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Choline Catabolism in *Burkholderia thailandensis* Is Regulated by Multiple Glutamine Amidotransferase 1-Containing AraC Family Transcriptional Regulators

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ABSTRACT

***Burkholderia thailandensis* is a soil-dwelling bacterium that shares many metabolic pathways with the ecologically similar, but evolutionarily distant, *Pseudomonas aeruginosa*. Among the diverse nutrients it can utilize is choline, metabolizable to the osmoprotectant glycine betaine and subsequently catabolized as a source of carbon and nitrogen, similar to *P. aeruginosa*. Orthologs of genes in the choline catabolic pathway in these two bacteria showed distinct differences in gene arrangement as well as an additional orthologous transcriptional regulator in *B. thailandensis*. In this study, we showed that multiple glutamine amidotransferase 1 (GATase 1)-containing AraC family transcription regulators (GATRs) are involved in regulation of the *B. thailandensis* choline catabolic pathway (*gbdR1*, *gbdR2*, and *souR*). Using genetic analyses and sequencing the transcriptome in the presence and absence of choline, we identified the likely regulons of *gbdR1* (*BTH_II1869*) and *gbdR2* (*BTH_II0968*). We also identified a functional ortholog for *P. aeruginosa souR*, a GATR that regulates the metabolism of sarcosine to glycine. GbdR1 is absolutely required for expression of the choline catabolic locus, similar to *P. aeruginosa* GbdR, while GbdR2 is important to increase expression of the catabolic locus. Additionally, the *B. thailandensis* SouR ortholog (*BTH_II0994*) is required for catabolism of choline and its metabolites as carbon sources, whereas in *P. aeruginosa*, SouR function can by bypassed by GbdR. The strategy employed by *B. thailandensis* represents a distinct regulatory solution to control choline catabolism and thus provides both an evolutionary counterpoint and an experimental system to analyze the acquisition and regulation of this pathway during environmental growth and infection.**

IMPORTANCE

Many proteobacteria that occupy similar environmental niches have horizontally acquired orthologous genes for metabolism of compounds useful in their shared environment. The arrangement and differential regulation of these components can help us understand both the evolution of these systems and the potential roles these pathways have in the biology of each bacterium. Here, we describe the transcriptome response of *Burkholderia thailandensis* to the eukaryote-enriched molecule choline, identify the regulatory pathway governing choline catabolism, and compare the pathway to that previously described for *Pseudomonas aeruginosa*. These data support a multitiered regulatory network in *B. thailandensis*, with conserved orthologs in the select agents *Burkholderia pseudomallei* and *Burkholderia mallei*, as well as the opportunistic lung pathogens in the *Burkholderia cepacia* clade.

Burkholderia thailandensis is a saprophytic, soil-dwelling bacterium common in tropical and subtropical regions, and it is an opportunistic pathogen of insects, plants, nematodes, and amoeba (1–3). *B. thailandensis* is used as a less virulent model for the select agents *B. pseudomallei* and *B. mallei*, the causative agents of melioidosis and glanders, respectively. The reduced virulence of *B. thailandensis* is due primarily to the absence of the major capsule locus, important for *B. pseudomallei* virulence in mammals (4), although it is used to study the effect of type III secretion systems on phagocytic escape and retains other genes associated with virulence (5, 6). Despite virulence differences, a great deal of the core genome is shared between *B. thailandensis* and its relatives, including many pathways for accessory metabolism.

Genes predicted to be involved in choline catabolism are found throughout the *B. pseudomallei* group (BPG) and *Burkholderia cepacia* complex (BCC) (7, 8). Many soil bacteria can use choline as a sole carbon and nitrogen source, and this catabolic pathway may be particularly important for bacteria associated with eukaryotes (9). Choline is part of the polar head group of both phosphatidylcholine and sphingomyelin, which together constitute the majority of lipids on the outer leaflet of eukaryotic cell membranes (10) and are also abundant in pulmonary surfactant (11). In addition to its role as a nutrient source, choline metabolism can generate glycine betaine (GB), an important osmoprotectant (12) and inducer of virulence factor production (13). The conversion of choline to GB has been shown to be important for *Escherichia coli* survival in urine (14) and *Pseudomonas aeruginosa* survival in

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the mammalian lung (15, 16). Many organisms, including *Burkholderiales*, maintain an intracellular GB pool, potentially as a hedge against future osmotic stress (17). Choline can also be found in the rhizosphere, exuded from plant roots, likely to influence populations of bacteria in this environment (18). In fact, many plants accumulate high levels of GB as an osmoprotectant, notably beets, from whence the appellation "betaine" was derived (19). In the environment, secreted or decaying organic matter provides a metabolic opportunity that holds much promise to microbial opportunists poised to exploit them.

Our laboratory has previously studied the choline catabolic pathway and its regulation in *Pseudomonas aeruginosa* (20–22), and examination of orthologous genes in *B. thailandensis* suggested an alternative, perhaps more complex, regulatory strategy. In *P. aeruginosa*, the critical regulator of GB catabolism is GbdR, a glutamine amidotransferase 1 (GATase 1)-like AraC family transcription regulator (GATR) that regulates the genes required for sequential demethylation of GB to glycine in *P. aeruginosa* (Fig. 1A, left) (22). In contrast to *P. aeruginosa*, *B. thailandensis* and other *Burkholderiales* appeared to have two GbdR orthologs (Fig. 1B and C) whose cooccurrence and conserved synteny suggested that they play nonoverlapping roles in *B. thailandensis* choline catabolism. In this study, we have investigated the contributions of the two *B. thailandensis* GbdR orthologs to choline and GB catabolism and to global gene expression in response to choline. These data and follow-up analyses reveal that the choline catabolism pathway in *B. thailandensis*, while bearing many similarities to *P. aeruginosa*, contains key differences in organization and regulation.

MATERIALS AND METHODS

Culture conditions. *Burkholderia thailandensis* E264 (ATCC 700388) (23) cultures were inoculated from −80°C glycerol stocks onto LB (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) with 1.5% agar and incubated at 37°C overnight. For protein expression and molecular cloning, *Escherichia coli* DH5α or *E. coli* T7 Express cells (New England BioLabs [NEB]) were then grown in LB broth containing 100 μg/ml carbenicillin, kanamycin, or trimethoprim (Tp) as appropriate.

Strain construction. Deletion strains (see Table S1 in the supplemental material) were generated as described in Thongdee et al., and the resistance markers were removed using the methods described in Choi et al. (24, 25). Briefly, a PCR product was amplified via splice overlap extension PCR that consisted of a trimethoprim resistance marker amplified from pUC18mini-TN7T-Tp, including the flanking Flp recombinase sites, and with regions of homology upstream and downstream of the gene of interest (see primers in Table S2). The resultant PCR product was then used to naturally transform *B. thailandensis* that had been grown in defined medium (DM) as previously described (24). To remove the resistance marker, pFLPe2 was electroporated into the Tp-resistant strain and plated onto low-salt LB (LSLB; 10 g/liter tryptone, 5 g/liter yeast extract, 3 g/liter NaCl, 1.5% agarose, pH 7.5, with 250 μg/ml zeocin and 0.2% L-rhamnose for Flp recombinase expression). Cells were cured of pFLPe2 by growth at 42°C. Resultant colonies were streaked onto plates with zeocin or Tp, or without antibiotics, to identify colonies where the trimethoprim marker and pFLPe2 had been lost. The Δ*gbdR1*, Δ*gbdR2*, and Δ*souR* phenotypes were complemented by cloning each gene and ~250 bp of the upstream regulatory region into pUC18mini-TN7T-Zeo and integration into one of the two *att*Tn*7* sites in *B. thailandensis* (26, 27) (see Table S1). Appropriate control strains were generated by *att*Tn*7* integration with the empty pUC18mini-TN7T-Zeo vector (see Table S1).

Growth assays. Growth assays were performed by starting overnight cultures in morpholinepropanesulfonic acid (MOPS) minimal medium supplemented with 20 mM pyruvate and 5 mM glucose incubated at 37°C (28). Cultures were diluted in MOPS medium to an optical density at 600 nm (OD_{600}) of 0.07, and 30 μl was used to inoculate 470 μl of MOPS medium supplemented with carbon sources as described in the figure legends in a flat-bottom 48-well plate (Costar). These plates were incubated at 37°C with agitation and OD_{600} measured using a Biotek Synergy 2 plate reader.

Alignments and phylogenetic tree construction. Sequence alignments and phylogenetic analysis were preformed using MEGA version 6 (29). Amino acid sequences of the relevant GATRs were aligned using MUSCLE (30). The *ftrA* sequence, a GATR homolog from *Escherichia coli*, was included as an outgroup to root the phylogenetic tree.

RNA-Seq. For RNA sequencing (RNA-Seq), wild-type (WT), Δ*gbdR1*, Δ*gbdR2*, and Δ*gbdR1* Δ*gbdR2* strains were streaked from frozen stocks onto LB agar plates and incubated overnight at 37°C. These plates were used to inoculate 3 ml MOPS with 20 mM pyruvate and 5 mM glucose cultures, which were incubated for 18 h at 37°C on a rotary wheel. To initiate the experiment, 1 ml of these cultures was added to 2 ml of prewarmed MOPS with 30 mM pyruvate or 2 ml MOPS with 30 mM pyruvate and 1.5 mM choline, resulting in final concentrations of 20 mM pyruvate and 1 mM choline. These cultures were incubated for 4 h at 37°C. Cells from 1.5 ml of culture were collected by centrifugation, and the resultant pellets were resuspended in RNA Protect bacterial reagent (Qiagen) and incubated at room temperature for 10 min. Cells were collected by centrifugation and resuspended in 50 μl 10 mM Tris, 1 mM EDTA (TE) buffer with 3 mg/ml lysozyme and incubated at room temperature for 5 min. To each of these resuspensions, 1 μl 20 mg/ml proteinase K, 0.5 μl of 10% SDS, 1 μl of DNase I (RNase free; Ambion), and 2 μl of 50 mM $MgCl_2$ were added, and samples were incubated for 5 min at room temperature. RNA was then prepared using the Qiagen RNeasy kit according to the manufacturer's instructions. Eluate from this purification was further treated with 11 μl 10× DNase I buffer and 2 μl DNase I (RNase free; Ambion), incubated at 37°C for 20 min to remove contaminating DNA, and repurified using the Qiagen RNeasy protocol with the optional on-column DNase I step. The resulting purified RNA was assessed for purity by PCR and quantified by a Bioanalyzer chip, depleted of 16S and 23S rRNA by using MICROBExpress bacterial mRNA enrichment (Ambion), and subsequently reassessed by Bioanalyzer (Agilent Technologies). RNA-Seq DNA libraries were prepared by the Vermont Cancer Center–College of Medicine Massively Parallel Sequencing Facility using an Illumina TruSeq stranded total RNA library preparation kit and were run on an Illumina Hi-Seq 1500 to generate single end reads. Read quality was checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Samples were aligned to the NC_007650 and NC_007651 chromosomes using BWA (31), duplicate reads were marked using Picard (version 1.110; Broad Institute of MIT and Harvard [http://broadinstitute.github.io/picard/]), and gene counts were calculated using summarizeOverlaps from the GenomicRanges package (32) before differential expression was called using the DESeq package (33) in R (version 3.0.1; R Development Core Team [https://www.r-project.org/]), with assistance from the University of Vermont College of Medicine Bioinformatics Shared Resource group.

Quantitative reverse transcription-PCR (qRT-PCR) to confirm RNA-Seq findings. Wild-type, Δ*gbdR1*, Δ*gbdR2*, and Δ*gbdR1* Δ*gbdR2* strains were grown and induced as described for the RNA-Seq experiment. RNA was extracted similarly to the procedure for RNA-Seq, except that a third DNase I treatment was performed using the Ambion DNA-free kit (AM1906) per the manufacturer's protocol. cDNA was generated using a Superscript IV first-strand synthesis system (Invitrogen) using 24 ng of RNA combined with the 5′-NSNSNSNSNS-3′ primer previously described (34). Quantitative PCR (qPCR) was performed as described in Willsey et al. (35). Briefly, 0.2× SYBR green I nucleic acid gel stain (Thermo Fisher Scientific [TFS]) was used with Q5 high-fidelity 2× master mix (NEB) and amplified using cycle conditions of 98°C for 2 min and 98°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 20 s, repeated by going back to the second step 39 times. Reactions were per-

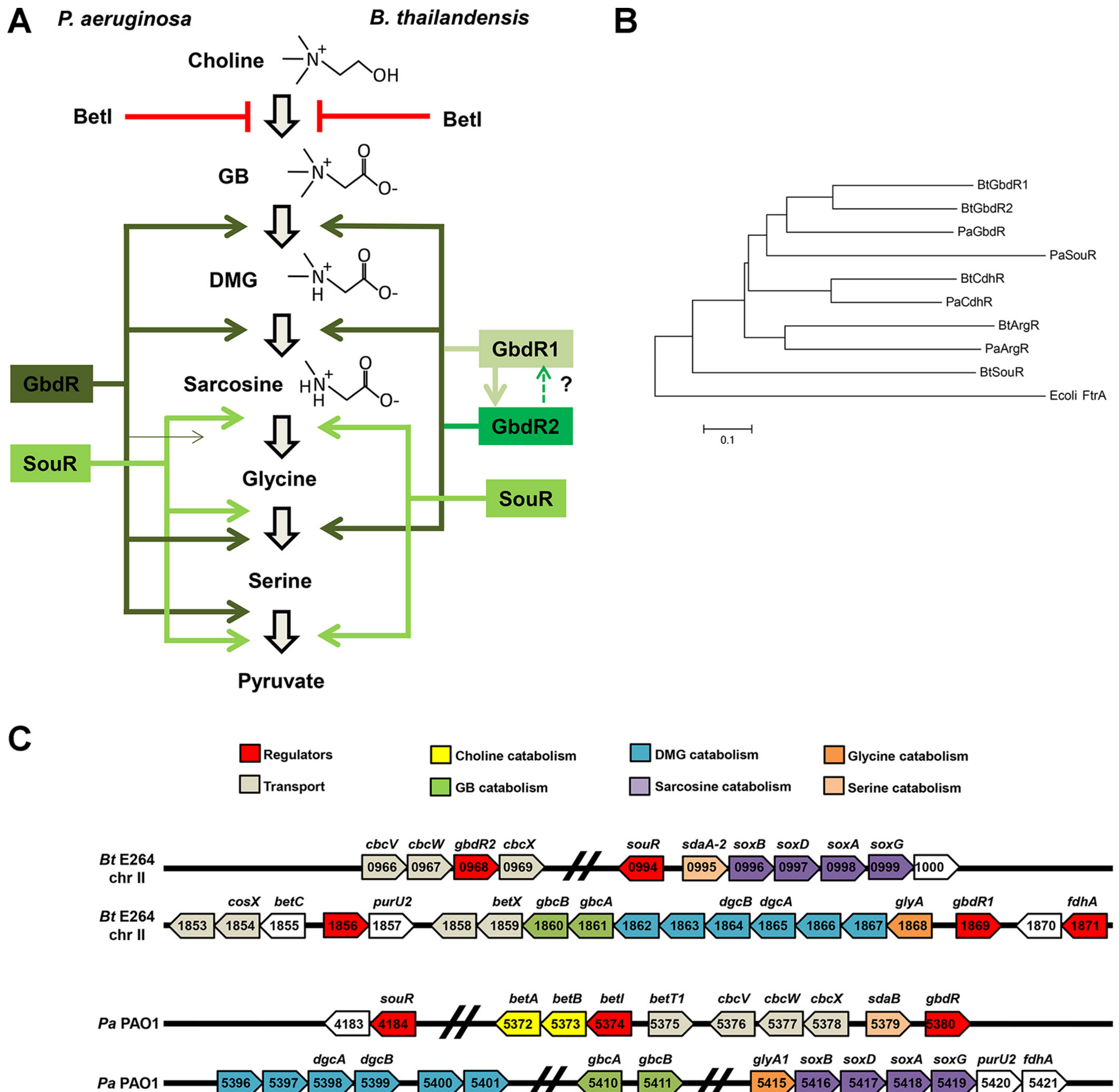

FIG 1 Comparison of *B. thailandensis* and *P. aeruginosa* choline metabolism and associated regulators. (A) Model of choline catabolic regulation in *P. aeruginosa* (left) and *B. thailandensis* (right). (B) Phylogenetic tree of relevant GATRs in *B. thailandensis* and *P. aeruginosa* rooted to FtrA, a GATR present in *E. coli*. (C) Diagram of the chromosomal context of *gbdR*, *gbdR1*, *gbdR2*, *sour*, and major catabolic genes for the catabolism of GB and subsequent metabolites for *B. thailandensis* and *P. aeruginosa*.

formed in technical duplicate and biological triplicate with RT primers designed for *BTH_I1140* (*rplU*), *BTH_II1869* (*gbdR1*), *BTH_II1861* (*gbcA*), *BTH_II1853* (putative porin), *BTH_II0968* (*gbdR2*), *BTH_II0966* (*cbcV*), *BTH_II0994* (*souR*), and *BTH_II0996* (*soxB*). A dilution series was used to generate a standard curve for each primer set. Each reaction was normalized to its respective *rplU* value, and then induced values were divided by uninduced values to derive a fold effect value.

Purification of GbdR1, GbdR2, and SouR. *gbdR1*, *gbdR2*, and *souR* were each cloned into the pMAL-C2X expression vector, generating an N-terminal maltose-binding protein (MBP) tag. The constructs were transformed into *E. coli* T7 Express, transformants were selected on LB agar plus 100 μg/ml carbenicillin, and positive colonies were used to inoculate 20-ml cultures of LB plus 100 μg/ml carbenicillin. After 18 h, these cultures were used to inoculate 500-ml flasks of LB plus 100 μg/ml LB carbenicillin, grown to an OD_{600} of 0.3, and induced for 4 h at 30°C with 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were collected by centrifugation. Cells were then washed and collected by centrifugation, and the resulting cell pellets were flash frozen in liquid nitrogen.

Cell pellets were resuspended on ice in 10 ml of chilled chromatography and lysis buffer (20 mM Tris, pH 7.4, 500 mM NaCl, 20 mM imidazole, 1× HALT protease inhibitor [TFS]). Ten microliters of DNase I was added to the resuspended cell pellet, and the mixture was incubated for 10 min on ice. The resuspended cells were lysed with a French cell press using three 1,100-lb/in^2 passages, and NP-40 was then added to a final concentration of 0.05%. The lysate was clarified by centrifugation for 15 min at 15,000 rpm at 4°C. The supernatant was passed over a column containing a 1-ml bed volume of amylose resin beads (NEB). The column was then washed with 10 ml of column buffer 300 (20 mM Tris, pH 7.4, 300 mM KCl, 1 mM EDTA, 0.05% NP-40, 0.7 μl/ml β-mercaptoethanol) and then 10 ml column buffer 150 (20 mM Tris, pH 7.4, 150 mM KCl, 1 mM EDTA, 0.05% NP-40, 0.7 μl/ml β-mercaptoethanol). Protein was eluted in column buffer 150 containing 10 mM maltose, and 12 300-μl fractions were collected. Fractions were analyzed by SDS-PAGE acrylamide gels stained with Coomassie. Since GATR proteins tend to precipitate at higher concentrations, fractions of lower concentration were pooled and dialyzed in Slide-A-Lyzer dialysis cassette G2 (TFS) against 2 liters of column buffer 150. Aliquots were flash frozen in liquid nitrogen and stored at −80°C.

EMSA. Biotinylated or unlabeled primers were used to generate DNA probes from appropriate templates containing the putative promoter regions using Q5 high-fidelity DNA polymerase (NEB) and then subjected to a QIAquick PCR purification kit (Qiagen) and quantified using a NanoDrop 1000 (TFS). Twenty-microliter reactions were assembled using 2× binding buffer (10 mM Tris, pH 7.4, 200 mM KCl, 2 mM EDTA, 2 mM dithiothreitol [DTT], 10% glycerol, and 200 μg/μl bovine serum albumin [36]), 5 ng/μl poly(dI-dC), 25 to 75 nM purified MBP-tagged protein, 1 nM biotinylated DNA probe, and (optionally) 20 nM unlabeled competitor DNA probe. Reaction mixtures were incubated in a 30°C water bath for 20 min, 5 μl 5× electrophoretic mobility shift assay (EMSA) loading buffer (1× Tris-borate-EDTA [TBE], 20% glycerol, 0.01% xylene cyanol, 0.01% bromophenol blue) was added, and samples were loaded onto a 5% acrylamide 0.5× TBE gel. Gels were run for 1 h at 100 V and then transferred to a BioDyne-B nylon membrane (Pierce) for 1 h at 80 V in 0.5× TBE at 4°C. DNA was cross-linked to the membrane using a UV Stratalinker 2400 (Stratagene) on the auto-cross-link setting, and the biotin-labeled probe was visualized using the Pierce LightShift chemiluminescent EMSA kit (TFS) and imaged on a ChemiDoc XRS+ molecular imager (Bio-Rad). Density of resulting bands was quantified using Quantity One software from Bio-Rad.

Generation of reporter constructs and β-galactosidase assays. Yeast homologous recombination was used to replace the kanamycin resistance gene in the shuttle vector pMQ131 (37) (*apaHA3*) with the Tp resistance gene (*dhrFII*), resulting in pAN1. Yeast recombination was also used to replace the *lacZ*α fragment with the full *lacZYA* operon, resulting in pAN7. The intergenic region between *gbdR1* and *glyA*, as well as truncations of this region, were ligated into the SphI site proximal to the *lacZYA* ribosomal binding site, and transformants were selected on LB agar plus 100 μg/ml Tp at 37°C. Tp-resistant colonies were used to inoculate 3 ml MOPS medium with 20 mM pyruvate, 5 mM glucose, and 100 μg/ml Tp, which were incubated for 48 h at 37°C. Cells from these cultures were collected by centrifugation at 13,000 rpm for 2 min at room temperature, washed with 1 ml MOPS medium, and collected again by centrifugation, and the pellets were resuspended in MOPS medium. Thirty microliters of this resuspension was used to inoculate each well in a 48-well plate that contained 470 μl MOPS medium with 20 mM pyruvate with or without 1 mM choline. These cultures were incubated for 48 h at 37°C on an orbital shaker at 170 rpm in a humidified chamber. The extended induction time was shown to be necessary for detectable β-galactosidase activity empirically in our hands and as indicated by Kang et al. (38). β-Galactosidase activity was quantified as previously described (21).

Accession number(s). RNA-Seq data have been deposited in NCBI GEO under record GSE81652.

RESULTS

Organization of predicted *B. thailandensis* orthologs of *P. aeruginosa* choline catabolic genes. The choline catabolic pathway and its regulation in *P. aeruginosa* have been described previously (20, 22). Briefly, in *P. aeruginosa* the TetR family transcription factor BetI represses expression of *betIBA* and is derepressed by choline, allowing production of the BetA and BetB enzymes that convert choline into GB (39–41). The GATase 1-like AraC family transcription regulator (GATR) GbdR responds to GB and dimethylglycine (DMG) by inducing expression of catabolic genes, including *gbcA-B*, the *dgc* operon (*PA5376*, *PA5377*, *dgcA*, *dgcB*, *PA5400*, and *PA5401*), and the sarcosine oxidase genes, which together contribute to the sequential demethylation of GB to DMG, sarcosine, and finally glycine (Fig. 1A and C). SouR, also a GATR, controls transcriptional induction of the sarcosine oxidase genes in response to sarcosine (35). Homologs of *betI* and *betBA* in *B. thailandensis* have been described previously (38).

Sequence analysis revealed two likely GbdR orthologs in *B. thailandensis*, *BTH_II1869* (*gbdR1*) and *BTH_II0968* (*gbdR2*), with strong amino acid sequence homology to *P. aeruginosa* GbdR (48.2% and 47.0% identity and 75.5% and 71.3% similarity, respectively, using BLOSUM50 along the entire protein length). Alignments of the characterized *P. aeruginosa* GATRs (ArgR, CdhR, and SouR), and their putative *B. thailandensis* orthologs, revealed that the *B. thailandensis* GbdR orthologs were more similar to each other than to *P. aeruginosa* GbdR or any other GATRs (Fig. 1B). This suggested that they also have similar functions or play separate roles within the same catabolic pathway. The presence of a more distantly related GATR divergently transcribed from the sarcosine oxidase operon (Fig. 1B and C) led us to predict that *BTH_II0994* was a functional ortholog of *P. aeruginosa* SouR, although the phylogenetic tree suggests that they share no recent paralogy.

On the *P. aeruginosa* chromosome, *gbdR* is located one gene away from the *cbcXWV* transport genes (Fig. 1C), previously implicated in the transport of choline and its immediate metabolites into the cytosol from the periplasm (42) and adjacent to the *sdaB* gene known to participate in the pathway (43). In *B. thailandensis*, *gbdR1* is located adjacent to a putative operon containing the bulk of predicted orthologous genes encoding enzymes needed for GB catabolism to sarcosine, while *gbdR2* is present within a locus containing the putative *cbcXWV* orthologs (Fig. 1C). The homology of GbdR1 and GbdR2 with *P. aeruginosa* GbdR, combined with their chromosomal locations adjacent to genes involved in choline metabolism, strongly suggested that both transcription factors were involved in the regulation of this pathway. The synteny of the *gbdR1* and *gbdR2* loci in *B. thailandensis* E264 is conserved throughout the various clades of *Burkholderiales*, including species such as *B. pseudomallei*, *B. mallei*, *B. cenocepacia*, *B. ambifaria*, and *B. multivorans*. This conservation in synteny suggests that both GbdR1 and GbdR2 play roles in the regulation of choline metabolism in *Burkholderia*.

***gbdR1* and *gbdR2* contribute differentially to choline catabolism.** *B. thailandensis*, like *P. aeruginosa* (44), can use choline as a sole source of carbon and/or nitrogen (Fig. 2A). Based on our predictions of *gbdR1* and *gbdR2* involvement in choline metabolism, we tested the ability of each single-deletion strain and the double-deletion strain to grow using choline as a sole carbon source. Wild-type *B. thailandensis* grows on choline as a sole car-

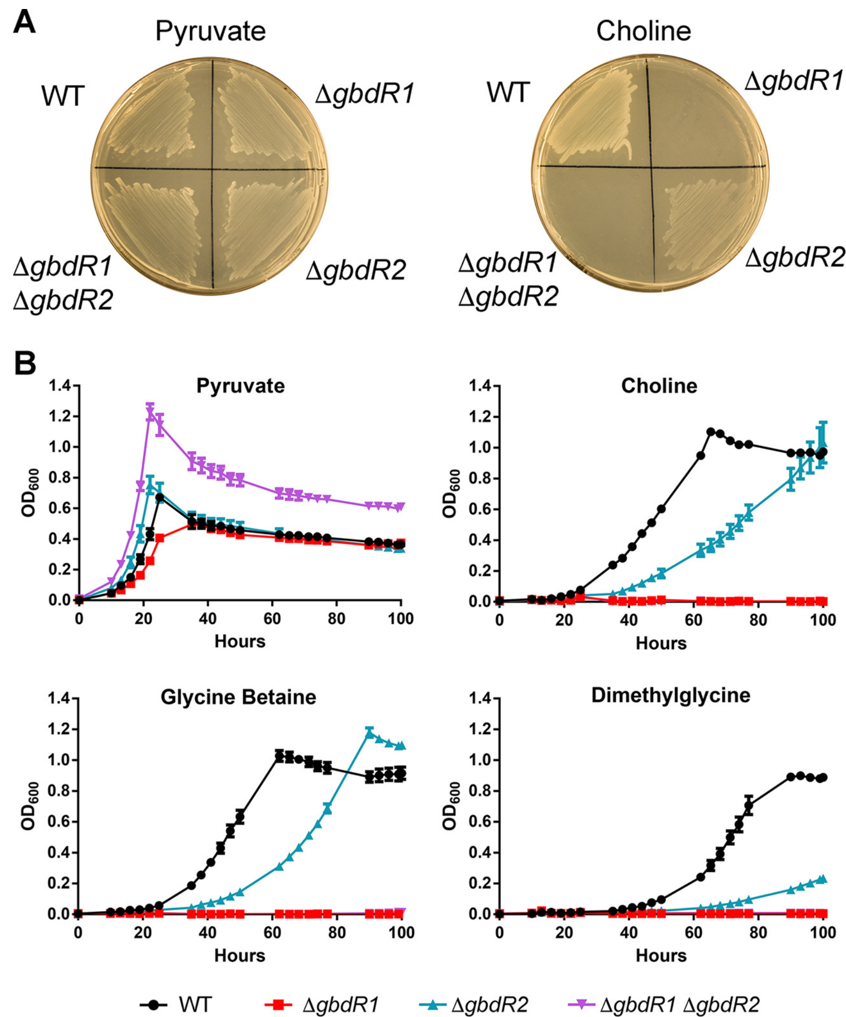

FIG 2 Deletion of *gbdR1* and *gbdR2* results in altered growth on choline and its metabolites as sole carbon sources. (A) Growth of *B. thailandensis* deletion strains on MOPS minimal medium agar plates supplemented with either 20 mM pyruvate or 20 mM choline and incubated at 37°C for 72 h. Δ*gbdR1* and Δ*gbdR1* Δ*gbdR2* strains fail to grow on choline, while the Δ*gbdR2* strain exhibits less growth. (B) Growth curves reveal the inability of Δ*gbdR1* and Δ*gbdR1* Δ*gbdR2* strains to grow on choline or its metabolites, while the Δ*gbdR2* strain exhibits a slow-growth phenotype on choline, glycine betaine, and dimethylglycine. Growth curves are representative of at least three experiments per condition at each time point, each with biological triplicates, and the error bars represent standard deviations.

bon source, while a Δ*gbdR1* or Δ*gbdR1* Δ*gbdR2* strain cannot (Fig. 2A, right). Deletion of *gbdR2* alone resulted in reduced growth on choline as a sole carbon source (Fig. 2A, right). On plates, none of the manipulations appeared to alter growth on pyruvate as a sole carbon source (Fig. 2A, left). The impaired but visible growth of the Δ*gbdR2* strain led us to examine the growth kinetics of these strains on choline and its downstream metabolites, GB and DMG. Deletion of *gbdR2* results in slower growth than that of the wild type on choline, GB, and DMG, comprised of both an extended lag phase and a lower growth rate during exponential phase (Fig. 2B). Deletion of these transcription factors did not substantially alter growth kinetics using pyruvate as a sole carbon source but did alter maximal yield in liquid culture in the double deletion strain (Fig. 2B), although this difference was not apparent on solid media (Fig. 2A, left). The Δ*gbdR1* and Δ*gbdR1* Δ*gbdR2* strains showed no measurable growth on any of the choline-related metabolites tested within 100 h.

Integration of *gbdR1* at the *att*Tn*7* site restored growth of a Δ*gbdR1* strain on choline, GB, and DMG (Fig. 3A). Growth in GB and DMG was very similar to that of the wild type, while growth of the complemented strain on choline exceeded that of the wild type on choline (Fig. 3A). Similarly, integration of *gbdR2* at the *att*Tn*7* site restored growth of a Δ*gbdR2* strain on choline-related carbon sources and resulted in more rapid growth than that in the wild type for choline, GB, and DMG (Fig. 3B). We also tested complementation of the Δ*gbdR1* Δ*gbdR2* strain with either *gbdR1* or *gbdR2*. The Δ*gbdR1* Δ*gbdR2* *att*Tn*7*::*gbdR1* strain was able to grow on choline as a sole carbon source in MOPS minimal medium, although not at wild-type levels, whereas the Δ*gbdR1* Δ*gbdR2* *att*Tn*7*::*gbdR2* strain was not capable of growth on choline (see Fig. S1 in the supplemental material). Taken together, these data suggest that *gbdR1* and *gbdR2* regulate the choline catabolic pathway, with *gbdR1* absolutely required and *gbdR2* playing an accessory role.

GbdR1 and GbdR2 regulate transcription of genes involved in the choline catabolic pathway. To identify the GbdR1 and GbdR2 regulons, we exposed our deletion strains to minimal medium with pyruvate as a primary carbon source and with or with-

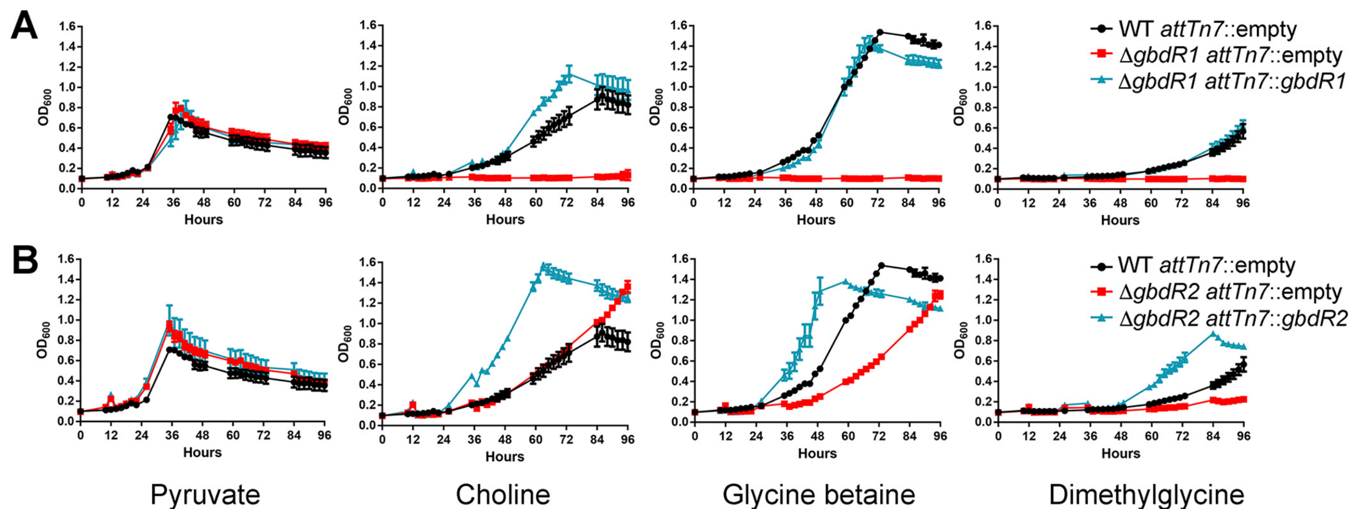

FIG 3 Growth phenotypes of Δ*gbdR1* and Δ*gbdR2* strains can be complemented. (A) Complementation of Δ*gbdR1* strain at *att*Tn*7* with *gbdR1* and its putative promoter results in restoration of ability to grow on choline and its metabolites. (B) Complementation of Δ*gbdR2* strain at *att*Tn*7* with *gbdR2* results in an increased growth rate on choline and its metabolites, surpassing the growth rate of the wild type. Growth curves are representative of at least three experiments per condition at each time point, each with biological triplicates, and the error bars represent standard deviations.

out 1 mM choline and analyzed the transcriptomes by RNA-Seq. Exposure of wild-type *B. thailandensis* to choline resulted in 57 transcripts being induced or repressed more than 2.3-fold, with many predicted to be involved in quaternary amine catabolism and transport (Table 1). A prominent cluster of induced genes from *BTH_II1868* to *BTH_II1853* includes orthologs to *P. aeruginosa glyA* (*BTH_II1868*), the *dgc* operon (*BTH_II1867*, *BTH_II1866*, *BTH_II1865*, *BTH_II1864*, *BTH_II1863*, and *BTH_II1862*), *gbcA* (*BTH_II1861*), *gbcB* (*BTH_II1860*), and *betX* (*BTH_II1859*). Based on these predictions, the putative operon consisting of *glyA* to *BTH_II1858* (Fig. 1C) carry the genes likely to be responsible for the breakdown of GB to sarcosine. Also induced was a nearby predicted operon consisting of *BTH_II1855-BTH_II1853*, carrying likely orthologs of *P. aeruginosa betC*, *cosX*, and a predicted porin. Another GATR, *BTH_II0994*, and the divergently transcribed operon consisting of *BTH_II0995* (*sdaA-2*), *BTH_II0996* (*soxB*), *BTH_II0997* (*soxD*), *BTH_II0998* (*soxA*), *BTH_II0999* (*soxG*), and *BTH_II1000*, were also induced, and we hypothesized that these are the *B. thailandensis souR* and sarcosine oxidase genes responsible for the breakdown of sarcosine to glycine (35).

Homologs of *cbcXWV* in *P. aeruginosa*, an operon important for the transport of choline, are present in *B. thailandensis* and are also induced by choline. The *gbdR2* gene is situated in this operon between *BTH_II0967* (*cbcW*) and *BTH_II0969* (*cbcX*). The aforementioned genes are induced by choline in the wild-type strain but are not in either the Δ*gbdR1* or Δ*gbdR1* Δ*gbdR2* strain, suggesting an absolute requirement of *gbdR1* in the regulation of these genes. Cells lacking *gbdR2* express all of the genes in the major catabolic operon, *glyA-BTH_II1858*, in response to choline but to a lower level than the wild type. Interestingly, the relative induction of the transcripts in the transport operon containing *gbdR2* is unaffected by the absence of *gbdR2*, suggesting that there is no autoregulation of the *gbdR2* operon.

To confirm our RNA-Seq results, we performed qRT-PCR on a subset of induced genes and the transcription factors of interest, normalized to *rplU* transcript levels (see Fig. S2 in the supplemental material). The wild-type *B. thailandensis* response to choline in our qRT-PCR experiment reflected our RNA-Seq results. *gbcA*, *cbcV*, and *soxB* are all induced in response to choline, suggesting overall induction of their putative operons. The qRT-PCR data also show that transcripts of the GATRs *gbdR1* and *souR* were slightly induced in response to choline, while *gbdR2* was induced at a higher level. This slight increase in *gbdR1* expression observed may be negligible; therefore, it is unclear if induction of *gbdR1* is a mechanism by which GbdR2 regulates choline catabolism. Overall, we interpret the matching trends in expression between the RNA-Seq and qRT-PCR experiments as a validation of our findings.

The *glyA* promoter is induced by choline under the control of GbdR1 and GbdR2. pAN27 is a reporter plasmid containing the full 408-bp intergenic region between *gbdR1* and *glyA*, such that the *glyA* promoter drives *lacZYA* expression. This reporter was used to assess the transcriptional control of the *glyA* promoter. This putative promoter likely controls the expression of *BTH_II1868* to *BTH_II1857* and thus governs the bulk of genes needed to convert GB to sarcosine. In a *B. thailandensis* wild-type background, pAN27 *lacZ* expression is induced by choline, GB, and DMG but not by ethylcholine (Fig. 4A). Ethylcholine is a nonmetabolizable inducer of GbdR-dependent transcription in *P. aeruginosa* (22). The inability of this choline analog to elicit similar effects in a GbdR/GbdR2-dependent promoter suggests specificity differences between GbdR and GbdR1 or GbdR2, a metabolite transport difference, or a difference in the specificity of choline oxidase. No β-galactosidase activity was detected when pAN27 in *B. thailandensis* was induced using 1 mM sarcosine, similar to findings in *P. aeruginosa* for *gbdR*-dependent induction of *choE*, a choline esterase (22), or for the phospholipase *plcH* (13, 45, and data not shown).

Δ*gbdR1*, Δ*gbdR2*, and Δ*gbdR1* Δ*gbdR2* deletion strains carrying pAN27 failed to show significant choline-induced β-galactosidase induction compared to the wild-type control (Fig. 4B), and basal expression was also low. This suggests that both *gbdR1* and *gbdR2* are required for robust induction of the *glyA* promoter. We

TABLE 1 Transcript changes in *B. thailandensis* in response to choline

Locus ID	Gene[b]	Linear fold change in transcript over pyruvate alone[a]			
		WT	Δ*gbdR1*	Δ*gbdR2*	Δ*gbdR1* Δ*gbdR2*
BTH_I0192		−2.6	NC	NC	NC
BTH_I0393		−4.8	NC	NC	NC
BTH_I0394		−5.1	NC	NC	NC
BTH_I0395		−8.3	NC	NC	NC
BTH_I0396		−4.3	NC	NC	NC
BTH_I0398		−3.9	NC	NC	NC
BTH_I0687		23.8	NC	10.5	NC
BTH_I0688		19.3	NC	NC	NC
BTH_I0698		11.3	NC	NC	NC
BTH_I0700		12.0	NC	NC	NC
BTH_I0866		5.8	5.3	NC	NC
BTH_I0959		−2.6	NC	−5.3	−5.0
BTH_I1406		−2.3	NC	−4.4	NC
BTH_I1620		5.7	NC	4.4	4.4
BTH_I1621		61.2	NC	18.3	NC
BTH_I1622		52.7	NC	9.7	NC
BTH_I1623		40.0	NC	9.3	NC
BTH_I1624		43.7	NC	5.0	NC
BTH_I1625		5.5	NC	NC	NC
BTH_I3016		−2.8	NC	−3.6	−3.4
BTH_I3017		−3.1	NC	−4.0	−3.9
BTH_II0001		5.4	NC	9.0	6.5
BTH_II0643		2.4	NC	NC	NC
BTH_II0694		−3.4	NC	−2.4	NC
BTH_II0695		−2.6	NC	NC	NC
BTH_II0964		2.9	NC	NC	NC
BTH_II0966	*cbcV*	45.4	NC	NC	NC
BTH_II0967	*cbcW*	94.0	NC	NC	NC
BTH_II0968	*gbdR2*	81.7	NC	NC	NC
BTH_II0969	*cbcX*	136.6	NC	NC	NC
BTH_II0970		37.7	NC	NC	NC
BTH_II0971		8.8	NC	NC	NC
BTH_II0994	*souR*	3.9	NC	4.7	NC
BTH_II0995	*sdaA-2*	90.8	NC	32.3	NC
BTH_II0996	*soxB*	130.6	NC	39.3	NC
BTH_II0997	*soxD*	172.0	NC	102.3	NC
BTH_II0998	*soxA*	122.7	NC	58.6	NC
BTH_II0999	*soxG*	24.9	NC	11.7	NC
BTH_II1000		7.6	−3.2	8.7	−3.5
BTH_II1546		4.3	NC	3.7	NC
BTH_II1853		5.4	NC	NC	NC
BTH_II1854	*cosX*	7.1	NC	NC	NC
BTH_II1855	*betC*	3.8	NC	NC	NC
BTH_II1856		11.5	NC	NC	NC
BTH_II1857	*purU2*	8.4	NC	NC	NC
BTH_II1858		6.5	NC	NC	NC
BTH_II1859	*betX*	175.6	NC	38.6	NC
BTH_II1860	*gbcB*	115.8	NC	28.9	NC
BTH_II1861	*gbcA*	110.8	NC	23.7	NC
BTH_II1862		218.7	NC	44.4	NC
BTH_II1863		163.4	NC	97.2	NC
BTH_II1864	*dgcB*	154.1	NC	28.9	NC
BTH_II1865	*dgcA*	199.7	NC	23.4	NC
BTH_II1866		223.9	NC	29.8	NC
BTH_II1867		135.3	NC	47.4	NC
BTH_II1868	*glyA*	162.9	NC	20.7	NC
BTH_II1869	*gbdR1*	2.6	NC	NC	NC

[a] Constraints on inclusion in the table were a ±2.3-fold change and P value of ≤0.005 based on the wild type. NC, no change due to significance, lower fold change, or experimental deletion of the gene.
[b] Gene names are putative based on homology to *P. aeruginosa*.

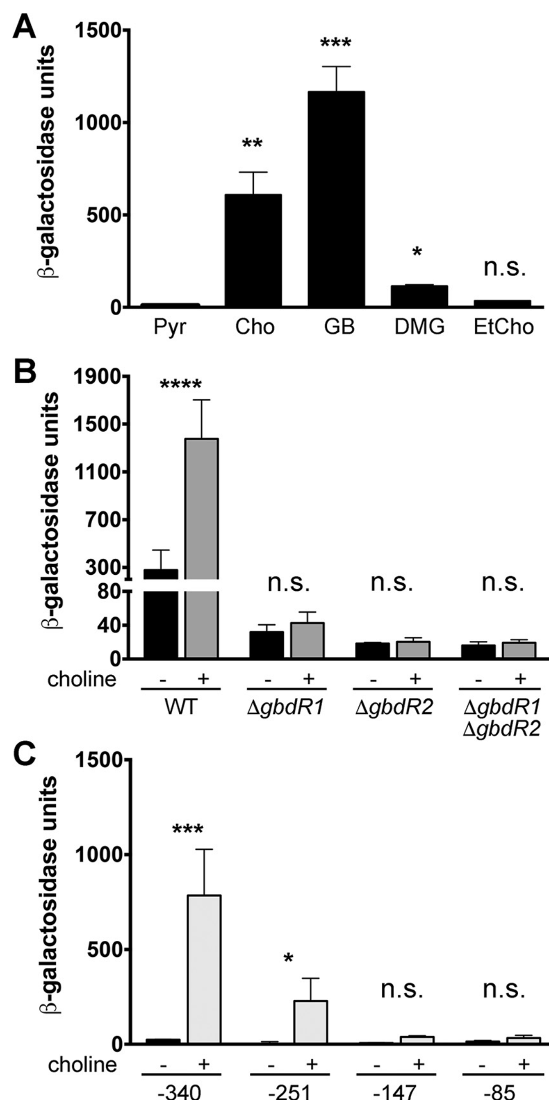

FIG 4 *glyA* promoter is induced by choline and its metabolites under the combined control of *gbdR1* and *gbdR2*. (A) β-Galactosidase activity from pAN27 is induced in the presence of choline and its metabolites but not pyruvate. (B) *gbdR1* and *gbdR2* are both required for significant β-galactosidase induction in the presence of choline. (C) Promoter truncations of the *glyA* promoter reveal that the critical region is located between −147 and −340 with respect to the predicted translational start site of *glyA*. In each case, data presented represents the standard errors of the means determined from three independent experiments, each with three technical replicates. Statistical notations in panel A are based on one-way analysis of variance (ANOVA) with Dunnett's posttest using the pyruvate group as the comparator, while panels B and C were analyzed by two-way ANOVA with Sidak's posttest comparing conditions of with versus without choline. Abbreviations and symbols: Pyr, pyruvate; Cho, choline; GB, glycine betaine; DMG, dimethylglycine; EtCho, ethylcholine; n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

would have predicted that the Δ*gbdR2* strain would produce some β-galactosidase activity in response to choline, as the Δ*gbdR2* strain can use choline as a sole carbon source (Fig. 2). The incongruity may be due to the unusually long time it takes to observe β-galactosidase activity in *B. thailandensis* combined with the long delay in growth of the Δ*gbdR2* strain on choline (Fig. 2). As a follow-up experiment, we transformed pAN27 into a Δ*souR* strain

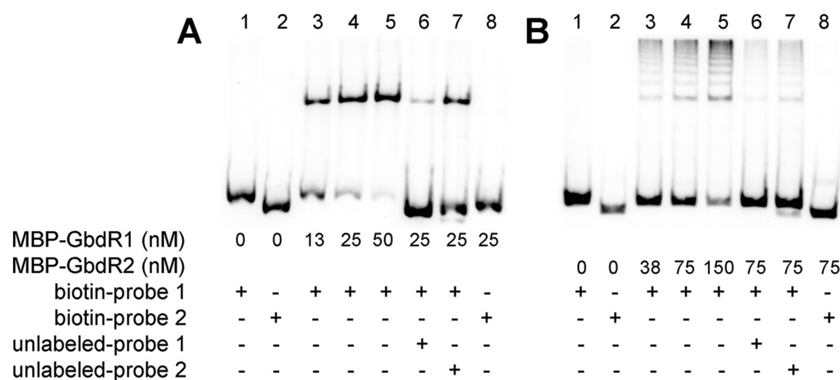

FIG 5 MBP-GbdR1 and MBP-GbdR2 bind to the regulatory region of the major GB catabolic operon (*BTH_II1868-BTH_II1858*). (A) MBP-GbdR1 binds to biotin probe 1 in a dose-dependent manner (lanes 3 to 5). The interaction is specific, as it does not bind to biotin probe 2 (lane 8) and can be competed off biotin probe 1 with unlabeled probe 1 (compare lane 6 to lane 4, 17.2% shift versus 75.2% shift, respectively) but less so with unlabeled probe 2 (compare lane 7 to lane 4, 51.5% shift versus 75.2% shift, respectively). (B) MBP-GbdR2 also binds specifically to probe 1 in a dose-dependent manner (lanes 3 to 5) while failing to bind to biotin probe 2 (lane 8). The binding between MBP-GbdR2 and biotin probe 1 is also specific, as it can be competed off by unlabeled probe 1 (compare lane 6 to lane 4, 4.3% shift versus 16%, respectively) but less so by unlabeled probe 2 (compare lane 7 to lane 4, 7.7% shift versus 16%, respectively).

to determine if SouR is required for *glyA* promoter induction in *B. thailandensis* (see Fig. S3 in the supplemental material). β-Galactosidase activity in the Δ*souR* strain in response to choline is robust, higher than that in the wild type, potentially due to blockage of the catabolic pathway and subsequent buildup of intermediate metabolites. This indicates that SouR is not required for induction of *glyA* but also could point to a positive feedback control between sarcosine metabolism and regulation of upstream steps.

Reporter plasmids derived from pAN27 with progressively smaller portions of the intergenic region between *gbdR1* and *glyA* were produced (−340, −251, −147, and −85 with respect to the *glyA* putative translational start site) in order to map the choline-dependent portion of the *glyA* regulatory region. Exposure of cells containing these constructs to choline resulted in choline-dependent induction of β-galactosidase activity for −340 and −251 but not −147 or −85 (Fig. 4C). This suggests that the region from bp −251 to the translation start site of *glyA* is necessary for a response to choline.

GbdR1 and GbdR2 bind directly to the *glyA* promoter. N-terminally tagged MBP-GbdR1 and MBP-GbdR2 were produced and assayed by EMSA using biotinylated DNA probes representing sections of the intergenic region between *gbdR1* and *glyA*. Probe 1 is 216 bp, covering the bp −320 to −104 region with respect to the *glyA* translational start site, while probe 2 is 178 bp, covering the −160 to +18 region. MBP-GbdR1 could bind probe 1 in a concentration-dependent manner, and binding to biotinylated probe 1 could be significantly competed off with unlabeled probe 1 (Fig. 5A). MBP-GbdR1 did not interact with probe 2 (Fig. 5A, rightmost lane), and unlabeled probe 2 was markedly less able to compete with MBP-GbdR1 binding to labeled probe 1. These data support a GbdR1 binding site within probe 1. Similar results were obtained using MBP-GbdR2, which was also able to bind to probe 1, not probe 2, and could be similarly competed from labeled probe 1 using unlabeled probe 1 (Fig. 5B) and, again, much less so with the unlabeled probe 2. The shift produced by MBP-GbdR2 consists of multiple bands that are likely the result of oligomerization. Results using 6×His-N-terminally tagged versions of GbdR1 and GbdR2 produced the same results and banding patterns, although the solubility of the 6-His version was much lower than that of the MBP fusions (data not shown). These results provide evidence that both GbdR1 and GbdR2 can bind to the *glyA* promoter. When combined with results from our reporter assays (Fig. 4), we infer that the binding site for GbdR1 and GbdR2 is contained within the bp −251 to −160 region with respect to the *glyA* translational start site. As we have previously shown for *P. aeruginosa* GbdR and SouR, addition of their presumptive ligand did not impact binding to DNA (data not shown).

***B. thailandensis* SouR regulates sarcosine metabolism.** Sarcosine is a downstream metabolite of choline, and orthologs of *P. aeruginosa* sarcosine oxidase genes were identified in our RNA-Seq results (Table 1 and Fig. 1C). *P. aeruginosa* uses sarcosine as a sole carbon and nitrogen source, and this is regulated by the transcription factor SouR (35). While *B. thailandensis* can use choline as a sole carbon or sole carbon and nitrogen source, *B. thailandensis* is unable to grow on sarcosine as a sole carbon source or as a sole carbon and nitrogen source (see Fig. S4 in the supplemental material). We hypothesize that the ability of *B. thailandensis* to transport extracellular sarcosine is limited compared to that of *P. aeruginosa*, and the rate of import is insufficient to support growth as a sole carbon source. It is, however, able to utilize sarcosine as a sole nitrogen source when pyruvate is available as a primary carbon source, and it does so in an SouR-dependent manner (Fig. 6A). Deletion of *gbdR1* or *gbdR2* has no major effect on the ability of *B. thailandensis* to utilize sarcosine as a nitrogen source, but the Δ*souR* strain does show a reduced level of growth.

BTH_II0994 is a GATR divergently transcribed from the sarcosine oxidase operon, and we hypothesized that it is functionally orthologous to *souR* in *P. aeruginosa* (Fig. 1C), despite BTH_II0994 and SouR not sharing a high degree of similarity compared to the other GATR orthologous pairs (Fig. 1B). To test if *BTH_II0994* was a functional *souR*, we generated a *B. thailandensis* Δ*souR* strain and used it to determine if *souR* is required for growth on choline. The Δ*souR* strain fails to grow on choline, GB, or DMG as a sole carbon source (Fig. 6B). This is contrary to regulation in *P. aeruginosa*, where choline, GB, and DMG can still be used as sole carbon sources for growth in the absence of *souR*, although at a diminished growth rate (35). This result also suggests that GbdR1 and GbdR2 are insufficient to cause induction of the sarcosine oxidase genes by themselves or together, contrary to

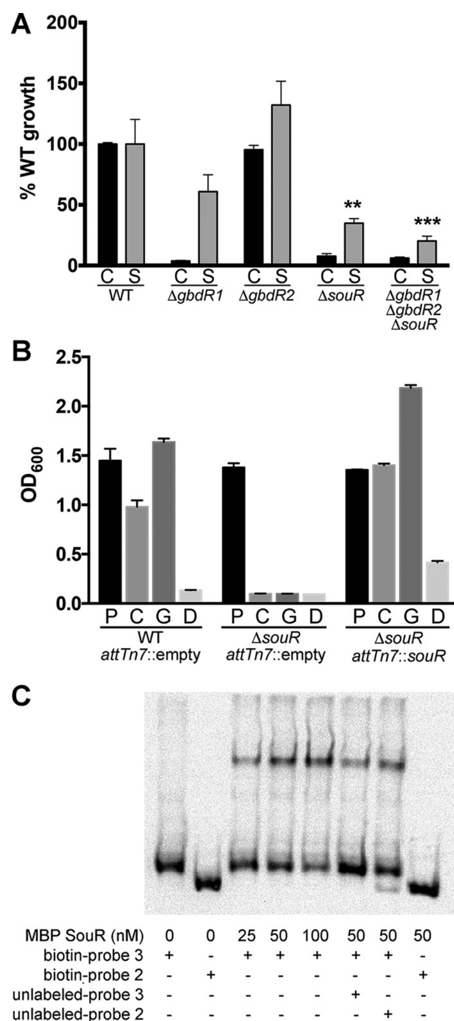

FIG 6 SouR is a critical regulator of the choline catabolic pathway. (A) *B. thailandensis* can utilize choline as a nitrogen source. Strains were grown on 20 mM pyruvate with either 5 mM choline (C) or sarcosine (S) for 72 h at 37°C. Δ*gbdR1*, Δ*souR*, and Δ*gbdR1* Δ*gbdR2* Δ*souR* strains were significantly diminished in their ability to utilize choline as a nitrogen source. The Δ*gbdR2* strain utilized both choline and sarcosine as a nitrogen source at levels similar to that of the wild type. The Δ*souR* strain used sarcosine as a nitrogen source at reduced rates compared to the wild type. OD_{600} measurements were normalized to basal growth without a nitrogen source and then normalized to the wild type and are represented as percent growth. (B) Deletion of *souR* prevents *B. thailandensis* from using choline as a carbon source. Complementation of *souR* at an *att*Tn*7* site with *souR*, under the control of its putative native promoter, restores the ability of *B. thailandensis* to utilize choline and its metabolites as carbon sources. Strains were grown with either 40 mM pyruvate (P), choline (C), GB (G), or DMG (D) as a sole carbon source in MOPS minimal medium for 72 h at 37°C. (C) MBP-SouR binds to the putative promoter of the sarcosine oxidase operon represented by biotin probe 3 in a dose-dependent manner (lanes 3 to 5). The interaction is specific, as MBP-SouR does not bind to biotin probe 2 (lane 8) and can be competed off by unlabeled probe 3 (compare lane 6 to lane 4, 22% shift versus 37.1%, respectively) but not by unlabeled probe 2 (compare lane 7 to lane 4, 38.2% shift versus 37.1%, respectively). Data in panels A and B are averaged from three experiments each with three biological replicates, and error bars represent SEM. These data were analyzed by two-way ANOVA with Tukey's posttest testing for strain effect for each carbon source separately, not comparing the carbon sources. For panel A, except for the Δ*gbdR2* strain, all choline changes are different from the WT and are not noted. Only the sarcosine comparisons different from the WT are noted. For panel B, pyruvates were not significantly different but all other comparisons were, except for WT versus the mutant in dimethylglycine. **, $P < 0.01$; ***, $P < 0.001$.

the regulation scheme in *P. aeruginosa* (Fig. 1A). This phenotype can be complemented by chromosomal integration of *souR* at an *att*Tn*7* site under the control of its native promoter (Fig. 6B).

MBP-SouR was produced and its DNA binding assessed using EMSA with a biotinylated 230-bp oligonucleotide designated probe 3, covering the region of bp −208 to +22 relative to the putative translation start site of *sdaA-2*, the first gene in the likely sarcosine oxidase operon of *B. thailandensis*. MBP-SouR was able to bind the biotinylated probe 3 but not the probe 2 negative control. The binding of MBP-SouR to biotinylated probe 3 could be competed off with unlabeled probe 3 but not unlabeled probe 2, suggesting that this interaction is specific (Fig. 6C). The binding of MBP-SouR to this putative promoter region coupled to the phenotype of the Δ*souR* strain suggests that SouR is responsible for the regulation of the sarcosine oxidase genes in *B. thailandensis*.

DISCUSSION

The ability to utilize GB as a compatible solute, providing protection against osmotic and other stresses without creating significant disruption of normal cell processes, is nearly ubiquitous among bacteria; however, the ability to catabolize GB is not as widespread (46). *B. thailandensis* and other species in this genus possess the enzymes for utilizing choline and its metabolites as carbon and nitrogen sources, taking advantage of this widely available biomolecule as befits their description as metabolically adaptable bacteria. *P. aeruginosa*, another soil-dwelling and metabolically adaptable microorganism, shares this ability and was our original model for the study of the GB catabolic pathway and its regulation (20). *B. thailandensis* and *P. aeruginosa* inhabit similar environments, possess similarly sized genomes, and encode diverse metabolic pathways; thus, they are considered to have similar generalist strategies, despite *B. thailandensis* and *P. aeruginosa* belonging to the betaproteobacteria and gammaproteobacteria classes, respectively. The metabolic enzymes in the choline catabolic pathway are well conserved, and the orthologous genes in different species are readily identifiable. However, the identification of *gbdR2* and the alternative gene organization in *B. thailandensis* prompted us to ask if the choline regulatory network was as well conserved. We show here that *B. thailandensis* uses an alternative regulatory solution to control this pathway compared to that of *P. aeruginosa*. Both *B. thailandensis* and *P. aeruginosa* can store pools of GB (17) and metabolize it as a carbon and nitrogen source; therefore, the different regulatory networks suggest evolution of alternative strategies to control the decision to store or catabolize.

Although the data presented here indicate that choline catabolism is regulated differently in *B. thailandensis* than in *P. aeruginosa* (Fig. 1A), the basic components of the pathway are conserved; thus, the overall scheme of the pathway is the same. In both organisms, choline catabolism begins with choline-dependent derepression of the catabolic genes responsible for the conversion of choline to GB, *betBA*, mediated by the choline-sensing TetR family transcription factor BetI. These genes have been described previously (39), and evidence suggests they function in *B. thailandensis* as they do in *P. aeruginosa* (38). In *P. aeruginosa*, GB is subsequently metabolized to DMG by GbcA and GbcB heterodimer (47, 48) and from DMG to sarcosine by enzymes in the *dgc* operon (49), all under the regulation of GbdR (20). In *B. thailandensis* the same enzymatic steps are under the control of

GbdR1, modulated by GbdR2. Sarcosine is then demethylated to glycine by components of the sarcosine oxidase operon and *glyA1*, controlled in tandem by GbdR and SouR in *P. aeruginosa* and controlled separately by SouR as well as GbdR1 and GbdR2, respectively, in *B. thailandensis* as described below.

In this study, we determined that both GbdR1 and GbdR2 participate in the regulation of GB catabolism and characterized their regulons. Both regulators are GATRs with strong amino acid sequence similarity to GbdR in *P. aeruginosa*, and deletion strains confirmed that both play roles in the regulation of choline catabolism and catabolism of its downstream metabolites (Fig. 2). Combining the transcriptional data with the growth phenotypes of the deletion strains supports *gbdR1* being required for expression of the major catabolic cluster of genes and the transport operon that includes *gbdR2*. *gbdR2*, on the other hand, is important for a robust response to choline, enhancing expression of many genes required to import and catabolize choline and its metabolites, particularly the major catabolic cluster (Fig. 1C and Table 1). The operon containing *gbdR2* and the putative *cbcWVX* orthologs is likely regulated solely by GbdR1. Although the presence of *gbdR1* is sufficient for growth (Fig. 2), reporter assays and growth studies showed that both *gbdR1* and *gbdR2* are required for a robust transcriptional response (Fig. 4A) and growth on choline (Fig. 2). In agreement with promoter-mapping results (Fig. 4C), EMSA data suggest that GbdR1 and GbdR2 share a similar binding site, raising the question of whether or not they compete for the same binding site or act synergistically. A possibility is that GbdR1 and GbdR2 possess differential DNA binding affinities that result in differential responses to choline and its metabolites under different physiological conditions.

In addition to *gbdR1* and *gbdR2*, our transcriptome analysis revealed a third GATR induced in response to choline, *BTH_II0994*, which we confirmed as a functional *souR* ortholog in *B. thailandensis*. In *P. aeruginosa*, SouR regulates the expression of the sarcosine oxidase catabolic operon, the components of which are responsible for converting sarcosine to glycine (35). SouR in *B. thailandensis* is less similar to GbdR, GbdR1, and GbdR2 than is SouR in *P. aeruginosa* (Fig. 1B). *B. thailandensis souR* (*BTH_II0994*) is divergently transcribed from the sarcosine oxidase operon, whereas *P. aeruginosa souR* (*PA4184*) is distantly located and part of a two-gene operon. These factors suggest a divergence in how *souR* was acquired or evolved in both organisms. The inability of *B. thailandensis* to grow on sarcosine as a sole carbon source was surprising given that choline is readily catabolized and the enzymes for sarcosine catabolism are expressed (Fig. 6A). We found that *souR* in *B. thailandensis* is required for sarcosine catabolism, unlike in *P. aeruginosa*, where GbdR can induce the sarcosine oxidase operon in the absence of *souR* (35). The complementation analysis and EMSA for the sarcosine oxidase operon putative promoter provides further evidence that *BTH_II0994* is indeed the functional *souR* ortholog in *Burkholderia* (Fig. 6B and C). It is not clear if SouR is capable of autoregulation of *souR* or if GbdR1 controls its expression directly.

GbdR1, GbdR2, and SouR are all GATRs implicated in the regulation of genes involved in the catabolism of quaternary amines or their metabolites. GATRs are members of the AraC transcription factor family, grouped by their canonical C-terminal DNA binding domain, but unlike classic AraC proteins they contain a GATase 1-like N-terminal domain. The GATase 1-like domain is characterized by its homology to class I glutamine amidotransferases, which bind glutamine or ammonia and participate in the amidation/deamidation reaction (50, 51). Many of the GATR N termini, notably excluding SouR in *P. aeruginosa*, also retain a bioinformatically identified cysteine residue that would be part of the functional catalytic triad of GATase 1 family enzymes. The N-terminal domain of AraC proteins typically is involved in ligand binding and dimerization, which is a characteristic important for their functionality, and dimerization is often affected by the binding of ligand (52). *P. aeruginosa* possesses other GATRs implicated in the catabolism of N-substituted amines, including *argR*, the regulator of arginine catabolism (53), and *cdhR*, the regulator of carnitine catabolism (20). Putative *argR* and *cdhR* orthologs are also present in *B. thailandensis* (unpublished data). We hypothesize that the GATase 1-like domain has been combined with the AraC-style DNA binding domain to enable detection of charged amine-containing compounds and that GATRs as a whole may represent a subfamily of transcription factors that regulate metabolism of accessory nitrogen sources, including choline and its metabolites.

The transport of choline and derivative molecules also appears to be handled differentially between *B. thailandensis* and *P. aeruginosa* based on genomic information. *P. aeruginosa* possesses an array of BCCT and ABC family transporters that have been implicated in choline and GB transport that are effective under different osmotic conditions or primarily utilized when choline is in such abundance that it can be used as a carbon or nitrogen source (42). *B. thailandensis* possesses fewer transporters, with only one putative homolog of the BCCT family transporter (*BTH_II1109*), compared to three in *P. aeruginosa* (*betT1*, *betT2*, and *betT3*). *B. thailandensis* possesses a putative amino acid permease, *BTH_II1858*, as part of the major GB catabolic operon with no obvious ortholog in *P. aeruginosa*. The placement of *BTH_II1858* and conserved synteny among *Burkholderia* species suggests a role as a transporter for choline or its derivatives, but there is no functional evidence as of yet. Some pseudomonads have a putative ortholog of *BTH_II1858* identified as an ethanolamine transporter, ethanolamine being similar to choline in its role as a head group for phospholipids, suggesting the possibility of a link to general fatty acid metabolism. Both *B. thailandensis* and *P. aeruginosa* also possess orthologs of the choline/GB transporter, *opuC*, that was described in *P. syringae* and found to function under hyperosmolar conditions (54). *B. thailandensis* orthologs to many of the *P. aeruginosa* periplasmic binding proteins mediate ABC transporter-dependent import of quaternary amines (*cbcX* [*BTH_II0969*], *caiX* [*BTH_II1849*], *betX* [*BTH_II1859*], and *cosX* [*BTH_II1853*]), but apparently these are not sufficient for efficient sarcosine transport (see Fig. S4 in the supplemental material). This result is perhaps not surprising, as limited current data suggest that sarcosine does not compete well with choline for these transporters in *P. aeruginosa* (55). To date, the sarcosine transporter has not been identified, and the functional differences between *P. aeruginosa* and *B. thailandensis* for sarcosine utilization in the background of a similar metabolic pathway may provide a platform for identification of this transporter.

In this study, we have identified an alternative model for the regulation of choline catabolism that incorporates multiple GATRs and examined their respective regulons. The conservation of *gbdR1* and *gbdR2* throughout the *Burkholderia* genus suggests that differences in regulation of choline catabolism outlined here represent a model that can be extrapolated to the more pathogenic

strains, as well as those strains associated with the rhizosphere. Contrasting the models of choline catabolism in *B. thailandensis* and *P. aeruginosa* will serve as a useful tool to probe the remaining unanswered questions concerning the pathway.

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