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Identification of genes required for *Pseudomonas aeruginosa* carnitine catabolism

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Summary

Carnitine is a quaternary amine compound prevalent in animal tissues and a potential carbon, nitrogen, and energy source for pathogens during infection. Characterization of activities from *Pseudomonas aeruginosa* cell lysates had previously shown that carnitine is converted to 3-dehydrocarnitine (3-dhc) which is metabolized to glycine betaine (GB), an intermediate metabolite in catabolism of carnitine to glycine. However, the identities of the enzymes required for carnitine catabolism were not known. We used a genetic screen of the *P. aeruginosa* PA14 transposon mutant library to identify genes required for growth on carnitine. We identified two genomic regions and their adjacent transcriptional regulators that are required for carnitine catabolism. The *PA5388-PA5384* region contains the predicted *P. aeruginosa* carnitine dehydrogenase homologue along with other genes required for growth on carnitine. The second region identified, *PA1999-PA2000*, encodes the alpha and beta subunits of a predicted 3-ketoacid CoA-transferase, an enzymatic activity hypothesized to be involved in the first step of deacetylation of 3-dhc. Furthermore, we confirmed that an intact glycine betaine catabolic pathway is required for growth on carnitine. The PA5389 and PA1998 transcription factors are required for growth on carnitine. PA5389 is required for induction of the *PA5388-PA5384* transcripts. The *PA1999-PA2000* transcripts are induced in a PA1998-dependent manner and induction appears to depend on a carnitine catabolite, possibly 3-dhc. These results provide important insight into elements required for carnitine catabolism in *P. aeruginosa* and likely in other bacteria.

Introduction

Carnitine is a quaternary amine compound required for transport of long chain fatty acids into the mitochondria of animals (Bremer, 1983). This important physiological role results in a relatively large amount of carnitine present in fat-metabolizing tissues which could be utilized by microorganisms during tissue necrosis or infection. Additionally, carnitine and acylcarnitine derivatives are found at high micromolar concentrations in the circulating and extracellular fluids (Bremer, 1983). Most bacteria can use carnitine as an osmoprotectant, where it functions as a compatible solute (Peluso *et al.*, 2000). Some bacteria, such as *E. coli*

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can synthesize carnitine to supply this osmoprotective function (Eichler *et al.*, 1994; Jung *et al.*, 1989). Other bacteria, including *Pseudomonas aeruginosa*, can utilize carnitine as a sole source of carbon and nitrogen (Aurich & Lorenz, 1959). The genes encoding the enzymes required for aerobic carnitine catabolism to glycine betaine (GB) have not been described for *P. aeruginosa*.

In *Pseudomonas* and *Arthrobacter*, carnitine has been shown to be converted to glycine betaine (GB), which is then catabolized to glycine (Meskys *et al.*, 2001; Strack *et al.*, 1964). While a requirement for the *Sinorhizobium* dimethylglycine catabolic genes has been demonstrated with mutant growth (Burnet *et al.*, 2000; Goldmann *et al.*, 1991), it has not been experimentally verified with specific catabolic mutants in *P. aeruginosa*.

Published data provides insight into the catabolism of carnitine to GB (Lindstedt *et al.*, 1967). The first step is the oxidation of the hydroxyl group on carbon three, forming 3-dehydrocarnitine (3-dhc). The *P. aeruginosa* enzyme involved in the conversion of carnitine to 3-dhc, L-(-)-carnitine dehydrogenase (CDH) (EC 1.1.1.108), has been biochemically characterized (Aurich *et al.*, 1968; Kleber & Aurich, 1967). The gene encoding a bacterial CDH was first cloned from *Xanthomonas* (Mori *et al.*, 1988), and an available sequence from *Alcaligenes* (E05045) (Houriyoun *et al.*, 1991) allowed Uanschou and colleagues to identify a predicted conserved operon containing the CDH homologue in a variety of organisms known to utilize carnitine as a sole carbon and nitrogen source (Uanschou *et al.*, 2005). Based on this bioinformatics analysis, the putative *P. aeruginosa* CDH homologue is predicted to be encoded by *PA5386*, although the role of this gene has not been confirmed in any Pseudomonad to date. The second predicted step in the catabolism of carnitine is the conversion of 3-dhc to GB. In *P. aeruginosa* cytosolic extracts, this reaction was shown to be CoA and ATP dependent but the responsible enzyme was not characterized (Lindstedt *et al.*, 1967) and no gene or protein associated with this activity has been described previously in any bacterium.

To identify the genes involved in carnitine catabolism, we have screened the PA14 non-redundant transposon mutant library (Liberati *et al.*, 2006) for strains capable of growth on GB but incapable of growth on carnitine. From this screen we identified genes in the predicted CDH operon and confirmed the role of these genes in *P. aeruginosa* carnitine catabolism. Our screen also enabled us to identify a two gene operon encoding the two subunits of a predicted 3-ketoacid CoA-transferase, the enzymatic activity of which is hypothesized to be required to for the conversion of 3-dhc to GB (Lindstedt *et al.*, 1967). Based on our screen and the analysis of mutants defective in GB catabolism in *P. aeruginosa* (Wargo *et al.*, 2008), we were able to demonstrate that, as predicted, mutant strains incapable of growth on GB were similarly unable to grow on carnitine. Finally, while conditions that induce the *P. aeruginosa* CDH enzyme activity were previously determined, the molecular basis of this regulation was not known. Through these studies, we identified two transcriptional regulators that are essential for *P. aeruginosa* growth on carnitine due to regulation of the neighboring catabolic genes, shedding light on the transcriptional control of the carnitine metabolism.

Methods

Strains and growth conditions

P. aeruginosa PA14 and PAO1 wild-type strains (Rahme *et al.*, 1995; Stover *et al.*, 2000), transposon mutants, and deletion strains, as well as *E. coli* strains (Table 1) were maintained on LB medium. For experiments with single carbon sources, MOPS minimal medium (Neidhardt *et al.*, 1974), with addition of the specified carbon source to 20 mM, was used unless otherwise noted. When necessary, gentamicin was added to a final concentration of 10 µg/ml for *E. coli*, 25 µg/ml for *P. aeruginosa* in MOPS, and 50 µg/ml for *P. aeruginosa* in LB.

Genetic screen

The PA14 non-redundant transposon mutant library was replicated as described (Liberati *et al.*, 2006). The library was replicated onto LB agar master plates overnight at 37 °C. Strains were subsequently replicated onto MOPS carnitine plates and incubated at 37 °C for 24 hours. Strains defective for growth on MOPS carnitine were recovered from the LB master plates and a secondary screen conducted for growth on MOPS agar plates with 20 mM of carnitine, GB, glucose, or pyruvate, and incubated at 37 °C for 24 h. Transposon insertion sites were confirmed using gene specific primers and the PMFLGM.GB-4a primer (Liberati *et al.*, 2006).

Growth assays

Growth assays were conducted in 1.0 ml volumes in 24-well polystyrene plates inoculated using overnight cultures grown in MOPS containing 25 mM pyruvate and 5 mM glucose that were washed resuspended in MOPS medium with the appropriate carbon source to an OD₆₀₀ of 0.05. Plates were shaken at 37 °C, and growth was measured by OD₆₀₀ every hour.

During the course of this study we have noted significantly better growth of all strains on MOPS carnitine plates compared to MOPS carnitine liquid medium. For the *PA5386* rescue construct described below, cells showed no growth after 24 hours in liquid MOPS carnitine, but we did see robust growth of the same strain streaked on MOPS carnitine agar plates.

Construction of deletion strains and complementation constructs

Gene numbers in this paper refer to the numbers from the PAO1 genome. There is at least 98% identity at both the nucleotide and amino acid levels when PAO1 and PA14 sequences are compared for all the genes described in this report. Organization of both regions and the surrounding genomic context of the genes of interest were also identical between the two strains.

Some internal segments of the *PA5388* to *PA5384* region proved difficult to amplify from genomic DNA using various primer sets with a large range of melting temperatures. To bypass this issue we constructed a library containing partial Sau3A1 digest fragments of *P. aeruginosa* PA14 genomic DNA, size selected for 4-15 kb, and cloned into BamHI digested pUCP22. The library contained approximately 11,000 clones. We pooled these clones and isolated the plasmid DNA. This pooled plasmid library was used to transform the

PA5386::Tn strain and we selected for growth on carnitine as a sole carbon source. After secondary verification, this selection yielded a single clone containing the genomic region corresponding to the PAO1 genome base pairs 6057482 to 6067212, as verified by sequencing the ends of the clone. This clone is referred to as pMW79 and served as a cloning template for this genomic region. This clone also rescues the growth defects of all deletions and transposons in the *PA5388* to *PA5385* region (data not shown).

Deletion constructs for *PA1998*, *PA1999-PA2000*, *PA5386-PA5387*, and *PA5388* were made using the pMQ30 plasmid (Shanks *et al.*, 2006) and the *PA5389* deletion construct was made using the pEX18Gm plasmid. Deletions in *P. aeruginosa* were made by recombination as described previously (Schweizer, 1993; Wargo *et al.*, 2008). With the exception of the *PA5386-PA5387* deletion construct, upstream and downstream regions of the genes of interest were amplified by PCR from genomic DNA or from the pMW79 construct. The splice overlap extension PCR products were cut and ligated into the suicide vector. After transformation into *E. coli* DH5 α and verification of the correct sequence, constructs in pMQ30 were transformed into *E. coli* S17/ λ pir. The *E. coli* were mated with the recipient *P. aeruginosa* strain and single crossover mutants selected for growth on gentamicin. Recombinants were screened by PCR after selecting for double crossover events by growth on 5% sucrose LB plates with no NaCl. Primers for the deletion constructs are listed in Table S1. Construction of the *PA5386-PA5387* deletion construct was done by restriction fragment cloning the appropriate SacI EcoRI fragment from pMW79 into pUCP22 generating pMW91 (Fig. 1A), followed by cloning of the appropriate HindIII BamHI fragment into pMW91. The HindIII EcoRI fragment from the resulting construct was ligated into a similarly cut pMQ30 to generate pMW99.

Complementation plasmids for *PA1998*, *PA1999-PA2000*, *PA5386-PA5385*, and *PA5389* were constructed in the pMQ80 vector using the primers listed in Table S1. Each of these constructs incorporates an N-terminal 6 \times His tag on the first gene in the construct. The *PA5385::Tn* insertion was rescued by pMW91, which contains the entire *PA5385* sequence and part of the *PA5384* sequence (Fig. 1A). The *PA5388* rescue construct, pMW96, was generated by cloning the appropriate KpnI BamHI fragment from pMW79 into pUCP22 (Fig. 1A). In addition to these individual gene rescue constructs, we subcloned pMW79 to generate a plasmid containing only the *PA5388-PA5385* region intact, named pMW89 (Fig. 1A).

RNA isolation and quantitative real time RT-PCR (qRT-PCR)

For RNA samples, cells were grown overnight in MOPS with 25 mM pyruvate and 5 mM glucose, harvested by centrifugation, resuspended in MOPS with 25 mM pyruvate and 2 mM of the listed carbon source, and grown for two hours at 37 °C. RNA was isolated and cDNA created as previously described (Wargo *et al.*, 2008). Primers are listed in Table S1. Quantitative real-time RT-PCR (qRT-PCR) was conducted with SYBR Green and AmpliTaq Gold DNA polymerase according to the manufacturer's instructions (Applied Biosystems). The amplification conditions were 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s using an Applied Biosystems 7500 instrument. Only one

PCR product was obtained for all primers and all samples based on melting curve analysis. Transcript levels were normalized to the *rpIU* transcript (Kuchma *et al.*, 2005).

Reporter constructs

We generated promoter fusions to *lacZ*YA in pMW5 (Wargo *et al.*, 2009) by amplification with promoter specific primers listed in Table S1. Numbering from the translational start, the *PA5388* promoter fusion contained the -422 to -21 segment, and the *PA1999* promoter fusion contained the -258 to -11 segment. Briefly, each PCR product was gel purified, digested with *Hind*III and *Kpn*I, and ligated into similarly digested pMW5. Ligation mixtures were transformed into *E. coli* DH5 α cells (Invitrogen) by chemical transformation. Plasmid preparations from *E. coli* clones were transformed into *P. aeruginosa* by electroporation (Choi *et al.*, 2006) and selected for growth on gentamicin. After overnight growth in MOPS pyruvate glucose medium as described above, cells were pelleted and resuspended in MOPS with 25 mM pyruvate and 2 mM of the inducing compound unless otherwise specified. Cells were grown in inducing conditions for three hours at 37 °C. The β -galactosidase assays were conducted according to Miller (Miller, 1972).

Results

Identification of mutants defective for growth on carnitine

The *P. aeruginosa* PA14 non-redundant library was screened to find mutants unable to grow in MOPS minimal medium with carnitine as a sole carbon source. To sort our mutants into functional categories and eliminate auxotrophs, we performed secondary screens for growth on glucose, pyruvate, and glycine betaine (GB) as sole carbon sources. Mutants that failed to grow on either pyruvate or glucose were not studied further. The 14 mutants unable to grow on carnitine could be separated into two groups comprised of strains that grew on neither carnitine nor GB and those that were only defective in growth on carnitine. The strains defective for growth on both carnitine and GB were found to have transposon insertions in genes required for GB catabolism in *P. aeruginosa* including *gbcA*, *gbcB*, *dgcAB*, *soxB*DAG and *gbdR* (Wargo *et al.*, 2008) indicating that GB is an essential intermediate in the catabolism of carnitine, consistent with data from previous studies of carnitine catabolism (Lindstedt *et al.*, 1967).

Mutants defective for growth on carnitine, but not GB, included mutants with transposon insertions in a predicted thioesterase (*PA5385*), a predicted periplasmic component of an ABC transporter (*PA5388*), two genes of a predicted operon (*PA1999-PA2000*), and a putative transcriptional regulatory protein (*PA1998*). The two regions identified as being involved in carnitine catabolism are shown in Figs. 1A-B.

The roles of the *PA5388-PA5384* genes in carnitine catabolism

P. aeruginosa strain PA14 mutants with transposon insertions into the *PA5385* and *PA5388* genes resulted in the inability of *P. aeruginosa* to grow on carnitine, while retaining growth on pyruvate (Fig. 1A and Table S2). The *P. aeruginosa* strain PAO1 transposon library contained strains with transposon insertions in each ORF of this region (Jacobs *et al.*, 2003). PAO1 mutants with transposon insertions in each of the *PA5388*, *PA5387*, *PA5386*, and

PA5385 genes were unable to grow on carnitine (Fig. 1A and Table S2). The measured growth rate for one representative transposon mutant strain from each gene is shown in Table S2. PA14 and PAO1 strains with transposon insertions in *PA5384* were capable of normal growth on carnitine, suggesting that this gene is not necessary for growth on free carnitine.

Deletion of *PA5388* was sufficient to abrogate growth on carnitine supporting a role for this ProX-homologue in carnitine uptake. This deletion could be rescued by the pMW96 construct (Fig. 1A and Table S2). Further characterization of the PA5388 protein and its role in transport will be reported elsewhere (G. Beattie, personal communication).

Complementation analysis of transposon and deletion mutants provided insight into the genes necessary for growth on carnitine. The growth defect of *PA5385*:Tn strains was rescued by complementation with *PA5385* on a plasmid (pMW91) (Fig. 1A and Table S2) indicating the importance of this gene for carnitine catabolism. However, this gene is not sufficient as none of the other transposon insertions in *PA5386*, *PA5387*, or *PA5388* were rescued by this plasmid. The rescue of growth of the *PA5386*:Tn strain using pMW95, which contains 6×His-tagged *PA5386* and untagged *PA5385* under control of an arabinose-inducible promoter was observed on solid medium with carnitine as the sole source of carbon (see Methods section). This plasmid, pMW95, was not able to rescue the growth phenotype resulting from transposon insertion in the *PA5387* gene, suggesting that *PA5387* is also required for growth on carnitine (Fig. 1A). While we were unable to construct deletion strains of either *PA5386* or *PA5387* via multiple independent constructs for reasons that remain unclear, we were able to generate a strain with a deletion eliminating the majority of the *PA5386-PA5387* coding sequences, which resulted in loss of growth on carnitine (Table S2). The pMW95 plasmid, which contains *PA5385* and *PA5386*, was not able to rescue this growth phenotype.

The requirement for rescue of the *PA5386*:Tn strains using MOPS carnitine plates was also noted for a non-His tagged construct driven by an arabinose-inducible promoter, and a construct containing a portion of the *PA5387* gene in addition to *PA5386-PA5385* and driven by the *lac* promoter (pMW102, data not shown). The pMW89 construct, however, rescued all of the *PA5386* and *PA5387* mutant strains to near wild-type growth levels in both liquid and plate-based assays suggesting the possibility that spatial co-transcription or co-translation of this region is important for wild-type level of enzymatic activity.

We next tested the requirement for the adjacent, divergently transcribed, AraC-family transcription factor, *PA5389*. Deletion of the *PA5389* gene prevents growth on carnitine (Fig. 1A and Table S2). The carnitine growth defect of the *PA5389* mutant was rescued by complementation with a 6×His-tagged *PA5389* construct (pMW19) (Table S2).

Based on the reported induction conditions for carnitine dehydrogenase enzymatic activity (Aurich *et al.*, 1967), we predicted that the *PA5388-PA5384* transcripts would be induced upon growth in carnitine but not pyruvate or GB. The transcripts for these genes were induced in carnitine only, with fold inductions measured by quantitative real time RT-PCR (qRT-PCR) reported in Table 2.

The genes of the *PA5384-PA5388* region are highly homologous to the CDH-containing region of other Gram-negative bacteria (Uanschou *et al.*, 2005). In addition, the N-terminal sequence of a purified *P. putida* CDH (Goulas, 1988) aligns with the predicted N-terminus of PA5386, matching 12 of 18 residues identically. Our screen and subsequent growth analyses confirm the predictions that this is the *cdh* region in *P. aeruginosa*, and that *PA5386* is the *cdh* homologue in *P. aeruginosa* that is required for the first step in carnitine degradation (Fig. 2). We therefore designate *PA5386* as *cdhA*. In some bacterial species, the proteins encoded by *PA5386* and *PA5385* are fused and encoded by one gene responsible for the CDH activity (Uanschou *et al.*, 2005). Transposon insertion into the *PA5385* gene, downstream of the canonical *cdh* homologue, also results in loss of growth on carnitine and this mutant is rescued by the *PA5385* coding sequence (pMW91) provided in trans (Fig. 1A and Table S2). These data support a role for PA5385 in carnitine catabolism, therefore *PA5385* is here named *cdhB*. The *PA5387* gene, based on the growth analyses presented here is required for growth on carnitine but has no known function or homology to proteins with known function. Based on the genetic data presented here, *PA5387* is here named *cdhC*. Deletion of the *PA5388* gene via an in-frame non-polar excision results in lack of growth on carnitine. The protein encoded by *PA5388* shows high similarity to ProX homologues and we predict it may function as part of a carnitine ABC-family transport system, however we will refrain from naming until the range of PA5388 ligands are identified. The *PA5389* gene, required for induction of the *PA5388-PA5384* genes, is renamed *cdhR* based on its regulatory role described here.

The role of the *PA1999-2000* operon in carnitine catabolism

Transposon insertion into the *PA1999* and *PA2000* genes resulted in the inability of *P. aeruginosa* to grow on carnitine, while retaining growth on pyruvate (Fig. 1B and Table S2). We constructed a deletion of the *PA1999* and *PA2000* genes (*PA1999-2000*) in both PA14 and PAO1. Deletion of this predicted operon resulted in failure to grow on carnitine (Fig. 1B and Table S2). This deletion could be rescued by pMW50, encoding a 6×His-tagged *PA1999* and untagged *PA2000*. Transposon insertion into, or deletion of, the adjacent, divergently transcribed LysR-family transcription factor, *PA1998*, also resulted in a carnitine growth deficiency (Fig. 1B and Table S2). The deletion of *PA1998* could be rescued by providing 6×His-tagged *PA1998* on a plasmid (pMW37) as well as by arabinose-induced overexpression of *PA1999-PA2000* (pMW50) (Fig. 1B).

We verified the operonic nature of the *PA1999-2000* genes by RT-PCR from carnitine-induced RNA using primers spanning these two reading frames. Based on this analysis (data not shown), we will now refer to the region as the *PA1999-2000* operon.

Like the *PA5388-PA5384* genes, the transcripts for the *PA1999* and *PA2000* genes were induced in carnitine only, with fold inductions measured by quantitative real time RT-PCR (qRT-PCR) reported in Table 2.

The *PA1999* and *PA2000* genes encode the alpha and beta subunits of a predicted 3-ketoacid CoA-transferase, which is the enzymatic function predicted to be required as the first step in deacetylation of 3-dhc (a 3-ketoacid) to GB and acetyl-CoA (Fig. 2). The growth phenotype and induction data of this operon, along with a predicted function corresponding to the

suspected enzyme activity for this step, strongly support the role of the *PA1999* and *PA2000* genes in conversion of 3-dhc to GB. Therefore, we designate *PA1999* and *PA2000* as *dhcA* and *dhcB*, respectively. The *PA1998* gene, required for induction of the *dhcAB* operon, is renamed *dhcR* based on its regulatory role described here.

Transcription factors required for growth on carnitine

In our screen and subsequent analyses we identified two transcription factors required for *P. aeruginosa* growth on carnitine; CdhR, encoded by *PA5389*, and DhcR, encoded by *PA1998*. We predicted that CdhR would be required for induction of *PA5388* promoter and DhcR would be required for induction of *dhcAB* promoter. To investigate these hypotheses, we used reporter gene constructs of the upstream regions from *PA5388* and *dhcA* fused to *lacZYA* (Fig. 3).

Both the *PA5388::lacZYA* (pMW46) and *dhcA::lacZYA* (pMW34) promoter fusions were induced strongly in response to carnitine in WT cells, whereas pyruvate and GB did not result in induction (Fig. 3A). In the *cdhR* strain, neither the *PA5388::lacZYA* nor *dhcA::lacZYA* constructs were induced in response to carnitine (Fig. 3B). We hypothesize that regulatory control of carnitine catabolism is a two-step process, where carnitine induces transcription from the *PA5388* promoter via CdhR and 3-dhc induces transcription from the *PA1999* promoter via DhcR. This model of carnitine catabolic regulation is shown in Fig. 2. Based on this model, inability to catabolize carnitine to 3-dhc would prevent induction of the *dhcA::lacZYA* in the presence of carnitine. To test this we transformed the *cdhA::Tn* strain of PAO1 with the *PA5388::lacZYA* and *dhcA::lacZYA* reporter fusions. As predicted, without the carnitine dehydrogenase activity to convert carnitine to 3-dhc, encoded by *cdhA*, the *dhcA* reporter was not induced in response to carnitine, while this mutation did not alter transcription from the *PA5388::lacZYA* fusion (Fig. 3).

Discussion

Despite significant work on the enzymology of carnitine catabolism, the aerobic carnitine catabolic pathway to glycine betaine (GB) in Gram-negative bacteria has yet to be fully described. We have used a genetic screen to identify both the CDH homologue and the genes predicted to be involved in conversion of 3-dhc to GB. This screen also led to the discovery of the transcriptional regulators of both sets of catalytic genes, shedding light on the regulation of carnitine catabolism in *P. aeruginosa*.

While the genes encoding CDH in *P. aeruginosa* had been predicted as such previously (Uanschou *et al.*, 2005), the genes encoding the enzymes for conversion of 3-dhc to GB have not been described in any bacterium. Here we identified the *dhcAB* operon as required for carnitine catabolism and propose that these genes encode subunits of the enzyme required to convert 3-dhc to GB. The *dhcAB* genes encode a predicted 3-ketoacid CoA-transferase, which gives us insight into the possible mechanism for deacetylation of 3-dhc.

Based on the enzymatic activities and general reaction mechanisms, the enzymatic steps between 3-dhc and GB are similar to the catabolism of β -hydroxybutyrate and acetoacetate in ketone bodies. Based on that analogy, the catabolism of carnitine to GB would follow the

pathway shown in Fig. 2. According to this scheme, we have identified the genes encoding enzymes required for the first and second steps. The thiolase predicted to be required for the third step may be encoded by *atoB* (*PA2001*) which is predicted to encode the *P. aeruginosa* acetoacyl CoA-thiolase involved in phospholipid metabolism. The *PA2001* transcript is slightly induced in response to carnitine and transposon insertion into *atoB* results in slow growth on carnitine (data not shown). This slow growth, versus lack of growth, may be explained by the presence of at least two alternate acetyl-CoA thiolases in *P. aeruginosa* encoded by *PA3589* and *PA3925*. If AtoB functions as the primary thiolase in this reaction scheme, the remaining unidentified enzyme is that required for step 4; conversion of glycine betaine-CoA to GB and CoA.

The CDH-encoding genomic region, as previously described (Uanschou *et al.*, 2005), is remarkably conserved in a variety of bacteria, and contains three core components: the CDH encoding enzyme (*cdhA*), the predicted thioesterase (*cdhB*), and a protein of unknown function DUF849 (*cdhC*). We have determined the necessity of these three genes in carnitine degradation, experimental verification of the hypothesis that all three of these coding regions are required for carnitine catabolism (Uanschou *et al.*, 2005). In *P. aeruginosa*, as described in the materials and methods section, the *cdhC* region is particularly problematic to amplify by PCR, however it is possible that the *cdhC* homologue in other organisms will be more amenable to molecular techniques.

The *cdhR* homologue is located adjacent to the CDH region of nearly all Gram negative bacteria that catabolize carnitine (Uanschou *et al.*, 2005). CdhR, an AraC-family transcription factor, has high similarity with the ArgR and GbdR transcription factors of *P. aeruginosa*, which induce transcription of target genes in response to the charged amine-containing compounds arginine and glycine betaine, respectively (Park *et al.*, 1997; Wargo *et al.*, 2008). Data presented here shows that CdhR induces transcription in response to carnitine, a specificity that is likely conserved in the CdhR-homologues of other bacteria.

Carnitine dehydrogenase activity is required for carnitine-dependent induction of the *dhcAB* operon, as a transposon insertion into *cdhA* (*PA5386*) abrogates this induction. We hypothesize that DhcR binds 3-dhc or a derivative, which causes induction of the *dhcAB* operon. Additionally, mutations in either *cdhA* or *dhcR* result in hyper-induction of the *PA5388::lacZYA* reporter fusion (Fig. 3), as one would predict due to backup of the catabolic pathway leading to higher concentrations of free carnitine in the cytosol.

The *dhcAB* genes, encoding a predicted carnitine CoA-transferase, show similarity to a number of different 3-ketoacid CoA-transferases, suggesting that either the similarity is due to conserved structure unrelated to the binding site residues or that these enzymes are somewhat promiscuous. These two possibilities are not mutually exclusive, but would have impacts on the study of *dhcAB* transcriptional regulation. If DhcAB and their homologues are capable of acting on a variety of 3-ketoacids, we would predict that the DhcR homologue has a similar range of inducing ligands. Understanding the regulation of this catabolic step could shed light on control of flux through this step in the presence of multiple 3-ketoacids.

This study provides a significant step forward in understanding carnitine catabolism in *P. aeruginosa*. The identification of DhcAB (PA1999 & PA2000) as the predicted 3-ketoacid CoA-transferase involved in 3-dhc catabolism gives support to the hypothesis that 3-dhc deacetylation proceeds in a manner similar to ketone body metabolism. Future studies will focus on biochemical examination of the reaction steps as well as exploration of the transcriptional regulation of the *cdh* and *dhcAB* genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

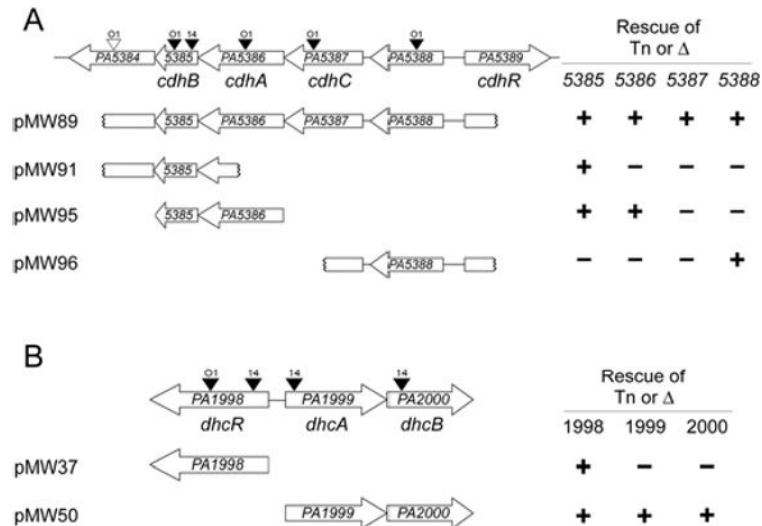
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**Fig. 1.**

Two regions of the *P. aeruginosa* genome contain genes required for carnitine catabolism to glycine betaine. A) The *PA5384* to *PA5389* region contains genes involved in the first step of carnitine catabolism, conversion to 3-dhc. B) The *PA1998* to *PA2000* region contains genes predicted to be involved in conversion of 3-dhc to glycine betaine. Transposon insertions are marked with triangles above the corresponding gene with a labeled to show the strain carrying the transposon; O1 = PAO1, 14 = PA14. Black triangles represent transposon insertions that could not grow on carnitine and white triangles represent insertions that did not alter growth on carnitine. Complementation constructs are diagramed below each genomic region with notation at the left denoting capability (+) or failure (-) to rescue a deletion or transposon insertion into the gene at the top of the column. Growth rates for strains summarized here can be seen in Table S2. The gene names below each genomic region are based on the naming scheme proposed in this report.

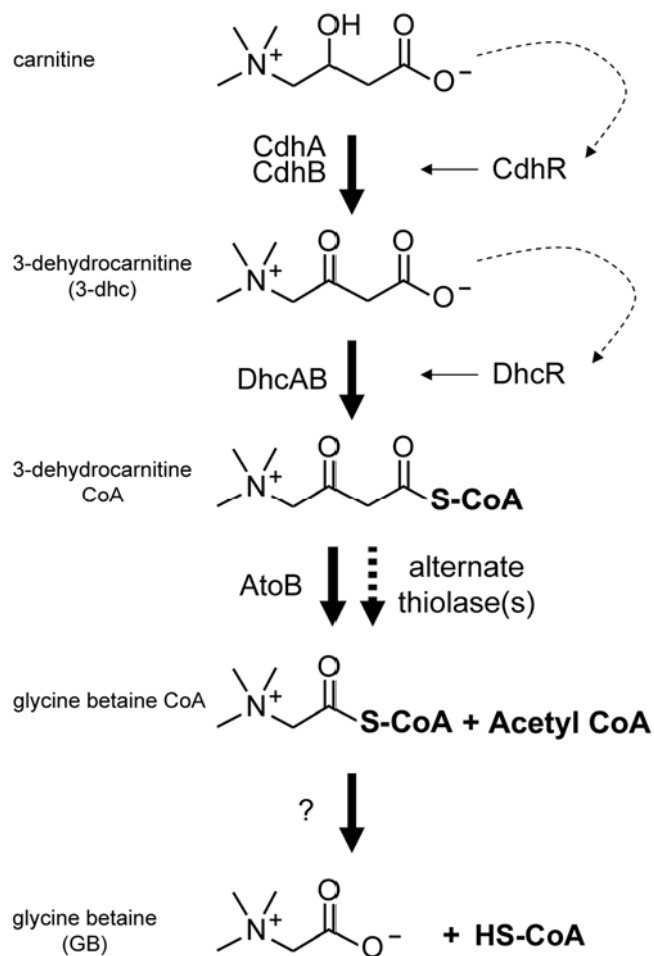
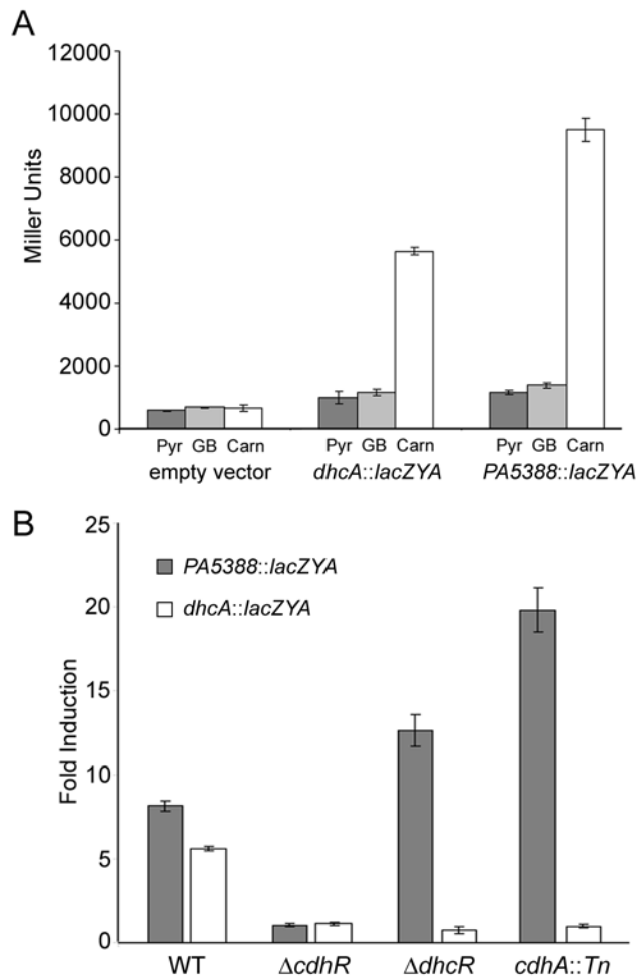


Fig. 2.

The proposed pathway for carnitine catabolism to GB in *P. aeruginosa*. Dashed arrows represent ligand activation of the respective transcription factor, with the solid arrows leading from the transcription factors representing transcriptional induction. Step 1: using NAD⁺, carnitine dehydrogenase (CDH) catalyzes the oxidation at carbon 3. Step 2: the predicted 3-ketoacid CoA-transferase encoded by *dhcAB*, along with a source of activated CoA, catalyzes the sulfur attack of carbon 1. Step 3: a thiolase, possibly encoded by *atoB*, catalyzes the deacetylation reaction. Step 4: an unknown enzyme catalyzes the removal of CoA, leaving glycine betaine.

**Fig. 3.**

CdhR (PA5389) and DhcR (PA1998) are transcriptional regulators of carnitine catabolic genes. A) *lacZYA* reporter fusions to the *PA5388* (pMW46) and *dhcA* (pMW34) promoters are both induced in response to carnitine (Carn) and not in the presence of pyruvate (Pyr) or glycine betaine (GB). B) *cdhR* is required for the induction of both the *PA5388::lacZYA* (dark bars) and *dhcA::lacZYA* fusions (white bars). *dhcR* is only required for induction of the *dhcA::lacZYA* fusion. When *P. aeruginosa* is unable to convert carnitine to 3-dhc (*cdhA::Tn*), carnitine does not lead to induction of the *dhcA::lacZYA* fusion. Error bars show standard deviation.

Table 1

Strains and Plasmids

Strain (gene name ¹)	Strain# ²	Description
<u><i>P. aeruginosa</i> PAO1-derived strains</u>		
PAO1	DH20	<i>P. aeruginosa</i> WT
<i>betB</i> ::Tn		PAO1 transposon mutant # ³
<i>PA1997</i> ::Tn		PAO1 transposon mutant 478
<i>PA1998</i> ::Tn (<i>dhcR</i>)		PAO1 transposon mutant 3
<i>PA1999-PA2000</i> (<i>dhcAB</i>)	MJ99	clean <i>PA1999-PA2000</i> deletion
<i>PA2001</i> ::Tn (<i>atoB</i>)		PAO1 transposon mutant 59
<i>PA5384</i> ::Tn		PAO1 transposon mutant 47947
<i>PA5385</i> ::Tn (<i>cdhB</i>)		PAO1 transposon mutant 10906
<i>PA5386</i> ::Tn (<i>cdhA</i>)		PAO1 transposon mutant 52057
<i>PA5387</i> ::Tn (<i>cdhC</i>)		PAO1 transposon mutant 14404
<i>PA5388</i> ::Tn		PAO1 transposon mutant 37920
<u><i>P. aeruginosa</i> PA14 derived strains</u>		
PA14	DH122	<i>P. aeruginosa</i> WT
<i>betA</i> ::Tn		PA14 transposon mutant 40757 ⁴
<i>gbcA-B</i>	DH802	<i>PA5410-PA5411</i> deletion (Wargo <i>et al.</i> , 2008)
<i>dgcA</i>	DH848	<i>PA5398</i> deletion (Wargo <i>et al.</i> , 2008)
<i>PA1998</i> ::Tn (<i>dhcR</i>)		PA14 transposon mutant 26061
<i>PA1999</i> ::Tn (<i>dhcA</i>)		PA14 transposon mutant 30401
<i>PA2000</i> ::Tn (<i>dhcB</i>)		PA14 transposon mutant 40012
<i>PA1999-PA2000</i> (<i>dhcAB</i>)	MJ98	clean <i>PA1999-PA2000</i> deletion
<i>PA5385</i> ::Tn (<i>cdhB</i>)		PA14 transposon mutant 27032
<i>PA5386-PA5387</i> (<i>cdhBC</i>)	MJ262	in frame <i>PA5386-PA5387</i> deletion
<i>PA5388</i>	MJ192	clean <i>PA5388</i> deletion
<i>PA5389</i> (<i>cdhR</i>)	MJ11	clean <i>PA5389</i> deletion
<u>Plasmids</u>	<u>Description</u>	
pUCP22	high-copy <i>Pseudomonas</i> stabilized vector; Gm ^R (Schweizer, 1991)	
pEX18-Gm	Gm ^R , integrating vector in <i>P. aeruginosa</i> (Schweizer, 1993)	
pMQ30	Suicide vector; Gm ^R (Shanks <i>et al.</i> , 2006)	
pMQ80	high-copy <i>Pseudomonas</i> /Yeast shuttle vector (Shanks <i>et al.</i> , 2006)	
pMW5	pUCP22 containing <i>lacZYA</i> from pRS415 (Wargo <i>et al.</i> , 2009)	
pMW19	6×His-tagged <i>PA5389</i> in pMQ80	
pMW34	<i>PA1999</i> promoter fusion in pMW5	
pMW37	6×His-tagged <i>PA1998</i> in pMQ80	
pMW40	<i>PA1998</i> deletion plasmid	
pMW41	<i>PA1999-2000</i> deletion plasmid	

Strain (gene name ¹)	Strain# ²	Description
pMW46	<i>PA5388</i> promoter fusion in pMW5	
pMW50	6×His-tagged <i>PA1999</i> and untagged <i>PA2000</i> in pMQ80	
pMW56	<i>PA5388</i> deletion plasmid	
pMW79	PA14 genomic clone containing the <i>PA5388-PA5384</i> region	
pMW85	<i>PA5389</i> deletion plasmid	
pMW89	subclone of pMW79 containing only the <i>PA5388-PA5384</i> region	
pMW91	<i>PA5385</i> and partial <i>PA5384</i> sequence in pUCP22	
pMW95	6×His-tagged <i>PA5386</i> and untagged <i>PA5385</i> in pMQ80	
pMW96	<i>PA5388</i> coding sequence in pMQ80	
pMW99	<i>PA5386-PA5387</i> deletion plasmid	
pMW102	<i>PA5386</i> and <i>PA5385</i> coding region subcloned from pMW79	

¹Gene names refer to the *P. aeruginosa* database nomenclature or are specified in this report.

²Strain numbers for non-transposon strains refers to the database designation used in our laboratory to facilitate strain requests.

³PAO1 transposon mutants numbered according to the two-allele library (Jacobs *et al.*, 2003). All strains are Tn5?? strains, except for ##. All PAO1 transposon strains are tetracycline resistant.

⁴PA14 transposon mutants numbered according to the database identification numbers (Liberati *et al.*, 2006). All carry they ## transposon and are gentamicin resistant.

Table 2

qRT-PCR quantification of transcript induction by carnitine.

Transcript	Fold induction^I (+/- S.D.)
PA1998	1.1 (0.57)
PA1999	178.8 (7.75)
PA2000	128.5 (24.8)
PA2001	2.2 (0.37)
PA5384	158.1 (24.5)
PA5385	266.1 (14.9)
PA5386	399.5 (71.9)
PA5387	286.9 (30.6)
PA5388	412.4 (12.6)
PA5389	2.2 (0.16)

^IFold induction calculated by dividing carnitine-induced transcript levels by levels of transcript in pyruvate-grown cells after normalization to the *rplU* transcript.

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