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MINIREVIEW

Homeostasis and Catabolism of Choline and Glycine Betaine: Lessons from *Pseudomonas aeruginosa*

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Most sequenced bacteria possess mechanisms to import choline and glycine betaine (GB) into the cytoplasm. The primary role of choline in bacteria appears to be as the precursor to GB, and GB is thought to primarily act as a potent osmoprotectant. Choline and GB may play accessory roles in shaping microbial communities, based on their limited availability and ability to enhance survival under stress conditions. Choline and GB enrichment near eukaryotes suggests a role in the chemical relationships between these two kingdoms, and some of these interactions have been experimentally demonstrated. While many bacteria can convert choline to GB for osmoprotection, a variety of soil- and water-dwelling bacteria have catabolic pathways for the multistep conversion of choline, via GB, to glycine and can thereby use choline and GB as sole sources of carbon and nitrogen. In these choline catabolizers, the GB intermediate represents a metabolic decision point to determine whether GB is catabolized or stored as an osmo- and stress protectant. This minireview focuses on this decision point in *Pseudomonas aeruginosa*, which aerobically catabolizes choline and can use GB as an osmoprotectant and a nutrient source. *P. aeruginosa* is an experimentally tractable and ecologically relevant model to study the regulatory pathways controlling choline and GB homeostasis in choline-catabolizing bacteria. The study of *P. aeruginosa* associations with eukaryotes and other bacteria also makes this a powerful model to study the impact of choline and GB, and their associated regulatory and catabolic pathways, on host-microbe and microbe-microbe relationships.

There is a general consensus that *Pseudomonas aeruginosa* did not evolve to be a human pathogen; rather, it is an opportunist that exploits environments where effective innate immune clearance has broken down (1–5). The cited studies also pointed out that virulence factor production and resistance to phagocytes are also not indicative of it being a pathogen, but as with many environmental bacteria, a set of responses likely evolved to evade phagocytic eukaryotes such as amoeba. *P. aeruginosa* is prevalent in the environment primarily as a member of aquatic communities, including freshwater, estuarine, and marine systems (1, 6–9), and in rivers appears to be more prevalent in human-impacted stretches (10). *P. aeruginosa* can be isolated from the soil, but is often absent or at low abundance in many soil samples (10, 11). However, during liquid broth enrichment of cultures from soil and aquatic environments, *P. aeruginosa* and many of its nonpathogenic relatives such as *P. putida*, *P. fluorescens*, and *P. stutzeri* are capable of rapid growth and often outcompete numerically abundant microbes from the original system, leading them to be categorized as optimal exploiters of nutrient pulses (12, 13). For *P. aeruguinosa*, the ability to utilize diverse carbon and nitrogen sources and its high growth rate under benign conditions likely contribute to its broad distribution and infectious potential (1, 14, 15).

Life in the soil is tough, particularly from the perspective of water availability. Water activity can range dramatically from extremely hypotonic at the soil surface during a massive rainfall to extremely hypertonic with very low water activity after the sun and wind dry the top layer of soil and impacts the survival of environmental pseudomonads (7, 16). To survive these extremes, bacteria must be able to make physiologic adjustments in response to these swings in the osmolarity of the soil. While *P. aeruginosa* may not be prevalent in many soils (as discussed above), its presence suggests some ability to survive these extremes. The life of *P. aeruginosa* in estuarine settings also exposes it to regular cycles of salinity, requiring the cells to undergo daily adaptation to span the activity range from hypotonic (low tide) to hypertonic (high tide). Under these conditions, while the range of salinities may be high, the change is slow relative to the bacterial life span and in comparison to the potential time scale of osmotic changes in the soil. Conversely, marine *P. aeruginosa* is subject to constitutively hyperosmotic conditions. To protect cell function and turgor under conditions that include any of these osmotic insults, osmoprotectants (also called compatible solutes) are imported or synthesized and accumulate in the cytosol (reviewed in references 17 and 18). Their import, export, and synthesis are tightly regulated, and most environmental bacteria can make use of diverse compatible solutes, including glycine betaine (GB), carnitine, choline-O-sulfate, dimethylsulfoniopropionate (DMSP), proline, stachydrine, taurine, ectoine, mannitol, trehalose, and *N*-acetylglutaminylglutamine amide (NAGGN) (18). The ability of bacteria to accumulate or synthesize these osmoprotectants is likely important for their survival and competition in any environment where the external osmolarity is not constant and relatively isotonic. Different species, even within the same genus, do not always have the same preferences for which of these osmoprotectants they import or synthesize under a given condition. Nutrient limitations can also drive cells to favor particular osmolytes; for instance, nitrogen limitation drives *Corynebacterium* to accumulate the compatible solute trehalose, a carbohydrate, which does not contain nitrogen (19). In this review, I focus on the role of GB and its precursor, choline, in *P. aeruginosa* osmoprotection, as GB can be utilized as

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an osmoprotectant by all pseudomonads studied to date. I also discuss regulation of choline and GB concentrations in the cytosol, and their additional roles as regulators of transcription. While this review is devoted to *P. aeruginosa*, much of the content is relatable to other pseudomonads, and many of the general concepts are relevant for nearly any bacterium from similarly varied environments.

DISTRIBUTION AND BIOLOGICAL ROLES OF CHOLINE AND GB

Glycine betaine (GB) is an important osmoprotectant for many species in all domains of life (17, 18, 20). The widespread use of GB has been explained, in part, by the superior osmoprotection offered by GB as a compatible solute for many organisms (21). GB functions as a compatible solute in these organisms and also plays important roles in methyl group metabolism (22–24). Soluble GB is at low concentrations in most animal fluids (~35 μM in serum [23]), and concentrations are also thought to be low in most environments, although no data could be found for soluble GB concentrations in soil or water. However, its metabolic precursor choline is predicted to be more abundant, predominately as a moiety on larger molecules. Evidence from experimental and natural systems points to rapid bacterial uptake of choline (25–28). This rapid uptake suggests that, while choline release rates may be high, free choline concentrations may be low. Soluble choline is measurable in some environments (~8 μM in serum, 0 to 45 nM in seawater [23, 29]), but the most abundant source is thought to be the choline headgroup moiety on the eukaryotic phospholipids phosphatidylcholine and sphingomyelin, which together typically comprise 50 to 90% of the outer leaflet of eukaryotic plasma membranes (reviewed in reference 30). Phosphatidylcholine and sphingomyelin are also abundant in human serum, at 2 mM and ~0.5 mM, respectively (31). Bacteria commonly produce secreted phospholipases C and/or D, which liberate phosphorylcholine or choline from choline phosphate-containing phospholipids and sphingolipids, respectively (32, 33). These phospholipases are often considered virulence factors, when studied in opportunistic pathogens (reviewed in reference 33), that can alter bacterial survival, cause cell damage, induce inflammatory cytokine production, and suppress antibacterial responses of innate immune cells (34–37). However, they could also be categorized as choline/nutrient acquisition systems beneficial for niche survival (38), with the side effects of host cell damage and potential hemolysis. Bioinformatic analyses predict that the overwhelming majority of soil- and water-dwelling bacteria sequenced to date possess one or more biochemical pathways to convert choline to GB (data not shown, based on the J. Craig Venter Institute Comprehensive Microbial Resource [JCVI CMR] [39] and NCBI BLAST and PSI-BLAST [40, 41]). The nearly ubiquitous nature of the choline oxidation module underlies the assumption that choline is a critical precursor of GB in many environments. This review focuses on *Pseudomonas aeruginosa* regulation of the steps controlling import and catabolism of choline and GB.

CHOLINE AND GB IMPORT VERSUS *DE NOVO* SYNTHESIS

As described above, choline and GB transport systems are nearly ubiquitous in bacteria, suggesting selection of their maintenance in diverse lineages. While choline is ubiquitous in eukaryotes, the ability to synthesize choline or GB *de novo* from phosphatidylethanolamine or glycine is less prevalent in bacteria and evidence suggests that it is absent in the pseudomonads (42). While *de novo* choline synthesis has not been demonstrated in the pseudomonads, transporters for exogenous choline import are ubiquitous within this group based on analyses of current genome sequences (43). Import of choline and GB has been well studied in these and other bacteria, and I direct readers to relevant reviews on the topic (44–46). This portion of the review briefly summarizes the transporters in *P. aeruginosa*, focusing on their impact on the homeostasis of choline and GB. The *P. aeruginosa* genome encodes three members of the BCCT (betaine-choline-carnitine transport) family and a single specific ABC (ATP binding cassette) family transporter predicted to be involved in quaternary amine transport. The BCCT family was largely thought to transport compatible solutes for the purpose of osmoprotection, while the ABC transporter has been shown to be important for bulk transport of choline as a primary carbon source (27, 47, 48). Recent work has elaborated the model of choline and GB uptake by *P. aeruginosa*, where it was shown that the four active transporters (BetT1, BetT2, BetT3, and CbcXWV) function to span the range of physiologic osmolarities to provide optimal uptake from the environment (48, 117) (Fig. 1). These data also support a model where initial uptake by BetT1 or BetT3 primes the induction of CbcXWV, creating a series of transport steps that can be regulated by choline and GB-sensitive transcription factors, as discussed below (48).

The osmotic induction of the BCCT family of transporters has been thoroughly studied and recently reviewed (44), and the data show that initial compatible solute import can occur without transcriptional intervention, as uptake is initiated in the time frame of seconds to a few minutes. While these transporters are transcriptionally induced by osmostress (reviewed in reference 44), the rapid initial uptake is not dependent on *de novo* transcription. This section of the review focuses only on transport regulation by the osmolytes themselves, which occurs after the initial osmostress response and under nonstress conditions, and I refrain from review of sensing osmolarity and cell envelope stress, which has been well reviewed elsewhere (18, 49, 50). Choline and GB transporters in *P. aeruginosa* can be regulated at the transcriptional level (48). In most bacteria, one of the BCCT-family transporters is close to, and generally divergently transcribed from, the choline oxidase genes and its associated choline-responsive transcription factor (51–53). In *P. aeruginosa*, this transporter is named BetT1, and it is divergently transcribed from the *betIBA* operon, where *betI* encodes the BetI choline-responsive transcriptional repressor (53, 54) (Fig. 2). When choline concentrations increase, choline binds to BetI, releasing it from the overlapping and divergent *betIBA* and *betT1* promoters (54). This means that choline uptake initiates a positive-feedback loop to promote further uptake of choline. Release of BetI repression also induces the choline oxidase that reduces the cytoplasmic choline concentration and eventually reestablishes BetI repression, as described in the catabolism section of this review. Recent work in *Bacillus subtilis* has demonstrated negative feedback of cytosolic GB upon activation of the *B. subtilis* choline-sensing repressor, GbsR, through an unknown mechanism (55). It is not known whether BetI in *P. aeruginosa* is similarly regulated by GB levels, but this would be an effective strategy to limit choline import in the presence of ample cytosolic GB.

In *P. aeruginosa*, GB-responsive transcripts are induced via the AraC-family transcriptional activator GbdR (56) (Fig. 2). AraC-family transcription regulators generally function as dimers to

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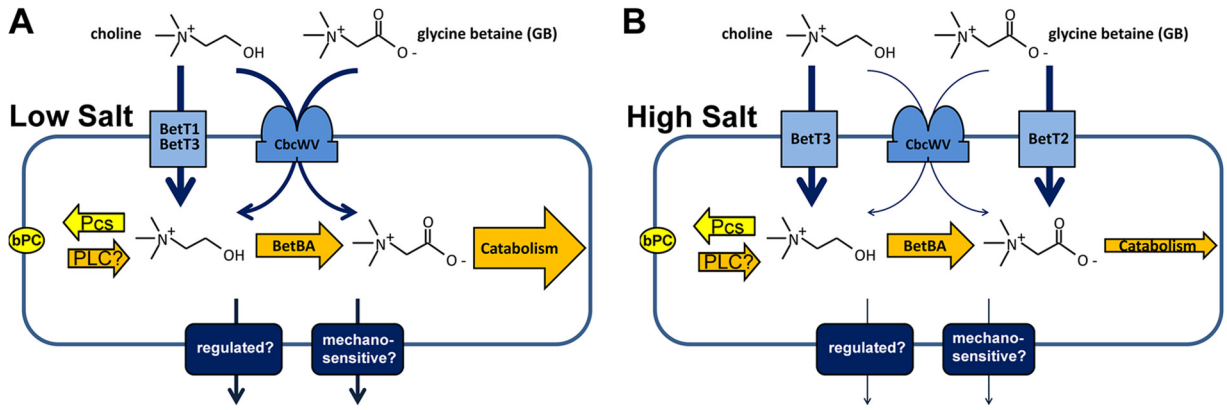

FIG 1 Schematic representation of choline and glycine betaine homeostasis and metabolism in *P. aeruginosa* under conditions of (A) low salt and (B) high salt. In *P. aeruginosa*, choline and GB import are mediated by the BCCT-family transporters BetT1, BetT2, and BetT3 and the ABC-family transporter CbcWV, but the importance of each changes based on the external osmolarity. The full choline catabolic pathway is depicted in Fig. 2 and simplified here. The potential for bacterial phosphatidylcholine (bPC) to be used as a source of choline is discussed in the text. There may be multiple export systems, but export has not been examined in *P. aeruginosa*. The outer membrane has been left out for clarity, but choline and betaine are predicted to access the periplasm via outer membrane porins. Abbreviations: Pcs = phosphatidylcholine synthase; PLC = phospholipase C.

either activate or repress transcription at a given locus (57, 58). The AraC family members canonically regulate genes involved in metabolism (e.g., AraC and XylS for regulation of arabinose and xylene/benzoate metabolism, respectively [59, 60]) and/or virulence (61) (e.g., ExsA regulation of type III secretion in *P. aeruginosa* [62] and ToxT regulation of cholera toxin and the toxin-coregulated pilus in *Vibrio cholerae* [63]). Evidence suggests that GbdR senses GB and dimethylglycine levels in the cell and induces transcription of genes involved in virulence, GB transport, GB catabolism, and detoxification of the catabolic byproducts, hydrogen peroxide and formaldehyde (48, 56, 64–68) (Fig. 2). There are strongly supported GbdR orthologues and orthologues of the entire *P. aeruginosa* choline degradation pathway in all other sequenced pseudomonads, suggesting similar capabilities across the genus. GbdR controls transcription of the *cbcXWV* operon in *P. aeruginosa*, thus stimulating increased transport as part of a positive-feedback loop (48). CbcW is the transmembrane-spanning subunit that assembles to form the transport pore, and CbcX is the

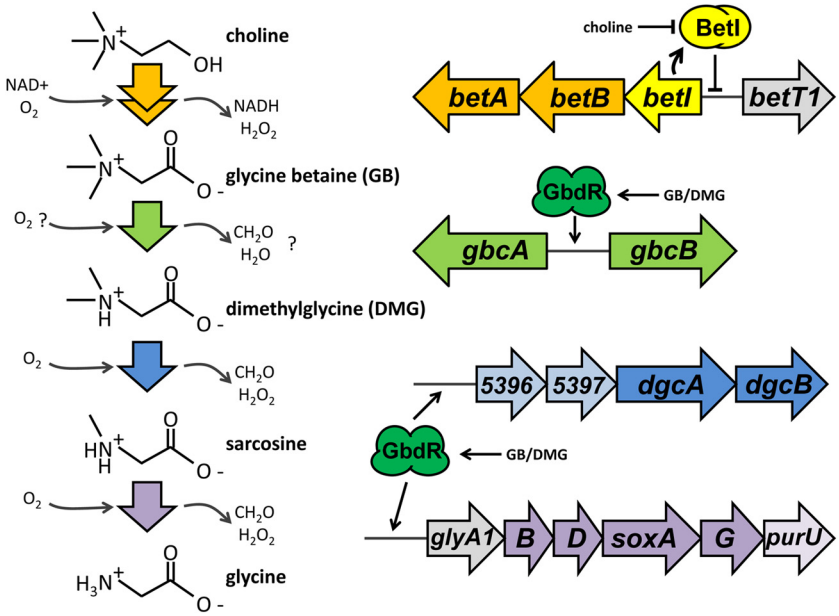

FIG 2 The *P. aeruginosa* choline catabolic pathway and schematic representation of the loci encoding the associated catabolic enzymes. The colored arrows in the metabolic pathway correspond to the similarly colored arrows in the diagrams of the loci; a color match indicates the genes encoding required components of the catabolic enzyme. Similar, but lighter, colors indicate genes associated with the process that do not participate directly in catabolism or whose exact function is unknown. The indicated identities of the reactants and products of GbcAB-dependent demethylation are not known (hence the question mark) but are based on predictions of homologous monooxygenases. For transcriptional control, BetI is a repressor whose repression of the *betI* and *betT1* promoters is released by binding choline; i.e., choline represses the BetI repressor function. GbdR is an activator that induces transcription from the *gbcA*, *gbcB*, *PA5396*, and *glyA1* promoters in response to sensing GB and dimethylglycine (DMG). The *soxA* gene is labeled, and the other genes coding for the SoxBDAG heterotetrameric sarcosine demethylase are noted only by the corresponding letters.

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ATPase subunit, and those subunits are most important for growth on choline, GB, and carnitine as both carbon and nitrogen sources (Fig. 1), while these solutes have specialized periplasmic binding subunits (CbcX, BetX, and CaiX, respectively) (47). The periplasmic solute binding proteins use a ligand binding site similar to that for compatible solute receptors in other bacteria (69, 70). This binding site, composed of the planar hydrophobic residues tryptophan and phenylalanine, has been proposed to comprise a conserved quaternary amine binding pocket that may be used in proteins other than the periplasmic solute binding proteins to bind these compounds (71).

Exogenous choline can be used as a precursor to the formation of bacterial phosphatidylcholine (bPC) in pseudomonads via the *pcs* biosynthetic pathway (72) (Fig. 1). In the presence of choline, laboratory and clinical isolates of *P. aeruginosa* synthesize bPC to achieve approximately 5% bPC in the cell membrane (73). bPC likely localizes to the inner membrane, but the partitioning of bPC to a specific membrane leaflet has not been determined, and neither has its contribution to the inner leaflet of the outer membrane been examined. bPC has been hypothesized to have a role as an inactive store of choline in the membrane that could be liberated when GB is needed (42, 74) (Fig. 1). This liberation could be a consequence of the presence of one or more bacterial phospholipase Cs (PLCs), but how this process is regulated is not known. Such a utilization of lipid headgroups is plausible; bacteria under conditions of phosphate starvation can selectively hydrolyze phospholipids to support the intracellular phosphate requirements (75). The potential role for bPC as a choline store has not been tested in *P. aeruginosa*, but lack of strong phenotypes for the *P. aeruginosa* Δ*pcs* mutant under a variety of conditions does suggest that the role of bPC, if any, manifests only in situations not yet tested (73).

Regardless of the method of acquisition, choline is readily converted to GB in all of the pseudomonads studied to date, and this conversion pathway is predicted to be functional for all sequenced pseudomonads, based on analysis of their genomes. Once choline or GB is in the cytosol, a choice must be made between catabolism, export, and storage or some balance of two or more of these processes. I examine each of these processes to understand what we know and what we would like to know about the homeostasis and catabolism of choline and GB in the pseudomonads.

REGULATION OF AEROBIC CHOLINE AND GB CATABOLISM IN PSEUDOMONADS

Many soil- and water-dwelling bacteria can metabolize choline as a sole carbon source either aerobically or anaerobically. The pseudomonads exclusively use the aerobic pathway for growth on choline as a sole carbon source (as diagrammed in Fig. 2), and this review covers only the aerobic pathway. Readers are directed to reference 76 for a review of anaerobic choline metabolism. Pseudomonads oxidize choline to GB using a pair of enzymes encoded in the same operon, choline oxidase (BetA) and betaine aldehyde dehydrogenase (BetB). The GB resulting from choline oxidation is then demethylated, a reaction that is predicted to use the oxygenase activity of GbcAB to demethylate GB to dimethylglycine and formaldehyde (56). The demethylation of dimethylglycine is carried out by a heterodimeric flavin-linked oxidoreductase comprised of DgcA and DgcB (56, 64), which are homologous to the proteins encoded by dimethylglycine catabolic genes of *Arthrobacter* spp. (77). Sarcosine demethylation is conducted by a heterotetrameric enzyme, SoxBDAG, which is homologous to the proteins encoded by sarcosine oxidase genes in *Corynebacterium* and *Stenotrophomonas* (formerly *Pseudomonas*) *maltophilia* (78, 79). The genes in this catabolic pathway in *P. aeruginosa* have been established on the basis of genetic analysis (56), and those findings are supported by protein induction data (80) (Fig. 2). These genes also have well-supported orthologues in all other pseudomonads (www.pseudomonas.com). Apart from the identification of a novel GB demethylase system based on a monooxygenase, this pathway is similar to that described for other choline degraders, particularly in the *Rhizobiaceae* (81, 82).

In the *Rhizobiaceae*, a GB methyl transferase (GBMT) mechanism has been proposed for GB demethylation and enzyme activity was shown to correspond to growth on GB (24). In the pseudomonads, there is evidence for GBMT enzymatic activity in *P. aeruginosa* (80, 83), and one group showed evidence for its effects on betaine growth in a nonstandard isolate of *P. aeruginosa* (83). Subsequently, I and others demonstrated that this mechanism was not important for GB catabolism as a sole carbon source in the *P. aeruginosa* laboratory isolates PAO1 and PA14 (56); thus, the biological role of GBMT activity in *P. aeruginosa* is not known. I note that the bacteria with a biochemically characterized GBMT system (*Sinorhizobium meliloti*, *Agrobacterium tumafaciens*, *P. aeruginosa*) also have well-supported GbcA and GbcB homologues (64), but to date, genetic analysis has not examined the necessity or sufficiency of the GbcA and -B homologues outside *P. aeruginosa*. More research is needed to determine whether the GbcA-B homologues function to demethylate GB in other bacterium and also to determine what role GB transmethylase activity plays in the GB pathway, even if the activity is not directly involved in utilization of GB as a carbon source.

In *P. aeruginosa*, both choline and GB can be used as sole carbon sources in the presence of substantial osmostress (750 mM NaCl) (65). This leads to the following question: how does *P. aeruginosa* simultaneously catabolize its osmoprotectant and still survive the osmostress? There are two answers to this question, one regulatory and one grounded in the arithmetic of growth.

Let us start with the calculations, which show that, in theory, the cell should have no problem balancing growth and osmoprotection. Using past experiments as a guide (56, 65), ~20 mM choline or GB as a sole source of carbon yields an optical density at 600 nm (OD_{600}) of ~1.0 from a starting OD_{600} of 0.05. If we make some simplifying assumptions (OD_{600} of 1.0 = 10^9 cells/ml, OD_{600} of 0.05 = 5×10^7 cells/ml, 1 cell [dry weight] in C and N = 2×10^{-13} g], this ~20-fold increase in biomass necessitates accumulation of ~0.2 mg of mass. Assuming a conservative conversion efficiency of ~20%, ~1 mg of C/N source would be required. A 20 mM concentration of choline is ~1.5 mg per ml of biosynthetic equivalents (the methyl carbons likely do not contribute substantially to biosynthesis [80]), leaving sufficient GB to achieve osmoprotection in the relatively small remaining cytoplasmic volume represented by this biomass (300 mM cytoplasmic GB in the cytoplasmic volume represented by this number of cells [1 μl] could be generated by ~35 μg of choline). This situation is, of course, an optimal one and likely does not model a soil or water niche well. However, the basic reasoning may still apply, given the fact that while choline concentrations are likely lower in the environment, so too are local cell populations and net growth capacity. Given the struggle for nutrients in the environment, these latter

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two terms are likely small enough that choline may simultaneously function as a nutrient and osmoprotectant in *P. aeruginosa*.

The calculation presented above would be similar for *Rhizobium*; however, it does not grow well on GB under high-salt conditions (82). Therefore, the pseudomonads must have some way to maintain intracellular osmoprotectant at or above a critical threshold to survive the osmotic insult while still permitting growth. In other words, *P. aeruginosa* has a way to license its GB catabolic enzymes even under conditions of salt stress. This careful partitioning implies a regulatory mechanism governing GB utilization.

There are two straightforward regulatory strategies to maintain osmoprotection during growth on the osmoprotectant. The first and most straightforward strategy is to synthesize an alternative osmoprotectant from the GB catabolic products and proceed with catabolism of the remaining GB. This approach has advantages, and it is obvious that an organism that can grow solely on choline and GB can use GB for synthesis of all cellular components, likely including alternate osmoprotectants. However, the synthesis of alternate compounds would further reduce the maximum growth yield on an already poor carbon source. Accumulation of GB is also known to alter or inhibit the synthesis of some alternate osmoprotectants, such as trehalose, in *P. aeruginosa* (80, 84, 85), potentially limiting the choice of the alternate protectant.

The second strategy for simultaneous growth and osmoprotection is to actively regulate flux through the GB pool to maintain a set concentration of the cytoplasmic pool. Our recent data in *P. aeruginosa* show that cells do maintain a GB pool during growth on choline as a sole carbon source, even under conditions of high salinity (65). The presence of this pool is not absolute, as it was drained during the stationary phase, likely to augment growth or maintenance, and may be accompanied by synthesis of alternative osmoprotectants (65). Depletion of the cytoplasmic GB pool was experimentally achieved by overexpression of the GB demethylase (GbcAB), supporting a model where enzyme production, and not enzyme regulation, is likely the step targeted by regulation (65). However, the identity of the regulatory step(s) that monitors and maintains GB levels is currently unknown. Choline and GB catabolism are under catabolite repression control by NtrBC and CbrAB (86), in a manner similar to histidine utilization in *P. fluorescens* (87). Massimelli et al. showed that, in addition to GbdR, growth on choline as a sole carbon source was dependent on CbrB, whereas NtrC and CbrB were both required to utilize choline as a sole nitrogen source (66). While there is no direct evidence that these catabolite repression regulators play a role in controlling flux through the choline and GB pools, their impact on metabolism suggests a potential control system for regulating one or more steps in the pathway to balance flux through the GB pool.

One way *P. aeruginosa* detects choline is through the BetI transcriptional repressor (52, 54). BetI is a member of the TetR family of transcriptional repressors that bind palindromic sequences as homodimers and are generally involved in resistance to antimicrobials and stress, including osmotic stress (88). Detection of GB by GbdR mediates the transcriptional response involved in GB catabolism (56, 67) (Fig. 2). These two transcriptional regulators are our starting point for understanding regulation of choline and GB metabolism, and therefore homeostasis, in *P. aeruginosa*. In *Rhizobium*, some of the enzymes in the GB catabolic pathway have decreased activity at high salt concentrations, presumably to drive accumulation of cytoplasmic GB (82). In *P. aeruginosa*, high salt concentrations do not appear to directly impact the catabolic pathway (65, 80); therefore, I hypothesize that the regulation at all salt concentrations occurs via regulation either at the transcriptional or the translational level.

BetI functions as a choline-sensitive repressor, releasing repression in the presence of choline. Due to the genetic architecture of the *betT1-betIBA* region (Fig. 2), release of repression through detection of choline results in two competing activities. First, loss of BetI repression leads to *betT1* induction (48), which provides for a higher rate of choline transport when choline remains in the extracellular environment. Second, loss of BetI repression stimulates transcription of *betIBA*, a single polycistronic transcript encoding the BetI repressor and the BetA and BetB enzymes for oxidation of choline to GB (52, 53, 89) (Fig. 2). The combination of increased repressor production (due to BetI synthesis) and the increase in choline catabolic capacity (due to BetA and BetB synthesis) eventually reestablishes BetI repression at this and other BetI-controlled promoters. Said another way, the simultaneous increase in BetI repressor concentration and decrease in the antirepressing ligand (choline) concentration results in greater repression of the BetI-controlled promoters. In this way, autoregulation of the choline pool can be achieved by the integrated activities of the proteins encoded at the *betIBA* and *betT* loci. *P. aeruginosa* maintains a cytoplasmic choline pool under a variety of conditions and even under conditions in which extracellular choline is depleted by active transport (65). It is not known if this choline pool is maintained by regulation of BetA and BetB enzyme activity directly, via balance of BetI repression, or if GB acts to repress expression or activity of this catabolic step as GbsR does in *B. subtilis* (55). In *P. aeruginosa*, the choline and GB pools are linked, but their regulation is not completely coupled. For instance, nearly complete loss of the GB pool does not substantially alter the levels of the choline pool (65). In addition to the *betIBA-betT1* promoters, BetI also regulates *betT3* (48). These promoters are the only strong matches for the *Escherichia coli* and *Sinorhizobium meliloti* BetI consensus binding sites in the PAO1 genome (48).

GB transport and catabolism is controlled by the AraC-family activator GbdR (56). GbdR induces transcription in response to GB and dimethylglycine in the cytosol, leading to induction of choline and GB acquisition components (*plcH*, *pchP*, *cbcXWV*, and *betX*) and induction of the components of the GB catabolic pathway (*gbcA* and *-B*, the *dgc* operon, *soxBDAG*, *glyA1*, and *sdaB*) (47, 48, 56, 64–67) (Fig. 2). Therefore, like BetI, GbdR promotes both the accumulation and depletion of its inducing ligands. *P. aeruginosa* maintains a cytoplasmic GB pool in the presence of various primary carbon sources, salt concentrations, and growth temperatures (65); however, the direct contribution of GbdR to homeostasis of this pool is unknown. Overexpression of the *gbcA* and *-B* genes using an engineered induction system was sufficient to deplete intracellular GB (65), but we do not know if hyperactivation of GbdR would accomplish the same goal. It is interesting that, while Diab et al. observed abundant GbcB (PA5411) protein in response to growth on GB, they did not detect GbcA (PA5410) (80). We showed that both the *gbcA* and *gbcB* transcripts are strongly induced in response to growth on GB (56), and unpublished quantitative reverse transcription-PCR (RT-PCR) data suggest that the *gbcA* transcript is present at higher copy numbers than the *gbcB* transcript (M. J. Wargo, unpublished data). Using these two observations, one can postulate a model where GbcA

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levels are controlled translationally or posttranslationally. Because GbcA is the predicted catalytic subunit, titration of this component by control of the protein levels would be an effective means of establishing and maintaining a cytoplasmic GB pool.

GB EXPORT: TOO MUCH OF A GOOD THING?

GB accumulation comes with some apparent drawbacks. Such large amounts of a solute (sometimes up to ~1 M during osmotic stress) can alter cell physiology (21, 90). In a number of bacteria, accumulated GB is rapidly exported upon release of the osmostress, often via one or more mechanosensitive channels (91–95). A mechanosensitive channel of this type was identified in *Corynebacterium glutamicum* and shown to contribute to the balance of osmoprotectants in the cytosol by efflux of GB (96), similar to the MscS channels in *E. coli* (97). Recently, a choline and GB exporter, EmrE, was experimentally demonstrated to reduce the ability of *E. coli* to grow under high-salt conditions when overexpressed, due to hyperefflux of GB (98). In *P. aeruginosa*, I strongly suspect the presence of an exporter, as a mutant in the GB demethylase does not accumulate substantially more GB than a wild-type (WT) cell (Wargo, unpublished) (Fig. 1). Thus, I hypothesize that in WT cells, the balance between catabolism and export sets a relatively narrow window that slides higher or lower according to the environmental conditions, as described for *C. glutamicum* (96). We currently do not know the identity or the physiological characteristics of such an exporter in *P. aeruginosa* (Fig. 1). There is only a poor homologue of the *C. glutamicum* MscCG-type exporter in *P. aeruginosa* (PA1408; 31% identical, 50% positive). However, there is a well-supported *P. aeruginosa* homologue of EmrE encoded by *PA4990* (46% identical and 66% positive over the entire amino acid sequences of both proteins). PA4990 is a good candidate to regulate levels of intracellular GB, although demonstration of this effect and the conditions under which it acts have not been reported. We know that at least one *P. aeruginosa* isolate from the cystic fibrosis (CF) lung appears to hypersecrete GB in favor of trehalose synthesis and accumulation (99). Both EmrE and PA4990 are part of the SMR family of exporters that transport molecules out of the cell using the proton motive force. Many of these transporters, typified by EmrE and Qac, provide low-level resistance to antibiotics and quaternary amine biocides and have moderate flexibility in substrate specificity (100). Given the relatively broad specificity of these transporters and their role in antibiotic and biocide resistance, it is tempting to speculate that this hyper-GB secretion strain (99) may hyperexport GB as a consequence of adaptation to strong antibiotic selection and microbial competition in the CF lung; in other words, adaptation for antibiotic efflux may result in the side effect of increased GB efflux.

The coexistence of choline import, choline oxidation to GB, and predicted regulated export of GB suggests that acquisition of abundant choline by one cell population may result in efflux of GB capable of providing osmoprotectants to other cells within the community, as described in work with *Vibrio cholerae* and other *Vibrio* species (101) and discussed by Welsh (102). In a mechanistic examination of this phenomenon, Hoffmann and colleagues recently reported the sharing and recycling of proline between cells of *Bacillus subtilis*, providing direct evidence of osmoprotectants as a piece of microbial communal property (103). In the same report, Hoffmann et al. communicate that *B. subtilis* is capable of sharing GB within a population (103). It remains to be demonstrated what impact such resource sharing of GB has on intraspecies survival or the extent to which interspecies sharing of GB occurs within microbial communities beyond *Vibrio*. Both of these issues could have interesting consequences for development of microbial communities in stressful environments where eukaryotes provide the bulk of the metabolizable biomass or in systems dominated by bacteria capable of synthesizing GB (104, 105).

GB STORAGE: PROTECTION FOR THE FUTURE?

P. aeruginosa maintains intracellular pools of choline and GB (>12 ng/mg [dry weight] and >45 ng/mg [dry weight], respectively) under conditions of exposure to choline regardless of the osmotic conditions (65), in contrast to what happens in the enteric bacteria studied to date. I and others have also shown that other *Pseudomonas* species, *P. syringae*, *P. fluorescens*, and *P. putida*, as well as *Burkholderia cepacia*, maintain choline and GB pools (65), suggesting that this is a common phenomenon in the pseudomonads and perhaps in other organisms from similar environments. However, the ubiquity of these pools in the pseudomonads remains to be examined. Why do the pseudomonads (and other soil- and water-dwelling bacteria [106, 107]) maintain a choline and GB pool in the absence of apparent stress? Here, I present three potential roles for these choline and GB pools that are not mutually exclusive. First, these bacteria may maintain these pools as preemptive hedges against future insults (102). GB is a protectant against osmostress, temperature stress, and oxidative stress, and has a role in maintenance of intracellular pH (98, 108–110). Therefore, perhaps GB is stored in these bacteria to provide enhanced survival and recovery from unexpected insults that provide a survival benefit versus bacteria that do not have such storage systems. A second possibility is that choline and GB represent relatively innocuous means of storing a high-N/C-ratio compound to provide a readily utilizable nitrogen source (102). For this role, it is important that catabolism of choline and GB is not under catabolite repression control during nitrogen starvation (86). Examining the size and presence of the choline and GB pools under different levels of nitrogen stress should shed light on this second possibility. Such a role has been studied for GB versus trehalose accumulation in the phototrophic haloalkalophile *Ectothiorhodospira halochloris* (111, 112). These first two possibilities represent anticipatory storage, which, while observed in the relatively innocuous environs of the laboratory, would be expected to be often required in the extremely variable natural environment. Both anticipatory storage mechanisms also fit well with the nature of *Pseudomonas* as a nutrient pulse exploiter in the natural environment, where pulses of choline or GB could be accumulated for later use.

A third possibility is that storage of choline and GB has a positive impact on nonstress physiology. While studies of GB alteration of physiology during stress abound, I know of no study providing strong evidence for *in vivo* effects under nonstress conditions. However, there is both theoretical and experimental evidence that physiologic concentrations of osmoprotectants can change protein shape and activity (113, 114). The biotechnology industry has also long used GB as a protein stabilizer and a stabilization agent for single-stranded nucleic acids based on basic research findings (115, 116). One can imagine GB accumulation modifying mRNA secondary structures, perhaps making them less restrictive to expression, or altering proteins to modify half-life and function. While there is currently no *in vivo* evidence for this role in bacteria, I find the idea attractive.

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CONCLUSIONS

Choline and GB represent an important source of osmoprotectants in terrestrial and freshwater ecosystems and likely play an accessory role or a role comparable with that of dimethylsulfoniopropionate (DMSP) as osmoprotectants in marine systems. Given the nearly ubiquitous nature of choline and GB uptake systems and the prevalence of choline oxidases, these molecules are likely to play crucial roles in many environments and impact processes from symbiosis and virulence to biofilm formation and nutrient cycling. The pseudomonads and other choline- and GB-catabolizing bacteria could play an interesting role in the flow of these quaternary amines through the environment. In addition to using choline and GB as osmoprotectants, these bacteria, and their anaerobic choline-catabolizing counterparts, can remineralize such resources, and this catabolic pathway might play an important role in the establishment and maintenance of multispecies communities.

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