184:**345-354.
19. **Wargo MJ, Hogan DA.** 2009. Identification of genes required for *Pseudomonas aeruginosa* carnitine catabolism. Microbiology **155:**2411-2419.
20. **Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS.** 2011. *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res. **39:**D596-600.
21. **Takahashi M, Ueda S.** January 31, 1995. 1995. Method of assyaing for acyl-L-carnitine and short-chain acyl-carnitine. Japan.
22. **Tomita K, Sakurada S, Minami S.** 2001. Enzymatic determination of acetylcarnitine for diagnostic applications. J. Pharm. Biomed. Anal. **24:**1147-1150.
23. **Kleber HP, Seim H, Aurich H, Strack E.** 1978. Interrelationships between carnitine metabolism and fatty acid assimilation in *Pseudomonas putida* (author's transl). Arch. Microbiol. **116:**213-220.
24. **Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM.** 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science **268:**1899-1902.
25. **Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959-964.
26. **LaBauve AE, Wargo MJ.** 2012. Growth and laboratory maintenance of *Pseudomonas aeruginosa*. Curr. Protoc. Microbiol. **Chapter 6:**Unit 6E 1.
27. **Shanks RM, Caiazza NC, Hinsa SM, Toutain CM, O'Toole GA.** 2006. *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria. Appl. Environ. Microbiol. **72:**5027-5036.
28. **Schweizer HD.** 1993. Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. Biotechniques **15:**831-834.

www.manaraa.com

29. **Wargo MJ, Szwergold BS, Hogan DA.** 2008. Identification of two gene clusters and a transcriptional regulator required for *Pseudomonas aeruginosa* glycine betaine catabolism. J. Bacteriol. **190:**2690-2699.
30. **Choi KH, Schweizer HP.** 2005. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. BMC Microbiol. **5:**30.
31. **Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenthner D, Bovee D, Olson MV, Manoil C.** 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U S A **100:**14339-14344.
32. **Choi KH, Schweizer HP.** 2006. mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. Nature protocols **1:**153-161.
33. **Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP.** 2005. A Tn7-based broad-range bacterial cloning and expression system. Nature methods **2:**443-448.
34. **Caiazza NC, O'Toole GA.** 2004. SadB is required for the transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **186:**4476-4485.
35. **Wargo MJ, Ho TC, Gross MJ, Whittaker LA, Hogan DA.** 2009. GbdR regulates *Pseudomonas aeruginosa* plcH and pchP transcription in response to choline catabolites. Infect. Immun. **77:**1103-1111.
36. **Miller JH.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Habor, NY.
37. **Kurioka S, Matsuda M.** 1976. Phospholipase C assay using p-nitrophenylphosphoryl-choline together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. Anal. Biochem. **75:**281-289.
38. **Chen C, Malek AA, Wargo MJ, Hogan DA, Beattie GA.** 2010. The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds. Mol. Microbiol. **75:**29-45.
39. **Rey S, Acab M, Gardy JL, Laird MR, deFays K, Lambert C, Brinkman FS.** 2005. PSORTdb: a protein subcellular localization database for bacteria. Nucleic Acids Res. **33:**D164-168.
40. **Yu NY, Laird MR, Spencer C, Brinkman FS.** 2011. PSORTdb-an expanded, auto-updated, user-friendly protein subcellular localization database for Bacteria and Archaea. Nucleic Acids Res. **39:**D241-244.
41. **Aragozzini F, Manzoni M, Cavazzoni V, Craveri R.** 1986. D,L-carnitine resolution by *Fusarium oxysporum*. Biotechnology Letters **8:**95-98.
42. **Matsumoto K, Takahashi M, Takiyama N, Misaki H, Matsuo N, Murano S, Yuki H.** 1993. Enzyme reactor for urinary acylcarnitines assay by reversed-phase high-performance liquid chromatography. Clin. Chim. Acta. **216:**135-143.
43. **Takahashi M, Ueda S.** 1991. Acyl-carnitine-esterase, method for measuring acylcarnitine. Japan.

www.manaraa.com

44. **Sage AE, Vasil AI, Vasil ML.** 1997. Molecular characterization of mutants affected in the osmoprotectant-dependent induction of phospholipase C in *Pseudomonas aeruginosa* PAO1. Mol. Microbiol. **23:**43-56.
45. **Sage AE, Vasil ML.** 1997. Osmoprotectant-dependent expression of plcH, encoding the hemolytic phospholipase C, is subject to novel catabolite repression control in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **179:**4874-4881.
46. **Kappes RM**, **Bremer E.** 1998. Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and gamma-butyrobetaine via the ABC transport system OpuC. Microbiology **144:**83-90.
47. **Bayles DO, Wilkinson BJ.** 2000. Osmoprotectants and cryoprotectants for *Listeria monocytogenes*. Lett. Appl. Microbiol. **30:**23-27.
48. **Verheul A, Rombouts FM, Beumer RR, Abee T.** 1995. An ATP-dependent L-carnitine transporter in *Listeria monocytogenes* Scott A is involved in osmoprotection. J. Bacteriol. **177:**3205-3212.
49. **Schweizer HP.** 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene **97:**109-121.

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CHAPTER 3:

THE ROLE OF *cbcWV* AND *caiX* IN THE TRANSPORT OF *O*-ACYLCARNITINES IN *PSEUDOMOANS AERUGINOSA*

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3.1. Abstract

The opportunistic pathogen *P. aeruginosa* can metabolize the compounds carnitine and acylcarnitines, which are abundant in host muscle and cardiac tissues. Acylcarnitines can be degraded to carnitine, where the liberated carnitine and its catabolic product glycine betaine can be used as osmoprotectants, to induce the virulence factor phospholipase C, and can function as sole carbon, nitrogen, and energy sources. *P. aeruginosa* is incapable of *de novo* synthesis of carnitine and acylcarnitines and they therefore must be imported from exogenous source. In this study we present the first characterization of acylcarnitine transport. Short-chain acylcarnitines are imported by the ABC transporter CaiX and CbcWV. Medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically and the free carnitine is transported through CaiX-CbcWV.

3.2. Introduction

Carnitine and acylcarnitines are quaternary amine compounds (QAC) that are present in all domains of life and are found in soil [1, 2] and plants [3, 4], and are abundant in animal tissues [5, 6]. The majority of carnitine is in its free form, but a portion is acylated, with acetylcarnitine being the most abundant acylcarnitine [7]. In animals, fatty acids are brought into the mitochondria via the carnitine shuttle where they undergo beta oxidation to produce energy for the cell. There are no animal enzymes capable of degrading carnitine, therefore any breakdown is from bacteria [8].

Pseudomonas aeruginosa is a Gram-negative bacterium that is nearly ubiquitous in nature and is an opportunistic pathogen that is capable of metabolizing carnitine [9]

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and acylcarnitines [10]. Short-chain acylcarnitines are hydrolyzed in the cytosol by HocS [10], but the medium- and long-chain acylcarnitine hydrolase(s) remains unidentified. Acylcarnitine hydrolysis yields a free fatty acid and carnitine. The resulting free carnitine and its catabolic product glycine betaine function as osmoprotectants in hyperosmotic environments [10-12], induce the virulence factor phospholipase C, *plcH* [11], and can be used as sole carbon, nitrogen, and energy sources [10, 13, 14]. The liberated fatty acid can also be used as a sole carbon source [15] and the *faoAB* (*fadBA5*) genes responsible for oxidation of fatty acids twelve carbons and longer were found to be strongly induced in cystic fibrosis sputum [16] and important for *P. aeruginosa* fitness in mouse wound models [17].

To date, de novo synthesis of carnitine has not been demonstrated in bacteria [18], therefore it must be acquired from the environment. There are two routes to carnitine transport: first, a BCCT (betaine/choline/carnitine) transporter, driven by sodium motive force, proton motive force, or substrate:product antiport [19]; and second, an ATP-binding cassette (ABC) transporter powered by ATP hydrolysis. In bacteria, ABC transporters are comprised of multiple proteins: a transmembrane domain, a nucleotide binding domain (ATPase), and a substrate binding protein (periplasmic binding protein) [20]. In *P. aeruginosa*, the genes encoding the ABC transporter for carnitine import are located in two different operons. The genes encoding the transmembrane domain (*cbcW*) and the nucleotide binding domain (*cbcV*) are located in the *cbcXWV* operon (*PA5378-PA5376*) [21], while the *caiX* gene, encoding the carnitine substrate binding protein is located in the carnitine catabolism operon, *caiXcdhCABhocS*

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(*PA5388-PA5384*) [22]. In *P. aeruginosa* CbcWV interacts with three different substrate binding proteins, CaiX, BetX, and CbcX, all of which bind ligand that are QACs: carnitine, glycine betaine, and choline (Fig. 1) [21]. *caiX* and *cbcWV* were previously shown to be required for growth on carnitine [21, 22], but the mechanism for acylcarnitine import and utilization in *P. aeruginosa* had not been studied.

Here we report the route of acylcarnitine transport in *P. aeruginosa* (Fig. 7). Through growth and transcriptional reporter assays, we demonstrate that medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically by an unknown hydrolase(s) to yield carnitine and a fatty acid. Carnitine and short-chain acylcarnitines are imported by the ABC transporter CbcWV and the substrate binding protein CaiX. *cbcWV* is only necessary for utilization of medium- and long-chain acylcarnitines as sole nitrogen sources but not osmoprotectants. *cbcWV* is necessary for short-chain acylcarnitines to function as osmoprotectants, but carnitine, medium-, and long-chain acylcarnitines are imported by an undefined transporter to provide osmoprotection.

3.3 Materials and Methods

3.3.1. Strain, growth conditions, and compounds

P. aeruginosa PA14 wild type [23], Δ*caiX* [22], and Δ*cbcV* [24] strains were maintained on PIA plates or in LB liquid and when necessary with 50 µg ml^{-1} or 40 µg ml^{-1} of gentamicin, respectively. Carnitine and acylcarnitines were purchased from the following vendors: L-carnitine (Acros); L-acetylcarnitine and DL-palmitoylcarnitine

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(Sigma); L-butyrylcarnitine (Crystal Chem Inc); and DL-hexanoyl-, DL-octanoyl-, DL-decanoyl-, DL-lauroyl-, and DL-myristoylcarnitine (BioTrends).

3.3.2. Growth assays

To test the growth of strains carrying deletions in transport components on carnitine and acylcarnitines (2-6 and 10-16 fatty acid chain length) as sole carbon sources, PA14 wild type, Δ*caiX*, or Δ*cbcV* were grown overnight at 37 °C in minimal media MOPS with 20 mM pyruvate and 5 mM glucose. Cells were then collected by centrifugation, washed, and inoculated at a final OD_{600} nm of 0.05 in MOPS with 20 mM of the individual acylcarnitine or carnitine. Cultures were grown at 37 °C in a 48-well plate on a horizontal shaker for 24 hours. OD_{600} was measured at the end of the assay on a BioTek plate reader. Due to insolubility, growth on palmitoylcarnitine growth was determined by CFU counts.

To test the use of carnitine and acylcarnitines as a sole nitrogen source, overnight cultures and preparation of the cells were the same as above. Cells were inoculated into minimal media M63 nitrogen free containing 20 mM pyruvate and 2 mM of the acylcarnitine. Palmitoylcarnitine samples had 0.75% deoxycholate added to prevent precipitation. Cultures were grown at 37 °C in a 48-well plate on a plate shaker for 24 hours. All acylcarnitine growth assays were measured on the BioTek plate reader at OD_{600}.

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3.3.3. Transcriptional reporter assay

To assess induction of *caiX* in response to acylcarnitines in the transport related deletion strains Δ*caiX* and Δ*cbcV*, we used the transcriptional reporter P*caiX-lacZYA* (pJAM22; construction described in Chapter 4). PA14WT, Δ*caiX*, or Δ*cbcV* carrying pJAM22 were grown overnight at 37 °C in MOPS medium, 20 mM pyruvate, 5 mM glucose, and 20 µg ml^{-1} gentamicin. Cells were collected by centrifugation, washed, and resuspended in MOPS. Cells were inoculated at a final OD_{600} of 0.05 into a 48-well plate containing MOPS medium, 20 mM pyruvate, 20 µg ml^{-1} gentamicin, and 1 mM of the induction compound (carnitine or acylcarnitines of 2-16 fatty acid chain length). Cells were incubated for 4 hours at 37 °C while shaking and then a β-galactosidase assay was performed according to Miller [25]. Due to insolubility of palmitoylcarnitine, OD_{600} for those wells was measured prior to inoculation with cells and subtracted from the final OD600 nm reading.

3.3.4. Osmoprotection assay

Osmoprotection assays were done as previously described with a few modifications [10]. Briefly, *P. aeruginosa* PA14 wild type and Δ*cbcV* were grown overnight in MOPS medium supplemented with 20 mM pyruvate and 5 mM glucose. Cells were collected by centrifugation, washed, and resuspended to an OD_{600} of 0.05 in MOPS medium with an additional 800 mM NaCl (850 mM total NaCl), 20 mM glucose, and 0.2 mM osmoprotectant (carnitine, acetylcarnitine, decanoylcarnitine, or

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myristoylcarnitine). 48-well plates were shaken at 37 °C for 48 hours and growth was determined by OD_{600}.

3.4. Results

3.4.1. *cbcV* is required for growth and induction of *caiX* in response to short-chain acylcarnitines.

In previous studies we showed that *P. aeruginosa* can grow on the short-chain acylcarnitines (SCAC) acetyl- and butyrylcarnitine [10]. The SCACs are hydrolyzed in the cytoplasm by HocS, therefore they must be imported to be used as sole carbon and nitrogen sources. It has been shown that the substrate binding protein *caiX* [22] and the core components of the ABC transporter *cbcWV* are required for growth on carnitine [21] (Fig. 2A). We hypothesized that this ABC carnitine transport system has broader specificity and can import short-chain acylcarnitines as well. The *cbcV* deletion strain was not able to grow on SCAC as the sole carbon source, (Fig. 2). Based on the growth data and HocS's cytoplasmic location, we predicted that *cbcWV* was necessary for SCAC induction of *caiX,* the first gene in the carnitine catabolism operon (Fig. 1). PA14WT and *cbcV* deletion mutant carrying the P*caiX-lacZ* plasmid (pJAM22) were induced with carnitine and SCACs, and *caiX* transcription was assessed by β-galactosidase assays. Carnitine and SCACs induced transcription of *caiX* in wild type but in the *cbcV* deletion mutant induction was greatly reduced due to the lack of the compounds not entering the cytoplasm (Fig. 2B). Taken together, the growth and reporter data indicate *cbcWV* is required for transport of SCACs.

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3.4.2. CaiX is the substrate binding protein for short-chain acylcarnitines.

CbcWV is the core ABC transporter for three QAC substrate binding proteins (SBP) – CaiX, BetX, and CbcX [21]. Since *cbcWV* is required for transport of SCACs (Fig. 2), this led us to investigate if CaiX was the SBP for SCACs, which the alternate possibility that there may be a separate SBP that interacts with CbcWV for SCAC transport. To determine if *caiX* is required for SCAC transport we compared *P. aeruginosa* PA14 wild type to the *caiX* mutant for their ability to grow on SCACs as sole nitrogen sources and β-galactosidase assays were performed as above on PA14 wild type and *caiX* carrying the P*caiX-lacZ* plasmid. The *caiX* mutant was unable to grow on SCACs (Fig. 3A) and induce the carnitine operon (Fig. 3B). These results support two conclusions, (i), *caiX* is required for growth on SCACs, and (ii), the ligand specificity of CaiX can be expanded to include acetyl- and butyrylcarnitine.

3.4.3. Growth on medium- and long-chain acylcarnitines does not require *cbcV*.

Animal and plant cells use medium- and long chain acylcarnitines as sources for and intermediate in beta-oxidation [26, 27], and we previously demonstrated that *P. aeruginosa* can use MCAC and LCAC as sole carbon sources [10]. We next tested if *cbcWV* was required for growth on MCAC and LCAC as sole carbon sources. PA14 wild type and the *cbcV* deletion mutant strain were able to grow in MOPS minimal media with acylcarnitines 6C and 10C-16C fatty acid chain length as the sole carbon source (Fig. 4). These data suggest that MCAC and LCAC require an entirely different transport system

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or are hydrolyzed in the outside of the cytosol where the free fatty acid can serve as a carbon source.

3.4.4. Medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically.

To determine the route of medium- and long-chain acylcarnitine being transport, we used the carnitine responsive transcriptional reporter P*caiX-lacZYA*. [22]. This rationale is that carnitine in the cytosol leads to *caiX* induction. If acylcarnitines can be transported into the cytoplasm and subsequently hydrolyzed, the free carnitine will induce *caiX*, whereas hydrolysis outside the cytosol would require the liberated carnitine to be transported via CaiX and CbcWV. Therefore, dependence on the free carnitine transporter components can allow us to identify the location of medium- and long-chain hydrolysis. Here we show that the induction of *caiX* is decreased by over three fold in a *caiX* and *cbcV* deletion mutant compared to the wild type for 6C-16C acylcarnitines, showing that *caiX* and *cbcV* are needed for the induction of *caiX* (Fig. 5A-B). The residual induction in these mutants will be addressed in the discussion section.

The lack of induction of *caiX* while retaining the ability to grow in a *cbcV* mutant when MCAC and LCAC were the sole carbon sources, led us to postulate that hydrolysis of these compounds occurs outside of the cytosol. A growth assay using 6C-16C acylcarnitines as sole nitrogen sources revealed that the *caiX* and *cbcV* deletion mutants failed to grow when compared to wild type (Fig. 5C). Along with the carbon source growth data and induction of *caiX* (Fig 5A-B, Fig. 4) indicates that hydrolysis of MCAC

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and LCAC occurs extracytoplasmically by an unidentified hydrolase and CaiX binds the liberated carnitine for transport through CbcWV as discussed in detail in the discussion.

3.4.5. Osmoprotection by short-chain acylcarnitines requires *cbcWV*.

It has been demonstrated that *cbcWV* is important for growth on choline but is not necessary for osmoprotection in a hyperosmotic environment when choline is the osmoprotectant [24]. We predict this is similar for carnitine and that another transporter is sufficient for osmoprotection. PA14 wild type and the *cbcV* mutant were grown in MOPS media with a final NaCl concentration of 850 mM, 20 mM of glucose as the carbon source, and 0.2 mM of the osmoprotectants carnitine, acetylcarnitine, decanoylcarnitine, or myristoylcarnitine. Carnitine, decanoylcarnitine, and myristoylcarnitine can be osmoprotectants in a *cbcV* mutant whereas acetylcarnitine cannot (Fig. 6). Carnitine and the free carnitine generated from hydrolysis outside the cytoplasm can circumvent *cbcV* by being imported through an unknown transporter to achieve osmoprotection. Acetylcarnitine, on the other hand, must first be imported into the cytoplasm by CbcWV for HocS hydrolysis in the cytosol in order to serve as an osmoprotectant [10].

3.5. Discussion

Acylcarnitines are valuable carbon and nitrogen sources, and its catabolic products carnitine and GB can function as osmoprotectants and induces the virulence factor phospholipase C, *plcH* [10]. In *P. aeruginosa* carnitine is transported by the substrate binding protein CaiX and the ABC transporter CbcWV but no work to date has

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examined acylcarnitine import [21, 22]. Our goal was to understand how acylcarnitines are imported and determine if there is any functional overlap between carnitine and acylcarnitine transport system. Using growth and reporter assays we show that MCAC and LCAC are hydrolyzed extracytoplasmically and the free carnitine is subsequently imported by CaiX and CbcWV to be utilized as a sole nitrogen source. Carnitine and the free carnitine generated from MCAC and LCAC hydrolysis can function as an osmoprotectant, but *cbcWV* is not required. SCAC require *caiX* and *cbcWV* for transport into the cytoplasm and are thus necessary for osmoprotection, expression of *plcH*, and use as carbon and nitrogen sources.

OpuC is an ABC transporter in *Bacillus subtilis* that imports the structurally similar QAC compounds carnitine, crotonobetaine, and gamma butyrobetaine [28]. The *P. aeruginosa* ABC transporter CbcWV is similar, and imports multiple QACs: glycine betaine, choline, and carnitine [21]. This led us to inquire if *cbcWV* imports other QACs and is involved in acylcarnitine import. Using growth assays with 2C-16C acylcarnitines (excluding octanoylcarnitine) as sole carbon sources, the *cbcV* mutant only had a defect on SCACs indicating that *cbcV* is not required for growth of MCAC and LCACs (Figs. 2 and 4). The substrate binding protein is the other essential component of an ABC transporter. Not all substrate binding proteins have a single ligand and some can promiscuously bind structurally similar compounds, as seen in *B. subtilis* [28, 29], *P. aeruginosa* [21], *E. coli* [30], and others [31]. In this study we have extended the binding specificity of CaiX to encompass acetyl- and butyrylcarnitine, as a *caiX* deletion mutant fails to grow on SCACs as sole carbon or nitrogen source. These data suggested two

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possible methods of transport of MCAC and LCACs: extracytoplasmic hydrolysis yielding carnitine and a fatty acid, or alternatively, import is through an unidentified transporter and cytoplasmic hydrolysis, in a manner similar to the SCACs [10].

Kappes and Bremer showed that *Bacillus subtilis* can hydrolyze acetyl- and octanoylcarnitine but were not able to determine if hydrolysis was on the cell surface or inside the cell [28]. Using the P*caiX-lacZ* reporter and nitrogen growth assays, we showed that MCAC and LCACs are hydrolyzed outside the cytoplasm and the liberated carnitine is transported through CbcWV (Figs. 4 and 5). The ability of the *cbcV* and *caiX* mutants to use MCAC and LCACs as sole carbon sources but not as sole nitrogen sources indicates that the fatty acid is the predominant carbon source in this compound (Fig. 5). In the *caiX* and *cbcV* mutants there is residual P*caiX-lacZ* induction in the carnitine and acylcarnitine samples, which we speculate is due to transport of carnitine through an unidentified BCCT transporter.

We previously reported that *P. aeruginosa* cannot utilize octanoylcarnitine as a sole carbon source [10]. Here we show octanoylcarnitine minimally induces *caiX* compared to carnitine in wild type *P. aeruginosa* (Fig. 5), indicating that hydrolysis is inefficient and is likely responsible for the inability of *P. aeruginosa* to grow on octanoylcarnitine [10]. Caprylic acid, the 8C chain fatty acid produced from octanoylcarnitine hydrolysis, has antibacterial properties and is commonly used in food preservation, medicine, cosmetics, and agriculture [32]. Caprylic acid and other fatty acids inhibit growth or kill bacteria, such as *Salmonella* Enteritidis [33], *E. coli* O157:H7 [34], *S. aureus* [35], and *P. aeruginosa* [35-37], by disrupting the cell membrane and

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interfering with processes associate within or at the membrane such as electron transport, oxidative phosphorylation [32]. The outer membrane acts as a semi-permeable barrier and defense mechanism [32, 38], but octanoylcarnitine may circumvent this natural barrier if hydrolysis is periplasmic, producing caprylic acid in the periplasm. *P. aeruginosa* may have evolved a hydrolase that excludes octanoylcarnitine as a substrate, in part to evade the antimicrobial effects of caprylic acid.

Acylcarnitine and carnitine transport into the cell for osmoprotection is well established [18]. Glycine betaine is the catabolic product of both carnitine and choline catabolism. Initial import of choline is through BetT1 and BetT3, with BetT3 having additional roles in growth and osmoprotection [24]. Choline is catabolized to glycine betaine which then induces *cbcWV* in a GbdR dependent manner, making *cbcWV* important for growth but not osmoprotection [24, 39]. Carnitine osmoprotection seems to functions similarly, with *cbcWV* being required for growth but not osmoprotection (Fig. 6). A carnitine transporter sufficient for osmoprotection has not been identified but may be a BCCT transporter, as with choline, and may even be one of the BetT family of transporters.

This study expands our knowledge on *P. aeruginosa* metabolism and transport of acylcarnitines. MCAC and LCACs are hydrolyzed extracytoplasmically by an unknown hydrolase to generate carnitine and a free fatty acid. Although *cbcWV* is not necessary for MC- and LCACs to be used as carbon sources, CbcWV provides a route for liberated carnitine to be used as a nitrogen source. The liberated carnitine can be used as an osmoprotectant via transport through an identified transporter likely a BCCT. We were

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also able to expand the ligand specificity of CaiX to include acetyl-, and butyrylcarnitine which are imported via the ABC transporter CbcWV, which is required for SCAC use an osmoprotectants and as carbon and nitrogen sources.

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3.6. Figures

Figure 1. Diagram of the operon configuration and ABC transporter in *P. aeruginosa* PA14 [21]. (A) Diagram of the *P. aeruginosa* PAO1 choline ABC transporter operon *cbcXWV*: choline substrate binding protein, *cbcX*; transmembrane protein, *cbcW*; and nucleotide binding protein, *cbcV*. (B) Diagram of the carnitine catabolism operon *caiXcdhCABhocS*: carnitine substrate binding protein, *caiX*; beta-keto cleavage acid enzyme, *cdhC*; carnitine dehydrogenase protein, *cdhAB*; and short-chain *O*-acylcarnitine hydrolase, *hocS*.

Figure 2. *cbcV* is necessary for growth and induction of *caiX* on short-chain acylcarnitines. (A) PA14WT and Δ*cbcV* cells were grown in MOPS minimal media supplemented with 20 mM carnitine, acetylcarnitine, or butyrylcarnitine. Cultures were grown at 37 °C for 24 hours prior to reading them at OD600 nm. (B) PA14 WT and Δ*cbcV* carrying *caiXlacZ* plasmid (pJAM22) were grown in MOPS, 20 mM pyruvate, 20 μg ml^{-1} gentamicin, and with or without 1 mM inducing compound carnitine, acetylcarnitine or butyrylcarnitine. Cells were induced for 4 hours at 37 °C before performing a Miller assay. AC is acetylcarnitine and BC is butyrylcarnitine. Two way ANOVA. ns, not significant; ****, $P < 0.0001$.

Figure 3. CaiX is the substrate binding protein for short-chain acylcarnitines. (A) PA14WT and Δ*caiX* cells were grown in MOPS minimal media supplemented with 20 mM carnitine, acetylcarnitine, or butyrylcarnitine. Cultures were grown at 37 °C for 24

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hours prior to reading them at OD600 nm. (B) PA14 WT and Δ*caiX* carrying *caiXlacZ* plasmid (pJAM22) were grown in MOPS, 20 mM pyruvate, 20 µg ml^{-1} gentamicin, and with or without 1 mM inducing compound carnitine, acetylcarnitine, or butyrylcarnitine. Cells were induced for 4 hours at 37 °C before performing a Miller assay. AC is acetylcarnitine and BC is butyrylcarnitine. Two way ANOVA. ns, not significant; ****, $P < 0.0001$

Figure 4. Medium- and long-chain acylcarnitines do not require *cbcV* for growth. PA14WT and *cbcV* deletion mutant were grown in MOPS with 20 mM of carnitine or acylcarnitines 6C, 10C-16C fatty acid chain length for 24 hours at 37 °C. OD600 nm was taken on a BioTek plate reader except for palmitoylcarnitine, which was determined by CFU due to micelle formation. One way ANOVA. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$

Figure 5. *caiX* and *cbcV* are required for growth when acylcarnitines are the sole nitrogen source. (A) PA14WT (white bars), Δ*cbcV* (gray bars), and Δ*caiX* (black bars) cells were grown at 37 °C in M63 minimal media supplemented with 20 mM pyruvate and 2 mM nitrogen source; ammonium, carnitine, or acylcarnitines 6C-16C fatty acid chain-length. Growth was assessed after 24 hours by OD600 nm. Two way ANOVA comparing carnitine to Δ*cbcV* and Δ*caiX*. ns, not significant; ****, $P < 0.0001$ (B) PA14WT was induced for 4 hours at 37 °C in MOPS with 20 mM pyruvate, 20 µg ml^{-1} gentamicin, and 1 mM carnitine, or 6C-16C acylcarnitines. Miller assays were

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performed. ns, not significant; **, P < 0.01 (C) PA14WT, Δ*cbcV*, and Δ*caiX* were induced for 4 hours at 37 °C in MOPS with 20 mM pyruvate, 20 µg ml^{-1} gentamicin, and 1 mM carnitine, or 6C-16C acylcarnitines. Miller assays were performed. ns, not significant; ****, P < 0.0001

Figure 6. Short-chain acylcarnitines require *cbcWV* for osmoprotection in hyperosmotic conditions. *P. aeruginosa* PA14WT and Δ*cbcV* were grown in MOPS supplemented with 800 mM NaCl, 20 mM glucose, and with or without 0.2 mM osmoprotectants carnitine, acetylcarnitine, decanoylcarnitine, or myristoylcarnitine for 48 hours before reading the OD600 nm. ns, not significant; ****, P < 0.0001

Figure 7. Model of acylcarnitine import in *P. aeruginosa* PA14.

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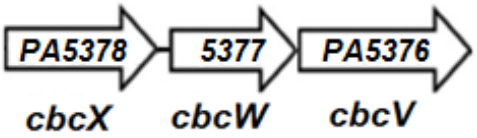
A



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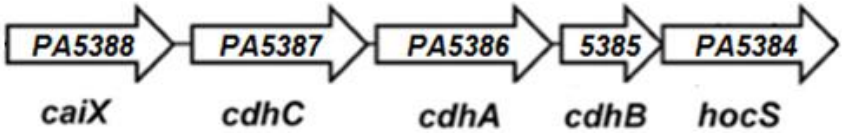

Figure 1. Diagram of the operon configuration and ABC transporter in *P. aeruginosa* PA14.

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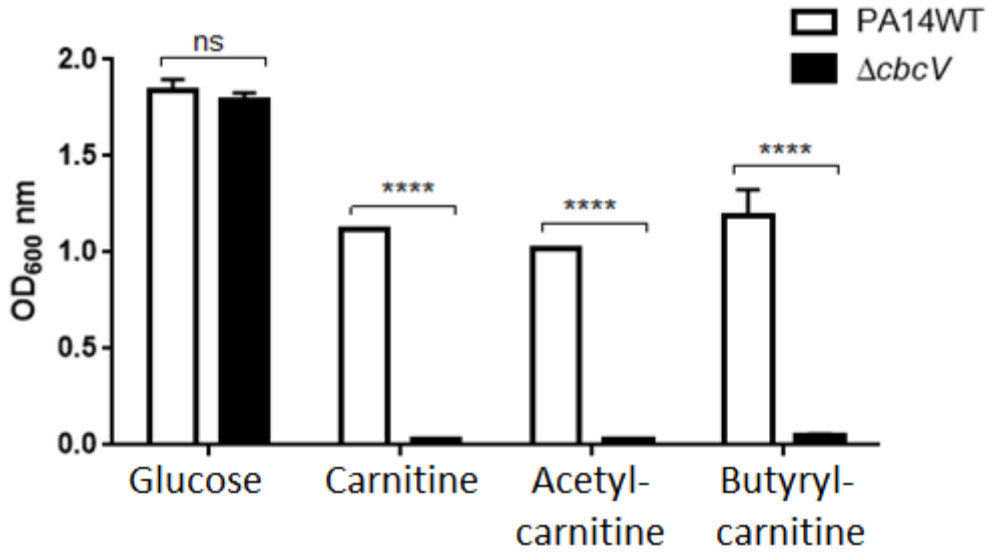

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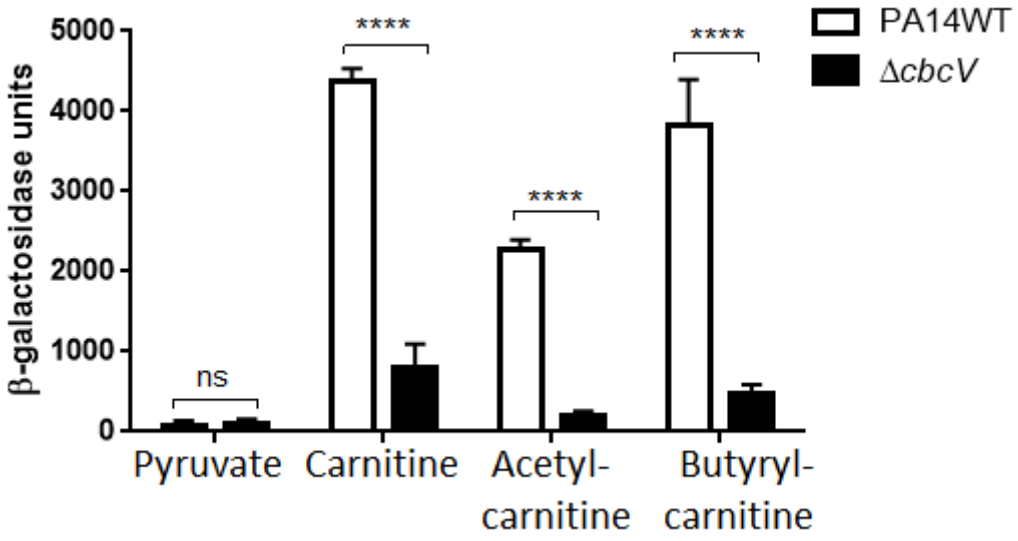

Figure 2. *cbcV* is necessary for growth and induction of *caiX* on short-chain acylcarnitines.

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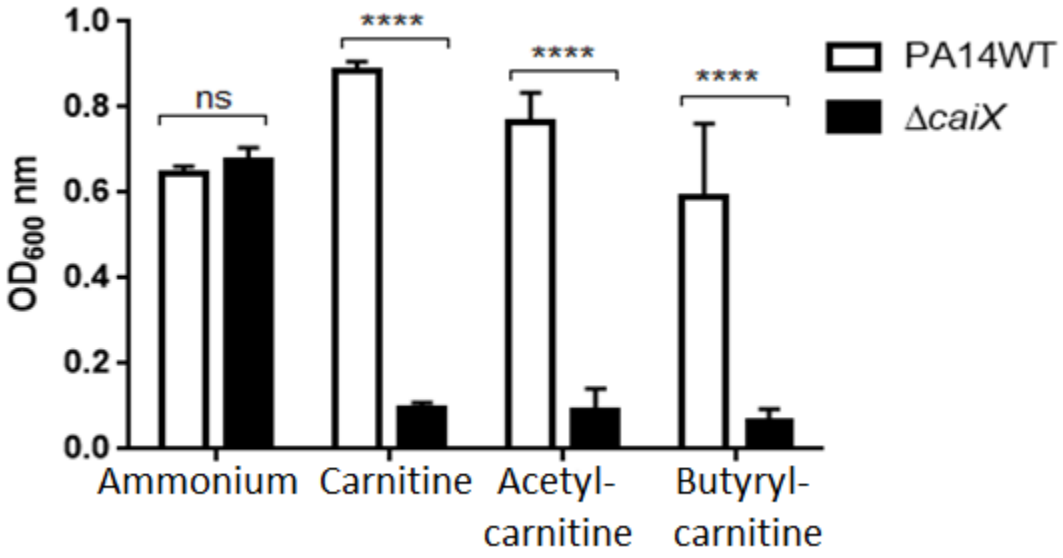
A.



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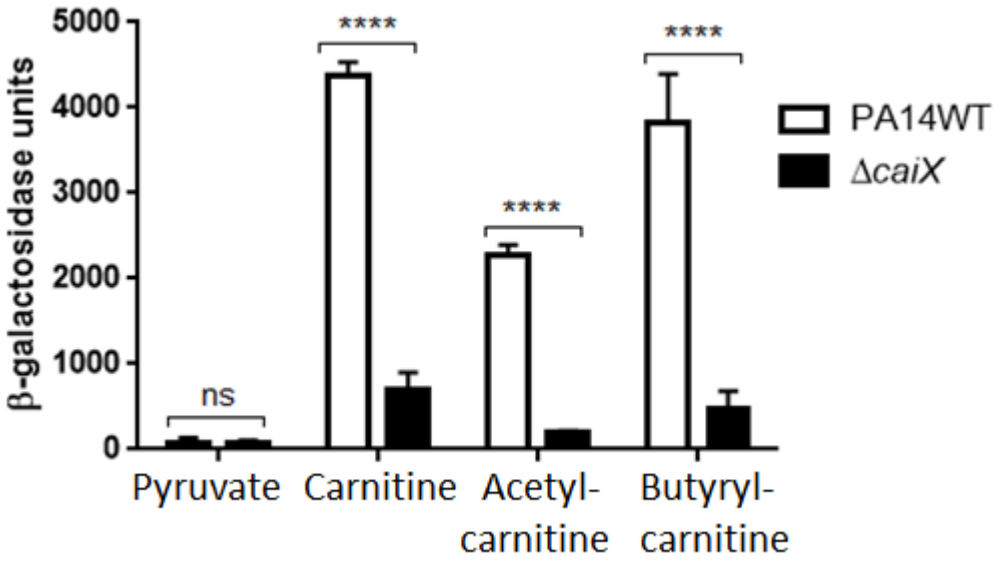

Figure 3. CaiX is the substrate binding protein for short-chain acylcarnitines.

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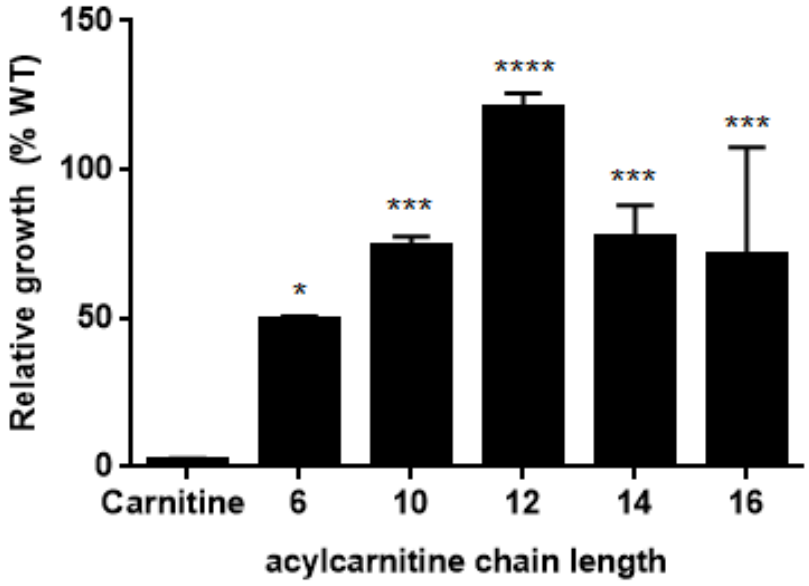

Figure 4. Medium- and long-chain acylcarnitines do not require *cbcV* for growth.

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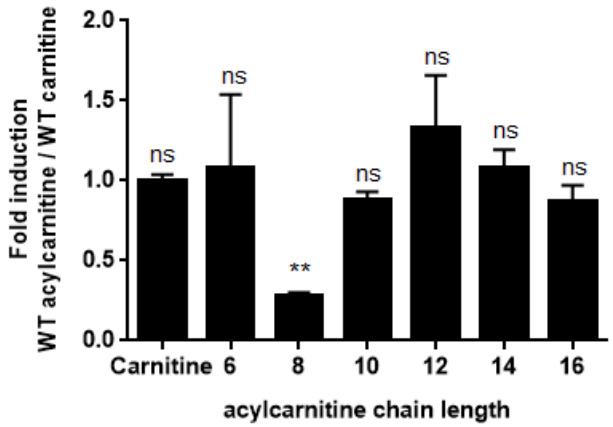
A.



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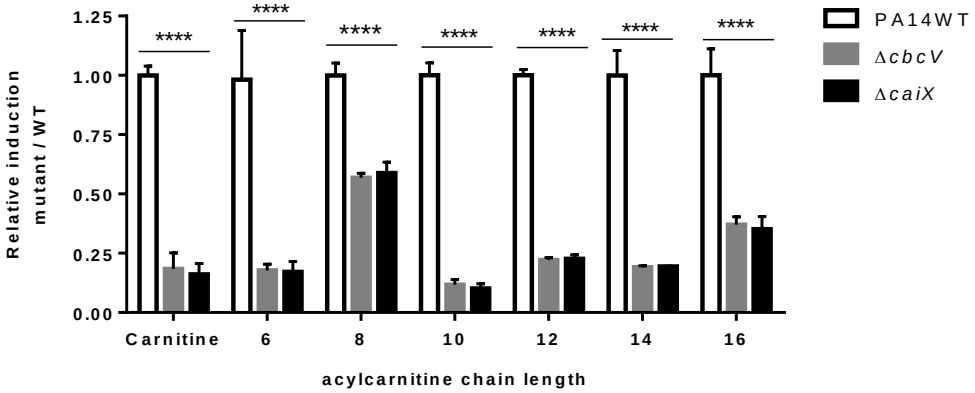

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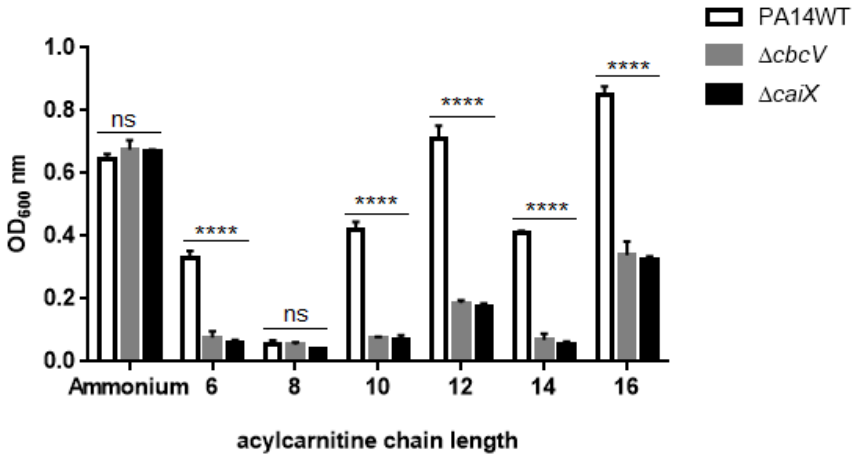

Figure 5. *caiX* and *cbcV* are required for growth when acylcarnitines are the sole nitrogen source.

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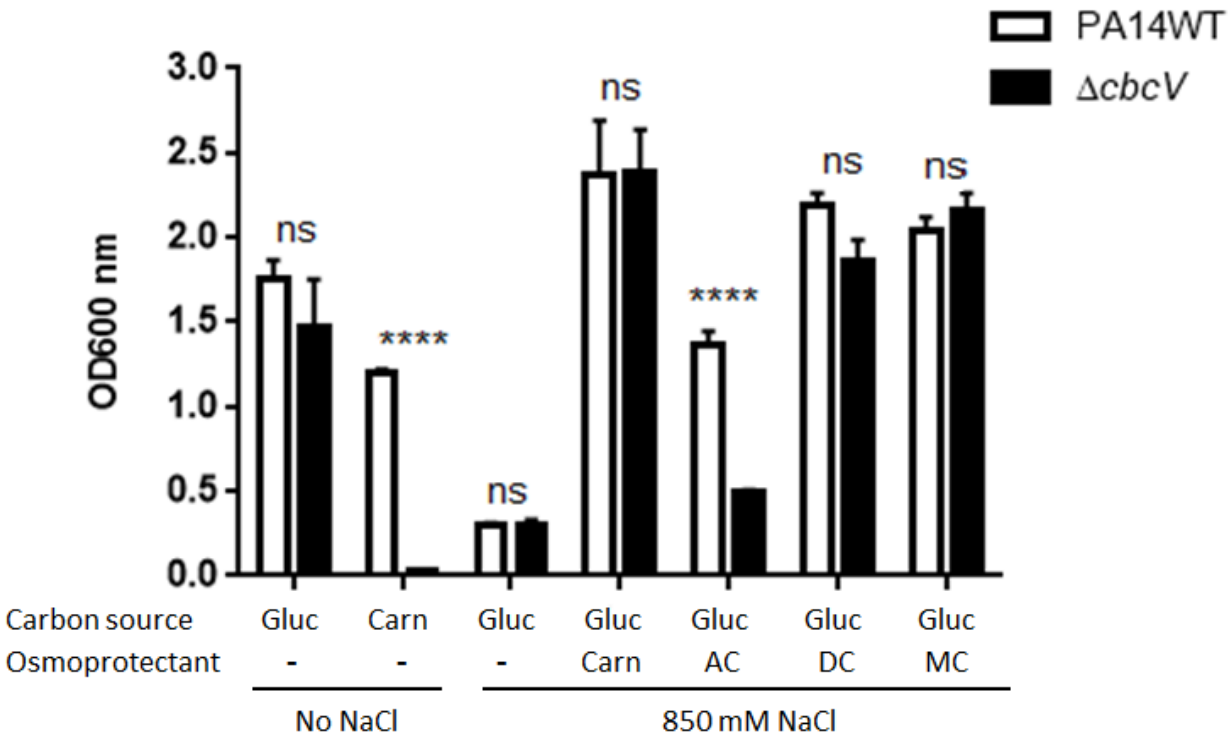

Figure 6. Short-chain acylcarnitines require *cbcWV* to function as osmoprotectants in hyperosmotic conditions.

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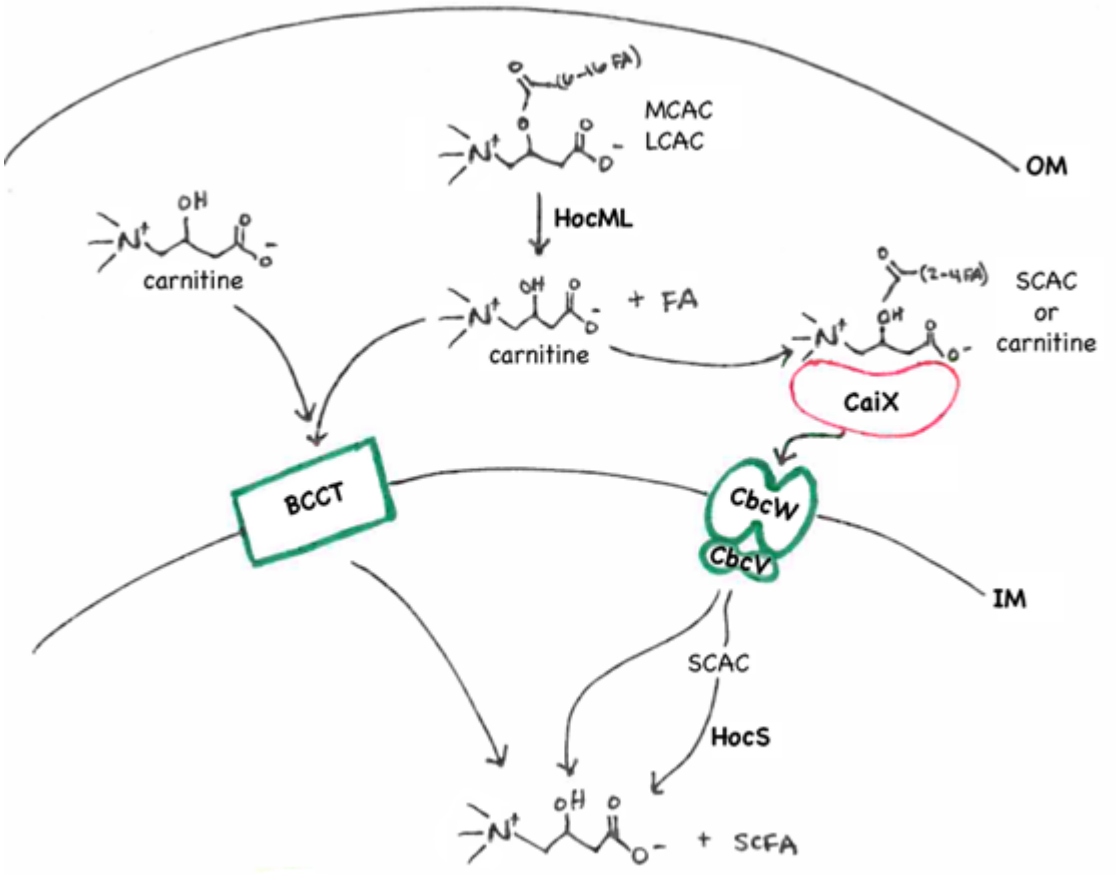

Figure 7. Model of *P. aeruginosa* transport of acylcarnitines.

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3.7. References

1. Warren, C. R. (2013) Quaternary ammonium compounds can be abundant in some soils and are taken up as intact molecules by plants, *The New phytologist.* **198**, 476-85.
2. Warren, C. R. (2013) High diversity of small organic N observed in soil water, *Soil Biol Biochem.* **57**, 444-450.
3. Fraenkel, G. (1951) Effect and distribution of vitamin BT, *Archives of biochemistry and biophysics.* **34**, 457-67.
4. Panter, R. A. & Mudd, J. B. (1969) Carnitine levels in some higher plants, *FEBS letters.* **5**, 169-170.
5. Bieber, L. L. (1988) Carnitine, *Annual review of biochemistry.* **57**, 261-83.
6. Borum, P. R. (1983) Carnitine, *Annual review of nutrition.* **3**, 233-59.
7. Rebouche, C. J. & Seim, H. (1998) Carnitine metabolism and its regulation in microorganisms and mammals, *Annual review of nutrition.* **18**, 39-61.
8. Rebouche, C. J. & Chenard, C. A. (1991) Metabolic fate of dietary carnitine in human adults: identification and quantification of urinary and fecal metabolites, *The Journal of nutrition.* **121**, 539-46.
9. Lindstedt, G., Lindstedt, S., Midtvedt, T., Tofft M. (1967) The Formation and Degradation of Carnitine in *Pseudomonas*, *Biochemistry.* **6**, 1262-1270.
10. Meadows, J. A. & Wargo, M. J. (2013) Characterization of *Pseudomonas aeruginosa* growth on O-acylcarnitines and identification of a short-chain acylcarnitine hydrolase, *Applied and environmental microbiology.* **79**, 3355-63.
11. Lucchesi, G. I., Lisa, T. A., Casale, C. H. & Domenech, C. E. (1995) Carnitine resembles choline in the induction of cholinesterase, acid phosphatase, and phospholipase C and in its action as an osmoprotectant in *Pseudomonas aeruginosa*, *Current microbiology.* **30**, 55-60.
12. D'Souza-Ault, M. R., Smith, L. T. & Smith, G. M. (1993) Roles of N-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress, *Applied and environmental microbiology.* **59**, 473-8.
13. Uanschou C., F. R., Pittner F. (2005) What to learn from comparative genomic sequence analysis of L-carnitine dehydrogenase, *Monatshefte fur Chemie.* **136**, 1365-1381.
14. Kleber, H. P. & Aurich, H. (1967) [Damped oscillations in the synthesis of carnitine dehydrogenase by *Pseudomonas aeruginosa*], *Hoppe-Seyler's Zeitschrift fur physiologische Chemie.* **348**, 1727-9.
15. Kang, Y., Nguyen, D. T., Son, M. S. & Hoang, T. T. (2008) The *Pseudomonas aeruginosa* PsrA responds to long-chain fatty acid signals to regulate the *fadBA5* beta-oxidation operon, *Microbiology.* **154**, 1584-98.
16. Son, M. S., Matthews, W. J., Jr., Kang, Y., Nguyen, D. T. & Hoang, T. T. (2007) In vivo evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients, *Infection and immunity.* **75**, 5313-24.

www.manaraa.com

17. Turner, K. H., Everett, J., Trivedi, U., Rumbaugh, K. P. & Whiteley, M. (2014) Requirements for *Pseudomonas aeruginosa* acute burn and chronic surgical wound infection, *PLoS genetics.* **10**, e1004518.
18. Meadows, J. A. & Wargo, M. J. (2015) Carnitine in bacterial physiology and metabolism, *Microbiology*.
19. Ziegler, C., Bremer, E. & Kramer, R. (2010) The BCCT family of carriers: from physiology to crystal structure, *Molecular microbiology.* **78**, 13-34.
20. Linton, K. J. (2007) Structure and function of ABC transporters, *Physiology.* **22**, 122-30.
21. Chen, C., Malek, A. A., Wargo, M. J., Hogan, D. A. & Beattie, G. A. (2010) The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds, *Molecular microbiology.* **75**, 29-45.
22. Wargo, M. J. & Hogan, D. A. (2009) Identification of genes required for *Pseudomonas aeruginosa* carnitine catabolism, *Microbiology.* **155**, 2411-9.
23. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S. & Olson, M. V. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen, *Nature.* **406**, 959-64.
24. Malek, A. A., Chen, C., Wargo, M. J., Beattie, G. A. & Hogan, D. A. (2011) Roles of three transporters, CbcXWV, BetT1, and BetT3, in *Pseudomonas aeruginosa* choline uptake for catabolism, *Journal of bacteriology.* **193**, 3033-41.
25. Miller, J. (1972) Experiments in molecular genetics in *Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, NY.
26. Bourdin, B., Adenier, H. & Perrin, Y. (2007) Carnitine is associated with fatty acid metabolism in plants, *Plant physiology and biochemistry : PPB / Societe francaise de physiologie vegetale.* **45**, 926-31.
27. Steiber, A., Kerner, J. & Hoppel, C. L. (2004) Carnitine: a nutritional, biosynthetic, and functional perspective, *Molecular aspects of medicine.* **25**, 455-73.
28. Kappes, R. M., Bremer E. (1998) Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and y-butyrobetaine via the ABC transport system OpuC *Microbiology.* **144**, 83-90.
29. Du, Y., Shi, W. W., He, Y. X., Yang, Y. H., Zhou, C. Z. & Chen, Y. (2011) Structures of the substrate-binding protein provide insights into the multiple compatible solute binding specificities of the *Bacillus subtilis* ABC transporter OpuC, *The Biochemical journal.* **436**, 283-9.
30. Trakhanov, S., Vyas, N. K., Luecke, H., Kristensen, D. M., Ma, J. & Quiocho, F. A. (2005) Ligand-free and -bound structures of the binding protein (LivJ) of the *Escherichia coli* ABC leucine/isoleucine/valine transport system: trajectory and dynamics of the interdomain rotation and ligand specificity, *Biochemistry.* **44**, 6597-608.

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31. Berntsson, R. P., Smits, S. H., Schmitt, L., Slotboom, D. J. & Poolman, B. (2010) A structural classification of substrate-binding proteins, *FEBS letters.* **584**, 2606-17.
32. Desbois, A. P. & Smith, V. J. (2010) Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential, *Applied microbiology and biotechnology.* **85**, 1629-42.
33. Skrivanova, E., Hovorkova, P., Cermak, L. & Marounek, M. (2015) Potential use of caprylic acid in broiler chickens: effect on *Salmonella enteritidis*, *Foodborne pathogens and disease.* **12**, 62-7.
34. Baskaran, S. A., Bhattaram, V., Upadhyaya, I., Upadhyay, A., Kollanoor-Johny, A., Schreiber, D., Jr. & Venkitanarayanan, K. (2013) Inactivation of *Escherichia coli* O157:H7 on cattle hides by caprylic acid and beta-resorcylic acid, *Journal of food protection.* **76**, 318-22.
35. Richter, F. L., Pedersen, D.E., Fredell, D.L. (1993) Food additive sanitizing compositions in (Patent, U. S., ed)
36. Rosenblatt, J., Reitzel, R. A. & Raad, I. (2015) Caprylic acid and glyceryl trinitrate combination for eradication of biofilm, *Antimicrobial agents and chemotherapy.* **59**, 1786-8.
37. Nakai, S., Siebert, K. (2004) Organic acid inhibition models for *Listeria innocua, Listeria ivanovii, Pseudomonas aeruginosa* and *Oenococcus oeni*, *Food Microbiology.* **21**, 67-72.
38. Osborn, M. J. & Wu, H. C. (1980) Proteins of the outer membrane of gram-negative bacteria, *Annual review of microbiology.* **34**, 369-422.
39. Hampel, K. J., LaBauve, A. E., Meadows, J. A., Fitzsimmons, L. F., Nock, A. M. & Wargo, M. J. (2014) Characterization of the GbdR regulon in *Pseudomonas aeruginosa*, *Journal of bacteriology.* **196**, 7-15.

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CHAPTER 4:

REGULATION OF CARNITINE METABOLISM IN *PSEUDOMONAS AERUGINOSA*

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4.1. Abstract

Pseudomonas aeruginosa is found in numerous environments and is an opportunistic pathogen affecting those who are immunocompromised. The large metabolically diverse genome and regulatory network that *P. aeruginosa* possesses make it versatile to adapting to various environments. We are interested in how *P. aeruginosa* senses and responds to host-derived compounds. Previously we identified CdhR and GbdR, members of the AraC transcription factor family that regulate catabolism of the quaternary amine compounds carnitine and glycine betaine (GB), respectively. Our goal was to further characterize regulation of carnitine catabolism by the TF CdhR. Here we report that CdhR binds in a concentration dependent manner to the enhancer site near the carnitine operon promoter (P*caiXcdhCABhocS*). We identified the CdhR binding site and compared with the known GbdR binding consensus, to show that the two binding sites overlap in the *caiX-cdhR* intergenic region. Carnitine catabolism is repressed by glucose and GB at the transcriptional level. Furthermore, using two different *cdhR* translational fusions we show that CdhR enhances its own expression and that GbdR contributes to *cdhR* expression by enhancing the level of basal expression.

4.2. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium found in soil, water, on skin and on surfaces, such as bed rails, catheters, and other medical devices [1, 2]. From the sites it can transition into being an opportunistic human pathogen that is the leading cause of infection in people with severe burns [3], and those with lung diseases like

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chronic obstructive pulmonary disease, ventilator-associated pneumonia, and cystic fibrosis, resulting in increased morbidity and mortality [4-7]. With antibiotic resistance on the rise [8], *P. aeruginosa* is difficult to treat and is a burden on our health care system [9]. The ability to transition from one environment to the next and cause disease is, in part, due to its large genome, with 5% of genes predicted to encode proteins related in carbon metabolism [10].

Carnitine and *O*-acylcarnitines are quaternary amine compounds that are abundant in host tissues and shuttle fatty acids in and out of the mitochondria for β-oxidation in animals [11]. There are no animal enzymes that can breakdown carnitine, consequently any degradation is due to bacteria [12], like *P. aeruginosa* [13]. No *de novo* synthesis of carnitine has been demonstrated in bacteria [14], therefore *P. aeruginosa* acquires it from the environment by import through the ABC transporter CbcWV [15]. *P. aeruginosa* contains a carnitine catabolism operon *caiXcdhCABhocS*, which encode enzymes to degrade short-chain acylcarnitines and carnitine for osmoprotection, and its catabolic product glycine betaine (GB) induces the virulence factor phospholipase C, *plcH* and be used as sole carbon, nitrogen, and energy source [16-19]. Medium- and long-chain acylcarnitines, with the exception of octanoylcarnitine, can be used as sole carbon sources as well, [16].

The ability to adapt to numerous environments can be attributed to the large regulatory repertoire that *P. aeruginosa* possesses. More than 9% of the genome is dedicated to transcriptional regulation, including regulation of metabolism, giving the bacterium metabolic flexibility [10]. One such regulator is CdhR, which is divergently

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transcribed from the carnitine catabolism operon and is required for growth on carnitine and induction of the carnitine operon [17]. Carnitine degradation leads to the formation of glycine betaine, whose catabolism is regulated by GbdR [20]. GbdR and CdhR not only regulate catabolism of quaternary amine compounds but both are AraC-family transcription factors that belong to the same GATase-1 subfamily and are highly similar in sequence (62% positive and 44% identities) [21].

In this study we expand our understanding of the regulation of carnitine catabolism, by identifying the enhancer site, essential residues, and showing catabolite repression from glucose and glycine betaine function at the level of transcription of the carnitine operon. Although an additional CdhR binding site was found upstream of *cbcWV* to which CdhR binds in a concentration dependent manner, we established that transcription from this locus is controlled solely by GbdR in response to glycine betaine. GbdR is also capable of binding the intergenic region of *caiX-cdhR* in an orientation suggesting regulation of *cdhR*. Additionally, CdhR positively regulates its own expression in the presence of carnitine but represses basal expression in the absence of ligand, however GbdR alleviates this repression. We propose that there is a hierarchy to CdhR and GbdR binding at their shared binding site with and without ligand.

4.3. Materials and methods

4.3.1. Strains and growth conditions

Pseudomonas aeruginosa strains PAO1 and PA14 were maintained on PIA plates or Lennox broth (LB) liquid, and when necessary 50 µg ml^{-1} or 40 µg ml^{-1}

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gentamicin was added to the media, respectively. *Escherichia coli* NEB5α or T7 Express *E. coli* (NEB C3016) were maintained on LB plates with 10 µg ml^{-1} gentamicin, LB liquid with 7 µg ml^{-1} gentamicin, LB plates/liquid with 125 µg ml^{-1} carbenicillin, or LB plates/liquid with 100 µg ml^{-1} kanamycin.

4.3.2. Deletion and complementation constructs

A clean deletion of *PA5389* (*cdhR*) in PAO1 was made in the wild type strain MJ79. The upstream and downstream regions of *PA5389* were PCR amplified from PAO1 genomic DNA with the primers 5389GOIF1KpnI, 5389SOEGOIR1, 5389SOEGOIF1, and 5389GOIR1BamHI. Splice overlap extension PCR product was ligated into the Zero Blunt™ plasmid pCR (Invitrogen), excised with EcoRI, and ligated into similarly cut pMQ30 to generate pJAM90. Donor *E. coli* S17λpir carrying pJAM90 were mated with PAO1 and screened as previously described [16], generating strain JM236. Deletion strain was verified by lack of growth on carnitine and by PCR.

To make a double mutant of *cdhR* and *gbdR* in PA14, the Δ*gbdR* PA14 strain MJ26 was used as the recipient strain for mating with *E. coli* S17λpir carrying pJAM90 as described above. Double mutant strain was verified by PCR.

4.3.3. Construction of transcriptional constructs

To determine the promoter of *PA5388* (*caiX*) four different truncations of the upstream region of *caiX* were cloned as transcriptional reporter constructs. pMW79 was used as the PA14 PCR template for amplifying the upstream region of *caiX*.

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PA5388promR is the reverse primer used in all four reactions and the forward primers are PA5388promF1, PA5388promF2, PA5388promF3, and PA5388promF4. The four PCR products were ligated into the Zero Blunt vector pCR, digested with KpnI and HindIII, and ligated into a similarly cut pMW5 to yield four different P*5388lacZYA* transcriptional fusions (pJAM22-pJAM25). Each was electroporated into PA14WT and selected on PIA gentamicin plates.

To assess which residues were essential for induction of *caiX*, seven different transcriptional reporters were constructed by amplifying the *caiX* binding site from pMW79 using the 5388promR primer and seven different forward primers: P5388pos, P5388mut1, P5388mut2, P5388mut3, P5388mut4, P5388mut5, P5388mut6. PCR products were digested with HindIII and KpnI, and ligated into similarly cut pMW5. The resulting plasmids pJAM122-pJAM127 and pJAM130 were electroporated into PA14WT and selected on PIA gentamicin.

4.3.4. Construction of translational constructs

To determine translational control of *cdhR* a divergent fluorescent translational reporter with YFP and CFP on either side of the intergenic region of *PA5388* and *PA5389* was made. YFP was amplified using primers YFP F Kpn Sal and YFP R HindIII from the template pUC18miniTn7T-Gm-*eyfp* [22]. The YFP fragment was ligated into pCR Zero Blunt plasmid, digested with HindIII and KpnI, and subsequently ligated into similarly cut pMQ80. The resulting plasmid was digested with EcoRI and SacI and ligated with a *P. aeruginosa* codon-biased CFP made by IDT, which was cut with EcoRI and SacI, to

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generate pJAM76. The *PA5388-PA5389* intergenic region was amplified using primers 88-89YC-DR#2ycF and 88-89YCDR#2ycR. pJAM76 was then digested with SalI to linearize the plasmid for recombination of the *PA5388-PA5389* intergenic regions using yeast cloning in *Saccharomyces cerevisiae* via the method of Shanks et al [23]. The resultant constructed plasmid was digested with HindIII and ligated into similarly cut pUC18-mini-Tn7T-Gm yielding pJAM86, which was coelectroporated with pTNS2 to insert CFP-PA5388-PA5389-YFP onto the chromosome at the *att*Tn7 site of strains PAO1 wild type, PAO1Δ*cdhR* and PAO1ΔgbdR.

A *PA5389* translational *lacZ* fusion was made by inserting a gBlock™ made by IDT that has 320 base pairs of the upstream region of *PA5389* to the translational start site of *PA5389* and then from the translational start site of *lacZ* to + 850 bases in the *lacZ* gene into pJAM131. pJAM131 was built by PCR amplifying the C-terminal end of *lacZ* with primers 2-lacZCtermFhindcla and 2-lacZCtermRsmakpn, digested with HindIII and KpnI, and ligated into a similarly cut pUCP22. The gBlock was digested with HindIII and ClaI and ligated into similarly cut pJAM131 to make pJAM135.

4.3.5. Cloning and expression of MBP-CdhR

The expression plasmid pJAM50, a maltose binding protein (MBP) fusion to the amino-terminus of CdhR was made by PCR amplifying *cdhR* with primers 5389Mal-C2XF and 5389Mal-C2XR, and the product was ligated into Zero Blunt pCR vector. *cdhR* was extracted with EcoRI and HindIII and ligated into the similarly cut pMal-C2X

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(NEB), transformed by electroporation into T7 Express *E. coli* (NEB C2566), and selected on LB carbenicillin to make strain JM153.

To express MBP-CdhR, 1L of JM153 was grown at 37 °C in LB with 75 µg ml^{-1} to an OD600 of 0.4. The culture was induced with 1 mM IPTG and grown for an additional 3 hours. Cells were collected by centrifugation and resuspended in 20 mM Tris HCl, 150 mM NaCl, pH 7.4 with 1X halt protease inhibitor cocktail (Thermo) and lysed by French press. DNaseI was added to the lysate and incubate room temperature for 15 minutes. Samples were centrifuged at 13K rpm for 30 minutes at 4 °C to separate the soluble and insoluble fractions. The soluble fraction was filtered through a 0.22 µm filter and applied to an Affi-Gel Heparin Gel (BioRad) column. The column was washed with column buffer (10 mM KH2PO4, 150 mM NaCl, pH 7.4 in 1XPBS) and MBP-CdhR was eluted from the column with column buffer plus 1.5 M NaCl. Fractions containing MBP-CdhR were run on a 12% SDS denaturing gel and stained with coomassie brilliant blue (Thermo) to verify purity and determine which fractions to use. Elutions containing MBP-CdhR were dialyzed in a 20K MWCO slidealyzer (Thermo) overnight at 4 °C in buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4).

4.3.6. Primer extension

Transcriptional start site of *caiX* and *cdhR* were determined by growing PA14WT carrying pMW79 [17] or PA14WT carrying pJAM135, respectively, in MOPS, 20 mM pyruvate, 5 mM glucose, and 20 µg ml^{-1} gentamicin overnight at 37 °C. Cells were washed, resuspended to an OD_{600} nm of 0.3 in MOPS supplemented with 20 mM L-

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carnitine or pyruvate and 20 µg ml^{-1} gentamicin in a 12 well-plate, and grown for 7 hours at 37 °C while shaking. Cells were collected by centrifugation and RNA was purified per the instructions of the RNeasy Mini Kit (Qiagen). Primer extension was completed using the purified RNA as the template for cDNA generation using the Superscript II RT (Invitrogen) and the 5'-fluorescein-labeled primers 5388primerextension or 5389primerextension. The cDNA products were analyzed by capillary electrophoresed on an ABI to determine the size of the DNA fragment of the induced carnitine sample verses the uninducing pyruvate and by comparing it to a known DNA size standard.

4.3.7. Beta-galactosidase assays

Strains were grown overnight at 37 °C in MOPS minimal media supplemented with 20 mM pyruvate, 5 mM glucose, and 20 µg ml^{-1} gentamicin. Cells were collected by centrifugation, washed, resuspended in MOPS and inoculated into MOPS with 20 mM pyruvate, 20 µg ml^{-1} gentamicin, and with or without 1 mM inducing compound carnitine. Induction was carried out for 4 hours at 37 °C, and β-galactosidase assays were performed according to Miller [24].

4.3.8. Catabolite repression

PA14WT carrying pJAM22 was grown overnight at 37 °C in MOPS with 20 mM pyruvate, 5 mM glucose, and 20 µg ml^{-1} gentamicin. Cells were collected by centrifugation, washed, and resuspended in MOPS, and inoculated into MOPS with 20 mM pyruvate and 20 µg ml^{-1} gentamicin at a final OD_{600} of 0.05. All samples had 1 mM

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of the inducting compound carnitine except for the noninducing control (pyruvate alone). Catabolite repression samples had either glucose at 2 mM, 4 mM, or 10 mM or glycine betaine at 20 mM or 40 mM. Cultures were induced for 4 hours at 37 °C, and β-galactosidase assays were performed according to Miller [24].

4.3.9. Electrophoretic Mobility Shift Assays (EMSA)

To determine binding of MBP-CdhR or MBP-GbdR to promoters, EMSAs were performed as previously described [21]. Briefly, *caiX*, *dhc*, *cbcX*, *cdhR*, or *cdhR* mutant promoter DNA fragments were made by PCR amplification from PA14 genomic DNA template with primers listed in the EMSA primer section in Table 2. The DNA probes were dialyzed in ¼ TE on a 0.025 µm filter for 20 minutes and verified by PCR. EMSAs were conducted by following the LightShift Chemiluminescent EMSA kit instructions (Pierce) as modified by Hampel et al [21]. Samples were electrophoresed on 5% TBE nondenaturing gels, transferred to BioDyne-B nylon membrane (Thermo), and detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo) per manufacturer's instructions.

4.3.10. DNaseI footprinting assay

DNaseI footprinting was performed as described previously [21]. Briefly, the target DNA was made by labeling the 5' end of one the primers PA5388promR or PA5388promF2 with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P]ATP, and amplifying the PA14 *caiX* upstream region. The radiolabeled PCR product was purified from a 5%

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polyacrylamide Tris-borate-EDTA gel. The MBP-CdhR footprinting assay was conducted as in Brenowitz [25] and Hampel et al [21].

4.3.11. Fluorescent microscopy

P. aeruginosa PAO1 strains WT, Δ*cdhR*, and Δ*gbdR* with the divergent fluorescent reporter *PA5388-PA5389* intergenic region CFPYFP-DR2 (pJAM86) at the *att*Tn7 site, were grown overnight in MOPS with 20 mM pyruvate and 5 mM glucose at 37 °C. Cells were centrifuged, washed, and resuspended in MOPS to an OD_{600} of 1.0. A 1/20 dilution was made and 1 µl was placed on the center of the agar pad (1.5% low melting agarose, MOPS, 20 mM pyruvate, and with or without 1 mM carnitine). The agar pad was placed upside down on a 50 mm glass bottom cell culture dish (Warner Instruments). Samples were imaged on a Nikon Ti-E, every 10 minutes for 6 hours, at 32 °C and exposure time was 100 msec for phase and 500 msec for YFP. Image stacks were imported into Fiji 2.0.0-rc-29/1.49s [26] using the Bio-Formats Importer 5.1.1 [27]. A rolling variance filter on the contrast channel was used to define background areas, and after an additional threshold was applied, the find maxima macro was used to define cell areas. Using masks from the contrast channel mean pixel intensity measurements were taken from the YFP channel. A framewise background intensity correction was performed for each cell using R 3.2.0 [28].

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4.4. Results

4.4.1. Promoter mapping reveals the CdhR binding site.

The carnitine catabolism operon, *caiX-cdhCAB-hocS* (Fig. 1), encodes proteins that are responsible for the hydrolysis of short-chain acylcarnitines [16] and degradation of carnitine to glycine betaine [17, 29]. *cdhR* is divergently transcribed from the carnitine catabolism operon (Fig. 1), and was previously shown to be required for induction of *caiX* and growth on carnitine [17]. To expand upon this basic understanding of CdhR's role in carnitine catabolism, we determined the binding site of CdhR by promoter mapping of P*caiX* using four sequentially shorter *lacZYA* transcriptional reporters. The constructs end at base pair (bp) -22 from the *caiX* translational start site and begin at bp -424 (pJAM22), bp -327 (pJAM23), bp -241 (pJAM24), and bp -137 (pJAM25)(Fig. 2). In the presence of carnitine all constructs except pJAM25 were induced, indicating that the binding site of CdhR is between bp -241 and -137 (Fig. 2). The binding site may also overlap with bp -137 with a partial site not sufficient to induce of *caiX*.

Primer extension was used to define the transcriptional start site of *caiX* and *cdhR*. Plasmids containing the target regions were used to increase RNA copy number, particularly for *cdhR*, as native transcripts are at low abundance [30]. The length of the caiX primer extension product place the transcriptional start site 25 bases upstream of the translational start site at a thymine residue. The length of the cdhR primer extension primer placed the transcriptional start site 63 bases upstream of the translational start site at a cytosine.

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4.4.2. Glucose and glycine betaine repress the transcription of *caiX*.

In *P. aeruginosa* the enzymatic activity of carnitine dehydrogenase (CDH) declines until no longer detectable if the cells are switched from carnitine to the preferred carbon source glucose, and the activity oscillates when carnitine is the sole carbon source. It is thought that oscillation occurs when the catabolic product glycine betaine is produced, resulting in initiation of a negative feedback oscillation loop [31]. To assess catabolite repression, we used the *caiX-lacZYA* transcriptional reporter (pJAM22) to determine if repression is transcriptionally regulated. Maximal catabolite repression is seen at 4 mM glucose, while GB represses transcription to a lesser extent and at a higher concentration (Fig. 3).

4.4.3. MBP-CdhR binds the *caiX* upstream activating site in a concentration-dependent manner.

Based upon CdhR-dependent transcriptional induction of *caiX*, we assayed the capability of purified MBP-CdhR to bind to the activing site (UAS) of *caiX*. Using biotin labeled *caiX* UAS as the DNA probe and purified MBP-CdhR, electrophoretic mobility shift assays revealed that MBP-CdhR binds the *caiX* UAS in a concentration dependent manner (Fig. 4 lanes 1-4). The binding interaction between MBP-CdhR and *caiX* probe can be competed with unlabeled *caiX* probe (lane 5) and MBP-CdhR does not shift a nonspecific UAS, *dhcA* (lanes 6-8). These data demonstrate that MBP-CdhR binds specifically to the *caiX* UAS (Fig. 4).

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4.4.4. Identification of the CdhR binding site sequence and bases required for induction of *caiX*.

Promoter mapping and EMSAs determined the region of DNA essential binding of CdhR and transcriptional induction of *caiX*. To further characterize the CdhR DNA contact site and binding site sequence within the *caiX* UAS, we employed DNaseI footprinting. Purified MBP-CdhR and radiolabeled *caiX* UAS demonstrated the characteristic and site specific protection of DNA as protein concentration increased. Comparing the zone of protection to the A+G ladder we were able to identify the CdhR contact region, stretching 34 bases (Fig. 5). The CdhR binding site reveals the half sites each contain the sequence GGTCGC with 15 bases in between, which is very similar to the binding sites of two other *P. aeruginosa* AraC transcription factors, GbdR [21] and ArgR [32]. In order to further characterize the CdhR binding site, we made mutations in the distal repeat by systematically changing partially overlapping two bases to adenosines (pJAM122-127). Using LacZ as the reporter, induction of the mutated *caiX* binding sites was assessed to determine that the second guanine residue in the repeat is essential for *caiX* expression but complete abolishment of expression requires an additional transversion mutation of the adjacent locus thymine residue (Fig. 5).

4.4.5. CdhR binds, but does not regulate the ABC transporter *cbcXWV*.

After establishing the CdhR binding sequence, GGTCGC-[N15]-GGTCGC, we wanted to identify if the sequence was located elsewhere in the *P. aeruginosa* PAO1 genome. Using the DNA motif search tool from the Pseudomonas Genome Database

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website [33], only two sites were identified within intergenic regions, *caiX-cdhR* and *cbcX-sdaB*, both of which are involved in carnitine metabolism [15, 17]. CbcXWV is an ABC transporter and the core transporter proteins CbcWV are required for growth on carnitine [15]. A MBP-CdhR EMSA with the *cbcX* promoter DNA probe shows MBP-CdhR binding in a concentration dependent manner (Fig. 6A), but the qRTPCR data reveals carnitine is not sufficient for induction of *cbcX* (Fig. 6B). We used the *cdhCA* deletion to eliminate production of GB from carnitine (Fig. 1) [17]. In order to induce *cbcX*, GB must be produced to allow induction of *cbcX* transcription via GbdR [21].

4.4.6. GbdR binds the intergenic region of *caiX-cdhR*.

The ability of CdhR to bind a known member of the GbdR regulon (*cbcX*) [21, 34], the detection of the GbdR binding consensus in the *caiX-cdhR* intergenic region [21], and the overlapping position of the CdhR and GbdR binding sites led us to further investigate the role of GbdR in carnitine regulation. We predicted that GbdR would be able to bind the *cdhR* UAS and an EMSA with purified MBP-GbdR in association with the *cdhR* UAS showed that MBP-GbdR binds the *cdhR* UAS in a concentration dependent manner (Fig. 7A). When the GbdR conserved residues CG in the distal half site were mutated to AA, binding was lost (Fig. 6), as seen with the *plcH* and *choE* mutated distal half site [21]. Since GbdR binds the *caiX-cdhR* intergenic region, induction of *caiX* (pJAM22) was assessed in relation to GbdR. A *gbdR* mutant has significantly less induction of *caiX* but this defect is likely due to a defect in carnitine import (as discussed in chapter 3). In addition, these data suggest that GbdR is not the

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transcriptional activator of *cdhR* since *caiX* is induced in a *gbdR* mutant, whereas in a *cdhR* mutant, *caiX* induction is completely abolished.

4.4.7. CdhR is required for induction of *cdhR* expression.

After establishing that CdhR and GbdR bind the intergenic region of *caiX-cdhR*, we wanted to determine expression of *cdhR* using a translational reporter in relation to CdhR and GbdR. Using two different translational fusions, one carried on a plasmid and one integrated into the chromosome at the *att*Tn7 site, it became apparent that CdhR has a role in its own expression. In wild type carnitine increased expression of *cdhR* compared to the basal expression level (uninduced) (Fig. 8). In the absence of *gbdR*, carnitine still induces *cdhR*, but basal expression of *cdhR* is decreased compared to wild type and a *cdhR* deletion, suggesting that GbdR functions to relieve repression at this locus (Fig. 8). These data analyzing the expression of *cdhR* may represent a hierarchy of the transcription factors GbdR and CdhR which we will discuss below.

4.5. Discussion

The metabolic diversity of *P. aeruginosa* is a product of the immense regulatory network encoded in the genome and it is this network of transcription factors (TF) that are a critical mechanism for sensing environmental cues [35]. One such family of transcription factors that controls carbon metabolism and virulence is the AraC transcription factor family. In this study we expand on our understanding of carnitine catabolism and how two AraC transcription factors, CdhR and GbdR, whose metabolic

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pathways converge at glycine betaine (Fig. 1), are intertwined. We identified the CdhR enhancer site and through reporter and DNA binding assays reveal this site as an additional GbdR binding site. Through reporter fusions we were able to show that CdhR regulates its own expression is responsive to carnitine. We also show that GbdR binding to *caiX-cdhR* region assists in regulating carnitine catabolism by inhibiting repression of *caiX* and *cdhR* transcription and maintaining a basal *cdhR* expression level.

The first question we addressed was how CdhR binds and regulates the carnitine operon. CaiF in *E. coli* is the only other carnitine regulator that has been characterized to date, and is a degenerate AraC TF that lacks the traditional N-terminal domain but maintains the helix-turn-helix DNA binding domains [36]. However, CaiF is transcribed in the opposite direction from the carnitine metabolism operon (*caiTABCDE)* and binds to inverted repeats in the enhancer site [36, 37]. *P. aeruginosa* and other Gram-negative bacteria that contain a carnitine catabolism operon capable of generating glycine betaine are organized in similar fashion to one another with an AraC-family transcription factor divergently transcribed from the catabolic operon [38]. CdhR binds to direct repeats and the binding site is upstream of the *caiX* promoter (Fig. 5), categorizing CdhR as a class I activator that requires both half sites for induction of *caiX* (Fig. 2).

Kleber and Aurich analyzed the activity of carnitine dehydrogenase (CDH) with respect to glucose and glycine betaine and showed that glucose, as a preferred carbon source, is catabolite repressive [31], whereas glycine betaine leads to repression of CDH activity resulting in oscillations of activity as carnitine is catabolized to GB [31]. Our data shows that repression by glucose and glycine betaine is controlled at the level of

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transcription (Fig. 3). Negative feedback by glycine betaine is likely GbdR dependent based on GbdR's capability to bind the *caiX-cdhR* intergenic region and GbdR's responsiveness to GB [39]. *P. aeruginosa* maintains intracellular glycine betaine pools and GbdR fine tuning carnitine catabolism may be directly related to sustaining the homeostatic levels of glycine betaine, as the pool has a physiological impact on nutrients, osmoprotection and virulence [40]. Chapter 5 address experiments to test whether this is the case.

We performed an alignment using the *Pseudomonas*.com DNA motif search tool of the newly identified CdhR binding sequence to the PAO1 genome and identified two intergenic regions: *caiX-cdhR* and *cbcX-sdaB* [33]. This led us to investigate if CdhR contributes to the regulation of carnitine import by the ABC transporter *cbcXWV,* which is required for growth on carnitine [15]. Even though CdhR binds to the *cbcX* upstream region *in vitro*, it does not contribute to *cbcX* expression (Fig. 6). We propose that an unknown BCCT transporter imports carnitine, it is degraded to GB, which drives expression of *cbcXWV* in a GbdR dependent manner allowing for a larger flux of carnitine which is needed for growth [15]. This is similar to the mechanism of choline import for *cbcXWV* induction, termed priming [34].

We previously characterized the GbdR regulon and identified the intergenic region of *caiX-cdhR* to have a GbdR binding site [21]. Here we report that GbdR binds the *caiX-cdhR* intergenic region in vitro and the conserved CG residues are necessary for GbdR binding, as mutating the residues to AA results in loss of binding (Fig. 7). Based upon the conserved residues being located in the distal half site [21], the orientation of

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GbdR binding is likely oriented towards *cdhR* activation and not *caiX*. Interestingly, the GbdR and CdhR binding sites overlap in the *caiX-cdhR* intergenic region, which led us to hypothesize that GbdR has a role in regulating carnitine catabolism and in particular *cdhR*.

Based on what we know about GbdR and what we presented here, we propose a schematic for the interplay of the two AraC TFs and how they regulate carnitine catabolism. In the absence of carnitine, CdhR binds to its target site in a manner that inhibits *cdhR* expression. GbdR can compete for the binding sites and prevent CdhR-dependent inhibition by CdhR, based on comparisons of basal expression levels (Fig. 8). Analyzing the DNA sequence up and downstream of the CdhR binding site reveals multiple CdhR half sites. These half sites could be participating in CdhR inhibition of basal expression. CdhR inhibition of its own expression may be similar to the AraC 'light-switch' mechanism (Figure in Chapter 1). In the absence of ligand, one AraC DNA binding domain binds an operator half site promoting DNA looping and prevents expression of the arabinose operon *araBAD*. Upon ligand binding AraC undergoes a conformational change promoting binding of both half sites of the enhancer and inducing transcription [41]. Another possibility is that CdhR oligomerizes along the DNA nucleated at these half sites and dampens *cdhR* basal expression. In this model, GbdR could compete for binding, supported by the lower basal expression in a *gbdR* mutant, where CdhR is binding and inhibiting *cdhR* expression. Furthermore, CdhR binding of carnitine causes a conformational change, allowing CdhR to increase expression of *cdhR* and *caiX* (Fig. 8). As the catabolic product GB builds up, the cell controls the flux of

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carnitine catabolism by GB-dependent transcriptional repression, which is likely regulated by GbdR. Fine tuning of this pathway is predominately controlled by GbdR, a principal regulator.

In conclusion, we show regulation of carnitine catabolism is controlled by two transcription factors that contribute to fine tuning of the system depending on when and if ligands are present. We put forward a hierarchy of regulation, where one transcription factor trumps the next depending on ligand: GbdR+GB > CdhR+carnitine > GbdR > CdhR. This system has likely evolved to control nutrient acquisition, virulence, GB pools, and possibly other metabolic bi-products like formaldehyde and peroxide, which are involved in carnitine and GB metabolism.

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4.6. Tables

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference
P. aeruginosa PAO1		
MJ79	wild type	[10]
MJ80	Δ*gbdR*	[20]
JM236	Δ*cdhR*	This study
JM253	wild type::88-89intYFPCFP-2	This study
JM339	Δ*cdhR*::88-89intYFPCFP-2	This study
JM340	Δ*gbdR*::88-89intYFPCFP-2	This study
P. aeruginosa PA14		
MJ101	wild type	[42]
MJ11	Δ*cdhR*	[17]
MJ26	Δ*gbdR*	[20]
MJ262	Δ*cdhCA*	[17]
JM179	Δ*cdhR* Δ*gbdR*	This study
E. coli		
MJ340	wild type S17λpir	
DH5α	NEB C2987	NEB
T7Express	NEB C3016	NEB
Plasmids		
pMQ30	suicide vector, Gm^r	[23]
pMQ80	high-copy number *Pseudomonas* vector, Gm^r	[23]
pMal-C2X	T7 Expressing vector, MBP N-terminal tag, Amp^r	NEB
pTNS2	plasmid carrying the *att*Tn7 transposase	[43]
pUC18-mini-Tn7T-Gm	Gm^r on mini-Tn7T	[22]
pUC18-mini-Tn7T-Gm-*eyfp*	Gm^r on mini-Tn7T with YFP	[22]
pUCP22	high copy # *Pseudomonas* stabilization vector, Gm^r	[44]
pMW5	*lacZYA* in pUCP22	[39]
pMW79	PA14 genomic clone of *PA5380-PA5389* in pMQ80	[17]
pJAM22	promoter *caiX-lacZYA* transcriptional fusion-A	This study
pJAM23	promoter *caiX-lacZYA* transcriptional fusion-B	This study
pJAM24	promoter *caiX-lacZYA* transcriptional fusion-C	This study
pJAM25	promoter *caiX-lacZYA* transcriptional fusion-D	This study
pJAM50	*PA5389* in pMal-C2X	This study
pJAM76	YFP-CFP in pMQ80	This study
pJAM86	CFP *PA5388-PA5389* intergenic region YFP in pUC18mini, DR2	This study

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pJAM90	*PA5389* deletion contruct in pMQ30	This study
pJAM122	promoter *caiX-lacZYA* transcriptional fusion	This study
pJAM123	promoter *caiX-lacZYA* mut 1 transcriptional fusion	This study
pJAM124	promoter *caiX-lacZYA* mut 2 transcriptional fusion	This study
pJAM125	promoter *caiX-lacZYA* mut 3 transcriptional fusion	This study
pJAM126	promoter *caiX-lacZYA* mut 4 transcriptional fusion	This study
pJAM127	promoter *caiX-lacZYA* mut 5 transcriptional fusion	This study
pJAM130	promoter *caiX-lacZYA* mut 6 transcriptional fusion	This study
pJAM131	C terminus of *lacZ* in pUCP22	This study
pJAM135	P*cdhRlacZYA* in pUCP22	This study

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Table 2. Primers used in this study

Primers 5' to 3'

Deletion constructs	
5389GOIF1KpnI	ATAGGGTACCgaagaacaccacccactgct
5389SOEGOIR1	aagtacgaaggcgactcgaCCATGGagaagcccattaccgagaagc
5389SOEGOIF1	gcttctcggtaatgggcttctCCATGGtcgagtcgccttcgtactt
5389GOIR1BamHI	ATCGTCTTCGCTGTTTTTCC
Protein expression construct	
5389Mal-c2xF	GCATCAgaattcTCCCAGGACTTCTGGTTTCT
5389Mal-c2xR	GCATCAaagcttTCAGCCTCGCTCAGCTCGA
Primer extension	
5388primerextension	5'-fluorescein 6 fam-ACTGGCCAGGATCAGCAGG
5389primerextension	5'-fluorescein 6 fam-AGACAGTATCGGCCTCAGGAA
EMSA probes	
PA5388promF3	aagcttGTGCCAGCGGTAGAGGTC
PA5388promR	TGAggtaccTTGATTGTTTTTCTGCGAGGT
PA5388promRbiot	biotin-TTGATTGTTTTTCTGCGAGGT
5389EMSA-F	ATGAaagcttGCAGCAGGAGAAACCAGAAG
5389EMSA-R-biot	biotin-TTGATTGTTTTTCTGCGAGGT
5389EMSA-Mut3F	GGACGGCGGCGAAGCGCACTGCGAAGACC
cbcXprom-F	CCGGCAAAGACCACTATGAT
cbcXprom-R-biot	biotin-GAACTCCTCTGCAGGGTAAGG
dhcprom-F-biot	biotin- GAGGCTTTCCTCCAGGCTCT
dhcprom-R	GGATggtaccCTCTTCCGGCTCTTGTGATT
dhcprom-F	GAACTCCTCTGCAGGGTAAGG
Transcriptional reporters	
PA5388promR	TGAggtaccTTGATTGTTTTTCTGCGAGGT
PA5388promF1	ATGAaagcttACAGCAGGTCGCCTTTCTT
PA5388promF3	aagcttGTGCCAGCGGTAGAGGTC
PA5388promF2	ATGAaagcttGCAGCAGGAGAAACCAGAAG
PA5388promF4	aagcttCTGCAGTGCAAGAGCTGGT
P5388pos	ATGAaagcttCGCTTGGCAATGGCCAGGTCGCT
P5388mut1	ATGAaagcttCGCTTGGCAATGGCCAAATCGCT
P5388mut2	ATGAaagcttCGCTTGGCAATGGCCAGAACGCT
P5388mut3	ATGAaagcttCGCTTGGCAATGGCCAGGAAGCT
P5388mut4	ATGAaagcttCGCTTGGCAATGGCCAGGTAACT
P5388mut5	ATGAaagcttCGCTTGGCAATGGCCAGGTCAAT

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P5388mut6	ATGAaagcttCGCTTGGCAATGGCCAGATCGCT
Translational reporters	
2-lacZCtermFhindcla	GCaagcttATTATCGATGAGCGTGGTGGTTATGC
2-lacZCtermRsmakpn	cggtacccggggatccTTATTTTTGACACCAGACC
YFP R HindIII	GCATCAaagcttATTACTTGTACAGCTCGTCCA
YFP F Kpn Sal	GACAGcggtaccAATCgtcgacCATatgctgagcaagggcgagg
88-89YC-DR#2ycF	gggcaccaccccggtgaacagctcctcgcccttgctcagcat-GGGGCGCTCCGGGGTTGA
88-89YC-DR#2ycR	cggcaccacgccggtgaacagctcctcgcccttgctcagcat-CGGTCTCCCCTCGTGCGG

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4.7. Figures

Figure 1. Diagram of the *P. aeruginosa* PAO1 carnitine catabolism operon and the catabolic pathway. (A) Arrows represent the individual open reading frames of the carnitine catabolism operon and the regulator *cdhR.* Below the arrow is the designated gene name. (B) A diagram of the converging carnitine and choline catabolism pathways. Black arrows represent an enzymatic step in the catabolic pathway and the gene names are italicized below. The blue arrows represent positive regulation by either CdhR or GbdR and the T bar represents repression by BetI.

Figure 2. Promoter mapping of the upstream region of *caiX* exposes the CdhR binding site. Transcriptional fusions of *lacZ* to the upstream region of *caiX* starts at base pair -22 from the *caiX* translational start site and end at base pair -424 for pJAM22, -327 for pJAM23, -241 for pJAM24, and -137 for pJAM25. *P. aeruginosa* PA14 carrying pJAM22, pJAM23, pJAM24, or pJAM25 were grown in MOPS with 20 mM pyruvate, 20 µg ml^{-1} gentamicin, and induced with 1 mM carnitine. The cultures were subjected to Miller assays to assess relative induction of the different *caiX* transcriptional fusions. Error bars represent standard deviations from three replicates, and results are representative of three independent experiments.

Figure 3. *caiX* transcription is repressed by glucose and glycine betaine. *P. aeruginosa* PA14 with *caiX-lacZ* (pJAM22) was grown in MOPS, 20 mM pyruvate and 20 µg ml^{-1} gentamicin. The positive control and samples have 1 mM carnitine. All

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Catabolite repression samples have additional compound added; 10 mM, 20 mM, 30 mM or 40 mM glycine betaine (pH 7), or 2 mM, 4 mM or 10 mM glucose. Cultures were induced for 4 hours prior to running a Miller assay. Error bars represent standard deviations from three replicates, and results are representative of three independent experiments. **, P < 0.001; ***, P < 0.001; ****, P < 0.0001.

Figure 4. MBP-CdhR binds the *caiX* upstream region in a concentration dependent manner. EMSA with biotin labeled *caiX* DNA probe alone (lane 1) or with increasing concentrations of purified MBP-CdhR (lanes 2-4). A cold *caiX* probe was used to compete for binding of MBP-CdhR from the labeled probe (lane 5). A nonrelated *dhc* DNA probe was used to show specificity of MBP-CdhR binding to *caiX* (lane 6-8).

Figure 5. The CdhR binding site sequence and key residues for induction are identified from DNaseI footprinting and Miller assays. (A) DNaseI footprinting assay was performed by taking the *caiX* promoter end labeled with P32 and adding increasing concentrations of MBP-CdhR. The samples were subjected to DNaseI treatment. The samples were run on a 5% polyacrylamide Tris-borate-EDTA gel and analyzed to determine the CdhR binding site. The first lane of the gel is the A+G sequencing ladder and the others are with or without MBP-CdhR. (B) The *caiX* enhancer site was mutated by changing two bases at a time in the *caiX* distal binding site to adenosines and fused to *lacZ* (pos, mut1-mut6). *P. aeruginosa* PA14 wild type carrying each of the plasmids were grown in MOPS with 20 mM pyruvate, 20 µg ml^{-1}, and induced with 1 mM carnitine for

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4 hours and then subjected to Miller assays. Error bars represent standard deviations from three replicates, and results are representative of three independent experiments. ****, P < 0.0001

Figure 6. MBP-CdhR binds but does not regulate *cbcX* expression. (A) EMSA with a biotin labeled *cbcX* upstream region and purified MBP-CdhR in increasing concentration. (B) Relative expression of *cbcX* was calculated based on the expression in WT pyruvate normalized to the rplU transcript. Three biological samples were run in triplicate and the graph represents the mean values and SEM. n.s., not significant; ***, P < 0.001.

Figure 7. MBP-GbdR binds the *caiX-cdhR* intergenic region but does not induce transcription of *caiX* or *cdhR*. (A) EMSAs were performed with purified MBP-GbdR in increasing concentration with either the biotin labeled *cdhR* binding site probe or a *cdhR* mutant binding site probe. The mutated probe has the distal half site CG residues in relation to *cdhR* changed to AA. (B) β-galactosidase assay with a *caiX-lacZ* reporter plasmid (pJAM22) in wild type, Δ*cdhR*, or Δ*gbdR* strains were grown in MOPS, 20 mM pyruvate, 20 μg ml^{-1} gentamicin, and induced cultures have an additional 1 mM carnitine. Error bars represent standard deviations from three replicates, and results are representative of three independent experiments.

Figure 8. CdhR promotes *cdhR* expression and GbdR dampens basal expression. (A) PA14WT, PA14Δ*cdhR*, PA14*ΔgbdR*, and PA14Δ*cdhR*Δ*gbdR* carrying a *cdhR-lacZ*

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translational plasmid reporter (pJAM135) were grown in MOPS with 20 mM pyruvate, 20 µg ml^{-1} gentamicin, and induced with 1 mM carnitine. The cultures were subjected to Miller assays. (B) PAO1WT, PAO1Δ*cdhR*, and PAO1*ΔgbdR,* all with the translational fusion *cdhRyfp* integrated at the *att*Tn7 site, were grown on MOPS agar pads with 20 mM pyruvate and with or without 1 mM carnitine. Cells were imaged under phase contrast and YFP fluorescence every 10 minutes for 6 hours at 32 °C. Cells were analyzed for mean fluorescence at each time point by subtracting the background fluorescence intensity for the cell fluorescence intensity.

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A.

B.

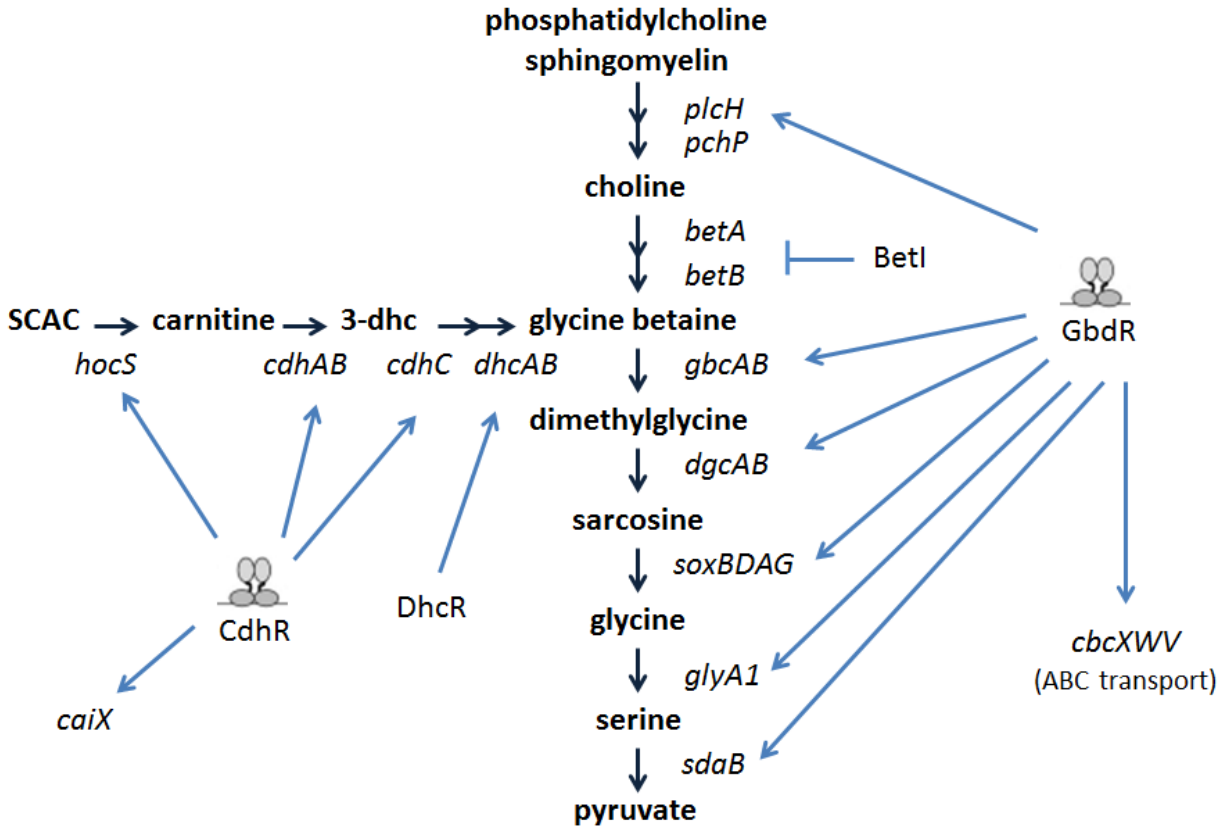

Figure 1. . Diagram of the *P. aeruginosa* PAO1 carnitine catabolism operon and the catabolic pathway.

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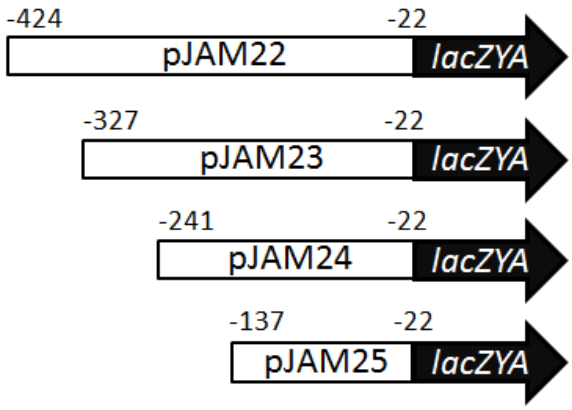
A.



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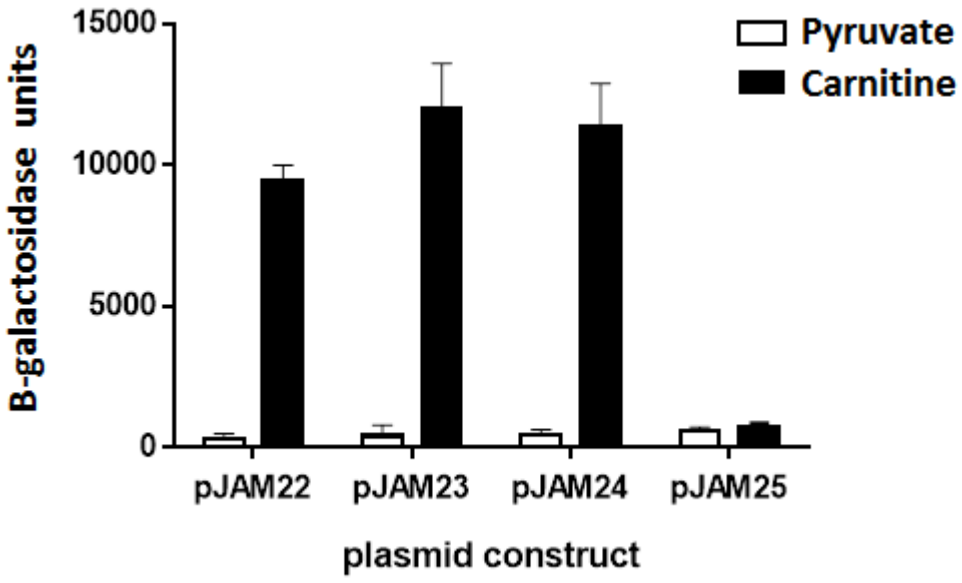

Figure 2. Promoter mapping of the upstream region of *caiX* exposes the CdhR binding site.

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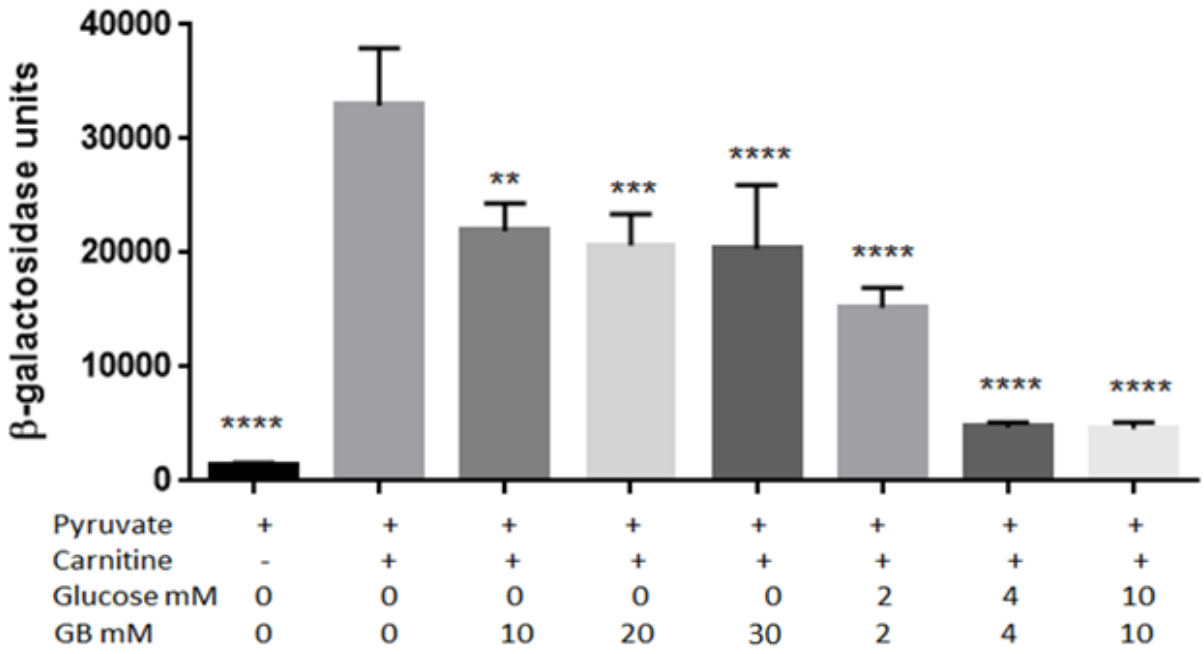

Figure 3. *caiX* transcription is repressed by glucose and glycine betaine.

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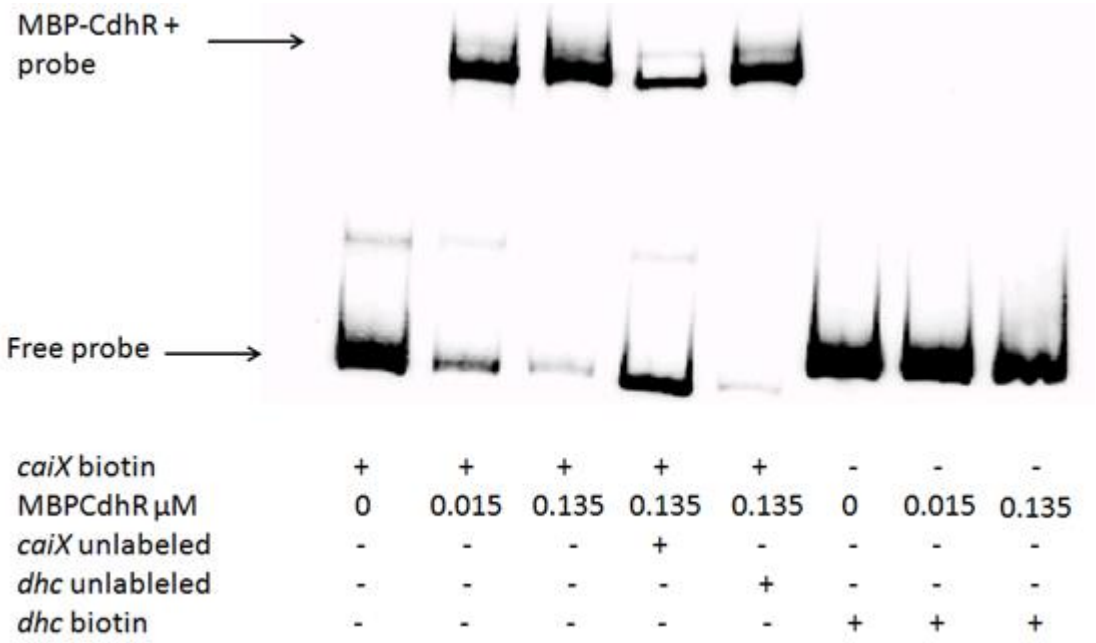

Figure 4. MBP-CdhR binds the *caiX* upstream region in a concentration dependent manner.

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A.

B.

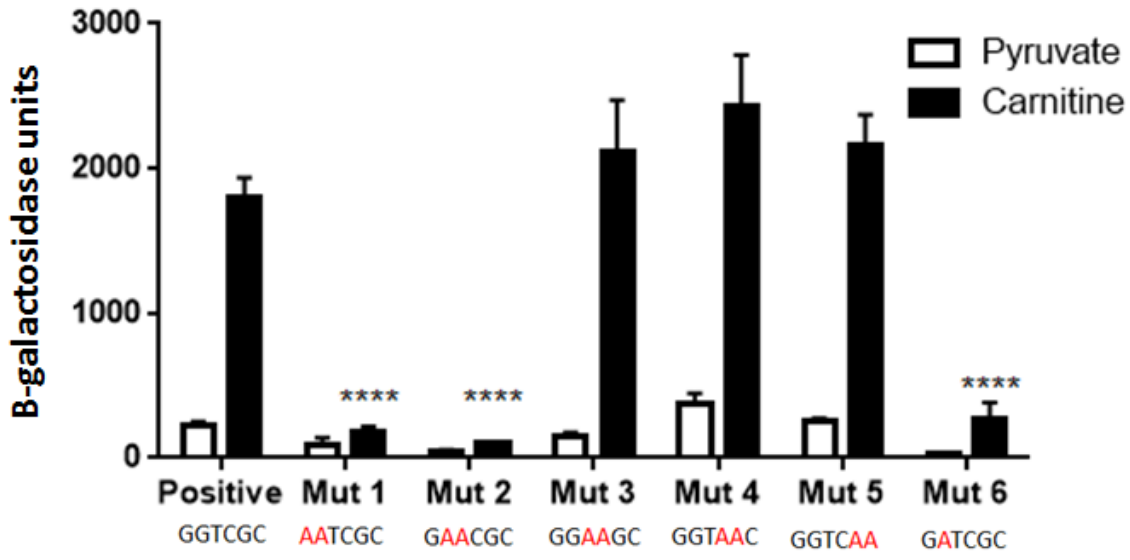

Figure 5. The CdhR binding site sequence and key residues for induction are identified from DNaseI footprinting and Miller assays.

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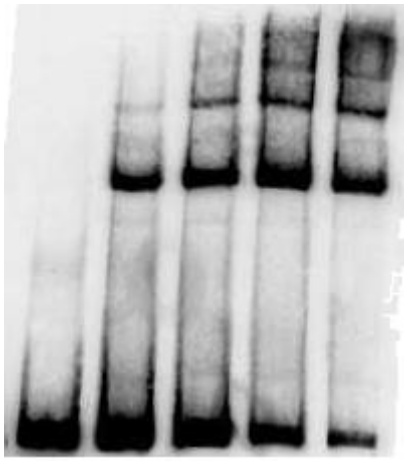
A.



cbcX biotin	+	+	+	+	+
MBP-CdhR µM	0	0.015	0.045	0.09	0.135

B.

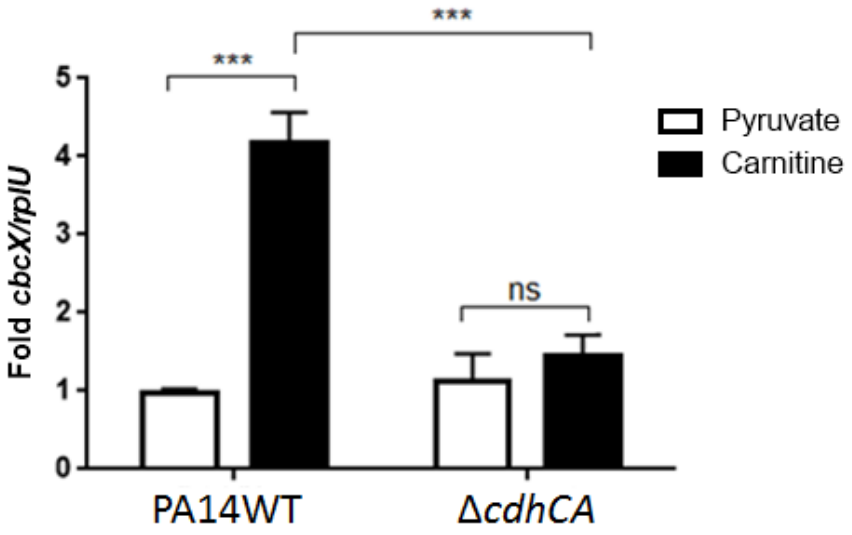


Figure 6. MBP-CdhR binds but does not regulate *cbcX* expression.

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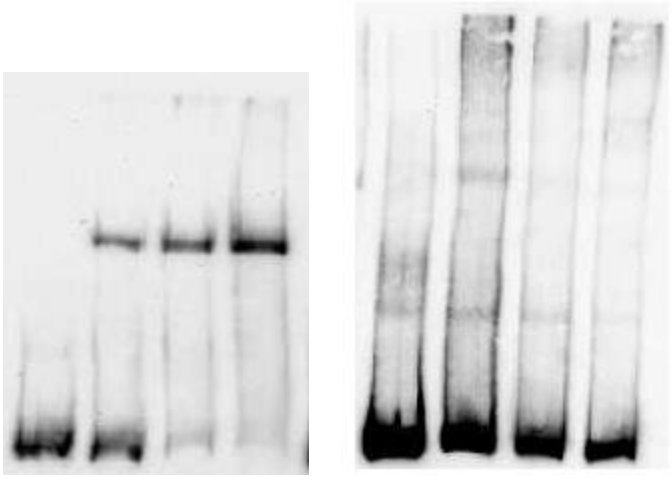
A.



cdhR biotin	+	+	+	+	-	-	-	-
cdhR mut biotin	-	-	-	-	+	+	+	+
MBP-GbdR µM	0	0.12	0.25	0.49	0	0.12	0.25	0.49

caiX---- ACGCCGCTGGTCGAGAACGTGACGTCGCTG ---- *cdhR*
**

B.

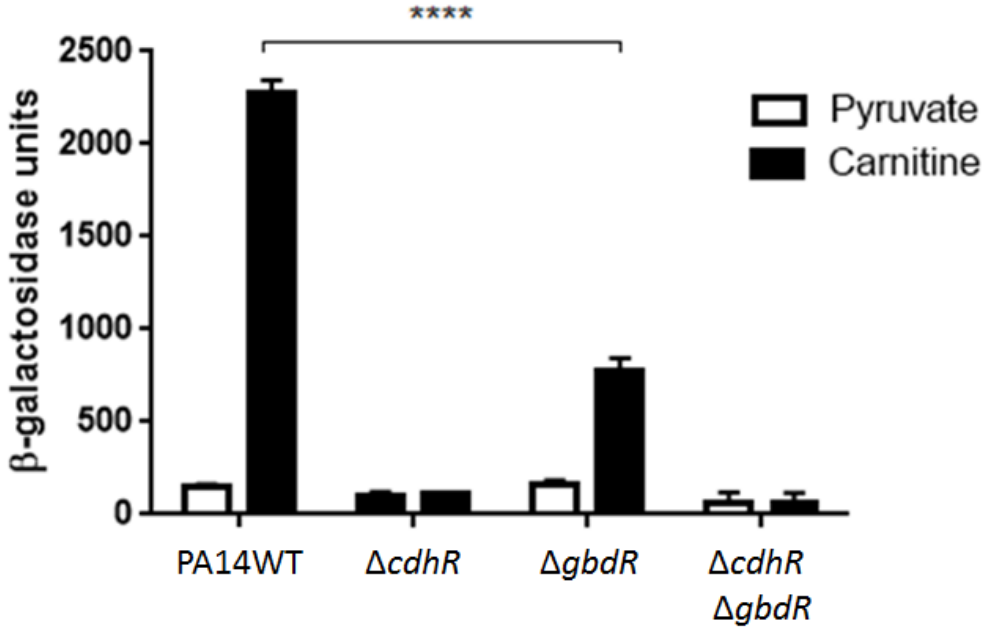


Figure 7. MBP-GbdR binds the *caiX-cdhR* intergenic region but does not induce transcription of *caiX* or *cdhR*.

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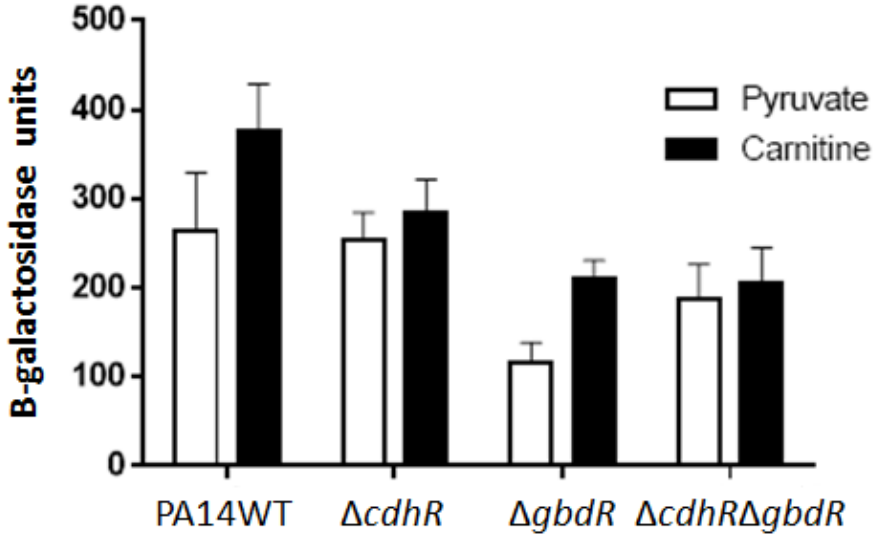
A.



B.

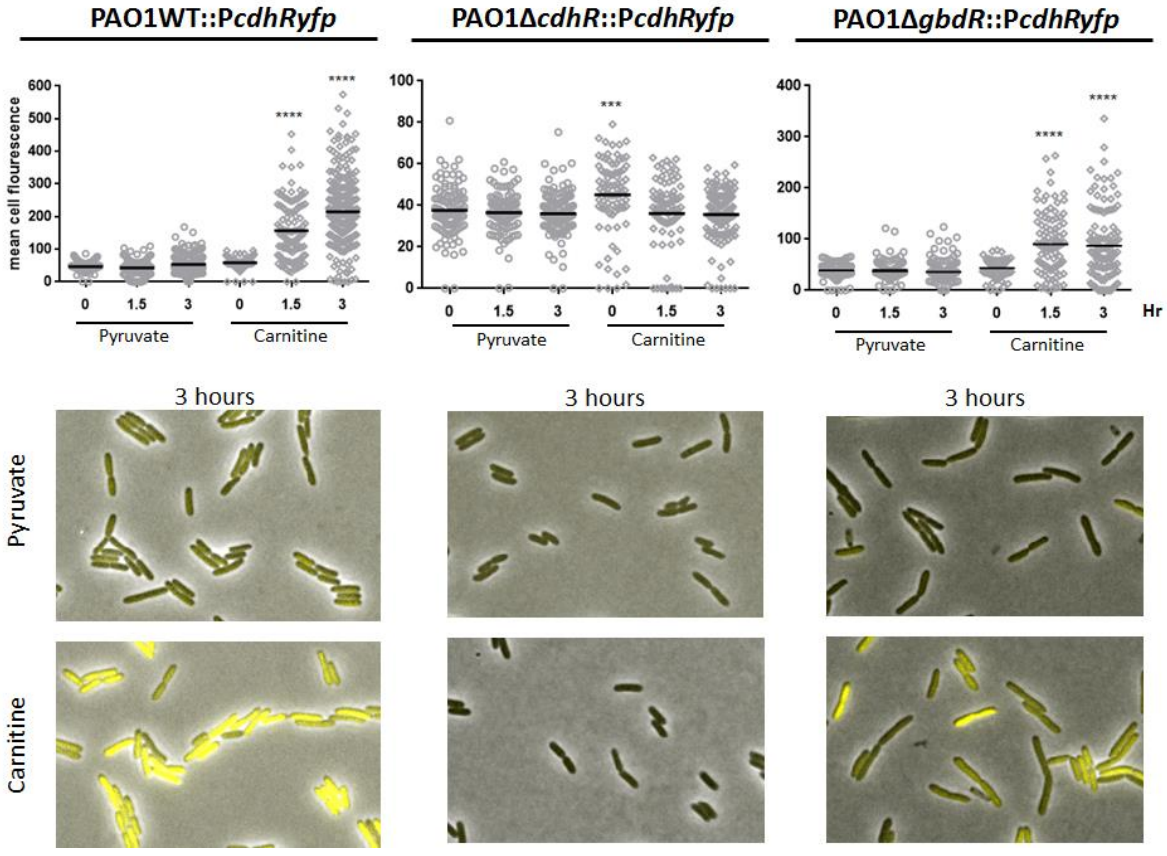


Figure 8. CdhR promotes *cdhR* expression and GbdR dampens basal expression.

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4.8. References

1. Green, S. K., Schroth, M. N., Cho, J. J., Kominos, S. K. & Vitanza-jack, V. B. (1974) Agricultural plants and soil as a reservoir for *Pseudomonas aeruginosa*, *Applied microbiology.* **28**, 987-91.
2. Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist, *Microbes and infection / Institut Pasteur.* **2**, 1051-60.
3. Church, D., Elsayed, S., Reid, O., Winston, B. & Lindsay, R. (2006) Burn wound infections, *Clinical microbiology reviews.* **19**, 403-34.
4. Emerson, J., Rosenfeld, M., McNamara, S., Ramsey, B. & Gibson, R. L. (2002) *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis, *Pediatric pulmonology.* **34**, 91-100.
5. Burns, J. L., Gibson, R. L., McNamara, S., Yim, D., Emerson, J., Rosenfeld, M., Hiatt, P., McCoy, K., Castile, R., Smith, A. L. & Ramsey, B. W. (2001) Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis, *The Journal of infectious diseases.* **183**, 444-52.
6. Montero, M., Dominguez, M., Orozco-Levi, M., Salvado, M. & Knobel, H. (2009) Mortality of COPD patients infected with multi-resistant *Pseudomonas aeruginosa*: a case and control study, *Infection.* **37**, 16-9.
7. Crouch Brewer, S., Wunderink, R. G., Jones, C. B. & Leeper, K. V., Jr. (1996) Ventilator-associated pneumonia due to *Pseudomonas aeruginosa*, *Chest.* **109**, 1019-29.
8. Breidenstein, E. B., de la Fuente-Nunez, C. & Hancock, R. E. (2011) *Pseudomonas aeruginosa*: all roads lead to resistance, *Trends in microbiology.* **19**, 419-26.
9. Sansgiry, S. S., Joish, V. N., Boklage, S., Goyal, R. K., Chopra, P. & Sethi, S. (2012) Economic burden of *Pseudomonas aeruginosa* infection in patients with cystic fibrosis, *Journal of medical economics.* **15**, 219-24.
10. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S. & Olson, M. V. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen, *Nature.* **406**, 959-64.
11. Bremer, J. (1983) Carnitine--metabolism and functions, *Physiological reviews.* **63**, 1420-80.
12. Rebouche, C. J. & Chenard, C. A. (1991) Metabolic fate of dietary carnitine in human adults: identification and quantification of urinary and fecal metabolites, *The Journal of nutrition.* **121**, 539-46.
13. Lindstedt, G., Lindstedt, S., Midtvedt, T., Tofft M. (1967) The Formation and Degradation of Carnitine in *Pseudomonas*, *Biochemistry.* **6**, 1262-1270.
14. Meadows, J. A. & Wargo, M. J. (2015) Carnitine in bacterial physiology and metabolism, *Microbiology*.

www.manaraa.com

15. Chen, C., Malek, A. A., Wargo, M. J., Hogan, D. A. & Beattie, G. A. (2010) The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds, *Molecular microbiology.* **75**, 29-45.
16. Meadows, J. A. & Wargo, M. J. (2013) Characterization of *Pseudomonas aeruginosa* growth on O-acylcarnitines and identification of a short-chain acylcarnitine hydrolase, *Applied and environmental microbiology.* **79**, 3355-63.
17. Wargo, M. J. & Hogan, D. A. (2009) Identification of genes required for *Pseudomonas aeruginosa* carnitine catabolism, *Microbiology.* **155**, 2411-9.
18. Lucchesi, G. I., Lisa, T. A., Casale, C. H. & Domenech, C. E. (1995) Carnitine resembles choline in the induction of cholinesterase, acid phosphatase, and phospholipase C and in its action as an osmoprotectant in *Pseudomonas aeruginosa*, *Current microbiology.* **30**, 55-60.
19. Kleber, H. P., Schopp, W., Sorger, H., Tauchert, H. & Aurich, H. (1967) [Formation of 3-dehydrocarnitine from L-carnitine through the action of a *Pseudomonas aeruginosa* enzyme], *Acta biologica et medica Germanica.* **19**, 659-67.
20. Wargo, M. J., Szwergold, B. S. & Hogan, D. A. (2008) Identification of two gene clusters and a transcriptional regulator required for *Pseudomonas aeruginosa* glycine betaine catabolism, *Journal of bacteriology.* **190**, 2690-9.
21. Hampel, K. J., LaBauve, A. E., Meadows, J. A., Fitzsimmons, L. F., Nock, A. M. & Wargo, M. J. (2014) Characterization of the GbdR regulon in *Pseudomonas aeruginosa*, *Journal of bacteriology.* **196**, 7-15.
22. Choi, K. H. & Schweizer, H. P. (2006) mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*, *Nature protocols.* **1**, 153-61.
23. Shanks, R. M., Caiazza, N. C., Hinsa, S. M., Toutain, C. M. & O'Toole, G. A. (2006) *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria, *Applied and environmental microbiology.* **72**, 5027-36.
24. Miller, J. (1972) *Experiments in molecular genetics.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. Brenowitz, M., Senear, D. F. & Kingston, R. E. (2001) DNaseI footprint analysis of protein-DNA binding, *Current protocols in molecular biology / edited by Frederick M Ausubel [et al]*. **Chapter 12**, Unit 12 4.
26. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. & Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis, *Nature methods.* **9**, 676-82.
27. Linkert, M., Rueden, C. T., Allan, C., Burel, J. M., Moore, W., Patterson, A., Loranger, B., Moore, J., Neves, C., Macdonald, D., Tarkowska, A., Sticco, C., Hill, E., Rossner, M., Eliceiri, K. W. & Swedlow, J. R. (2010) Metadata matters: access to image data in the real world, *The Journal of cell biology.* **189**, 777-82.
28. Team, C. (2015) R: A language and environment for statistical computing in *R Foundation for Statistical Computing* pp. URL http://www.R-project.org/Vienna, Austria.

www.manaraa.com

29. Bastard, K., Smith, A. A., Vergne-Vaxelaire, C., Perret, A., Zaparucha, A., De Melo-Minardi, R., Mariage, A., Boutard, M., Debard, A., Lechaplais, C., Pelle, C., Pellouin, V., Perchat, N., Petit, J. L., Kreimeyer, A., Medigue, C., Weissenbach, J., Artiguenave, F., De Berardinis, V., Vallenet, D. & Salanoubat, M. (2014) Revealing the hidden functional diversity of an enzyme family, *Nature chemical biology.* **10**, 42-9.
30. Kolodrubetz, D. & Schleif, R. (1981) Identification of *araC* protein and two-dimensional gels, its in vivo instability and normal level, *Journal of molecular biology.* **149**, 133-9.
31. Kleber, H. P. & Aurich, H. (1967) [Damped oscillations in the synthesis of carnitine dehydrogenase by *Pseudomonas aeruginosa*], *Hoppe-Seyler's Zeitschrift fur physiologische Chemie.* **348**, 1727-9.
32. Park, S. M., Lu, C. D. & Abdelal, A. T. (1997) Purification and characterization of an arginine regulatory protein, ArgR, from *Pseudomonas aeruginosa* and its interactions with the control regions for the *car*, *argF*, and *aru* operons, *Journal of bacteriology.* **179**, 5309-17.
33. Winsor, G. L., Lam, D. K., Fleming, L., Lo, R., Whiteside, M. D., Yu, N. Y., Hancock, R. E. & Brinkman, F. S. (2011) *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes, *Nucleic acids research.* **39**, D596-600.
34. Malek, A. A., Chen, C., Wargo, M. J., Beattie, G. A. & Hogan, D. A. (2011) Roles of three transporters, CbcXWV, BetT1, and BetT3, in *Pseudomonas aeruginosa* choline uptake for catabolism, *Journal of bacteriology.* **193**, 3033-41.
35. Yahr, T. L. & Greenberg, E. P. (2004) The genetic basis for the commitment to chronic versus acute infection in *Pseudomonas aeruginosa*, *Molecular cell.* **16**, 497-8.
36. Eichler, K., Buchet, A., Lemke, R., Kleber, H. P. & Mandrand-Berthelot, M. A. (1996) Identification and characterization of the *caiF* gene encoding a potential transcriptional activator of carnitine metabolism in *Escherichia coli*, *Journal of bacteriology.* **178**, 1248-57.
37. Buchet, A., Nasser, W., Eichler, K. & Mandrand-Berthelot, M. A. (1999) Positive co-regulation of the *Escherichia coli* carnitine pathway *cai* and *fix* operons by CRP and the CaiF activator, *Molecular microbiology.* **34**, 562-75.
38. Uanschou C., F. R., Pittner F. (2005) What to learn from comparative genomic sequence analysis of L-carnitine dehydrogenase, *Monatshefte fur Chemie.* **136**, 1365-1381.
39. Wargo, M. J., Ho, T. C., Gross, M. J., Whittaker, L. A. & Hogan, D. A. (2009) GbdR regulates *Pseudomonas aeruginosa plcH* and *pchP* transcription in response to choline catabolites, *Infection and immunity.* **77**, 1103-11.
40. Fitzsimmons, L. F., Hampel, K. J. & Wargo, M. J. (2012) Cellular choline and glycine betaine pools impact osmoprotection and phospholipase C production in *Pseudomonas aeruginosa*, *Journal of bacteriology.* **194**, 4718-26.
41. Schleif, R. (2010) AraC protein, regulation of the l-arabinose operon in *Escherichia coli*, and the light switch mechanism of AraC action, *FEMS microbiology reviews.* **34**, 779-96.

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42. Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995) Common virulence factors for bacterial pathogenicity in plants and animals, *Science.* **268**, 1899-902.
43. Choi, K. H., Gaynor, J. B., White, K. G., Lopez, C., Bosio, C. M., Karkhoff-Schweizer, R. R. & Schweizer, H. P. (2005) A Tn7-based broad-range bacterial cloning and expression system, *Nature methods.* **2**, 443-8.
44. Schweizer, H. P. (1991) *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19, *Gene.* **97**, 109-21.

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CHAPTER 5:

SUMMARY AND FUTURE DIRECTIONS

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5.1 Summary

This dissertation focuses on *Pseudomonas aeruginosa* sensing and utilization of the environmental and host-derived compound carnitine, as well as its acyl derivatives, acylcarnitines. Carnitine and acylcarnitines are widely distributed in nature and are especially abundant in animal tissues [1]. *P. aeruginosa* can catabolize carnitine [2] and we showed for first time that it is capable hydrolyzing acylcarnitines to use the liberated carnitine, and its catabolic product glycine betaine, as osmoprotectants, inducers of a virulence factor, and as sole carbon, nitrogen, and energy sources. We identified a short-chain acylcarnitine hydrolase, HocS, and determined how acylcarnitines are transported (Figure 1). Lastly, we expanded our understanding of how carnitine catabolism is regulated by the transcription factors CdhR and GbdR.

Figure 1 is a model representing this dissertation in its entirety. Although represented in the periplasm, medium- and long-chain acylcarnitines are hydrolyzed extracytoplasmically by an unknown hydrolase to yield free carnitine and a medium- or long-chain fatty acid. The fatty acid can be imported and used as a sole carbon source. The liberated carnitine and free carnitine, along with short-chain acylcarnitines are imported through the ABC transporter CaiXCbcWV for growth. Alternatively, carnitine can function as an osmoprotectant, whether from medium- and long-chain acylcarnitines or free, by being imported through an unknown transporter, likely a BCCT. Once in the cytoplasm, short-chain acylcarnitines are hydrolyzed by HocS. Finally, carnitine catabolism is controlled by the AraC family transcription factor, CdhR.

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Pseudomonas aeruginosa is found in soil, water, and can thrive on surfaces such as medical devices. It adapts to different environments by utilizing a wide range of compounds for nutrients and survival, such as carnitine and its acyl derivatives. For the most part, *P. aeruginosa* is thought to be a free-living noninfectious microbe, but in the right environment can cause disease, typically in people who are immunocompromised. What is often overlooked is *P. aeruginosa's* ability to cause infection in plants, insects, reptiles, and other mammals besides humans. Many pathogens cause disease in a specific host, whereas *P. aeruginosa* is a widespread and resourceful pathogen, likely owing to its large genome that encodes diverse metabolic pathways and a large regulatory repertoire [3]. *P. aeruginosa*'s ability to metabolize carnitine and acylcarnitines is not essential for survival, suggesting that metabolism of carnitine and acylcarnitine is an additional tool in the metabolism toolbox. The ability to adapt and change genetic profiles quickly provides *P. aeruginosa* an advantage for establishing and persisting in various niches, a trait that can drive disease.

We made large strides in understanding carnitine and acylcarnitine metabolism but there are still many questions remaining. The carnitine and acylcarnitine figure (Figure 1) has the majority of the transport and metabolism steps determined. Short-chain acylcarnitine transport and metabolism is complete, but the medium- and long-chain acylcarnitine hydrolase(s) still needs to be identified. The alternative carnitine transporter that confers osmoprotection, likely a BCCT, remains to be identified. And lastly, how carnitine is bound by the substrate binding protein, CaiX, and the carnitine regulator, CdhR, is still not understood.

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5.2 Medium- and long-chain acylcarnitines (MCAC- LCAC)

5.2.1. Identifying the MCAC-LCAC hydrolase(s)

We were the first to show that *P. aeruginosa* is capable of using acylcarnitines as sole carbon and nitrogen sources [4]. *Alcaligenes* sp., *Pseudomonas putida*, and *Acinetobacter calcoaceticus*, to name a few, all have been shown to catabolize acylcarnitines of varying length but no responsible gene has been identified [5-7]. The short-chain acylcarnitine hydrolase, *hocS*, was the first gene of any acylcarnitine hydrolase gene to be ascertained [4]. We still have yet to determine the medium- and long-chain acylcarnitine hydrolase(s). There are three methods that could be used to identify the hydrolase(s). The first approach is to take an already existing *P. aeruginosa* PAO1 [8] or *P. aeruginosa* PA14 [9] transposon mutant library and screen separately for growth on a MCAC and a LCAC as sole nitrogen sources. The second approach is to perform a genetic screen by transposon mutagenizing *P. aeruginosa* PA14 wild type strain carrying the P*caiX-lacZ* transcriptional reporter on the pJAM22 plasmid (strain JM92) and screen for loss of β-galactosidase activity in the presence of the MCAC or LCAC as a nitrogen source. The third would be microarray analysis on wild type *P. aeruginosa* grown on MCAC or LCAC as sole nitrogen sources to compare the expression profiles to the wild type strain grown with carnitine as the sole nitrogen source. Both MCAC and LCAC should be used in any of these screens, as there could be independent hydrolases for the two classes of acylcarnitines. *P. putida* can grow on 10C -16C fatty acid chain lengths [6] and *P. aeruginosa* may have long-chain acylcarnitine hydrolase similar to *P. putida* but acquired or maintained a MCAC hydrolase due to its

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pathogenic nature. The three proposed screens have inherent and known positive controls: carnitine catabolism genes (*cdhCAB*), transport genes (*caiXcbcWV*), carnitine regulator (*cdhR*), and genes required for glycine betaine catabolism to pyruvate (Figure 1, Chapter 4). In the β-galactosidase assay, those transposon mutants that are in the listed genes will be white, in the growth assay there will be no growth, and in the microarray they will be induced.

5.2.2. Why not octanoylcarnitine?

I think a fascinating aspect and question of acylcarnitine catabolism is why *P. aeruginosa* doesn't use octanoylcarnitine as a sole carbon or nitrogen source [4]; after identifying the MCAC-LCAC hydrolase(s), this question can be addressed. The transport data in Chapter 3 shows that octanoylcarnitine is not hydrolyzed as efficiently as the other acylcarnitines. Although other bacteria can hydrolyze octanoylcarnitine [10], physiologic utility was generally not addressed except for *B. subtilis* employing the liberated carnitine as an osmoprotectant [11]. Interestingly, *P. aeruginosa* may have evolved a selective hydrolase as a survival strategy to combat the antimicrobial effects of caprylic acid [12-14], the direct product of octanoylcarnitine hydrolysis. Determining the protein structure or using gain-of-function mutagenesis to restore octanoylcarnitine would provide insight into the mechanism of octanoylcarnitine exclusion. Specific amino acid residues in the hydrolase may provide steric hindrance by interacting with the fatty acid and occluding octanoylcarnitine from the binding pocket.

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5.3. Transport

5.3.1. Transporter for osmoprotection

The observation that carnitine induces *caiX* and functions as an osmoprotectant in the absence of the ABC transporter *cbcWV*, suggests that there is one or more additional carnitine transporters. We hypothesize that carnitine can be imported through a betaine/choline/carnitine transporter (BCCT) particularly at high salt concentrations. The only characterized BCCT that is known to transport carnitine is *caiT* in *E. coli* and *P. mirabilis* [15, 16]. Since CaiT does not confer osmoprotection [17] and no homologs are predicted in *P. aeruginosa*, it is unlikely that *P. aeruginosa* uses a BCCT similar to CaiT. *P. aeruginosa* does possess BetT1, BetT2, and BetT3; BCCTs that are driven by proton motive force, transport glycine betaine and choline, and provide osmoprotection [18, 19]. These BetT transporters are good candidates for the alternative carnitine transporter. The *betT1*, *betT3*, *cbcVbetT1*, *cbcVbetT3*, and *betT1betT3* [18] mutants are available and would be a great starting point to fully understand carnitine transport. If the BetT proteins are not the alternative carnitine transporter, transposon mutagenesis of the *cbcV* mutant can be performed. Screening for lack of growth in a hyperosmotic environment will identify the carnitine specific BCCT.

5.3.2. CaiX ligand specificity

The *P. aeruginosa* quaternary amine SBP CbcX binds choline and, to a lesser extent, glycine betaine [20]. Based on our growth data, CaiX is analogous to CbcX and binds multiple ligands: carnitine, acetylcarnitine, and butyrylcarnitine, and would be

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interesting to see which of these ligands is preferred for CaiX. Using radiolabeled compounds and performing transport assays [20], would give insight into ligand preference. Carnitine is more prevalent than short-chain acylcarnitines in the environment [21] and animal tissues [22], which may be mirrored by the ligand preference of CaiX.

5.4. Regulation of carnitine catabolism

5.4.1. Ligand binding

It has been shown through reporter assays and via analysis of epistasis that CdhR is responsive to carnitine, but ligand binding has not been definitively shown. Ligand binding studies of AraC-family TF have been difficult due to the unruly nature of AraC TFs but binding has been shown in a few instances [21-23]. Using recombinant CdhR and radioactive carnitine, far western or equilibrium dialysis assays would show ligand binding by CdhR. It would be important to extend ligand binding studies to GbdR to tease apart the role GbdR has as a dominant regulator of carnitine and choline catabolism.

5.4.2. Catabolite repression of carnitine catabolism

The role of GbdR in catabolite repression of carnitine catabolism is an intriguing question. Glucose and glycine betaine can both repress the transcription of *caiX*. For GB, this catabolite repression control is predicted to be important for negative feedback as carnitine is converted to GB. This repression may be regulated by GdbR, supported by our previous published data that GbdR responds to GB [26-28] and data presented here

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showing that GbdR binds the *caiX-cdhR* intergenic region. Reporter experiments in a *gbdR* mutant will help elucidate the source of transcriptional repression, but will need to take into account GbdR-dependent activation of the carnitine and GB transporter ABC transporter *cbcWV* [20]. To circumvent the defect of GB and carnitine transport in a *gbdR* mutant, placing a constitutive promoter at the native locus of *caiXcbcXWV*, or having the GB-carnitine transport system *in trans* would alleviate a GB and carnitine transport defect.

5.4.3. How is *cdhR* transcription regulated?

There are a few things we know about the regulation of *cdhR* transcription: GbdR is not an activator of *cdhR* transcription and CdhR plays a role in its own regulation. A *cdhR* mutant with stop codons truncating the ORF maintains the transcript but not the protein, which allows us to assess transcript levels by qRT-PCR in a *cdhR* mutant. In Chapter 4, I propose a mechanism for CdhR and GbdR influencing transcription by regulating CdhR binding to the *caiX-cdhR* DNA region, either by binding half sites and looping the DNA or by protein oligomerization (Figure 2) along the DNA. There are additional CdhR and GbdR half sites in the intergenic region and within *caiX* and *cdhR* (Figure 3). I hypothesize that they contribute to the observed transcriptional repression. By strategically adding a reporter in the CdhR half site rich area and/or mutating certain half sites we can elucidate if these half sites function as operator sites. This can be expanded to GbdR but may prove to be difficult or very time

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consuming because in Figure 3 I have marked the most common distal half site GbdR binding sequence, not the other possible GbdR binding sites.

5.5 Concluding remarks

Carnitine metabolism in *P. aeruginosa* has been known for over fifty years but it was not until recently that the genes responsible for carnitine catabolism were discovered; and it was not until this work that acylcarnitine metabolism was characterized in *P. aeruginosa* (Figure 1). We show how acylcarnitines are transported into the cell, and for the first time, identify the gene responsible for encoding a short-chain acylcarnitine hydrolase. Although the medium- and long-chain acylcarnitine hydrolase(s) is still unknown, there are clear benefits for *P. aeruginosa* maintaining an acylcarnitine degradation pathway, including the products being as an osmoprotectants, to induce virulence factors, and to function as carbon, nitrogen, and energy sources. We further expanded upon the regulation of this system to include characterization of the CdhR binding site, investigation of *cdhR* expression, and exploration of the regulatory hierarchy between the transcription factors CdhR and GbdR.

Utilization of carnitine and acylcarnitines by *P. aeruginosa* contributes to its metabolic versatility and likely fine--tunes adaptation to various environments. Carnitine and acylcarnitines in soil and plants provide nutrients and osmoadaptation capabilities to survive the ever--changing environment, but may also provide *P. aeruginosa* an advantage in the human host and establishment of infection. Long-chain fatty acids are a carbon and energy source in mouse chronic wound infections [29], and these fatty acids

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could partially be coming from long-chain acylcarnitine catabolism. Additionally, the carnitine catabolism genes are increased in expression by 5 to 15 fold compared to the succinate control in chronic wound infections [29]. These findings from Turner *et al.* lead to the hypothesis that carnitine and acylcarnitine metabolism are important for establishing infections where there is significant damage to areas high in carnitine, like muscle [1].

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5.6. Figures

Figure 1. Diagram of carnitine and acylcarnitine metabolism. Abbreviations: OM is outer membrane, IM is inner membrane, SCAC is short-chain acylcarnitine, MCAC is medium-chain acylcarnitine, LCAC is long-chain acylcarnitine, HocML is hydrolase of *O*-acylcarnitine medium long, HocS is hydrolase of *O*-acylcarnitine short chain, FA is fatty acid, and BCCT is betaine/choline/carnitine transporter.

Figure 2. Proposed mechanism of *cdhR* expression. Red and purple bars are CdhR and GbdR half sites, respectively. Red monomers are CdhR and purple monomers are GbdR. Black diamonds are carnitine and yellow triangles are glycine betaine (GB). Blue and green lines are representations of *cdhR* and *caiX* expression levels, respectively.

Figure 3. DNA sequence of *caiX* and *cdhR* with CdhR and GbdR half sites. The DNA sequence colored green is the *caiX* sequence, red is the intergenic region, and blue is the *cdhR* sequence. The underlined and yellow highlighted sequence is the identified CdhR binding site. The GCGACC is the CdhR half site binding sequence and is underlined in black. CGCTG the GbdR distal half site and is in bold orange [26].

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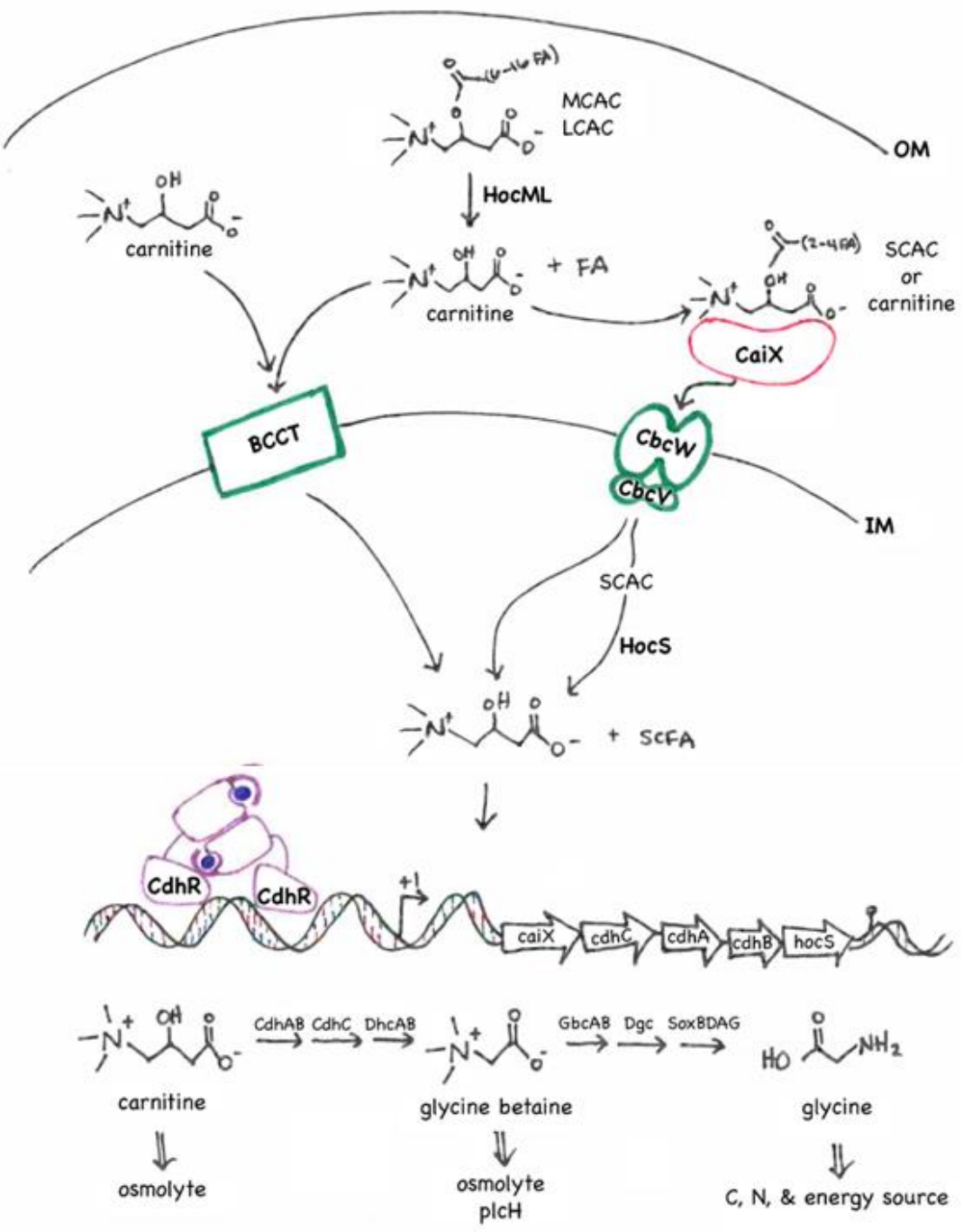

Figure 1. Diagram of carnitine and acylcarnitine metabolism.

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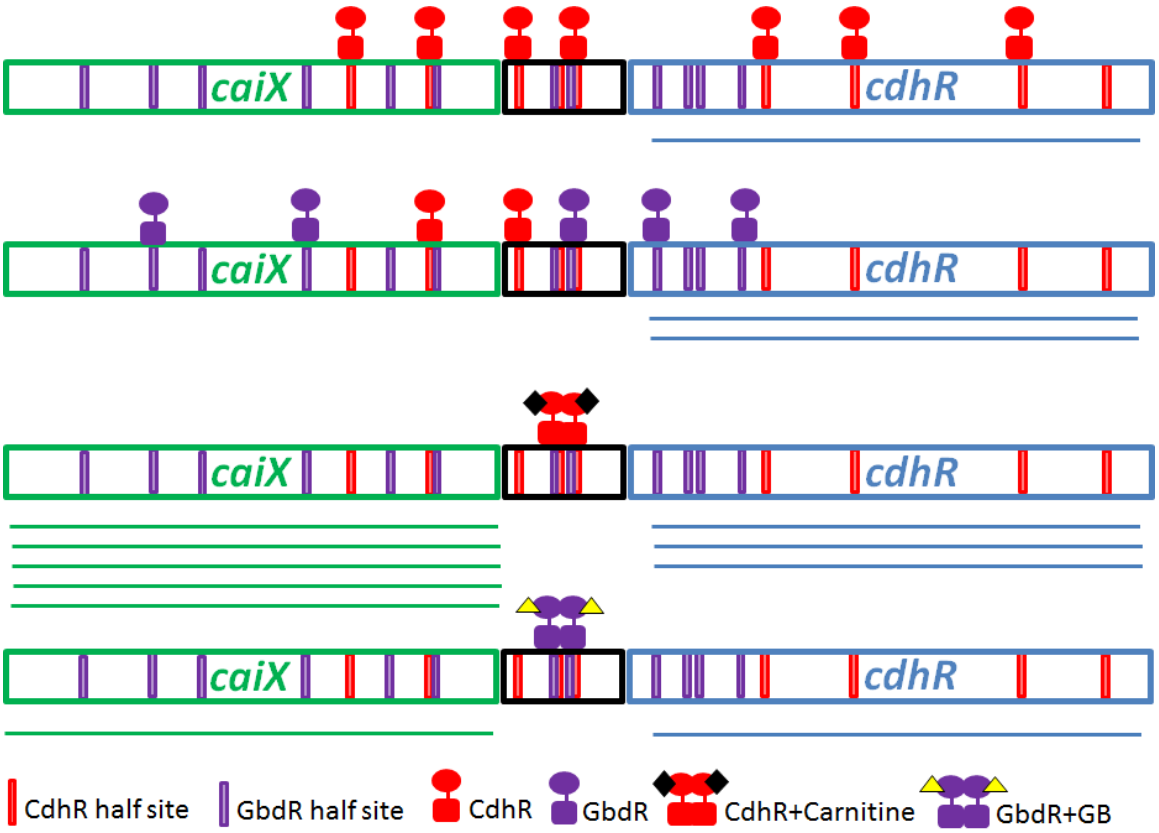

Figure 2. Proposed mechanism of *cdhR* expression.

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TTACTTCAGCGAGGCCTGGAGCGCGGCCAGGCCGTCCTTGCCGTCGAAGGTGCTCACGCCATC
CAGCCAGGCCTTCAGCGGCTCGGGGTTGGCCTTCAGCCACTGGCGCGCGGCGTCGAGGGGCT
GGGTGCGATCGAGGATCGGCGCCATCACCTGGCTGACCTGGGCGCTGCTGAAGCGCAGGTTG
TGCAGCAGCCGGGCGACGTTGCCGCACTGCGCCTGGTAGCCGGCGGCGGTCATGGTGGAGAC
GGTGGCCGCGCCCTCGTCGGGACCGAACACATCCTCGCTGCCGGTGAGGTAGGTCATGTCGA
TCTGCAGGTTCATCGGATGCGGCTTCCAGCCGAAGAACACCACCCACTGCTTGCGCTTGATCG
CCCGCTTCACCGCCGTGAGCATCCCGGCCTCGCTGGACTCCACCAGTTGGAAGCCCTTCAGGC
CGAAACGGTTGTCGTCGATCATCTTCCGGGTGATGCGGTTCGAGCCGCTGCCCGGCTCGATGG
CGTAGATGCGGCCGCCAAGCTTGTCCTTGAAGCGGGCGATGTCGGCGAAGGTCTTCAGCCCGC
CCTCGGCGACGTAGCTCGGCACCGCATAGGTGGACTGCGCGTCGGCCAGGGTCGGTGGCTCG
ATCACTTCGATCCGGCCTTTGTCCAGGTAAGGCCTGGCGACCGGCTGCATGGTCGGTTGCCAG
TAGCCGAGGAACAGGTCCAGCTGCTTGTCGGCCAGGCCGGCCAAAACGATCTGTTGCGAGGC
GCTGGTCTGCTTCACCTGGTAGCCCAGGCCATCGAGCAAGGCTTCGGCCACGGCGCTGCTGG
CGACCACGTCGGTCCAGTTCACCGTGCCCAGGCGCACCGTACGGCACTGCTCGGGTTCTGCGG
CCAGGGCGGCGCCACTGGCCAGGATCAGCAGGGCGCAGCCCAGCTGGCGAATCGTCGTCTTC
ATCGGTCTCCCCTCGTGCGGCTATTGATTGTTTTTCTGCGAGGTGGGCGCCGGCCCCTTGCGAG
ACCGACCCTGGCGACCAAGGTACGCAGCCGGGACGGCGGCGAAGCGCACTGCGGCGACCAG
CTCTTGCACTGCAGCGACCTGGCCATTGCCAAGCGCGGCGGGCCTGCGCTACACACAGGCTG
CGCAACCTCAACCCCGGAGCGCCCCATGTCCCAGGACTTCTGGTTTCTCCTGCTGCCCGGCTTC
TCGGTAATGGGCTTCGTTTCCGCCGTGGAGCCACTGCGCGTGGCCAACCGCTTCCACGCCGAC
CTCTACCGCTGGCACGTGCTCAGCGCCGACGGCGGTCCGGTGCTGGCCAGCAATGGCATGTC
GGTGAACAGCGACGGCGCGCTGGAGCCGCTGAAGAAAGGAGACCTGCTGTTCGTGGTGGCC
GGTTTCGAGCCGCTGCGGGCGGTCACCCCGGCACTGGTGCAGTGGCTGCGCAAGCTCGATCG
CAACGGCGTGACCCTCGGTGGCATCGATACCGGCAGCGTGGTGCTGGCCGAGGCCGGTCTGCT
CGACGGGCGCCGCGCGACCCTGCACTGGGAAGCCATCGACGCCTTCCAGGAGTCCTACCCGC
AACTGAGCGTGACCCAGGAGCTGTTCGAGATCGACGGTCCGCGCATCACCTCGGCCGGCGGC
ACCGCCTCCATCGACCTGATGCTCGACCTGATCGCCCAGGCCCACGGTCCGCAACTGGCGGTG
CAGGTTTCCGAGCAGTTCGTGCTCGGCCGCATCCGCCCGCGCCAGGACCACCAGCGCCTGCAG
GTGGCGACCCGCTATGGCGTGAGCAACCGCAAGCTGGTGCAGGTGATCGGCGAGATGGAGCG
GCATACCGAGCCGCCACTGACCACCCTGGAGCTGGCCGAGCGCATCCAGGTCACCCGCCGCC
AGCTGGAACGGCTGTTCCGCGTGCACCTGGACGACACGCCCTCGAACTTCTACCTCGGCCTGC
GCCTGGACAAGGCCCGCCAGTTGCTGCGCCAGACCGACCTCAGCGTGCTACAGGTGAGCCTG
GCCTGCGGCTTCGAGCGACCCTTCGTACTTCTCCCGCAGCTACCGCGCGCGCTTCGCCGCCAG
TCCCAGCCAGGACCGCGCGGTGCTGCCGCTCAAGGCTCCCGCAGCCACGCCGCCAGGCGCGC
CAGCAGGGCGCGACCACGCCTCGAGCTGAGCGAGGCTGA

Figure 3. DNA sequence of *caiX* and *cdhR* with CdhR and GbdR half sites.

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5.7. References

1. Bremer, J. (1983) Carnitine--metabolism and functions, *Physiological reviews.* **63**, 1420-80.
2. Aurich, H., Kleber, H. P. & Schopp, W. D. (1967) An inducible carnitine dehydrogenase from *Pseudomonas aeruginosa*, *Biochimica et biophysica acta.* **139**, 505-7.
3. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S. & Olson, M. V. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen, *Nature.* **406**, 959-64.
4. Meadows, J. A. & Wargo, M. J. (2013) Characterization of *Pseudomonas aeruginosa* growth on O-acylcarnitines and identification of a short-chain acylcarnitine hydrolase, *Applied and environmental microbiology.* **79**, 3355-63.
5. Takahashi M., U. S. (January 1995) Method of assaying for acyl-L- carnitine and short-chain acyl-carnitine, *US Patent.* **5,385,829**.
6. Kleber, H. P., Seim, H., Aurich, H. & Strack, E. (1978) [Interrelationships between carnitine metabolism and fatty acid assimilation in *Pseudomonas putida* (author's transl)], *Archives of microbiology.* **116**, 213-20.
7. Kleber, H. P., Seim H., Aurich H., Strack E. (1977) Verwertung von Trimethylammoniumverbindungen durch *Acinetobacter calcoaceticus*, *Arch Microbiol.* **112**, 201-206.
8. Jacobs, M. A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenthner, D., Bovee, D., Olson, M. V. & Manoil, C. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*, *Proceedings of the National Academy of Sciences of the United States of America.* **100**, 14339-44.
9. Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., Villanueva, J., Wei, T. & Ausubel, F. M. (2006) An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants, *Proceedings of the National Academy of Sciences of the United States of America.* **103**, 2833-8.
10. Meadows, J. A. & Wargo, M. J. (2015) Carnitine in bacterial physiology and metabolism, *Microbiology*.
11. Kappes, R. M., Bremer E. (1998) Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and y-butyrobetaine via the ABC transport system OpuC *Microbiology.* **144**, 83-90.
12. Richter, F. L., Pedersen, D.E., Fredell, D.L. (1993) Food additive sanitizing compositions in (Patent, U. S., ed)
13. Rosenblatt, J., Reitzel, R. A. & Raad, I. (2015) Caprylic acid and glyceryl trinitrate combination for eradication of biofilm, *Antimicrobial agents and chemotherapy.* **59**, 1786-8.

www.manaraa.com

14. Nakai, S., Siebert, K. (2004) Organic acid inhibition models for *Listeria innocua, Listeria ivanovii, Pseudomonas aeruginosa* and *Oenococcus oeni*, *Food Microbiology.* **21**, 67-72.
15. Eichler, K., Bourgis, F., Buchet, A., Kleber, H. P. & Mandrand-Berthelot, M. A. (1994) Molecular characterization of the cai operon necessary for carnitine metabolism in *Escherichia coli*, *Molecular microbiology.* **13**, 775-86.
16. Schulze, S., Koster, S., Geldmacher, U., Terwisscha van Scheltinga, A. C. & Kuhlbrandt, W. (2010) Structural basis of Na(+)-independent and cooperative substrate/product antiport in CaiT, *Nature.* **467**, 233-6.
17. Verheul, A., Wouters, J. A., Rombouts, F. M. & Abee, T. (1998) A possible role of ProP, ProU and CaiT in osmoprotection of *Escherichia coli* by carnitine, *Journal of applied microbiology.* **85**, 1036-46.
18. Malek, A. A., Chen, C., Wargo, M. J., Beattie, G. A. & Hogan, D. A. (2011) Roles of three transporters, CbcXWV, BetT1, and BetT3, in *Pseudomonas aeruginosa* choline uptake for catabolism, *Journal of bacteriology.* **193**, 3033-41.
19. Chen, C. & Beattie, G. A. (2008) *Pseudomonas syringae* BetT is a low-affinity choline transporter that is responsible for superior osmoprotection by choline over glycine betaine, *Journal of bacteriology.* **190**, 2717-25.
20. Chen, C., Malek, A. A., Wargo, M. J., Hogan, D. A. & Beattie, G. A. (2010) The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds, *Molecular microbiology.* **75**, 29-45.
21. Warren, C. R. (2013) High diversity of small organic N observed in soil water, *Soil Biol Biochem.* **57**, 444-450.
22. Flanagan, J. L., Simmons, P. A., Vehige, J., Willcox, M. D. & Garrett, Q. (2010) Role of carnitine in disease, *Nutrition & metabolism.* **7**, 30.
23. Luzader, D. H., Clark, D. E., Gonyar, L. A. & Kendall, M. M. (2013) EutR is a direct regulator of genes that contribute to metabolism and virulence in enterohemorrhagic *Escherichia coli* O157:H7, *Journal of bacteriology.* **195**, 4947-53.
24. Soisson, S. M., MacDougall-Shackleton, B., Schleif, R. & Wolberger, C. (1997) The 1.6 A crystal structure of the AraC sugar-binding and dimerization domain complexed with D-fucose, *Journal of molecular biology.* **273**, 226-37.
25. Soisson, S. M., MacDougall-Shackleton, B., Schleif, R. & Wolberger, C. (1997) Structural basis for ligand-regulated oligomerization of AraC, *Science.* **276**, 421-5.
26. Hampel, K. J., LaBauve, A. E., Meadows, J. A., Fitzsimmons, L. F., Nock, A. M. & Wargo, M. J. (2014) Characterization of the GbdR regulon in *Pseudomonas aeruginosa*, *Journal of bacteriology.* **196**, 7-15.
27. Wargo, M. J., Ho, T. C., Gross, M. J., Whittaker, L. A. & Hogan, D. A. (2009) GbdR regulates *Pseudomonas aeruginosa plcH* and *pchP* transcription in response to choline catabolites, *Infection and immunity.* **77**, 1103-11.
28. Wargo, M. J., Szwergold, B. S. & Hogan, D. A. (2008) Identification of two gene clusters and a transcriptional regulator required for *Pseudomonas aeruginosa* glycine betaine catabolism, *Journal of bacteriology.* **190**, 2690-9.

www.manaraa.com

29. Turner, K. H., Everett, J., Trivedi, U., Rumbaugh, K. P. & Whiteley, M. (2014) Requirements for *Pseudomonas aeruginosa* acute burn and chronic surgical wound infection, *PLoS genetics.* **10**, e1004518.

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CHAPTER 6:

APPENDIX

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6.1. Rationale

The data presented below is pertinent to this thesis but is not incorporated into Chapters 2-4. Therefore, this section has some experiments briefly described and the results are stated.

6.2. Induction of *caiX* with varying concentrations of carnitine

To determine the sensitivity of *caiX* induction by carnitine, the concentration of carnitine was titrated and a Miller assay was performed. We were able to assess the inducing limit of *caiX* by carnitine.

Pseudomonas aeruginosa PA14 wild type carrying P*caiX-lacZYA* (pJAM22) was grown overnight at 37 °C in MOPS minimal media supplemented with 20 mM pyruvate, 5 mM glucose, and 20 µg ml^{-1} gentamicin. Cells were collected by centrifugation, washed, and inoculated into MOPS minimal media with 20 mM pyruvate, 20 µg ml^{-1} gentamicin, and with or without carnitine ranging from 1 mM to 250 nM. Cells were induced for 4 hours at 37 °C while shaking. A β-galactosidase assay was performed according to the method of Miller in duplicate.

The induction of *caiX* is similar from 1 mM to 250 µM carnitine. 125 µM of carnitine induces *caiX* to about 70 % of 250 µM and there is a sharp decrease in induction of *caiX* at 1 µM (Figure 1). Therefore any of the experiments performed in this thesis, whether it is 1 mM or 500 µM of the inducing agent, does not alter the relative induction observed. This is noted because there are some experiments have inducing compounds at 1 mM of the L-isomer compound compared to 1 mM DL-racemic mixture.

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6.3. CdhR senses and responds to acetyl- and butyrylcarnitine

Short-chain acylcarnitines, acetyl- and butyrylcarnitine, are imported into the cell via the ABC transporter CaiXcbcWV as described in Chapter 4. Once in the cytoplasm the carnitine catabolism operon, *caiXcdhCABhocS*, is induced. The question addressed was does CdhR sense short-chain acylcarnitines and induce *caiX*, or do they need to be hydrolyzed first.

Pseudomonas aeruginosa PA14 wild type and Δ*hocS* carrying P*caiX-lacZYA* (pJAM22) were grown overnight at 37 °C in MOPS minimal media supplemented with 20 mM pyruvate, 5 mM glucose, and 20 µg ml^{-1} gentamicin. Cells were collected by centrifugation, washed, and inoculated into MOPS minimal media with 20 mM pyruvate, 20 µg ml^{-1} gentamicin, and induced with 1 mM carnitine, acetylcarnitine, or butyrylcarnitine. Cells were induced for 4 hours at 37 °C while shaking. A β-galactosidase assay was performed according to the method of Miller in triplicate, three independent times.

Both acetyl- and butyrylcarnitine induce *caiX* in a *hocS* mutant similar to wild type (Figure 2). These data indicate that short-chain acylcarnitines are likely sensed by CdhR and do not need to be hydrolyzed to induce *caiX*.

6.4. Purification of CdhR and GbdR

CdhR and GbdR are members of the AraC family transcription factors (TF). As described in Chapter 1, AraC TFs have an N-terminal ligand binding and dimerization domain. In order to study ligand binding and protein interactions, it is beneficial to have

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the protein in its native conformation and not have a protein fused to the N-termini. To purify the protein without any tags we perform affinity purification with heparin. Heparin is glycoaminoglycans that is negatively charged and structurally and charged similar to DNA. CdhR and GbdR are DNA binding proteins (the C-terminal domain is the DNA binding domain) and therefore heparin can be used to purify the TFs (Figure 3).

CdhR was constructed by PCR amplifying with primers 5'-TCAcataTGTCCCAGGACTTCTGGT and 5'- ACTggtacCGTCAGCCTCGCTCAGCTC using PA14 as template DNA. The product was digested with NdeI and KpnI and ligated into similarly cut pET30a. GbdR was constructed by amplifying with primers 5'-ACGcatATGACCACGTACGCGCCC and 5'-ACTggatccTCAGATCCGCACGCTGGCG using PA14 as the template DNA. GbdR PCR product was digested with NdeI and BamI and ligated into the similarly cut pET30a. Both CdhR and GbdR carrying plasmids, pJAM91 and pJAM100, respectively, were transformed into T7Express *E. coli* and selected on LB 100 µg ml^{-1} kanamycin plates. *E. coli* strain JM256 (CdhR) and JM273 (GbdR) were grown overnight in LB 100 µg ml^{-1} kanamycin and inoculated into 1 L LB with 75 µg ml^{-1} kanamycin. Cells were grown at 37 °C to an OD_{600} nm of 0.4, induced with 1 mM IPTG, and induced for 3 hours. Cells were collected by centrifugation and resuspended in 3 ml 20 mM TrisHCl with 150 mM NaCl, Halt cocktail protease inhibitor (Thermo), and lysed by French press. Lysate was spun at 4 °C for 30 minutes and the supernatant was syringe filter sterilized with a 0.22 µm filter and applied to a heparin column. The column was washed in wash buffer (100 mM PBS, 150 mM NaCl, pH 7.4) with an additional 350 mM NaCl and then eluted with wash buffer plus 500 mM NaCl. Elution fractions were

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dialyzed in 20 mM TrisHCl, 150 mM NaCl, 1 mM EDTA, at pH 7.4 overnight in a 20K MWCO slidealyzer (Thermo). A Bradford assay was performed to determine protein concentrations.

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6.5. Figures

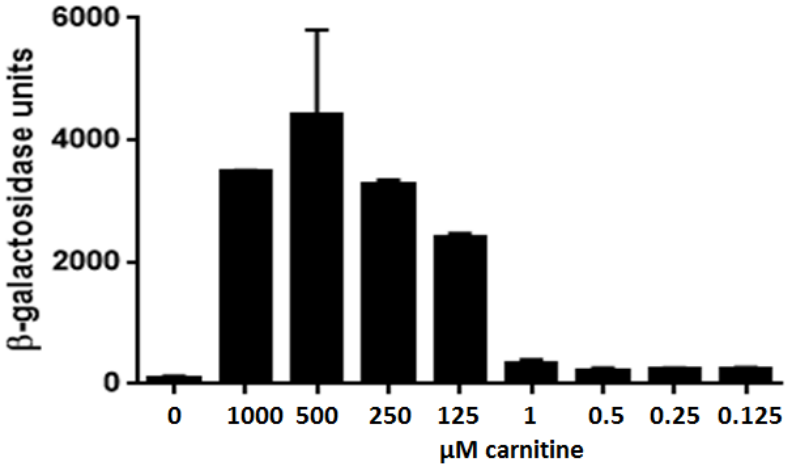

Figure 1. Titration of carnitine inducing *caiX*

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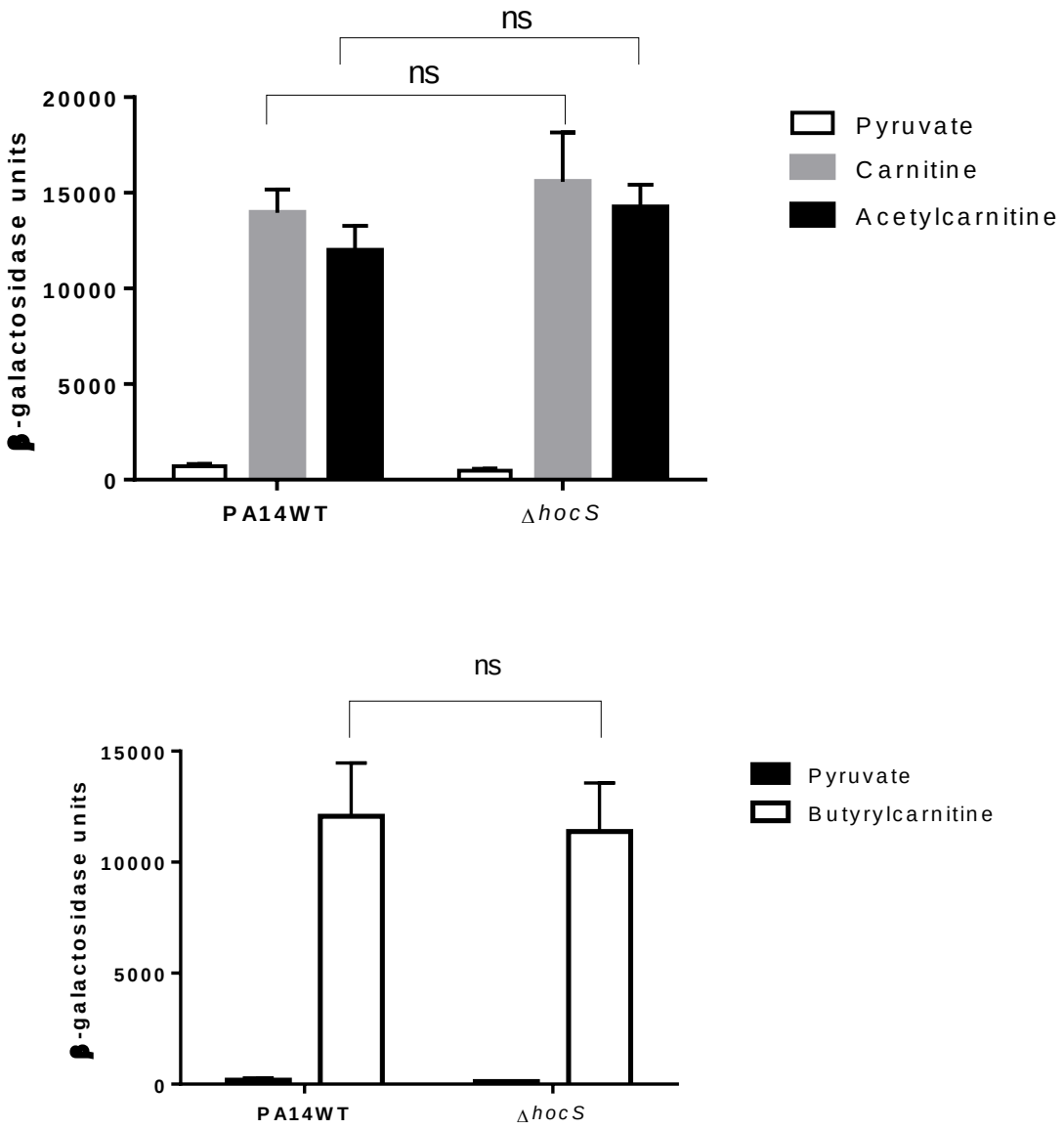

Figure 2. Acetyl- and butyrylcarnitine induce *caiX* in a *hocS* mutant

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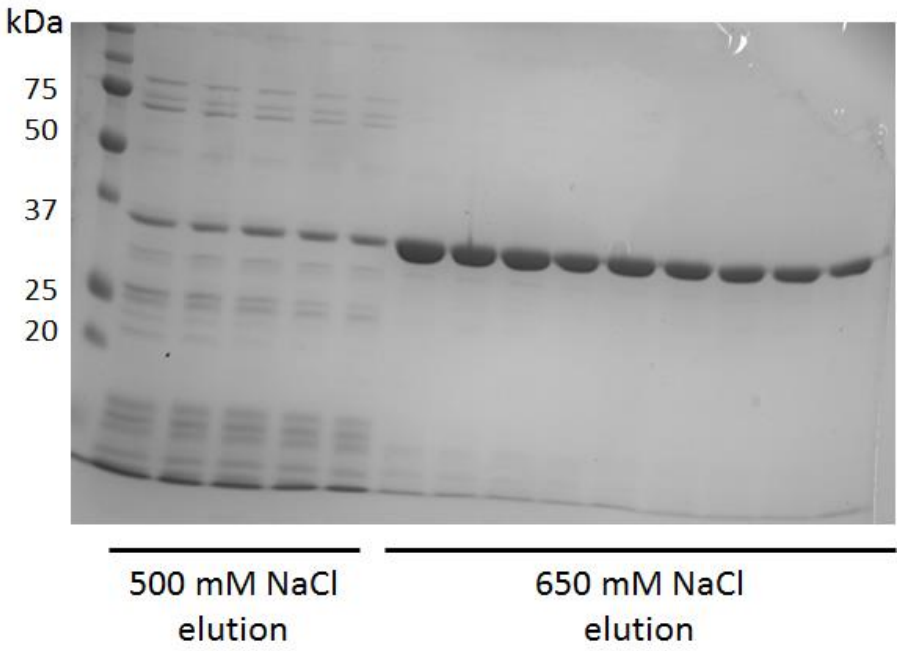

Figure 3. SDS-PAGE of purified CdhR

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COMPREHENSIVE BIBLIOGRAPHY

1. **Abuaita BH, and Withey JH**. Bicarbonate Induces Vibrio cholerae virulence gene expression by enhancing ToxT activity. *Infection and immunity* 77: 4111-4120, 2009.
2. **Achouak W, Christen R, Barakat M, Martel MH, and Heulin T**. Burkholderia caribensis sp. nov., an exopolysaccharide-producing bacterium isolated from vertisol microaggregates in Martinique. *International journal of systematic bacteriology* 49 Pt 2: 787-794, 1999.
3. **Alekshun MN, and Levy SB**. Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulon. *Antimicrobial agents and chemotherapy* 41: 2067-2075, 1997.
4. **Angelidis AS, and Smith GM**. Role of the glycine betaine and carnitine transporters in adaptation of Listeria monocytogenes to chill stress in defined medium. *Applied and environmental microbiology* 69: 7492-7498, 2003.
5. **Angelidis AS, Smith LT, Hoffman LM, and Smith GM**. Identification of opuC as a chill-activated and osmotically activated carnitine transporter in Listeria monocytogenes. *Applied and environmental microbiology* 68: 2644-2650, 2002.
6. **Aragozzini F. MM, Cavazzoni V., Craveri R.** D,L-carnitine resolution by Fusarium oxysporum. *Biotech Letters* 8: 95-98, 1986.
7. **Aragozzini F. MM, V. Cavazzoni and R. Craveri**. D,L-carnitine resolution by *Fusarium oxysporum*. *Biotechnology Letters* 8: 95-98, 1986.
8. **Arima J, Uesumi A, Mitsuzumi H, and Mori N**. Biochemical characterization of L-carnitine dehydrogenases from Rhizobium sp. and Xanthomonas translucens. *Bioscience, biotechnology, and biochemistry* 74: 1237-1242, 2010.
9. **Aurich H, Kleber HP, and Schopp WD**. An inducible carnitine dehydrogenase from *Pseudomonas aeruginosa*. *Biochimica et biophysica acta* 139: 505-507, 1967.
10. **Aurich H, and Lorenz I**. [On the catabolism of carnitine by *Pseudomonas pyocyanea*]. *Acta Biol Med Ger* 3: 272-275, 1959.
11. **Barbier M, Owings JP, Martinez-Ramos I, Damron FH, Gomila R, Blazquez J, Goldberg JB, and Alberti S**. Lysine trimethylation of EF-Tu mimics platelet-activating factor to initiate *Pseudomonas aeruginosa* pneumonia. *mBio* 4: e00207-00213, 2013.
12. **Barrett EL, and Kwan HS**. Bacterial reduction of trimethylamine oxide. *Annual review of microbiology* 39: 131-149, 1985.
13. **Baskaran SA, Bhattaram V, Upadhyaya I, Upadhyay A, Kollanoor-Johny A, Schreiber D, Jr., and Venkitanarayanan K**. Inactivation of *Escherichia coli* O157:H7 on cattle hides by caprylic acid and beta-resorcylic acid. *Journal of food protection* 76: 318-322, 2013.
14. **Bastard K, Smith AA, Vergne-Vaxelaire C, Perret A, Zaparucha A, De Melo-Minardi R, Mariage A, Boutard M, Debard A, Lechaplais C, Pelle C, Pellouin V, Perchat N, Petit JL, Kreimeyer A, Medigue C, Weissenbach J,**

www.manaraa.com

Artiguenave F, De Berardinis V, Vallenet D, and Salanoubat M. Revealing the hidden functional diversity of an enzyme family. *Nature chemical biology* 10: 42-49, 2014.
15. **Bayles DO, and Wilkinson BJ**. Osmoprotectants and cryoprotectants for *Listeria monocytogenes*. *Lett Appl Microbiol* 30: 23-27, 2000.
16. **Begley M, Gahan CG, and Hill C**. The interaction between bacteria and bile. *FEMS microbiology reviews* 29: 625-651, 2005.
17. **Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J, Allayee H, Lee R, Graham M, Crooke R, Edwards PA, Hazen SL, and Lusis AJ**. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell metabolism* 17: 49-60, 2013.
18. **Berk RS, Brown D, Coutinho I, and Meyers D**. In vivo studies with two phospholipase C fractions from *Pseudomonas aeruginosa*. *Infect Immun* 55: 1728-1730, 1987.
19. **Berka RM, Gray GL, and Vasil ML**. Studies of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*. *Infection and immunity* 34: 1071-1074, 1981.
20. **Bernal V, Arense P, Blatz V, Mandrand-Berthelot MA, Canovas M, and Iborra JL**. Role of betaine:CoA ligase (CaiC) in the activation of betaines and the transfer of coenzyme A in *Escherichia coli*. *Journal of applied microbiology* 105: 42-50, 2008.
21. **Berntsson RP, Smits SH, Schmitt L, Slotboom DJ, and Poolman B**. A structural classification of substrate-binding proteins. *FEBS letters* 584: 2606-2617, 2010.
22. **Berntsson RP, ter Beek J, Majsnerowska M, Duurkens RH, Puri P, Poolman B, and Slotboom DJ**. Structural divergence of paralogous S components from ECF-type ABC transporters. *Proceedings of the National Academy of Sciences of the United States of America* 109: 13990-13995, 2012.
23. **Beumer RR, Te Giffel MC, Cox LJ, Rombouts FM, and Abee T**. Effect of exogenous proline, betaine, and carnitine on growth of Listeria monocytogenes in a minimal medium. *Applied and environmental microbiology* 60: 1359-1363, 1994.
24. **Bieber LL**. Carnitine. *Annual review of biochemistry* 57: 261-283, 1988.
25. **Bjarnsholt T, and Givskov M**. The role of quorum sensing in the pathogenicity of the cunning aggressor *Pseudomonas aeruginosa*. *Analytical and bioanalytical chemistry* 387: 409-414, 2007.
26. **Borths EL, Locher KP, Lee AT, and Rees DC**. The structure of *Escherichia coli* BtuF and binding to its cognate ATP binding cassette transporter. *Proceedings of the National Academy of Sciences of the United States of America* 99: 16642-16647, 2002.
27. **Borum PR**. Carnitine. *Annual review of nutrition* 3: 233-259, 1983.
28. **Bourdin B, Adenier H, and Perrin Y**. Carnitine is associated with fatty acid metabolism in plants. *Plant physiology and biochemistry : PPB / Societe francaise de physiologie vegetale* 45: 926-931, 2007.

www.manaraa.com

29. **Breidenstein EB, de la Fuente-Nunez C, and Hancock RE**. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends in microbiology* 19: 419-426, 2011.
30. **Bremer E**. Crystal ball - 2011. *Environmental microbiology reports* 3: 1-26, 2011.
31. **Bremer J**. Carnitine--metabolism and functions. *Physiological reviews* 63: 1420-1480, 1983.
32. **Bremer J**. Carnitine-metabolism and functions. *Physiol Rev* 63: 1420-1480, 1983.
33. **Bremer KRMaE**. Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and gamma-butyrobetaine via the ABC transport system OpuC. *Microbiology* 144: 83-90, 1998.
34. **Brenowitz M, Senear DF, and Kingston RE**. DNaseI footprint analysis of protein-DNA binding. *Current protocols in molecular biology / edited by Frederick M Ausubel [et al]* Chapter 12: Unit 12 14, 2001.
35. **Brown AD, and Simpson JR**. Water relations of sugar-tolerant yeasts: the role of intracellular polyols. *Journal of general microbiology* 72: 589-591, 1972.
36. **Browning DF, and Busby SJ**. The regulation of bacterial transcription initiation. *Nature reviews Microbiology* 2: 57-65, 2004.
37. **Buchet A, Eichler K, and Mandrand-Berthelot MA**. Regulation of the carnitine pathway in *Escherichia coli*: investigation of the cai-fix divergent promoter region. *Journal of bacteriology* 180: 2599-2608, 1998.
38. **Buchet A, Nasser W, Eichler K, and Mandrand-Berthelot MA**. Positive co-regulation of the *Escherichia coli* carnitine pathway cai and fix operons by CRP and the CaiF activator. *Molecular microbiology* 34: 562-575, 1999.
39. **Burns JL, Gibson RL, McNamara S, Yim D, Emerson J, Rosenfeld M, Hiatt P, McCoy K, Castile R, Smith AL, and Ramsey BW**. Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. *The Journal of infectious diseases* 183: 444-452, 2001.
40. **Bustos SA, and Schleif RF**. Functional domains of the AraC protein. *Proceedings of the National Academy of Sciences of the United States of America* 90: 5638-5642, 1993.
41. **Caiazza NC, and O'Toole GA**. SadB is required for the transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas aeruginosa* PA14. *J Bacteriol* 186: 4476-4485, 2004.
42. **Canovas D, Castellar M.R., Obon T., Torroglosa C., Olivares J.L., Iborra J.L.** Racemisation of D(+)-carnitine into L(-)-carnitine by *Escherichia coli* strains. *Process Biochemistry* 39: 287-293, 2003.
43. **Cantisani M, Vitiello M, Falanga A, Finamore E, Galdiero M, and Galdiero S**. Peptides complementary to the active loop of porin P2 from Haemophilus influenzae modulate its activity. *International journal of nanomedicine* 7: 2361-2371, 2012.
44. **Carra JH, and Schleif RF**. Variation of half-site organization and DNA looping by AraC protein. *The EMBO journal* 12: 35-44, 1993.

www.manaraa.com

45. **Castellar MR, Canovas M, Kleber HP, and Iborra JL**. Biotransformation of D(+)-carnitine into L(-)-carnitine by resting cells of *Escherichia coli* O44 K74. *Journal of applied microbiology* 85: 883-890, 1998.
46. **Cave MC, Hurt RT, Frazier TH, Matheson PJ, Garrison RN, McClain CJ, and McClave SA**. Obesity, inflammation, and the potential application of pharmaconutrition. *Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition* 23: 16-34, 2008.
47. **Chen C, and Beattie GA**. Characterization of the osmoprotectant transporter OpuC from *Pseudomonas syringae* and demonstration that cystathionine-beta-synthase domains are required for its osmoregulatory function. *Journal of bacteriology* 189: 6901-6912, 2007.
48. **Chen C, and Beattie GA**. *Pseudomonas syringae* BetT is a low-affinity choline transporter that is responsible for superior osmoprotection by choline over glycine betaine. *Journal of bacteriology* 190: 2717-2725, 2008.
49. **Chen C, Malek AA, Wargo MJ, Hogan DA, and Beattie GA**. The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds. *Molecular microbiology* 75: 29-45, 2010.
50. **Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, and Schweizer HP**. A Tn7-based broad-range bacterial cloning and expression system. *Nature methods* 2: 443-448, 2005.
51. **Choi KH, and Schweizer HP**. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiol* 5: 30, 2005.
52. **Choi KH, and Schweizer HP**. mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. *Nature protocols* 1: 153-161, 2006.
53. **Church D, Elsayed S, Reid O, Winston B, and Lindsay R**. Burn wound infections. *Clinical microbiology reviews* 19: 403-434, 2006.
54. **Cole SD, and Schleif R**. A new and unexpected domain-domain interaction in the AraC protein. *Proteins* 80: 1465-1475, 2012.
55. **Combs Jr. GF**. *Vitamins Fundamental Aspects in Health and Nutrtion*. Academic Press, Elsevier Inc. , 2012.
56. **Cooper G**. *The Cell: A molecular approach*. Sunderland, MA: Sinauer Associates, Inc, 2000.
57. **Cowell BA, Chen DY, Frank DW, Vallis AJ, and Fleiszig SM**. ExoT of cytotoxic *Pseudomonas aeruginosa* prevents uptake by corneal epithelial cells. *Infection and immunity* 68: 403-406, 2000.
58. **Creasey EA, Delahay RM, Daniell SJ, and Frankel G**. Yeast two-hybrid system survey of interactions between LEE-encoded proteins of enteropathogenic *Escherichia coli*. *Microbiology* 149: 2093-2106, 2003.
59. **Crouch Brewer S, Wunderink RG, Jones CB, and Leeper KV, Jr.** Ventilator-associated pneumonia due to *Pseudomonas aeruginosa*. *Chest* 109: 1019-1029, 1996.

www.manaraa.com

60. **Curson AR, Todd JD, Sullivan MJ, and Johnston AW**. Catabolism of dimethylsulphoniopropionate: microorganisms, enzymes and genes. *Nat Rev Microbiol* 9: 849-859, 2011.
61. **D'Souza-Ault MR, Smith LT, and Smith GM**. Roles of N-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress. *Applied and environmental microbiology* 59: 473-478, 1993.
62. **Dalmastri C, Fiore A, Alisi C, Bevivino A, Tabacchioni S, Giuliano G, Sprocati AR, Segre L, Mahenthiralingam E, Chiarini L, and Vandamme P**. A rhizospheric Burkholderia cepacia complex population: genotypic and phenotypic diversity of Burkholderia cenocepacia and Burkholderia ambifaria. *FEMS microbiology ecology* 46: 179-187, 2003.
63. **Desbois AP, and Smith VJ**. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Applied microbiology and biotechnology* 85: 1629-1642, 2010.
64. **Dodge JA, Lewis PA, Stanton M, and Wilsher J**. Cystic fibrosis mortality and survival in the UK: 1947-2003. *The European respiratory journal* 29: 522-526, 2007.
65. **Doring G, Parameswaran IG, and Murphy TF**. Differential adaptation of microbial pathogens to airways of patients with cystic fibrosis and chronic obstructive pulmonary disease. *FEMS microbiology reviews* 35: 124-146, 2011.
66. **Du Y, Shi WW, He YX, Yang YH, Zhou CZ, and Chen Y**. Structures of the substrate-binding protein provide insights into the multiple compatible solute binding specificities of the *Bacillus subtilis* ABC transporter OpuC. *The Biochemical journal* 436: 283-289, 2011.
67. **Dubey AK, Baker CS, Suzuki K, Jones AD, Pandit P, Romeo T, and Babitzke P**. CsrA regulates translation of the *Escherichia coli* carbon starvation gene, cstA, by blocking ribosome access to the cstA transcript. *Journal of bacteriology* 185: 4450-4460, 2003.
68. **Eichler K, Bourgis F, Buchet A, Kleber HP, and Mandrand-Berthelot MA**. Molecular characterization of the cai operon necessary for carnitine metabolism in *Escherichia coli*. *Molecular microbiology* 13: 775-786, 1994.
69. **Eichler K, Buchet A, Bourgis F, Kleber HP, and Mandrand-Berthelot MA**. The fix *Escherichia coli* region contains four genes related to carnitine metabolism. *Journal of basic microbiology* 35: 217-227, 1995.
70. **Eichler K, Buchet A, Lemke R, Kleber HP, and Mandrand-Berthelot MA**. Identification and characterization of the caiF gene encoding a potential transcriptional activator of carnitine metabolism in *Escherichia coli*. *Journal of bacteriology* 178: 1248-1257, 1996.
71. **Eichler K, Schunck WH, Kleber HP, and Mandrand-Berthelot MA**. Cloning, nucleotide sequence, and expression of the *Escherichia coli* gene encoding carnitine dehydratase. *Journal of bacteriology* 176: 2970-2975, 1994.
72. **Elssner T, Engemann C, Baumgart K, and Kleber HP**. Involvement of coenzyme A esters and two new enzymes, an enoyl-CoA hydratase and a CoA-

www.manaraa.com

transferase, in the hydration of crotonobetaine to L-carnitine by *Escherichia coli*. *Biochemistry* 40: 11140-11148, 2001.

73. **Elssner T, Hennig L, Frauendorf H, Haferburg D, and Kleber HP**. Isolation, identification, and synthesis of gamma-butyrobetainyl-CoA and crotonobetainyl-CoA, compounds involved in carnitine metabolism of E. coli. *Biochemistry* 39: 10761-10769, 2000.
74. **Elssner T, Preusser A, Wagner U, and Kleber HP**. Metabolism of L(-)-carnitine by Enterobacteriaceae under aerobic conditions. *FEMS microbiology letters* 174: 295-301, 1999.
75. **Emerson J, Rosenfeld M, McNamara S, Ramsey B, and Gibson RL**. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatric pulmonology* 34: 91-100, 2002.
76. **Engemann C, Elssner T, and Kleber HP**. Biotransformation of crotonobetaine to L(-)-carnitine in Proteus sp. *Archives of microbiology* 175: 353-359, 2001.
77. **Engemann C, Elssner T, Pfeifer S, Krumbholz C, Maier T, and Kleber HP**. Identification and functional characterisation of genes and corresponding enzymes involved in carnitine metabolism of Proteus sp. *Archives of microbiology* 183: 176-189, 2005.
78. **Engemann C, and Kleber HP**. Epigenetic regulation of carnitine metabolising enzymes in Proteus sp. under aerobic conditions. *FEMS microbiology letters* 196: 1-6, 2001.
79. **Fitzsimmons LF, Hampel KJ, and Wargo MJ**. Cellular choline and glycine betaine pools impact osmoprotection and phospholipase C production in *Pseudomonas aeruginosa*. *Journal of bacteriology* 194: 4718-4726, 2012.
80. **Flanagan JL, Simmons PA, Vehige J, Willcox MD, and Garrett Q**. Role of carnitine in disease. *Nutrition & metabolism* 7: 30, 2010.
81. **Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Hoiby N, and Molin S**. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. *Nature reviews Microbiology* 10: 841-851, 2012.
82. **Fraenkel G**. Effect and distribution of vitamin BT. *Archives of biochemistry and biophysics* 34: 457-467, 1951.
83. **Fraenkel G, and Friedman S**. Carnitine. *Vitamins and hormones* 15: 73-118, 1957.
84. **Fraser CM, Norris SJ, Weinstock GM, White O, Sutton GG, Dodson R, Gwinn M, Hickey EK, Clayton R, Ketchum KA, Sodergren E, Hardham JM, McLeod MP, Salzberg S, Peterson J, Khalak H, Richardson D, Howell JK, Chidambaram M, Utterback T, McDonald L, Artiach P, Bowman C, Cotton MD, Fujii C, Garland S, Hatch B, Horst K, Roberts K, Sandusky M, Weidman J, Smith HO, and Venter JC**. Complete genome sequence of Treponema pallidum, the syphilis spirochete. *Science* 281: 375-388, 1998.
85. **Fraser KR, Harvie D, Coote PJ, and O'Byrne CP**. Identification and characterization of an ATP binding cassette L-carnitine transporter in Listeria monocytogenes. *Applied and environmental microbiology* 66: 4696-4704, 2000.

www.manaraa.com

86. **Fraser KR, and O'Byrne CP**. Osmoprotection by carnitine in a Listeria monocytogenes mutant lacking the OpuC transporter: evidence for a low affinity carnitine uptake system. *FEMS microbiology letters* 211: 189-194, 2002.
87. **Fritz IB**. Carnitine and Its Role in Fatty Acid Metabolism. *Adv Lipid Res* 1: 285-334, 1963.
88. **Furukawa S, Kuchma SL, and O'Toole GA**. Keeping their options open: acute versus persistent infections. *Journal of bacteriology* 188: 1211-1217, 2006.
89. **Gahan CG, and Hill C**. Listeria monocytogenes: survival and adaptation in the gastrointestinal tract. *Frontiers in cellular and infection microbiology* 4: 9, 2014.
90. **Gallegos MT, Schleif R, Bairoch A, Hofmann K, and Ramos JL**. Arac/XylS family of transcriptional regulators. *Microbiology and molecular biology reviews : MMBR* 61: 393-410, 1997.
91. **Geiser TK, Kazmierczak BI, Garrity-Ryan LK, Matthay MA, and Engel JN**. *Pseudomonas aeruginosa* ExoT inhibits in vitro lung epithelial wound repair. *Cellular microbiology* 3: 223-236, 2001.
92. **Gilljam H, Ellin A, and Strandvik B**. Increased bronchial chloride concentration in cystic fibrosis. *Scandinavian journal of clinical and laboratory investigation* 49: 121-124, 1989.
93. **Goldmann A, Boivin C, Fleury V, Message B, Lecoeur L, Maille M, and Tepfer D**. Betaine use by rhizosphere bacteria: genes essential for trigonelline, stachydrine, and carnitine catabolism in Rhizobium meliloti are located on pSym in the symbiotic region. *Molecular plant-microbe interactions : MPMI* 4: 571-578, 1991.
94. **Green SK, Schroth MN, Cho JJ, Kominos SK, and Vitanza-jack VB**. Agricultural plants and soil as a reservoir for *Pseudomonas aeruginosa*. *Applied microbiology* 28: 987-991, 1974.
95. **Gulewitsch W KR**. Uber das Carnitin. *Hoppe-Seyler's Z Physiol Chem* 45: 326-330, 1905.
96. **Hampel KJ, LaBauve AE, Meadows JA, Fitzsimmons LF, Nock AM, and Wargo MJ**. Characterization of the GbdR regulon in *Pseudomonas aeruginosa*. *Journal of bacteriology* 196: 7-15, 2014.
97. **Hancock RE**. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 27 Suppl 1: S93-99, 1998.
98. **Hanschmann H, Ehricht R, and Kleber HP**. Purification and properties of L(-)-carnitine dehydrogenase from Agrobacterium sp. *Biochimica et biophysica acta* 1290: 177-183, 1996.
99. **Hanschmann H, and Kleber HP**. Purification and characterization of D(+)-carnitine dehydrogenase from Agrobacterium sp.--a new enzyme of carnitine metabolism. *Biochimica et biophysica acta* 1337: 133-142, 1997.
100. **Harmer T, Wu M, and Schleif R**. The role of rigidity in DNA looping-unlooping by AraC. *Proceedings of the National Academy of Sciences of the United States of America* 98: 427-431, 2001.

www.manaraa.com

101. **Hartiala J, Bennett BJ, Tang WH, Wang Z, Stewart AF, Roberts R, McPherson R, Lusis AJ, Hazen SL, Allayee H, and Consortium CA**. Comparative genome-wide association studies in mice and humans for trimethylamine N-oxide, a proatherogenic metabolite of choline and L-carnitine. *Arteriosclerosis, thrombosis, and vascular biology* 34: 1307-1313, 2014.
102. **Hauser AR**. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nature reviews Microbiology* 7: 654-665, 2009.
103. **Hauser AR, Kang PJ, and Engel JN**. PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. *Molecular microbiology* 27: 807-818, 1998.
104. **Heijerman H**. Infection and inflammation in cystic fibrosis: a short review. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society* 4 Suppl 2: 3-5, 2005.
105. **Higgins CF, and Linton KJ**. The ATP switch model for ABC transporters. *Nature structural & molecular biology* 11: 918-926, 2004.
106. **Hoffmann T, Wensing A, Brosius M, Steil L, Volker U, and Bremer E**. Osmotic control of opuA expression in *Bacillus subtilis* and its modulation in response to intracellular glycine betaine and proline pools. *Journal of bacteriology* 195: 510-522, 2013.
107. **Hung K, Kleber, H.P.** Vorkommen und Regulation der carnitindehydrogenase *Pseudomonas*-species. *Wiss Z Karl-Marx-Univ Leipzig, Math-Nat R* 34: 293-296, 1985.
108. **Hwang K-C, Won-Gi Bang**. Optimal resolution of L-carnitine from racemic DL-carnitine by *Enterobacter* sp. assimilating D-carnitine. *J Microbiol Biotechn* 7: 318-322, 1997.
109. **Ivanova EP, Gorshkova NM, Bowman JP, Lysenko AM, Zhukova NV, Sergeev AF, Mikhailov VV, and Nicolau DV**. Shewanella pacifica sp. nov., a polyunsaturated fatty acid-producing bacterium isolated from sea water. *International journal of systematic and evolutionary microbiology* 54: 1083-1087, 2004.
110. **Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenthner D, Bovee D, Olson MV, and Manoil C**. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 100: 14339-14344, 2003.
111. **Jaenicke R**. Protein structure and function at low temperatures. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 326: 535-551; discussion 551-533, 1990.
112. **Jander G, Rahme LG, and Ausubel FM**. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *Journal of bacteriology* 182: 3843-3845, 2000.
113. **Jebbar M, Champion C, Blanco C, and Bonnassie S**. Carnitine acts as a compatible solute in Brevibacterium linens. *Research in microbiology* 149: 211-219, 1998.

www.manaraa.com

114. **Johri AM, Heyland DK, Hetu MF, Crawford B, and Spence JD**. Carnitine therapy for the treatment of metabolic syndrome and cardiovascular disease: Evidence and controversies. *Nutrition, metabolism, and cardiovascular diseases : NMCD* 2014.
115. **Jumpertz T, Holland, B., Schmitt, L.** *ABC tansporter in microorganisms*. Norfolk, UK: Caister Academic Press, 2009.
116. **Jung H, Buchholz M, Clausen J, Nietschke M, Revermann A, Schmid R, and Jung K**. CaiT of *Escherichia coli*, a new transporter catalyzing L-carnitine/gamma -butyrobetaine exchange. *The Journal of biological chemistry* 277: 39251-39258, 2002.
117. **Jung H, Jung K, and Kleber HP**. L-carnitine uptake by *Escherichia coli*. *Journal of basic microbiology* 30: 507-514, 1990.
118. **Jung H, Jung K, and Kleber HP**. Purification and properties of carnitine dehydratase from *Escherichia coli*--a new enzyme of carnitine metabolization. *Biochimica et biophysica acta* 1003: 270-276, 1989.
119. **Jung H. KHP**. Metabolism of D(+)carnitine by *Escherichia coli*. *Appl Microbiol Biotechnol* 35: 191-395, 1991.
120. **Jung K, Jung H, and Kleber HP**. Regulation of L-carnitine metabolism in *Escherichia coli*. *Journal of basic microbiology* 27: 131-137, 1987.
121. **Kakayama K, Honda, H., Ogawa, Y., Ohta, T., Ozawa, T.** Method of producing carnitine. *US Patent* 5,041,375: August 1991.
122. **Kalayil S, Schulze S, and Kuhlbrandt W**. Arginine oscillation explains Na+ independence in the substrate/product antiporter CaiT. *Proceedings of the National Academy of Sciences of the United States of America* 110: 17296-17301, 2013.
123. **Kang Y, Nguyen DT, Son MS, and Hoang TT**. The *Pseudomonas aeruginosa* PsrA responds to long-chain fatty acid signals to regulate the fadBA5 beta-oxidation operon. *Microbiology* 154: 1584-1598, 2008.
124. **Kappes RM, Bremer E.** Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and y-butyrobetaine via the ABC transport system OpuC *Microbiology* 144: 83-90, 1998.
125. **Kelley LA, and Sternberg MJ**. Protein structure prediction on the Web: a case study using the Phyre server. *Nature protocols* 4: 363-371, 2009.
126. **Kets EPW, Galinski, E.A., de Bont, J.A.M.** Carnitine: a novel compatible in Lactobacillus plantarum. *Arch Microbiol* 162: 243-248, 1994.
127. **Klagsbrun M, and Furano AV**. Methylated amino acids in the proteins of bacterial and mammalian cells. *Archives of biochemistry and biophysics* 169: 529-539, 1975.
128. **Kleber HP**. Bacterial carnitine metabolism. *FEMS microbiology letters* 147: 1-9, 1997.
129. **Kleber HP, and Aurich H**. [Damped oscillations in the synthesis of carnitine dehydrogenase by *Pseudomonas aeruginosa*]. *Hoppe-Seyler's Zeitschrift fur physiologische Chemie* 348: 1727-1729, 1967.

www.manaraa.com

130. **Kleber HP, Schopp W, Sorger H, Tauchert H, and Aurich H**. [Formation of 3-dehydrocarnitine from L-carnitine through the action of a *Pseudomonas aeruginosa* enzyme]. *Acta biologica et medica Germanica* 19: 659-667, 1967.
131. **Kleber HP, Seim H, Aurich H, and Strack E**. Interrelationships between carnitine metabolism and fatty acid assimilation in *Pseudomonas putida* (author's transl). *Arch Microbiol* 116: 213-220, 1978.
132. **Kleber HP, Seim H, Aurich H, and Strack E**. [Interrelationships between carnitine metabolism and fatty acid assimilation in *Pseudomonas putida* (author's transl)]. *Archives of microbiology* 116: 213-220, 1978.
133. **Kleber HP, Seim H., Aurich H., Strack E.** Verwertung von Trimethylammoniumverbindungen durch Acinetobacter calcoaceticus. *Arch Microbiol* 112: 201-206, 1977.
134. **Kluttermann K, Tauchert, H., Kleber, H.P.** Synthesis of Poly-beta-hydroxybutyrate by *Agrobacterium radiobacter* after Growth on D-carnitine. *Acta biotechnol* 22: 261-269, 2002.
135. **Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, Smith JD, DiDonato JA, Chen J, Li H, Wu GD, Lewis JD, Warrier M, Brown JM, Krauss RM, Tang WH, Bushman FD, Lusis AJ, and Hazen SL**. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nature medicine* 19: 576-585, 2013.
136. **Kolodrubetz D, and Schleif R**. Identification of araC protein and two-dimensional gels, its in vivo instability and normal level. *Journal of molecular biology* 149: 133-139, 1981.
137. **Koulenti D, Lisboa T, Brun-Buisson C, Krueger W, Macor A, Sole-Violan J, Diaz E, Topeli A, DeWaele J, Carneiro A, Martin-Loeches I, Armaganidis A, Rello J, and Group E-VCS**. Spectrum of practice in the diagnosis of nosocomial pneumonia in patients requiring mechanical ventilation in European intensive care units. *Critical care medicine* 37: 2360-2368, 2009.
138. **Krall R, Sun J, Pederson KJ, and Barbieri JT**. In vivo rho GTPase-activating protein activity of *Pseudomonas aeruginosa* cytotoxin ExoS. *Infection and immunity* 70: 360-367, 2002.
139. **Kuka J, Liepinsh E, Makrecka-Kuka M, Liepins J, Cirule H, Gustina D, Loza E, Zharkova-Malkova O, Grinberga S, Pugovics O, and Dambrova M**. Suppression of intestinal microbiota-dependent production of pro-atherogenic trimethylamine N-oxide by shifting L-carnitine microbial degradation. *Life sciences* 117: 84-92, 2014.
140. **Kula MR, Joeres, U., Stelkes-Ritter, U.** New Microbial Amidases. *Annals of the New York Academy of Sciences* 799: 725-728, 1996.
141. **Kula MRJ, U.** L-carnitine amidase produced by a microorganism. *US Patent* 5,238,838: 1993.
142. **Kunin CM, Hua TH, Van Arsdale White L, and Villarejo M**. Growth of *Escherichia coli* in human urine: role of salt tolerance and accumulation of glycine betaine. *J Infect Dis* 166: 1311-1315, 1992.

www.manaraa.com

143. **Kurioka S, and Matsuda M**. Phospholipase C assay using p-nitrophenylphosphoryl-choline together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. *Anal Biochem* 75: 281-289, 1976.
144. **Kutscher F**. Zur Kenntnis des Novains. *Z Physiol Chem* 4947-49: 1905.
145. **LaBauve AE, and Wargo MJ**. Growth and laboratory maintenance of *Pseudomonas aeruginosa*. *Curr Protoc Microbiol* Chapter 6: Unit 6E 1, 2012.
146. **Lambert PA**. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *Journal of the Royal Society of Medicine* 95 Suppl 41: 22-26, 2002.
147. **Lau GW, Hassett DJ, Ran H, and Kong F**. The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends in molecular medicine* 10: 599-606, 2004.
148. **Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, and Ausubel FM**. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proceedings of the National Academy of Sciences of the United States of America* 103: 2833-2838, 2006.
149. **Lindstedt G, and Lindstedt S**. Cofactor requirements of gamma-butyrobetaine hydroxylase from rat liver. *The Journal of biological chemistry* 245: 4178-4186, 1970.
150. **Lindstedt G, Lindstedt S, Midtvedt T, and Tofft M**. Inducible gamma-butyrobetaine-degrading enzymes in Pseudomonas species AK 1. *Journal of bacteriology* 101: 1094-1095, 1970.
151. **Lindstedt G, Lindstedt S, Olander B, and Tofft M**. Alpha-ketoglutarate and hydroxylation of gamma-butyrobetaine. *Biochimica et biophysica acta* 158: 503-505, 1968.
152. **Lindstedt G, Lindstedt S, and Tofft M**. Gamma-butyrobetaine hydroxylase from Pseudomonas sp AK 1. *Biochemistry* 9: 4336-4342, 1970.
153. **Lindstedt G, Lindstedt, S., Midtvedt, T., Tofft M.** The Formation and Degradation of Carnitine in Pseudomonas. *Biochemistry* 6: 1262-1270, 1967.
154. **Linkert M, Rueden CT, Allan C, Burel JM, Moore W, Patterson A, Loranger B, Moore J, Neves C, Macdonald D, Tarkowska A, Sticco C, Hill E, Rossner M, Eliceiri KW, and Swedlow JR**. Metadata matters: access to image data in the real world. *The Journal of cell biology* 189: 777-782, 2010.
155. **Linton KJ**. Structure and function of ABC transporters. *Physiology* 22: 122-130, 2007.
156. **Linton KJ, and Higgins CF**. Structure and function of ABC transporters: the ATP switch provides flexible control. *Pflugers Archiv : European journal of physiology* 453: 555-567, 2007.
157. **Lister PD, Wolter DJ, and Hanson ND**. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clinical microbiology reviews* 22: 582-610, 2009.
158. **Lowden MJ, Skorupski K, Pellegrini M, Chiorazzo MG, Taylor RK, and Kull FJ**. Structure of Vibrio cholerae ToxT reveals a mechanism for fatty acid

www.manaraa.com

regulation of virulence genes. *Proceedings of the National Academy of Sciences of the United States of America* 107: 2860-2865, 2010.
159. **Lu X, Zhang P, Li Q, Liu H, Lin X, and Ma X**. [Cloning, expression and characterization of a gamma-butyrobetaine hydroxylase gene bbh from Pseudomonas sp. L-1]. *Wei sheng wu xue bao = Acta microbiologica Sinica* 52: 602-610, 2012.
160. **Lucchesi GI, Lisa TA, Casale CH, and Domenech CE**. Carnitine resembles choline in the induction of cholinesterase, acid phosphatase, and phospholipase C and in its action as an osmoprotectant in *Pseudomonas aeruginosa*. *Current microbiology* 30: 55-60, 1995.
161. **Luzader DH, Clark DE, Gonyar LA, and Kendall MM**. EutR is a direct regulator of genes that contribute to metabolism and virulence in enterohemorrhagic *Escherichia coli* O157:H7. *Journal of bacteriology* 195: 4947-4953, 2013.
162. **Lyczak JB, Cannon CL, and Pier GB**. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes and infection / Institut Pasteur* 2: 1051-1060, 2000.
163. **Mahajan-Miklos S, Tan MW, Rahme LG, and Ausubel FM**. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-Caenorhabditis elegans pathogenesis model. *Cell* 96: 47-56, 1999.
164. **Malek AA, Chen C, Wargo MJ, Beattie GA, and Hogan DA**. Roles of three transporters, CbcXWV, BetT1, and BetT3, in *Pseudomonas aeruginosa* choline uptake for catabolism. *Journal of bacteriology* 193: 3033-3041, 2011.
165. **Mao B, Pear MR, McCammon JA, and Quiocho FA**. Hinge-bending in L-arabinose-binding protein. The "Venus's-flytrap" model. *The Journal of biological chemistry* 257: 1131-1133, 1982.
166. **Marciani P, Lindi C, Marzo A, Arrigoni Martelli E, Cardace G, and Esposito G**. L-carnitine and carnitine ester transport in the rat small intestine. *Pharmacological research : the official journal of the Italian Pharmacological Society* 23: 157-162, 1991.
167. **Martin RG, and Rosner JL**. The AraC transcriptional activators. *Current opinion in microbiology* 4: 132-137, 2001.
168. **Massimelli MJ, Beassoni PR, Forrellad MA, Barra JL, Garrido MN, Domenech CE, and Lisa AT**. Identification, cloning, and expression of *Pseudomonas aeruginosa* phosphorylcholine phosphatase gene. *Current microbiology* 50: 251-256, 2005.
169. **Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW, and Boucher RC**. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 95: 1005-1015, 1998.
170. **Matsumoto K, Takahashi M, Takiyama N, Misaki H, Matsuo N, Murano S, and Yuki H**. Enzyme reactor for urinary acylcarnitines assay by reversed-phase high-performance liquid chromatography. *Clin Chim Acta* 216: 135-143, 1993.

www.manaraa.com

171. **Meadows JA, and Wargo MJ**. Carnitine in bacterial physiology and metabolism. *Microbiology* 2015.
172. **Meadows JA, and Wargo MJ**. Characterization of *Pseudomonas aeruginosa* growth on O-acylcarnitines and identification of a short-chain acylcarnitine hydrolase. *Applied and environmental microbiology* 79: 3355-3363, 2013.
173. **Mekalanos JJ**. Environmental signals controlling expression of virulence determinants in bacteria. *Journal of bacteriology* 174: 1-7, 1992.
174. **Meyers DJ, and Berk RS**. Characterization of phospholipase C from *Pseudomonas aeruginosa* as a potent inflammatory agent. *Infect Immun* 58: 659-666, 1990.
175. **Miller J**. Experiments in molecular genetics. In: *Cold Spring Harbor Laboratory Press*. Cold Spring Harbor, NY: 1972.
176. **Miller J**. *Experiments in molecular genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1972.
177. **Miller JH**. Experiments in molecular genetics. Cold Spring Habor, NY: Cold Spring Harbor Laboratory, 1972.
178. **Miller TR, Hnilicka K, Dziedzic A, Desplats P, and Belas R**. Chemotaxis of Silicibacter sp. strain TM1040 toward dinoflagellate products. *Applied and environmental microbiology* 70: 4692-4701, 2004.
179. **Miura-Fraboin J, Kleber, H.P., Englard, S.** Assimilation of gamma-butyrobetaine, and D- and L-carnitine by resting cell suspensions of *Acinetobacter calcoaceticu*s and *Pseudomonas putida*. *Arch Microbiol* 133: 217-221, 1982.
180. **Monnich K. HH, Kleber HP.** Utilization of D-carnitine by Pseudomonas sp. AK 1. *FEMS microbiology letters* 132: 51-55, 1995.
181. **Montero M, Dominguez M, Orozco-Levi M, Salvado M, and Knobel H**. Mortality of COPD patients infected with multi-resistant *Pseudomonas aeruginosa*: a case and control study. *Infection* 37: 16-19, 2009.
182. **Moore KN, Day RA, and Albers M**. Pathogenesis of urinary tract infections: a review. *Journal of clinical nursing* 11: 568-574, 2002.
183. **Mori N, Kasugai, T., Kitamoto, Y., Ichikawa, Y.** Purification and Some Properties of Carnitine Dehydrogenase from Xanthomonas translucens. *Agric Biol Chem* 52: 249-250, 1988.
184. **Mourez M, Hofnung M, and Dassa E**. Subunit interactions in ABC transporters: a conserved sequence in hydrophobic membrane proteins of periplasmic permeases defines an important site of interaction with the ATPase subunits. *The EMBO journal* 16: 3066-3077, 1997.
185. **Murakami KS, and Darst SA**. Bacterial RNA polymerases: the wholo story. *Current opinion in structural biology* 13: 31-39, 2003.
186. **Murray TS, Egan M, and Kazmierczak BI**. *Pseudomonas aeruginosa* chronic colonization in cystic fibrosis patients. *Current opinion in pediatrics* 19: 83-88, 2007.

www.manaraa.com

187. **Naidu G, Lee, IY., Cho, OK., Park YH**. Conversion of gamma-butyrobetaine to L-carnitine by *Achromobacter cycloclast*. *J Ind Microbiol Biotechnol* 26: 309-315, 2001.
188. **Nakai S, Siebert, K.** Organic acid inhibition models for *Listeria innocua, Listeria ivanovii, Pseudomonas aeruginosa* and *Oenococcus oeni*. *Food Microbiology* 21: 67-72, 2004.
189. **Nenoff P, Paasch U, and Handrick W**. [Infections of finger and toe nails due to fungi and bacteria]. *Der Hautarzt; Zeitschrift fur Dermatologie, Venerologie, und verwandte Gebiete* 65: 337-348, 2014.
190. **Nguyen UT, Wenderska IB, Chong MA, Koteva K, Wright GD, and Burrows LL**. Small-molecule modulators of Listeria monocytogenes biofilm development. *Applied and environmental microbiology* 78: 1454-1465, 2012.
191. **Nikaido H**. Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and molecular biology reviews : MMBR* 67: 593-656, 2003.
192. **Nikaido H, and Vaara M**. Molecular basis of bacterial outer membrane permeability. *Microbiological reviews* 49: 1-32, 1985.
193. **Nikaido K, and Ames GF**. One intact ATP-binding subunit is sufficient to support ATP hydrolysis and translocation in an ABC transporter, the histidine permease. *The Journal of biological chemistry* 274: 26727-26735, 1999.
194. **Nobile S, and Deshusses J**. Transport of gamma-butyrobetaine in an Agrobacterium species isolated from soil. *Journal of bacteriology* 168: 780-784, 1986.
195. **Nudler E, and Mironov AS**. The riboswitch control of bacterial metabolism. *Trends in biochemical sciences* 29: 11-17, 2004.
196. **O'Toole GA, and Kolter R**. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular microbiology* 30: 295-304, 1998.
197. **Obon JM, Maiquez JR, Canovas M, Kleber HP, and Iborra JL**. High-density *Escherichia coli* cultures for continuous L(-)-carnitine production. *Appl Microbiol Biotechnol* 51: 760-764, 1999.
198. **Osborn MJ, and Wu HC**. Proteins of the outer membrane of gram-negative bacteria. *Annual review of microbiology* 34: 369-422, 1980.
199. **Ostroff RM, Vasil AI, and Vasil ML**. Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa*. *J Bacteriol* 172: 5915-5923, 1990.
200. **Palmer GC, Jorth PA, and Whiteley M**. The role of two *Pseudomonas aeruginosa* anthranilate synthases in tryptophan and quorum signal production. *Microbiology* 159: 959-969, 2013.
201. **Panter RA, and Mudd JB**. Carnitine levels in some higher plants. *FEBS letters* 5: 169-170, 1969.
202. **Park S, Smith LT, and Smith GM**. Role of Glycine Betaine and Related Osmolytes in Osmotic Stress Adaptation in Yersinia enterocolitica ATCC 9610. *Applied and environmental microbiology* 61: 4378-4381, 1995.

www.manaraa.com

203. **Park SM, Lu CD, and Abdelal AT**. Purification and characterization of an arginine regulatory protein, ArgR, from *Pseudomonas aeruginosa* and its interactions with the control regions for the car, argF, and aru operons. *Journal of bacteriology* 179: 5309-5317, 1997.
204. **Pollack M**. The role of exotoxin A in pseudomonas disease and immunity. *Reviews of infectious diseases* 5 Suppl 5: S979-984, 1983.
205. **Potvin E, Sanschagrin F, and Levesque RC**. Sigma factors in *Pseudomonas aeruginosa*. *FEMS microbiology reviews* 32: 38-55, 2008.
206. **Preusser A, Wagner U, Elssner T, and Kleber HP**. Crotonobetaine reductase from *Escherichia coli* consists of two proteins. *Biochimica et biophysica acta* 1431: 166-178, 1999.
207. **Privalov PL, and Gill SJ**. Stability of protein structure and hydrophobic interaction. *Advances in protein chemistry* 39: 191-234, 1988.
208. **Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, and Ausubel FM**. Common virulence factors for bacterial pathogenicity in plants and animals. *Science* 268: 1899-1902, 1995.
209. **Ran H, Hassett DJ, and Lau GW**. Human targets of *Pseudomonas aeruginosa* pyocyanin. *Proceedings of the National Academy of Sciences of the United States of America* 100: 14315-14320, 2003.
210. **Rebouche CJ**. Carnitine function and requirements during the life cycle. *FASEB J* 6: 3379-3386, 1992.
211. **Rebouche CJ**. Kinetics, pharmacokinetics, and regulation of L-carnitine and acetyl-L-carnitine metabolism. *Annals of the New York Academy of Sciences* 1033: 30-41, 2004.
212. **Rebouche CJ**. *Modern Nutrition in Health and Disease*. Woldters Kluwer, Lippincott Williams and Wilkins, 2014.
213. **Rebouche CJ, and Chenard CA**. Metabolic fate of dietary carnitine in human adults: identification and quantification of urinary and fecal metabolites. *The Journal of nutrition* 121: 539-546, 1991.
214. **Rebouche CJ, and Seim H**. Carnitine metabolism and its regulation in microorganisms and mammals. *Annual review of nutrition* 18: 39-61, 1998.
215. **Reddy JK, and Hashimoto T**. Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annual review of nutrition* 21: 193-230, 2001.
216. **Rees DC, Johnson E, and Lewinson O**. ABC transporters: the power to change. *Nature reviews Molecular cell biology* 10: 218-227, 2009.
217. **Rey S, Acab M, Gardy JL, Laird MR, deFays K, Lambert C, and Brinkman FS**. PSORTdb: a protein subcellular localization database for bacteria. *Nucleic Acids Res* 33: D164-168, 2005.
218. **Ribet D, and Cossart P**. Post-translational modifications in host cells during bacterial infection. *FEBS letters* 584: 2748-2758, 2010.
219. **Rice AJ, Park A, and Pinkett HW**. Diversity in ABC transporters: type I, II and III importers. *Critical reviews in biochemistry and molecular biology* 49: 426-437, 2014.

www.manaraa.com

220. **Richter FL, Pedersen, D.E., Fredell, D.L.** Food additive sanitizing compositions. edited by Patent US1993.
221. **Robert H, Le Marrec C, Blanco C, and Jebbar M**. Glycine betaine, carnitine, and choline enhance salinity tolerance and prevent the accumulation of sodium to a level inhibiting growth of Tetragenococcus halophila. *Applied and environmental microbiology* 66: 509-517, 2000.
222. **Rosenblatt J, Reitzel RA, and Raad I**. Caprylic acid and glyceryl trinitrate combination for eradication of biofilm. *Antimicrobial agents and chemotherapy* 59: 1786-1788, 2015.
223. **Roth S, Jung K, Jung H, Hommel RK, and Kleber HP**. Crotonobetaine reductase from *Escherichia coli*--a new inducible enzyme of anaerobic metabolization of L(-)-carnitine. *Antonie van Leeuwenhoek* 65: 63-69, 1994.
224. **Rudolph AS, Crowe JH, and Crowe LM**. Effects of three stabilizing agents--proline, betaine, and trehalose--on membrane phospholipids. *Archives of biochemistry and biophysics* 245: 134-143, 1986.
225. **Ruetschi U, Nordin I, Odelhog B, Jornvall H, and Lindstedt S**. gamma-Butyrobetaine hydroxylase. Structural characterization of the Pseudomonas enzyme. *European journal of biochemistry / FEBS* 213: 1075-1080, 1993.
226. **Russell RM, Sharp FC, Rasko DA, and Sperandio V**. QseA and GrlR/GrlA regulation of the locus of enterocyte effacement genes in enterohemorrhagic *Escherichia coli*. *Journal of bacteriology* 189: 5387-5392, 2007.
227. **Ryser ET, Marth, E.H.** *Listeria, listeriosis, and food safety*. CRC Press, Taylor and Francis Group, LLC, 2007.
228. **Sage AE, Vasil AI, and Vasil ML**. Molecular characterization of mutants affected in the osmoprotectant-dependent induction of phospholipase C in *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* 23: 43-56, 1997.
229. **Sage AE, and Vasil ML**. Osmoprotectant-dependent expression of plcH, encoding the hemolytic phospholipase C, is subject to novel catabolite repression control in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 179: 4874-4881, 1997.
230. **Saier MH, Jr., and Paulsen IT**. Whole genome analyses of transporters in spirochetes: Borrelia burgdorferi and Treponema pallidum. *Journal of molecular microbiology and biotechnology* 2: 393-399, 2000.
231. **Sansgiry SS, Joish VN, Boklage S, Goyal RK, Chopra P, and Sethi S**. Economic burden of *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. *Journal of medical economics* 15: 219-224, 2012.
232. **Sato H, Okinaga K, and Saito H**. Role of pili in the pathogenesis of *Pseudomonas aeruginosa* burn infection. *Microbiology and immunology* 32: 131-139, 1988.
233. **Sawa T**. The molecular mechanism of acute lung injury caused by *Pseudomonas aeruginosa*: from bacterial pathogenesis to host response. *Journal of intensive care* 2: 10, 2014.
234. **Schiefner A, Breed J, Bosser L, Kneip S, Gade J, Holtmann G, Diederichs K, Welte W, and Bremer E**. Cation-pi interactions as determinants for binding of the compatible solutes glycine betaine and proline betaine by the periplasmic

www.manaraa.com

ligand-binding protein ProX from *Escherichia coli*. *The Journal of biological chemistry* 279: 5588-5596, 2004.
235. **Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, and Cardona A**. Fiji: an open-source platform for biological-image analysis. *Nature methods* 9: 676-682, 2012.
236. **Schirmer T**. General and specific porins from bacterial outer membranes. *Journal of structural biology* 121: 101-109, 1998.
237. **Schleif R**. AraC protein, regulation of the l-arabinose operon in *Escherichia coli*, and the light switch mechanism of AraC action. *FEMS microbiology reviews* 34: 779-796, 2010.
238. **Schleif R**. AraC protein: a love-hate relationship. *BioEssays : news and reviews in molecular, cellular and developmental biology* 25: 274-282, 2003.
239. **Schmitt L, Benabdelhak H, Blight MA, Holland IB, and Stubbs MT**. Crystal structure of the nucleotide-binding domain of the ABC-transporter haemolysin B: identification of a variable region within ABC helical domains. *Journal of molecular biology* 330: 333-342, 2003.
240. **Schulze S, Koster S, Geldmacher U, Terwisscha van Scheltinga AC, and Kuhlbrandt W**. Structural basis of Na(+)-independent and cooperative substrate/product antiport in CaiT. *Nature* 467: 233-236, 2010.
241. **Schweizer HD**. Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. *BioTechniques* 15: 831-834, 1993.
242. **Schweizer HP**. Escherichia-Pseudomonas shuttle vectors derived from pUC18/19. *Gene* 97: 109-121, 1991.
243. **Seedorff J, and Schleif R**. Active role of the interdomain linker of AraC. *Journal of bacteriology* 193: 5737-5746, 2011.
244. **Seim H, Loster H, Claus R, Kleber HP, and Strack E**. Stimulation of the anaerobic growth of Salmonella typhimurium by reduction of L-carnitine, carnitine derivatives and structure-related trimethylammonium compounds. *Archives of microbiology* 132: 91-95, 1982.
245. **Seim H, Loster, H., Claus R., Kleber H.P., Strack E.** Formation of gamma-butryobetaine and trimethylamine from quaternary ammonium compounds structure-related to L-carnitine and choline by Proteus vularis. *FEMS microbiology letters* 13: 201-205, 1982.
246. **Seim H. LH, Kleber H.P.** Reduktiver stoffwechsel des L-carnitins and strukturverwandter trimethylammoniumverbindugen in *Escherichia coli*. *Acta biologica et medica Germanica* 41: 1009-1019, 1982.
247. **Seymour JR, Simo R, Ahmed T, and Stocker R**. Chemoattraction to dimethylsulfoniopropionate throughout the marine microbial food web. *Science* 329: 342-345, 2010.
248. **Shanks RM, Caiazza NC, Hinsa SM, Toutain CM, and O'Toole GA**. Saccharomyces cerevisiae-based molecular tool kit for manipulation of genes

www.manaraa.com

from gram-negative bacteria. *Applied and environmental microbiology* 72: 5027-5036, 2006.
249. **Sheppard DE, and Englesberg E**. Further evidence for positive control of the L-arabinose system by gene araC. *Journal of molecular biology* 25: 443-454, 1967.
250. **Sikorski J, Stackebrandt E, and Wackernagel W**. Pseudomonas kilonensis sp. nov., a bacterium isolated from agricultural soil. *International journal of systematic and evolutionary microbiology* 51: 1549-1555, 2001.
251. **Silby MW, Winstanley C, Godfrey SA, Levy SB, and Jackson RW**. Pseudomonas genomes: diverse and adaptable. *FEMS microbiology reviews* 35: 652-680, 2011.
252. **Silo-Suh L, Suh SJ, Sokol PA, and Ohman DE**. A simple alfalfa seedling infection model for *Pseudomonas aeruginosa* strains associated with cystic fibrosis shows AlgT (sigma-22) and RhlR contribute to pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 99: 15699-15704, 2002.
253. **Skrivanova E, Hovorkova P, Cermak L, and Marounek M**. Potential use of caprylic acid in broiler chickens: effect on Salmonella enteritidis. *Foodborne pathogens and disease* 12: 62-67, 2015.
254. **Sleator RD, Banville N, and Hill C**. Carnitine enhances the growth of Listeria monocytogenes in infant formula at 7 degrees C. *Journal of food protection* 72: 1293-1295, 2009.
255. **Sleator RD, Francis GA, O'Beirne D, Gahan CG, and Hill C**. Betaine and carnitine uptake systems in Listeria monocytogenes affect growth and survival in foods and during infection. *Journal of applied microbiology* 95: 839-846, 2003.
256. **Sleator RD, and Hill C**. Compatible solutes: the key to Listeria's success as a versatile gastrointestinal pathogen? *Gut pathogens* 2: 20, 2010.
257. **Sleator RD, Wouters J, Gahan CG, Abee T, and Hill C**. Analysis of the role of OpuC, an osmolyte transport system, in salt tolerance and virulence potential of Listeria monocytogenes. *Applied and environmental microbiology* 67: 2692-2698, 2001.
258. **Smajs D, McKevitt M, Howell JK, Norris SJ, Cai WW, Palzkill T, and Weinstock GM**. Transcriptome of Treponema pallidum: gene expression profile during experimental rabbit infection. *Journal of bacteriology* 187: 1866-1874, 2005.
259. **Smiddy M, Sleator RD, Patterson MF, Hill C, and Kelly AL**. Role for compatible solutes glycine betaine and L-carnitine in listerial barotolerance. *Applied and environmental microbiology* 70: 7555-7557, 2004.
260. **Soisson SM, MacDougall-Shackleton B, Schleif R, and Wolberger C**. The 1.6 A crystal structure of the AraC sugar-binding and dimerization domain complexed with D-fucose. *Journal of molecular biology* 273: 226-237, 1997.
261. **Soisson SM, MacDougall-Shackleton B, Schleif R, and Wolberger C**. Structural basis for ligand-regulated oligomerization of AraC. *Science* 276: 421-425, 1997.

www.manaraa.com

262. **Son MS, Matthews WJ, Jr., Kang Y, Nguyen DT, and Hoang TT**. In vivo evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. *Infection and immunity* 75: 5313-5324, 2007.
263. **Steiber A, Kerner J, and Hoppel CL**. Carnitine: a nutritional, biosynthetic, and functional perspective. *Molecular aspects of medicine* 25: 455-473, 2004.
264. **Stock AM, Robinson VL, and Goudreau PN**. Two-component signal transduction. *Annual review of biochemistry* 69: 183-215, 2000.
265. **Stocker R, and Seymour JR**. Ecology and physics of bacterial chemotaxis in the ocean. *Microbiology and molecular biology reviews : MMBR* 76: 792-812, 2012.
266. **Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, and Olson MV**. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406: 959-964, 2000.
267. **Strom AR, Olafsen JA, and Larsen H**. Trimethylamine oxide: a terminal electron acceptor in anaerobic respiration of bacteria. *Journal of general microbiology* 112: 315-320, 1979.
268. **Takahashi M. US**. Method of assaying for acyl-L- carnitine and short-chain acyl-carnitine. *US Patent* 5,385,829: January 1995.
269. **Takahashi MUS**. Acyl-carnitine-esterase, method for measuring acylcarnitine. edited by IND AC. Japan: Asahi Kasei Kogyou Kabushiki, 1991.
270. **Tang L, Bai L, Wang WH, and Jiang T**. Crystal structure of the carnitine transporter and insights into the antiport mechanism. *Nature structural & molecular biology* 17: 492-496, 2010.
271. **Tars K, Leitans J, Kazaks A, Zelencova D, Liepinsh E, Kuka J, Makrecka M, Lola D, Andrianovs V, Gustina D, Grinberga S, Liepinsh E, Kalvinsh I, Dambrova M, Loza E, and Pugovics O**. Targeting carnitine biosynthesis: discovery of new inhibitors against gamma-butyrobetaine hydroxylase. *Journal of medicinal chemistry* 57: 2213-2236, 2014.
272. **Team C**. R: A language and environment for statistical computing.
273. **Terada LS, Johansen KA, Nowbar S, Vasil AI, and Vasil ML**. *Pseudomonas aeruginosa* hemolytic phospholipase C suppresses neutrophil respiratory burst activity. *Infect Immun* 67: 2371-2376, 1999.
274. **Tomita K, Sakurada S, and Minami S**. Enzymatic determination of acetylcarnitine for diagnostic applications. *J Pharm Biomed Anal* 24: 1147-1150, 2001.
275. **Trakhanov S, Vyas NK, Luecke H, Kristensen DM, Ma J, and Quiocho FA**. Ligand-free and -bound structures of the binding protein (LivJ) of the *Escherichia coli* ABC leucine/isoleucine/valine transport system: trajectory and dynamics of the interdomain rotation and ligand specificity. *Biochemistry* 44: 6597-6608, 2005.

www.manaraa.com

276. **Treptow NA, and Shuman HA**. Genetic evidence for substrate and periplasmic-binding-protein recognition by the MalF and MalG proteins, cytoplasmic membrane components of the *Escherichia coli* maltose transport system. *Journal of bacteriology* 163: 654-660, 1985.
277. **Turner KH, Everett J, Trivedi U, Rumbaugh KP, and Whiteley M**. Requirements for *Pseudomonas aeruginosa* acute burn and chronic surgical wound infection. *PLoS genetics* 10: e1004518, 2014.
278. **Uanschou C, Frieht R, and Pittner F**. What to learn from a comparative genomic sequence analysis of L-carnitine dehydrogenase. *Monatsh Chem* 136: 1365-1381, 2005.
279. **Uanschou C. FR, Pittner F.** What to learn from comparative genomic sequence analysis of L-carnitine dehydrogenase. *Monatshefte fur Chemie* 136: 1365-1381, 2005.
280. **Ueda MTaS**. Method of assyaing for acyl-L-carnitine and short-chain acyl-carnitine. edited by Patent US. Japan: 1995.
281. **Ulrich J. K, M.R.** Purification and characterisation of a microbial L-carnitine amidase. *Appl Microbiol Biotechnol* 40: 606-610, 1994.
282. **Unemoto T, Hayashi M, Miyaki K, and Hayashi M**. Formation of trimethylamine from DL-carnitine by Serratia marcescens. *Biochimica et biophysica acta* 121: 220-222, 1966.
283. **Ussher JR, Lopaschuk GD, and Arduini A**. Gut microbiota metabolism of L-carnitine and cardiovascular risk. *Atherosclerosis* 231: 456-461, 2013.
284. **van der Heide T, and Poolman B**. ABC transporters: one, two or four extracytoplasmic substrate-binding sites? *EMBO reports* 3: 938-943, 2002.
285. **van Veen HW, Callaghan R, Soceneantu L, Sardini A, Konings WN, and Higgins CF**. A bacterial antibiotic-resistance gene that complements the human multidrug-resistance P-glycoprotein gene. *Nature* 391: 291-295, 1998.
286. **Vasil ML, A. I. Vasil, and V. D. Shortridge.** Phosphate in Microorganisms: Cellular and Molecular Biology. In: *Phosphate and osmoprotectants in the pathogenesis of Pseudomonas aeruginosa*, edited by E. Torriani-Gorrini EY, and S. Silver. Washington D.C.: ASM Press, 1994.
287. **Vaz FM, and Wanders RJ**. Carnitine biosynthesis in mammals. *The Biochemical journal* 361: 417-429, 2002.
288. **Veesenmeyer JL, Hauser AR, Lisboa T, and Rello J**. *Pseudomonas aeruginosa* virulence and therapy: evolving translational strategies. *Critical care medicine* 37: 1777-1786, 2009.
289. **Verheul A, Glaasker E, Poolman B, and Abee T**. Betaine and L-carnitine transport by Listeria monocytogenes Scott A in response to osmotic signals. *Journal of bacteriology* 179: 6979-6985, 1997.
290. **Verheul A, Rombouts FM, Beumer RR, and Abee T**. An ATP-dependent L-carnitine transporter in Listeria monocytogenes Scott A is involved in osmoprotection. *Journal of bacteriology* 177: 3205-3212, 1995.

www.manaraa.com

291. **Verheul A, Wouters JA, Rombouts FM, and Abee T**. A possible role of ProP, ProU and CaiT in osmoprotection of *Escherichia coli* by carnitine. *Journal of applied microbiology* 85: 1036-1046, 1998.
292. **Vilhelmsson O, and Miller KJ**. Humectant permeability influences growth and compatible solute uptake by Staphylococcus aureus subjected to osmotic stress. *Journal of food protection* 65: 1008-1015, 2002.
293. **Walker TS, Bais HP, Deziel E, Schweizer HP, Rahme LG, Fall R, and Vivanco JM**. *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation. *Plant physiology* 134: 320-331, 2004.
294. **Walt A, and Kahn ML**. The fixA and fixB genes are necessary for anaerobic carnitine reduction in *Escherichia coli*. *Journal of bacteriology* 184: 4044-4047, 2002.
295. **Wanders RJ, and Waterham HR**. Biochemistry of mammalian peroxisomes revisited. *Annual review of biochemistry* 75: 295-332, 2006.
296. **Wargo MJ**. Homeostasis and catabolism of choline and glycine betaine: lessons from *Pseudomonas aeruginosa*. *Applied and environmental microbiology* 79: 2112-2120, 2013.
297. **Wargo MJ, Gross MJ, Rajamani S, Allard JL, Lundblad LK, Allen GB, Vasil ML, Leclair LW, and Hogan DA**. Hemolytic phospholipase C inhibition protects lung function during *Pseudomonas aeruginosa* infection. *American journal of respiratory and critical care medicine* 184: 345-354, 2011.
298. **Wargo MJ, Ho TC, Gross MJ, Whittaker LA, and Hogan DA**. GbdR regulates *Pseudomonas aeruginosa* plcH and pchP transcription in response to choline catabolites. *Infect Immun* 77: 1103-1111, 2009.
299. **Wargo MJ, and Hogan DA**. Identification of genes required for *Pseudomonas aeruginosa* carnitine catabolism. *Microbiology* 155: 2411-2419, 2009.
300. **Wargo MJ, Szwergold BS, and Hogan DA**. Identification of two gene clusters and a transcriptional regulator required for *Pseudomonas aeruginosa* glycine betaine catabolism. *J Bacteriol* 190: 2690-2699, 2008.
301. **Warren CR**. High diversity of small organic N observed in soil water. *Soil Biol Biochem* 57: 444-450, 2013.
302. **Warren CR**. Quaternary ammonium compounds can be abundant in some soils and are taken up as intact molecules by plants. *The New phytologist* 198: 476-485, 2013.
303. **Waters LS, and Storz G**. Regulatory RNAs in bacteria. *Cell* 136: 615-628, 2009.
304. **Watson D, Sleator RD, Casey PG, Hill C, and Gahan CG**. Specific osmolyte transporters mediate bile tolerance in Listeria monocytogenes. *Infection and immunity* 77: 4895-4904, 2009.
305. **Wei BL, Brun-Zinkernagel AM, Simecka JW, Pruss BM, Babitzke P, and Romeo T**. Positive regulation of motility and flhDC expression by the RNA-binding protein CsrA of *Escherichia coli*. *Molecular microbiology* 40: 245-256, 2001.

www.manaraa.com

306. **Welsh DT**. Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. *FEMS Microbiology Reviews* 24: 263-290, 2000.
307. **Wemekamp-Kamphuis HH, Sleator RD, Wouters JA, Hill C, and Abee T**. Molecular and physiological analysis of the role of osmolyte transporters BetL, Gbu, and OpuC in growth of Listeria monocytogenes at low temperatures. *Applied and environmental microbiology* 70: 2912-2918, 2004.
308. **Wemekamp-Kamphuis HH, Wouters JA, Sleator RD, Gahan CG, Hill C, and Abee T**. Multiple deletions of the osmolyte transporters BetL, Gbu, and OpuC of Listeria monocytogenes affect virulence and growth at high osmolarity. *Applied and environmental microbiology* 68: 4710-4716, 2002.
309. **Wiener-Kronish JP, Sakuma T, Kudoh I, Pittet JF, Frank D, Dobbs L, Vasil ML, and Matthay MA**. Alveolar epithelial injury and pleural empyema in acute *P. aeruginosa* pneumonia in anesthetized rabbits. *J Appl Physiol* 75: 1661-1669, 1993.
310. **Wilkens S**. Structure and mechanism of ABC transporters. *F1000prime reports* 7: 14, 2015.
311. **Winkler WC, and Breaker RR**. Regulation of bacterial gene expression by riboswitches. *Annual review of microbiology* 59: 487-517, 2005.
312. **Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, and Brinkman FS**. Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes. *Nucleic acids research* 39: D596-600, 2011.
313. **Wu M, and Schleif R**. Mapping arm-DNA-binding domain interactions in AraC. *Journal of molecular biology* 307: 1001-1009, 2001.
314. **Yahr TL, and Greenberg EP**. The genetic basis for the commitment to chronic versus acute infection in *Pseudomonas aeruginosa*. *Molecular cell* 16: 497-498, 2004.
315. **Yamamoto K, Hirao K, Oshima T, Aiba H, Utsumi R, and Ishihama A**. Functional characterization in vitro of all two-component signal transduction systems from *Escherichia coli*. *The Journal of biological chemistry* 280: 1448-1456, 2005.
316. **Yancey PH**. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *The Journal of experimental biology* 208: 2819-2830, 2005.
317. **Yu NY, Laird MR, Spencer C, and Brinkman FS**. PSORTdb--an expanded, auto-updated, user-friendly protein subcellular localization database for Bacteria and Archaea. *Nucleic Acids Res* 39: D241-244, 2011.
318. **Yu Y, Cheng AS, Wang L, Dunne WM, and Bayliss SJ**. Hot tub folliculitis or hot hand-foot syndrome caused by *Pseudomonas aeruginosa*. *Journal of the American Academy of Dermatology* 57: 596-600, 2007.
319. **Zhang L, Xu Z, and Patel BK**. Bacillus decisifrondis sp. nov., isolated from soil underlying decaying leaf foliage. *International journal of systematic and evolutionary microbiology* 57: 974-978, 2007.

www.manaraa.com

320. **Zhu Y, Jameson E, Crosatti M, Schafer H, Rajakumar K, Bugg TD, and Chen Y**. Carnitine metabolism to trimethylamine by an unusual Rieske-type oxygenase from human microbiota. *Proceedings of the National Academy of Sciences of the United States of America* 111: 4268-4273, 2014.
321. **Ziegler C, Bremer E, and Kramer R**. The BCCT family of carriers: from physiology to crystal structure. *Molecular microbiology* 78: 13-34, 2010.

www.manaraa.com