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Regulation of expression of the tricarballylate utilization operon (*tcuABC*) of *Salmonella enterica*

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Abstract

The *t*ric*a*rballylate *ut*ilization locus (*tcuRABC*) of *Salmonella enterica* serovar Typhimurium is comprised of a 3-gene operon (*tcuABC*) that encodes functions that allow this bacterium to use tricarballylate as a source of carbon and energy, and the *tcuR* gene, which encodes a putative LysR-type transcriptional regulator. In our studies, transcription of the *tcuABC* operon peaked at mid-log phase, and declined moderately during stationary phase. This pattern was not due to a change in the amount of TcuR in the cell, as *tcuR* expression did not change under the conditions tested, and TcuR did not control *tcuR* expression. Tricarballylate was the co-inducer. *tcuABC* expression was negatively affected by the cAMP receptor protein (Crp). Expression of *tcuABC* was one order of magnitude higher in a *crp* mutant strain than in the crp^+ strain; derepression of *tcuABC* expression was also observed in a strain lacking adenylate cyclase (Cya). At present, it is unclear whether the effect of Crp is direct or indirect. Studies with molecular mimics of tricarballylate showed that the co-inducer site restricts binding of structural mimics that contain a hydroxyl group. Two classes of TcuR constitutive variants were isolated. Class I variants responded to tricarballylate, while class II did not.

Keywords

Carbon metabolism; Tricarballylate utilization; Gene expression; LysR-type regulators

1. Introduction

Tricarballylate is a citrate analog that is considered the causative agent of grass tetany, a ruminant disease characterized by an acute hypomagnesia [28]. Tricarballylate is not catabolized by the ruminant or the rumen flora, hence it is excreted by the animal as a magnesium chelate [33]. Unlike the normal rumen flora, *Salmonella enterica* serovar Typhimurium LT2 (hereafter referred to as *S. enterica*) can use tricarballylate as a carbon and energy source [18]. The genome of *S. enterica* contains a 3-gene operon (*tcuABC*) dedicated to tricarballylate utilization (Fig. 4, supplemental material). The *tcuABC* operon contains all of the functions required for the catabolism of this tricarboxylic acid [24]. The first gene of the operon (*tcuA*) encodes a protein with tricarballylate dehydrogenase activity, which converts tricarballyate to *cis*-aconitate, a Krebs cycle intermediate. TcuA is the only enzyme needed to catabolize tricarballylate; the *tcuB* and *tcuC* genes encode an electron transfer protein (required

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to re-oxidize the flavin cofactor of TcuA) and a tricarballylate transporter, respectively [23, 24].

Immediately upstream of the *tcuABC* operon is the *tcuR* gene, which encodes a putative LysR-type transcriptional regulator (LTTR) [24] (Fig. 4, supplemental material); *tcuR* is not part of the *tcuABC* operon. LTTRs are among the most ubiquitous forms of transcriptional regulators, and hundreds have been found in bacteria and some archaea [29]. LTTRs have a distinct domain architecture (Fig. 5, supplemental information), with a helix-turn-helix in their N termini [35], while the C-terminal domains contain the co-inducer binding site and the oligomerization domain [1,9,11–13,21,22,25,35].

Here we provide in vivo evidence that the TcuR protein uses tricarballylate as its co-inducer for transcription of the *tcuABC* operon, and that the catabolite repression protein (Crp) negatively regulates expression of the operon. We also report the isolation and initial characterization of mutant alleles of *tcuR* that encode TcuR variants that activate *tcuABC* expression in the absence of tricarballylate.

2. Materials and methods

2.1. Bacterial strains, culture media, and growth conditions

A list of strains and plasmids used and their genotypes is provided in Table 4 (supplemental material). All chemicals were purchased from Sigma unless otherwise stated. *Escherichia coli* cultures were maintained in lysogenic (LB) broth [7,8] (Difco). Nutrient broth (NB; Difco) was used as rich medium for *S. enterica*. Antibiotic concentrations were (in μg/ml): ampicillin (Ap), 100; chloramphenicol (Cm), 20; kanamycin (Km), 50; tetracycline (Tc), 20. No-Carbon E (NCE) [6] was used as minimal medium, and was supplemented with $MgSO_4$ (1 mM), methionine (0.5 mM), and trace minerals [3,15]. Whenever used as sole carbon and energy sources, citrate was at 20 mM, acetate at 30 mM, succinate at 30 mM, glucose at 10 mM, and *cis*-aconitate at 20 mM. When used in combination, succinate, acetate, and formate were all at 20 mM. All experiments were performed aerobically with shaking.

2.2. Genetic crosses

Transductions involving phage P22 HT105 *int-201* [31,32] were performed using described protocols [10,15].

2.3. Construction of a nonpolar *tcuR* deletion

An in-frame deletion in *tcuR* was performed using a modification of the method described by Datsenko and Wanner [14]. Briefly, the *cat* cassette of pKD3 was amplified using a 5′ primer containing 51-bp identical to the 5′ end of *tcuR* and a 3′ primer containing 51-bp identical to the 3′ end of *tcuR*. Manipulations were performed in strain JE6692 (Table 1). Insertion of the *cat* gene into *tcuR* was verified by DNA sequencing. The *tcuR50::cat*$^+$ insertion was transduced into JE7212. Removal of the *cat* gene was performed as described [14].

2.4. Chemical mutagenesis

S. enterica strain JE7212 (*tcuA*::MudJ(*lacZ*$^+$ *kan*$^+$) was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NG) as described by [26]. Cells were incubated with NG for 40 min, and excess NG was removed by washing the cells with sterile phosphate buffer (0.1 M, pH 7.0) twice by centrifugation at 8000 *g* using a Beckman/Coulter Avanti J-25I microcentrifuge. Mutagenized cells were allowed to recover at 37°C until the culture reached an OD_{650} of ~1.5 before being plated for single colonies on NCE medium supplemented with lactose (10 mM). Large colonies were re-streaked on selective medium and saved. Mutant strains were reconstructed using phage P22 grown on the original mutant strains as donor, and strain

TR6583 (*tcuR*$^+$ *tcuABC*$^+$) as recipient on NB agar plates containing kanamycin. Km^r transductants were replica-printed onto NCE medium supplemented with lactose. Lac$^+$ colonies were freed of phage as described above. The *tcuR* gene from each Lac$^+$ strain was sequenced to identify the mutations. Additionally, the first 500 bases immediately upstream of the *tcuA* start codon (*tcuA* promoter region) were sequenced, and no additional mutations were found in the *tcuR* mutant strains.

2.5. Recombinant DNA techniques

Unless otherwise stated, restriction and modification enzymes were purchased from MBI Fermentas and were used according to manufacturer's instructions. All DNA manipulations were performed in *E. coli* DH5α/F′. Plasmids were transformed into *E. coli* cells by $CaCl_2$ heat-shock [20]. Plasmids isolated from *E. coli* were transformed into *S. enterica* via electroporation [27]. Plasmid DNA was isolated using the Wizard Plus SV Plasmid Miniprep kit from Promega as per manufacturer's instructions. DNA fragments were isolated from 1% (w/v) agarose gels and purified using the Qiaquick® gel extraction kit (Qiagen). PCR reactions were purified using the Qiaquick® PCR purification kit (Qiagen). Big-Dye® (ABI-PRISM) non-radioactive sequencing reactions were performed and the mixtures were resolved and analyzed at the Biotechnology Center of the University of Wisconsin-Madison.

2.6. Plasmid constructions

Plasmids were propagated in *E. coli* strain DH5α/F′ except where noted. Genomic DNA for PCR was prepared from *S. enterica* strain JE6583 using the Wizard SV Genomic Purification System from Promega. All primers used for PCR amplifications were purchased from Integrated DNA Technologies.

Plasmid pTCU86—The *tcuC* gene was sub-cloned from plasmid pTCU5 [24] using 5′ *Sac*I and 3′ *Hin*dIII sites and cloned into plasmid pBAD33 [19] using the same restriction sites. Plasmid pTCU86 is 6.8-kb long and confers chloramphenicol resistance.

Plasmid pTCU94—The 600-bp fragment immediately upstream of the ATG start site of *tcuR* was amplified using PCR primers containing a 5′ *Eco*RI site and 3′ *Bam*HI site. The PCR product was cloned into plasmid pRS551 [34] using the same restriction enzymes. Plasmid pTCU94 is13.1-kb long, and confers kanamycin and ampicillin resistance. pRS551 is a derivative of plasmid pBR322, which is an intermediate copy number vector (~20–30 copies per cell) [4].

2.7. β-Galactosidase activity assays

β-Galactosidase activity was measured using established protocols [16]. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per min. Specific activity is reported as the number of units per OD_{650} unit. Enzyme activity was measured in mid-log cultures (i.e., OD_{650} ~0.4–0.6), unless otherwise noted. Optical density was monitored with a Spectronic 20D spectrometer. Unless otherwise noted, 5-ml cultures were used.

3. Results and Discussion

3.1. Expression of the *tcuABC* operon requires TcuR

A reporter strain carrying both a *tcuA*::MudJ(*lacZ*$^+$) transcriptional fusion and an in-frame deletion of *tcuR* (strain JE9315) was used to assess whether TcuR was required for *tcuA* expression. Strain JE9315 was transformed with plasmid pTCU5, which contained the wild-type allele of *tcuC*, the gene that encodes the tricarballylate transporter. The resulting strain,

JE10458, was transformed with either plasmid pTCU26 (carries the wild-type *tcuR*$^+$ allele) or a vector-only negative control. Cells were grown in NCE-glycerol (30 mM) ± tricarballylate (100 μM) to mid-log phase (OD_{650} ~0.4–0.5). Tricarballylate induced expression of *tcuA*::MudJ(*lacZ*$^+$) by >22-fold when *tcuR*$^+$ was provided *in trans* (Table 1; lines 2, 4). In contrast, we measured a 50-fold induction of *tcuA* expression in strain JE7212 (*tcuA*::MudJ (*lacZ*$^+$), which carries a wild-type allele of *tcuR* on its chromosome, under the same growth conditions (NCE-glycerol + tricarballylate; Table 2, lines 5, 6). The observed differences in tricarballylate-induced *tcuA* expression likely reflect reduced expression of the transporter (*tcuC*) or the regulator (*tcuR*) genes. Support for this interpretation was obtained from experiments performed in the presence of high levels of arabinose (10 mM), which led to substantially higher induction of the *tcuA-lacZ* fusion by tricarballylate (111 ± 0.5 U/OD_{650}). Lowering the arabinose concentration to 1 mM lowered *tcuA-lacZ* expression levels to 23.5 ± 3.0 U/OD_{650} when cells were grown with tricarballylate, versus 7.0 ± 0.3 when cells were grown in glycerol alone. Further lowering of the concentration of arabinose (0.5 mM) in the medium led to lower expression of the *tcuA-lacZ* fusion (9.6 ± 0.6 U/OD_{650}) in medium containing tricarballylate, versus 5.8 ± 0.9 U/OD_{650} for glycerol alone.

Induction by tricarballylate depended on a functional TcuC protein. Expression of *tcuA* in strain JE7212 carrying the cloning vector (pBAD30) did not respond to tricarballylate (11 ± 3 U/OD_{650} in medium containing tricarballylate versus 4 ± 1 U/OD_{650} in glucose alone). In contrast, the same strain transformed with plasmid pTCU5 (*tcuC*$^+$) responded to tricarballylate (807 ± 196 U/OD_{650} in medium containing tricarballylate versus 4 ± 1 U/OD_{650} in glucose alone). Thus, both TcuR and TcuC functions were required for *tcuABC* induction by tricarballylate.

3.2. Micromolar levels of tricarballylate stimulate TcuR activity

We determined how much tricarballylate was required for maximal expression of the *tcuABC* operon. Strain JE7212 [*tcuA33*::MudJ(*lacZ*$^+$)] was transformed with plasmid pTCU5 (*tcuC*$^+$), the gene that encodes the tricarballylate transporter. A culture of the resulting strain (JE10614, Table 4, supplemental material) was grown to mid-log phase in NCE-glucose medium containing varying concentrations of tricarballylate, and β-galactosidase assays were performed. As shown in figure 1B, as little as 12 μM tricarballylate maximally induced *tcuA-lacZ* expression; clearly, the cell sensed and responded to extremely low levels of of tricarballylate in the medium. For all subsequent experiments, tricarballylate was added to 100 μM to ensure maximal *tcuA-lacZ* expression.

3.3. Insights into the co-inducer site of TcuR

The observation that tricarballylate induced *tcuA-lacZ* expression in the absence of tricarballylate dehydrogenase (TcuA) function suggested that tricarballylate, not a catabolite of it, was the direct co-inducer of TcuR. To gain insights into the tricarballylate binding site of TcuR in the absence of a three-dimensional structure, strain JE7212 (*tcuA33*::MudJ(*kan*$^+$)) was grown on different carbon sources (Table 2). Glucose, glycerol, citrate, isocitrate, and *cis*-aconitate failed to induce *tcuA-lacZ* expression. Lower but substantial levels of *tcuA-lacZ* expression were measured when succinate or acetate was used as carbon and energy source (Table 3). The effects of succinate and acetate depended on a functional TcuR protein, as strain JE9315 [Δ*tcuR61 tcuA33*::MudJ(*kan*$^+$)] containing a deletion of *tcuR* failed to activate *tcuA-lacZ* on either succinate or acetate (3.0 ± 1.1 and 3.1 ± 1.8 U/OD_{650}, respectively.) This result was consistent with succinate and acetate acting as co-inducers of TcuR. Because succinate is structurally similar to tricarballylate, (Fig. 3), we explained the stimulatory effect of succinate as molecular mimicry. To explore this idea further, we combined succinate with acetate and found that such a combination did not stimulate *tcuA-lacZ* expression relative to when succinate or acetate was used alone (Table 2). This result, while initially surprising, can possibly be explained if both succinate and acetate cannot simultaneously occupy the co-inducer site due

to steric hindrance. Perhaps succinate and acetate displaced each other from the co-inducer site, thus resulting in poor activation of TcuR. To investigate the merit of this explanation we tested the combination of succinate and formate, which contains one less methyl group than acetate. The latter combination stimulated *tcuABC* to higher levels than those measured when succinate was used alone (Table 2), lending support to the idea that the methyl group of acetate sterically hinders the combined binding of succinate and acetate to the co-inducer site of TcuR. When used in combination with tricarballylate, neither succinate nor acetate abrogated the ability of tricarballylate to fully induce *tcuA-lacZ* expression (data not shown), which most likely reflects a higher affinity of TcuR for tricarballylate in comparison to these molecular mimics.

In contrast to the above findings, citrate and isocitrate were poor co-inducers of TcuR, suggesting that the co-inducer binding site of TcuR does not allow the presence of polar functional groups on the carbons connected to the carbonyl carbons of the co-inducer. One prediction for this hypothesis was that malate (hydroxysuccinate, Fig. 3) would be a poor inducer relative to succinate. Indeed, malate induced *tcuA-lacZ* expression only 17-fold compared to 50-fold for succinate (Table 2, lines 14, 9). Furthermore, the malate/formate combination increased *tcuABC* expression only 15-fold over un-induced levels. In contrast, for the succinate/formate combination we measured a 93-fold increased in expression, further supporting the idea that polar functional groups adjacent to the carbonyl groups may exert a negative effect on co-inducer binding. Further *in vitro* analysis of TcuR will address the true binding affinities of both succinate and acetate for TcuR as compared to tricarballylate.

3.4. *tcuABC* operon expression peaks during exponential phase

Strain JE7212 was transformed with plasmid pTCU21, which carried the entire *tcuABC* wild-type operon; plasmid pTCU21 was previously shown to be able to complement the polar *tcuA*::MudJ(kan$^+$) mutation [24]. Plasmid pTCU21 contains 16 nucleotides immediately upstream of the *tcuA* start codon; the 16-bp region contains the *tcuA* Shine-Dalgarno sequence but lacks the *tcuA* promoter and putative TcuR binding sites. This construct allowed us to follow the temporal expression pattern of *tcuABC* expression while the cells are growing on tricarballylate. Notably, this construct also allowed us to maintain the proper stoichiometry of TcuR and TcuR-binding sites on the chromosome. To determine the temporal expression of the *tcuABC* operon, β-galactosidase assays were performed throughout the growth cycle when growing on tricarballylate (Fig. 1A). Expression of the *tcuA-lacZ* fusion peaked during exponential growth and dropped back to lag-phase levels when the cells began to enter stationary phase. These results suggested that the level of TcuR protein was not constant throughout the cell cycle, that proteins other than TcuR were involved in *tcuABC* operon regulation, or that a combination of these two possibilities occurred. We first tested whether TcuR regulated its own expression. Because expression of the *tcuA-lacZ* fusion peaked at mid-log phase, all experiments, including those shown in the preceding section above, were performed at mid-log phase to simplify comparisons.

3.5. TcuR does not regulate its own expression

The first 600 bp immediately upstream of the *tcuR* start codon were cloned into a promoter-less *lacZ*$^+$ fusion vector (pRS551; ~20–30 copies per cell) to generate plasmid pTCU94. Expression of P_{tcuR}-*lacZ* in strain TR6583 (*tcuR*$^+$) remained constant in NB medium with or without 100 μM tricarballylate (1540 ± 20 versus 1450 ± 50 U/OD_{650}, respectively). In addition, expression of P_{tcuR}-*lacZ* did not change when *tcuR* was inactivated (JE7213; 1490 ± 40 U/OD_{650}).)]. While most LTTRs repress their own transcription, those that do are most often divergently transcribed from the genes they regulate [29]. Because *tcuR* is not divergently transcribed from *tcuABC*, the observation that neither TcuR nor tricarballylate had an effect on P_{tcuR}-*lacZ*transcription was not surprising. We further tested whether the differences in

growth rate expression of the *tcuA-lacZ* fusion were caused by differences in the level of TcuR throughout the growth phase. Expression of of P_{tcuR}-*lacZ* (pTCU94) in the wild-type strain TR6583 was monitored at various time points when cells were growing on NCE-tricarballylate medium. The level of P_{tcuR}-*lacZ* expression did not change throughout the growth curve (data not shown), suggesting that the increase in transcription of the chromosomal *tcuA-lacZ* fusion during exponential phase was not due to changes in the level of *tcuR* expression. However, we cannot at this time exclude the possibility that TcuR levels are regulated post-transcriptionally via targeted degradation or other means. In addition, there is evidence that pBR322 derivatives (i.e. pRS551) increase in copy number during stationary phase [2]. Thus, it is possible that a decrease in P_{tcuR}-*lacZ* is masked by the increase in copy number. Further *in vitro* characterization of TcuR should establish whether TcuR can bind to its own promoter and repress transcription.

3.6. The cAMP-dependent global regulatory protein Crp negatively affects *tcuABC* expression

We assessed whether the *tcuABC* operon was controlled by catabolite repression. For this purpose, we tested the effects of mutations in either adenylate cyclase (Cya) or cAMP receptor protein (Crp). Tricarballylate-dependent expression of the chromosomal *tcuA-lacZ* fusion in a strain of *S. enterica* carrying a null allele of the *crp* gene [JE7830 *tcuA33*::MudJ(*lacZ*$^+$) *crp*::Tn*10*(*tet*$^+$)] was >10-fold higher than the level of *tcuA-lacZ* expression in the *crp*$^+$ strain (JE7212 *tcuA33*::MudJ(*lacZ*$^+$), Fig. 2). Consistent with the involvement of the Crp/cAMP complex, strain JE10114 [*tcuA33*::MudJ(*lacZ*$^+$) *cya*::Tn*10*(*tet*$^+$)] carrying a null allele of adenylate cyclase (*cya*) also showed a substantial increase (6-fold) in *tcuA-lacZ* expression relative to the level measured in the *cya*$^+$ strain. Addition of cAMP (1 mM) to the medium reduced *tcuA-lacZ* expression in the presence of tricarballylate >3.5-fold, and the introduction of plasmid pHY26-1, which carried an allele of *crp* that encoded a cAMP-independent Crp protein, reduced *tcuA-lacZ* expression ~2-fold. Reasons that pHY26-1 resulted in lower repression compared to the addition of cAMP may include improper gene dosage and/or partial activity of the constitutive Crp variant protein.

If Crp were acting as a repressor of the *tcuABC* operon, we would expect *tcuA-lacZ* expression to be activated during growth on glucose. Indeed, tricarballylate-induced *tcuA-lacZ* expression was higher in glucose-containing medium than either citrate or glycerol-containing medium (Table 2; lines 2, 4, 6). These results suggested that the Crp protein acted as a repressor of *tcuABC* expression. This was somewhat surprising, as Crp most commonly acts as an activator and only a few enzyme-encoding genes are repressed by Crp/cAMP [17]. It is not obvious why the cell would use Crp to repress *tcuABC* expression. We speculate that the involvement of Crp in *tcuABC* expression reflects conditions where *S. enterica* encounters tricarballylate in the environment. The extant regulatory circuit of *tcuABC* expression allows *S. enterica* to catabolize tricarballylate in the presence of glucose. If glucose repressed tricarballylate catabolism in a canonical catabolite repression fashion, any tricarballylate that entered the cell would inhibit aconitase, preventing the synthesis of essential amino acid precursors such as α-ketoglutarate, succinyl-CoA and oxaloacetate.

It is possible that Crp acts as a repressor of *tcuR* and that Crp repression of *tcuA-lacZ* expression is at least partially the result of Crp-mediated repression of *tcuR*. We tested whether P_{tcuR}-*lacZ* expression from plasmid pTCU94 differed in either a *crp* or *cya* background (strains JE10943 and JE10944, respectively) when the cells were grown in LB medium containing tricarballylate (100 μM). We measured no significant difference (1.2 fold) in P_{tcuR}-*lacZ* expression in the *crp* or *cya* strains compared to that of the wild-type strain TR6583 (1600 ± 70 and 1790 ± 40 versus 1450 ± 70 U/OD_{650}, respectively). Addition of cAMP (1 mM) to the medium reduced P_{tcuR}-*lacZ* in the *cya* strain by 1.3-fold (1790 ± 40 versus 1360 ± 70 U/

OD_{650}, respectively). Furthermore, there was slight evidence of glucose activation, as expression of P_{tcuR}-*lacZ* in the wild-type strain was only 1.1-fold increased for cells growing on glucose compared to LB (1620 ± 50 vesus 1450 ± 70 U/OD_{650}, respectively). These results suggested that the Crp protein did not have an effect on *tcuR* expression. While it is possible that Crp could have affected the copy number of pTCU94, we found no evidence that this may be the case in the literature. Additionally, there are no notable consensus motifs for Crp-binding sites in the *tcuR* promoter region (see below). Direct in vitro binding studies between Crp and the *tcuR* promoter region would firmly establish whether Crp directly interacts with the *tcuR* promoter.

Crp-dependent repression of *tcuA-lacZ* expression and the increase in the expression of *tcuA-lacZ* during mid-log growth suggest that *tcuABC* regulation is complex. At present, it is unclear whether the effects of Crp are direct or indirect, and whether any other global regulators also control either *tcuR* or *tcuABC* operon expression. Noteworthy, there are no notable consensus motifs for Crp-binding sites in either the *tcuR* or *tcuA* promoter regions. If the Crp effect were indirect, we would hypothesize that Crp would act as an activator of a protein that represses *tcuABC* expression. Hence in the absence of Crp, and consequently of the putative repressor, *tcuABC* expression would increase. In vitro biochemical studies are needed to determine whether the effect of Crp on *tcuABC* expression is direct.

3.8. Isolation of two classes of constitutive *TcuR* variants

Chemical mutagenesis with NG was performed on strain JE7212 (*tcuA33*::MudJ(*lacZ*$^+$) to isolate constitutive mutations in TcuR. We selected derivatives of strain JE7212 that formed large colonies on NCE-lactose medium lacking tricarballylate. Each mutant was reconstructed as described above, and in every case the Lac$^+$ phenotype was co-transducible with the *kan*$^+$ marker. We identified eight mutant *tcuR* alleles upon sequencing (Fig. 5, supplemental material). The promoter region of each *tcuR* strain was also sequenced, and no additional mutations were found. Several *tcuR* mutations were isolated between residues 241–244, the region of the protein containing the putative C-terminal multimerization domain. While the screen was likely non-saturating, several mutations were independently isolated twice (E42G, L264R) or thrice (A242T), highlighting the relevance of the position. Changes in the residues within the proposed co-inducer-binding domain were not isolated. Reasons why we did not find mutations in this domain could include the mutational bias of NG, the stringency of the screen and the need for multiple mutations to observe the desired phenotype.

Expression of the *tcuA-lacZ* fusion in the mutant strains was assessed during growth in rich medium with or without tricarballylate. While every mutant strain had at least a 25-fold increase in *tcuA-lacZ* expression in the absence of tricarballylate, there were two distinct phenotypes in the presence of tricarballylate. Class I mutations mapped within four residues of each other, and were defined as those alleles encoding TcuR variants whose activity was >2-fold higher when tricarballylate was added to the medium (Table 3). Class I mutations may bias TcuR towards achieving an active conformation in the absence of tricarballylate, but still allow a response to tricarballylate. Class II mutations, with the lone exception of S241C, were located elsewhere in TcuR, and responded poorly to tricarballylate (<2-fold induction of *tcuABC*). Class II mutations may enhance DNA-binding independently of the normally required conformational change. In fact, the E42G substitution (a class II mutation) falls within the putative helix-turn-helix, which would support this explanation. The L264R substitution (also class II) may also increase DNA-binding affinity, as substitutions near this residue in both NahR (regulator of naphthalene catabolism in *Pseudomonas putida*) and AmpR (regulator of β-lactamase expression in *Citrobacter freundii*) led to loss of DNA-binding in previous studies [5,30].

Substitution G241C may be an outlier, as it falls within the group of residues with a Class I phenotype. In this case, it is possible that this TcuR^{G241C} variant is locked in a conformation that prevents it from responding to tricarballylate. In vitro approaches are needed to analyze Class I and Class II mutations to determine their effects on DNA-binding and transcriptional activation.

This study establishes the in vivo basis for the regulation of the *tcuABC* operon by TcuR and its co-inducer, tricarballylate. We have shown that tricarballylate alone is sufficient for TcuR-mediated induction of *tcuABC*. Our results also demonstrate a role for the global regulator, Crp, in tricarballylate catabolism. The results described here should be valuable for future structure/function analyses of the important LTTR family. The response of TcuR to molecular mimics of tricarballylate including succinate and acetate may lead to increased understanding of how LTTRs recognize their co-inducers. In addition, the two classes of constitutively active mutants may lead to understanding of how LTTRs adopt a transcriptionally competent conformation. Current efforts are focused on obtaining a crystal structure of TcuR and performing detailed in vitro analysis.

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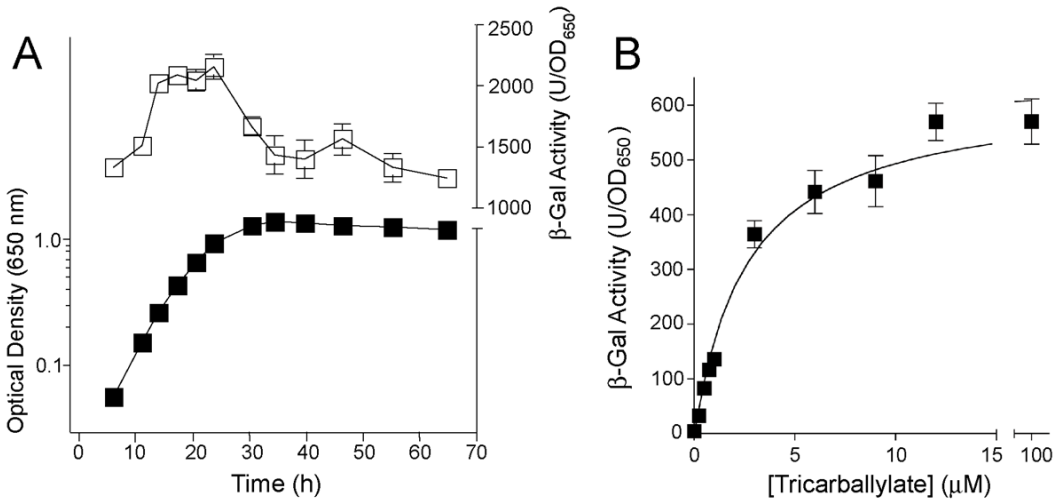

Fig. 1.

A. The *tcuABC* operon is maximally transcribed during exponential growth. Black squares represent the growth of strain JE7212 [*tcuA33*::MudJ(*kan*$^+$)] containing plasmid pTCU21 (*tcuABC*$^+$) on tricarballylate (20 mM) in the presence of L(+)-arabinose (500 μM) to induce expression of the *tcuABC* operon. Open squares represent β-galactosidase activity (U/OD_{650}) assayed from 1-ml samples taken at each point of the growth phase. **B. Low levels of tricarballylate fully induce *tcuABC* expression.** β-Galactosidase activity of strain JE7212 [*tcuA33*::MudJ(*kan*$^+$)] containing plasmid pTCU5 (*tcuC*$^+$) on NCE-glucose (10 mM) supplemented with 500 μM L-(+)-arabinose and varying amounts of tricarballylate. All experiments were performed using triplicate cultures, and error bars denote standard error.

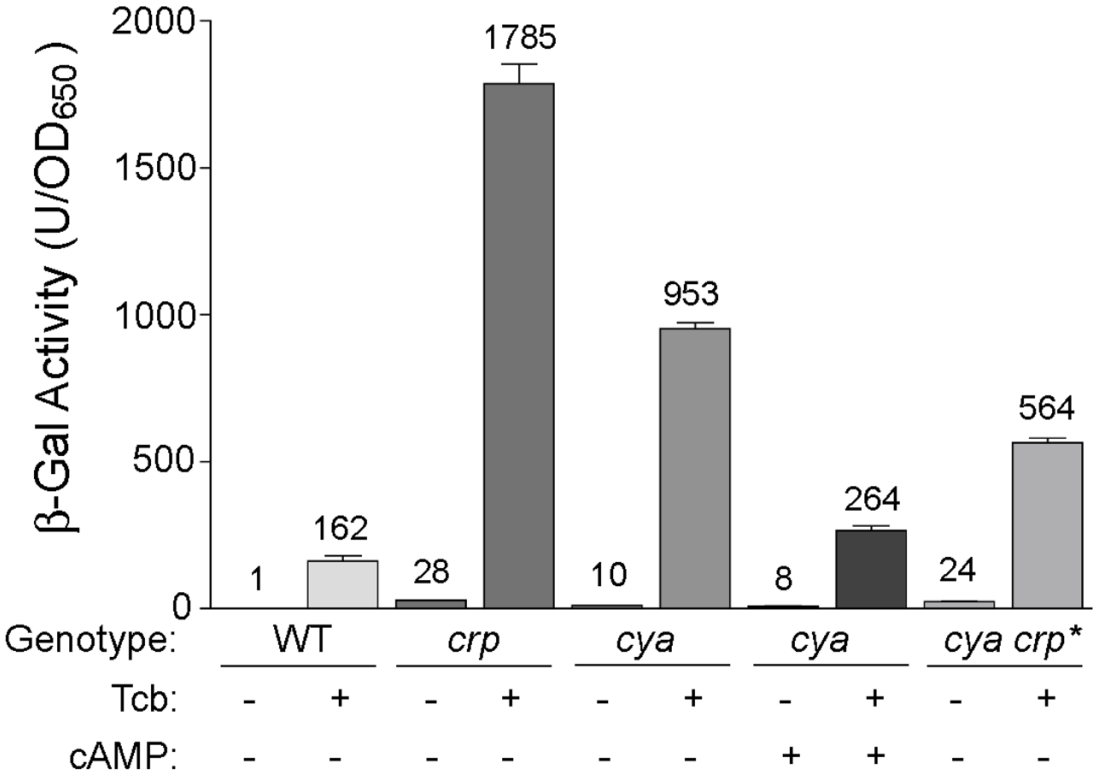

Fig. 2. Effect of Crp on the expression of a chromomosal *tcuA-lacZ* transcriptional fusion

Cultures were grown in LB media. All strains contained *metE205 ara-9 tcuA33*::MudJ(*kan*$^+$) mutations in the chromosome and contained plasmid pTCU86 (*tcuC*$^+$). *crp* identifies strain JE7830 [*crp*::Tn*10*(*tet*$^+$)]; *cya* identifies strain JE10114 [*cya*::Tn*10*(*tet*$^+$)] mutation; *cya crp** identifies strain JE10114 (JE10623/pHY26-1 (*crp**)]. Tcb and cAMP denote tricarballylate (100 μM) and cyclic AMP (1 mM) present in the medium, respectively. The experiment was performed using triplicate cultures, and error bars denote standard error.

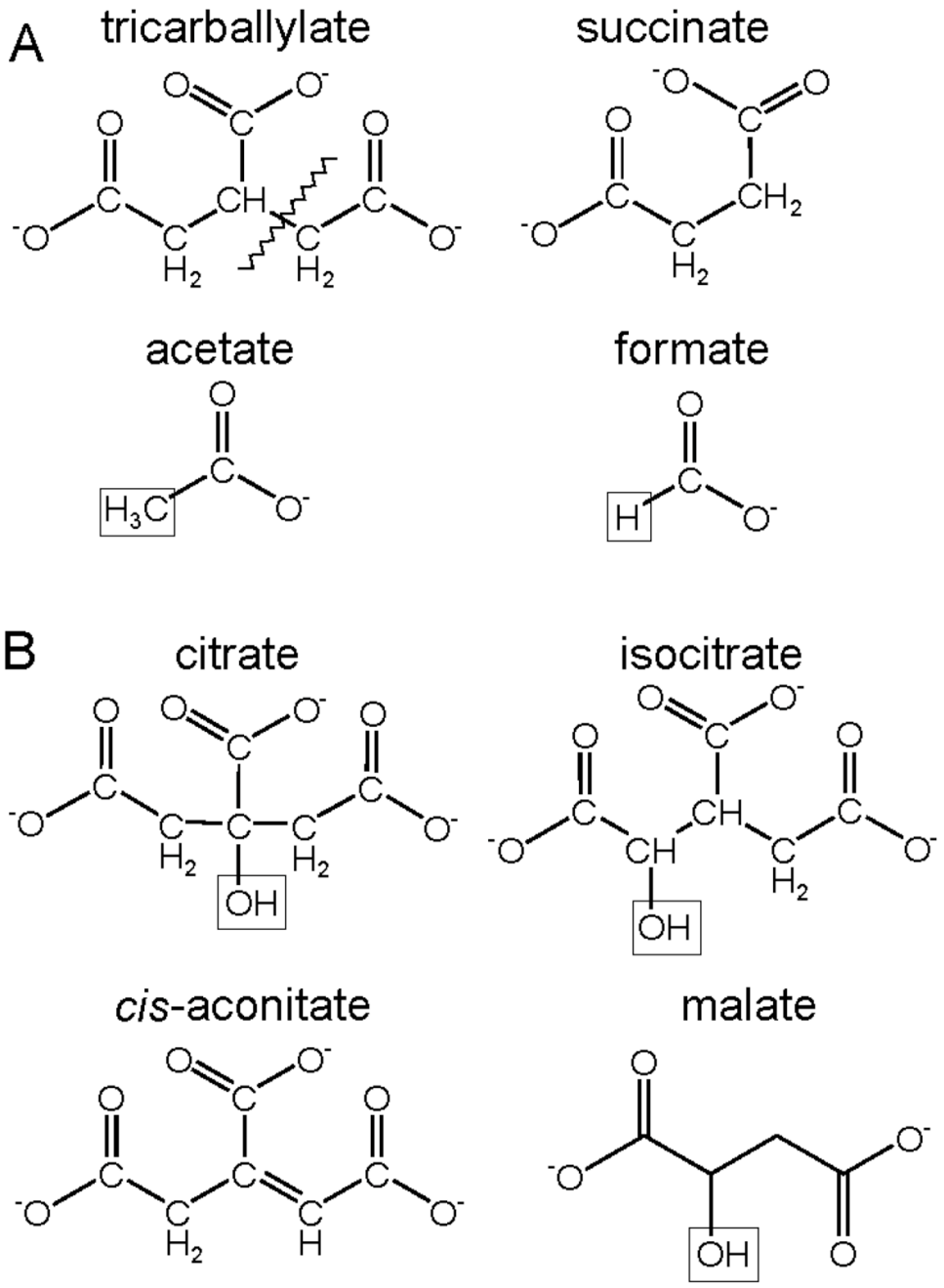

Fig. 3. Structural analogs of tricarballylate

A. Analogs of tricarballylate that activated *tcuA-lacZ* expression included succinate, acetate, and a combination of succinate and formate. **B**. Analogs that had no effect on *tcuA-lacZ* expression included citrate, isocitrate, and *cis*-aconitate.

Table 1

TcuR is required for tricarballylate-induced *tcuABC* transcription.

Line #	Relevant genotype[a]	NCE-glycerol medium[b] ± inducer[c]	β-Galactosidase activity[d] (U/OD_{650})	Fold increase (over line #1)
1	Δ*tcuR*/pBAD18-Cm[e]	None	2.5± 0.8	1
2	Δ*tcuR*/pBAD18-Cm	Tricarballylate	5.1± 0.1	2.0
3	Δ*tcuR*/pTCU26 (*tcuR*$^+$)	None	7.2 ± 0.3	2.9
4	Δ*tcuR*/pTCU26 (*tcuR*$^+$)	Tricarballylate	111 ± 0.5	44

[a] Strain JE9315 (*metE205 ara-9* Δ*tcuR61 tcuA33*::MudJ(*kan*$^+$) was used for these experiments and contained plasmid pTCU5 (*tcuC*$^+$).

[b] Strains were grown on NCE-glycerol (30 mM) medium supplemented with 10 mM L-(+)-arabinose to induce expression of *tcuC* and *tcuR* from their respective plasmids.

[c] When present in the medium, tricarballylate was at 100 μM.

[d] Cultures were grown to mid-log phase (OD_{650} ~0.4–0.5). Each experiment was performed using triplicate cultures; error reported as standard error.

[e] Empty vector control.

Table 2

Effect of various carbon sources on *tcuABC* operon expression.

Line #	Carbon source[a]	β-Galactosidase activity (U/OD_{650})	Fold increase over glucose (line #1)
1	glucose	3 ± 0.1	1
2	glucose + tricarballylate	960 ± 40	320
3	citrate	6 ± 0.1	2
4	citrate + tricarballylate	780 ± 10	260
5	glycerol	10 ± 1	3
6	glycerol + tricarballylate	500 ± 70	167
7	isocitrate	7 ± 0.4	2
8	*cis*-aconitate	8 ± 0.2	3
9	succinate	150 ± 15	50
10	acetate	120 ± 3	40
11	succinate + acetate	16 ± 1	5
12	glucose + formate	2 ± 0.1	1
13	succinate + formate	280 ± 40	93
14	malate	50 ± 2	17
15	malate + formate	45 ± 6	15

[a] Cultures of the *tcuA-lacZ* reporter strain JE10614 (*tcuA33*::MudJ(*kan*$^+$)/pTCU5 *tcuC*$^+$) were grown on NCE medium on the indicated carbon sources as the source(s) of carbon and energy to mid-log phase (OD_{650} ~0.4–0.5). L-(+)-arabinose (500 μM) was included in the medium to induce expression of *tcuC*. The concentrations of each carbon source individually were: citrate, 20 mM; acetate, 30 mM; succinate, 30 mM; glucose, 10 mM; *cis*-aconitate, 20 mM. When used in combination, succinate, acetate, and formate were all at 20 mM. Each experiment was performed using triplicate cultures. The error is reported as standard error.

Table 3

Constitutively active TcuR variants

Line #	TcuR Variant	NB medium ± inducer[a]	β-Galactosidase activity[b] (U/OD_{650})	Fold increase over the $TcuR^{WT}$ (line #1)
1	$TcuR^{WT}$	None	2 ± 0.5	1
2	$TcuR^{WT}$	Tricarballylate	310 ± 20	155
Class I TcuR constitutively active variants that still respond to tricarballylate				
3	$TcuR^{A242V}$	None	70 ± 7	35
4	$TcuR^{A242V}$	Tricarballylate	350 ± 45	175
5	$TcuR^{A242T}$	None	110 ± 10	55
6	$TcuR^{A242T}$	Tricarballylate	310 ± 30	155
7	$TcuR^{I244F}$	None	150 ± 20	75
8	$TcuR^{I244F}$	Tricarballylate	760 ± 30	380
9	$TcuR^{G241S}$	None	50 ± 5	25
10	$TcuR^{G241S}$	Tricarballylate	245 ± 25	123
Class II TcuR constitutively active variants that do not respond to tricarballylate				
11	$TcuR^{L264R}$	None	175 ± 20	88
12	$TcuR^{L264R}$	Tricarballylate	210 ± 10	110
13	$TcuR^{E42G}$	None	250 ± 10	125
14	$TcuR^{E42G}$	Tricarballylate	225 ± 15	113
15	$TcuR^{G241C}$	None	250 ± 10	125
16	$TcuR^{G241C}$	Tricarballylate	310 ± 30	155

[a] When added to the medium, tricarballylate was at 100 μM.

[b] Cultures were grown to mid-log phase (OD_{650} ~0.4–0.5). Each experiment was performed using triplicate cultures; error reported as standard error.