

Discovery of a Choline-Responsive Transcriptional Regulator in *Burkholderia xenovorans*

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Abstract

The search for effectors of novel transcriptional regulators is a challenging task. Here, we present the prediction and validation of an effector for a novel transcriptional regulator (TR). The clustering of genes around the gene coding for Bxe_A0425, a TR in *Burkholderia xenovorans* LB400 and its closest orthologs, suggests the conservation of a functional operon composed a several open reading frames from which a TR, a transporter, and two oxidoreductases can be easily identified. A search of operons containing these functional components revealed a remarkable resemblance of this system to the evolutionarily convergent and functionally conserved operons found in *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus xylosus* and *Pseudomonas aeruginosa*. These operons are involved in the uptake and catabolism of choline to create the potent osmo-protectant molecule glycine betaine. We used frontal affinity chromatography coupled to mass spectrometry to screen for the binding of choline and other intermediates of the glycine biosynthesis pathway to the TR Bxe_0425. We then used electrophoretic mobility shift assays to confirm our results. We found that choline was the sole metabolite binding to this TR and identified choline as an effector molecule for Bxe_A0425. These findings suggest that this operon in *B. xenovorans* is involved in the uptake and catabolism of choline to protect the organism from osmotic stress.

Keywords: FAC-MS, uptake and catabolism of choline, EMSA, osmo-protectant, *Burkholderia xenovorans*, osmoregulator, Bxe_A0425, transcriptional regulator

1. Introduction

As a group, *Burkholderiales* are well-adapted organisms capable of surviving in environments ranging from animal hosts to contaminated soils that subject them to wide array of stresses. Among the stresses that they are adapted to overcome is osmotic stress, which they can encounter in animal cells and fluids as well as soils. One of the most effective mechanisms that bacteria have developed to overcome osmotic stress is the accumulation of glycine betaine, an osmo-protectant, either through choline uptake or *de novo* synthesis from choline (Csonka & Hanson, 1991). Many organisms contain evolutionarily convergent operons that are involved in the uptake and conversion of choline to glycine betaine (Figure 1). These operons maintain remarkable functional similarity although in many cases they exhibit very little sequence homology. For example, in *Escherichia coli*, the operon encodes for a choline transporter (BetT), two oxidoreductases, a NADH-dependent glycine betaine aldehyde dehydrogenase (BetB), and a FADH-dependent choline dehydrogenase (BetA) (Lamark et al., 1991). The *Bacillus subtilis* operon exhibits the choline dehydrogenating function but the enzyme catalyzing the choline oxidation is different; the choline dehydrogenase (BetA) is replaced by a cholineoxidase (GbsB) (Boch, Kempf, Schmid, & Bremer, 1996) that belongs to a family of alcohol dehydrogenases commonly found in higher organisms.

We sought to identify the effector metabolite for a TR in *Burkholderia xenovorans*. This TR, Bxe_A0425, and the operon in which it resides have remained largely uncharacterized. We present data showing that Bxe_A0425 binds choline and that the operon in which Bxe_A0425 resides is of suitable composition to be involved in choline uptake and catabolism.

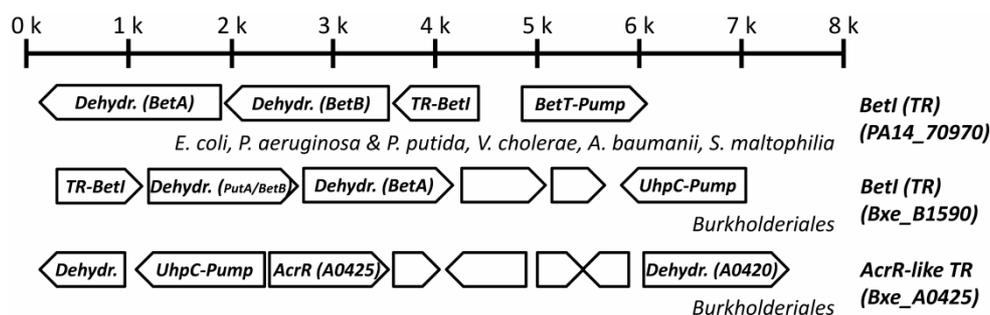


Figure 1. Genomic clustering for the known operon for choline uptake and catabolism in various organisms (row one) and in the *Burkholderiales* order (row two)

Row three presents the suggested secondary operon for choline uptake and catabolism in *Burkholderiales*. The organisms in row one are *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Vibrio cholerae*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*.

2. Methods

2.1 Gene Cluster Analysis

Physical gene clustering around the gene coding for Bxe_A0425 in *B. xenovorans* and its closest analogs was analyzed by utilizing an online integrated portal for comparative and functional genomics [Microbes Online (Dehal et al., 2010)]. We utilized a homolog approach similar to that used by Martí-Arbona et al. (2003), Hall et al. (2013) and Nguyen et al. (2013). Literature searches were performed using Google Scholar.

2.2 Cloning, Expression and Purification of the GST-Bxe_A0425 Protein

2.2.1 Cloning

The gene Bxe_A0425 was cloned from *B. xenovorans* as described in Nguyen and co-workers for Bxe_B2842 (Nguyen et al. 2013) with the following primer modifications: forward 5'-GATCCATGGATGTCCGACAGTGAAACCCC-3' and reverse 5'-GATCGGATCCTCAACCGTCCGAAGGCGCCG-3'.

2.2.2 Protein Expression and Purification

The affinity chromatography-based purification of the GST-Bxe_A0425 was performed as described by Nguyen and co-workers for GST-Bxe_B2842 (Nguyen et al. 2013).

2.3 Frontal Affinity Chromatography-Mass Spectrometry (FAC-MS)

2.3.1 Effector Library Design

The functional assignments of the genes clustering around the gene coding for the TR Bxe_A0425 suggest that this operon could be involved in the uptake and dehydrogenation of choline. Possible effectors were identified based on their occurrence in the metabolism of glycine, serine and threonine in the KEGG database (<http://www.genome.jp/kegg/>). Choline and several other compounds in the metabolism of glycine, serine and threonine were used to investigate the effector-binding specificity of Bxe_A0425. Ten μM solutions of commercially available compounds were prepared in buffer C and adjusted to pH 7.4. The compounds used in this screening library were glycerate-3-phosphate, AMP, phosphocholine, creatine, 5-aminolevulinate, hypotaurine, taurine, sarcosine, dihydroxy fumarate, L-threonine, L-serine, glycine, 2,3-diphospho-D-glycerate and choline.

2.3.2 FAC-MS Experiments

The FAC-MS approach to TR discovery used here was previously described by our group (Hall et al., 2013; Martí-Arbona et al., 2012; Nguyen et al., 2013). The preparation of the FAC-MS column was performed as described by Hall et al. (2013) for the protein GST-Bxe_A0736.

2.4 Screening for TR/DNA/Effector Interactions.

The binding of the effector molecule to the TR/DOS complex was monitored by electrophoretic mobility shift

assays (EMSA) as described by our group (Maity et al., 2012). dsDNA (1 μ M) was incubated with varying amounts of GST-Bxe_A0425 (0 to 20 μ M). Free and protein-bound DNAs were separated on a 1% TBE-agarose gel and then visualized by staining with an ethidium bromide solution. The DNA sequence of the DOS utilized in the EMSA experiments was 5'-GATGTTAGTTACTGATCGGTAACATG-3', which is located between 35 and 76 bp up-stream of the Bxe_A0425 gene.

3. Results and Discussion

3.1 Gene Cluster Analysis

The genes clustered around the gene coding for Bxe_A0425 in *B. xenovorans* and its closest orthologs suggest the conservation of a functional operon composed a several open reading frames (ORFs) from which a TR, a transporter/pump and two short-chain dehydrogenating enzymes can be easily identified. A literature search of functional operons containing these functional components revealed a remarkable resemblance of this system to the evolutionarily convergent and functionally conserved operons found in *E. coli* (Lamark et al., 1991), *B. subtilis* (Boch, Kempf, Schmid, & Bremer, 1996) and *S. xylosus* (Rosenstein, Futter-Bryniok, & Gotz, 1999). These reported functional operons have been linked to the uptake and dehydrogenation of choline to generate glycine betaine, a potent osmo-protectant molecule. Importantly, homology indicated that the genes named *betB*, *gbsA*, *putA*, *cutA* and possibly others all encode for BetB, a NAD-dependent dehydrogenase.

Choline uptake and catabolism operons are examples of convergent evolution, meaning that some organisms developed to utilize a set of bacterial NAD-dependent dehydrogenases while others developed to use FADH-dependent oxidases. Moreover, a comparative analysis of the genes in the Bet-operon in *Pseudomonales* (Figure 2) showed that divergence in gene homology is observed not only in the oxidoreductases in the operon but also in transporters. For example, *Pseudomonas aeruginosa* utilizes the BetT transporter; *Pseudomonas mendocina* uses a major facilitator superfamily transporter (MSF); *Pseudomonas fluorescens* uses an OpuD transporter (BCCT family transporter) and in *Pseudomonas syringae* the transporter is missing from the operon. *B. xenovorans* does not contain a BetT-like transporter; instead, it contains transporters in the UhpC family (MSF-type) of transporters (Bxe_A0426 and Bxe_B1595) like *P. mendocina*.

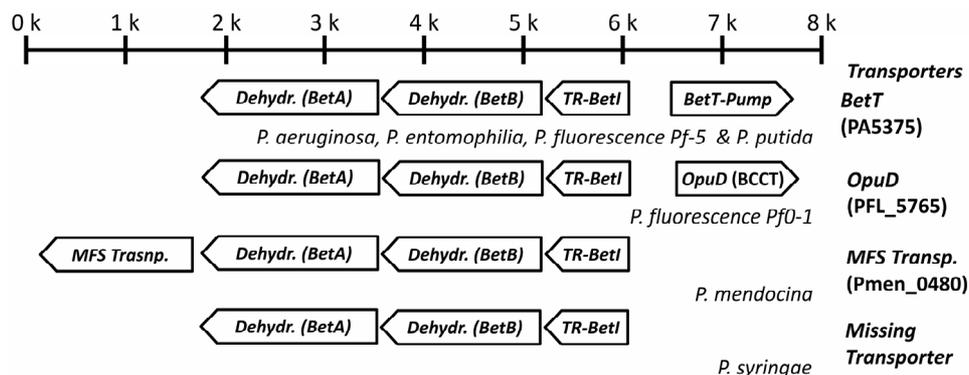


Figure 2. Genomic clustering for choline uptake and catabolism (Bet-operon) of choline in representative *Pseudomonales*

Each row represents a member(s) of the *Pseudomonales* with a variation in the transporter gene within the Bet-operon.

Figure 1 presents a comparison of the known operons encoding for the choline uptake systems in other bacteria with two operons in *B. xenovorans* of very similar composition, each apparently under the control of a single AcrR-like TR. The traditional Bet-operon is depicted in row two and shows the conservation of both dehydrogenating enzymes (BetA: Bxe_B1592 and PutA/BetB: Bxe_B1591) and introduces a new type of MSF-type transporter (UhpC family) similar to transporters observed in *P. mendocina*. The operon containing the TR Bxe_A0425 (Figure 1: row three) also contains a MFS transporter annotated as UhpC (Bxe_A0426), a putative oxidoreductase (Bxe_A0427) and a putative monooxygenase/dehydrogenase (Bxe_A0420). These observations suggest the possible involvement of this operon in the uptake and catabolism of choline.

3.2 Screening for the Effector Molecule Binding to the TR

Based on the implication of choline uptake and metabolism by the gene cluster analysis and the involvement of the general metabolic area of glycine, serine and threonine metabolism in glycine betaine generation, we generated a library of possible effectors from commercially available substrates and products from these pathways. This compound library contained glycerate-3-phosphate, AMP, phosphocholine, creatine, 5-aminolevulinate, hypotaurine, taurine, sarcosine, dihydroxyfumarate, L-threonine, L-serine, glycine, 2,3-diphospho-D-glycerate and choline. Analysis of this library by FAC-MS of the immobilized Bxe_A0425 TR showed a wide distribution of the elution time of the infused compounds. Compounds that quickly eluted from the column were cataloged as no-binders of Bxe_A0425 while those that were retained and eluted at a later time were be cataloged as binders. Large retention time implies high the affinity of Bxe_A0425 for the ligand. Each compound's ion intensity was normalized and an elution profile was created using Sigma Plot 12.3 (Figure 3). The affinity of Bxe_A0425 for these compounds was choline (black) >>> 2,3-diphospho-D-glycerate (dark grey) > glycine (yellow) > L-serine (green) > L-threonine (Brown) > dihydroxyfumarate (red) > sarcosine (dark red) > taurine (purple) > Hypotaurine (green) > 5-aminolevulinate (dark blue) > creatine (grey) > phosphocholine (orange) > AMP (cian) > glycerate-3-phosphate (blue). Compared with all the other compounds tested, the retention of choline was significant, suggesting that under the arbitrary conditions of our FAC-MS screen assay, cholinewas the best effector for Bxe_A0425.

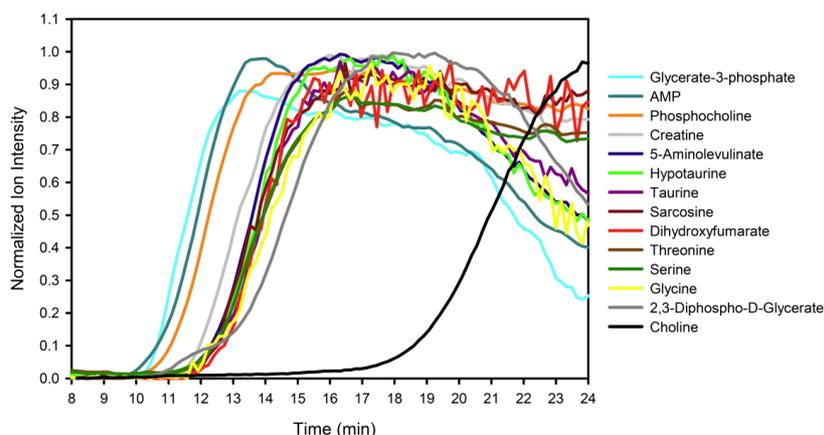


Figure 3. Screening for effectors of Bxe_A0425

The elution profiles of the library of potential effectors (10 μ M each) after flowing past immobilized GST-Bxe-A00425 are shown. The retention of choline (black line) in the column was much greater, suggesting that it is the best effector for Bxe_A0425.

3.3 Effector Binding to the TR/DOS Complex

Bxe_A0425 is a member of the TetR family of TRs. Maity et al. (2012) demonstrated that GST-Bxe_A0425 is capable of binding its DOS to create a TR/DOS complex in the absence of an effector molecule. It is therefore possible that it functions as a transcriptional repressor (Maity et al., 2012). We used EMSA experiments to test the ability of choline to disrupt the TR/DOS complex. Figure 4 shows a set EMSA gels. The gel in the left panel was run in the absence of the effector molecule (choline) and the gel in the right panel was run with 10 mM choline. The concentration of GST-Bxe_A0425 was varied from 0 (lane 1, the electrophoretic mobility of free DNA) to 20 μ M (lane 8, the electrophoretic mobility of the fully formed TR/DOS complex). Under the experimental conditions studied, an increase in the intensity of the free DNA band in lanes 3, 4 and 5 is observed when choline is added to the assay. These changes suggest that choline indeed affects the TR/DOS complex and causes the protein to release the DNA. This observation supports the results obtained from the FAC-MS experiments and indicates that Bxe_A0425 is involved in the regulation of this newly identified operon.

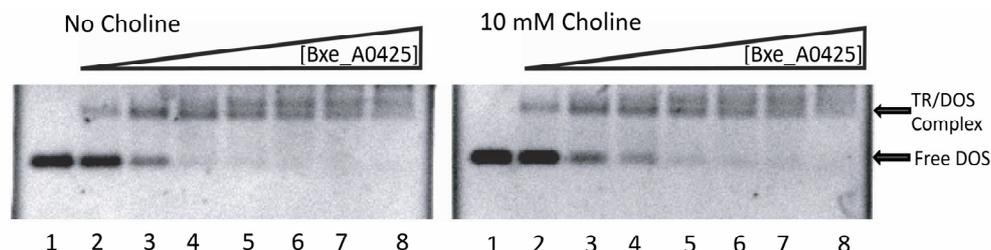


Figure 4. EMSA assays to test the ability of choline to disrupt the TR/DOS complex

Lane 1 shows the electrophoretic mobility of free DNA while lane 8 shows the electrophoretic mobility of the fully formed TR/DOS complex. Lanes 2 to 8 contain increasing concentrations of the GST-Bxe_A0425 TR, the concentration were 0.3, 0.6, 1.2, 2.5, 5.0, 10.0 and 20.0 μM , respectively. Lanes 3 to 5 show a change in the free DOS band intensity, suggesting that choline disrupts the TR/DOS binary complex.

3.4 Conclusions

We found Bxe_A0425 in a cluster of genes in *B. xenovorans* and other *Burkholderiales* containing close homologues of the TR and in an apparent operon. The general functional annotation of the genes in the clusters was consistent with the possibility that the operon and the TR are involved in choline uptake and metabolism to produce glycinebetaine. Bxe_A0425 bound choline when screened against a library of metabolites characteristic of this general metabolic area. Results from an EMSA assay were consistent with choline impacting the association of this TR with its DOS. Taken together, these findings suggest that TR Bxe_A0425 and the operon in which it resides are involved in choline uptake and conversion to glycine betaine.

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