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Research Letter

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Expression and activity of the Calvin–Benson–Bassham cycle transcriptional regulator CbbR from *Acidithiobacillus ferrooxidans* in *Ralstonia eutropha*

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One sentence summary: Microbes from extremely acidic conditions are important for environmental and industrial reasons and we report how their gene functions can be studied in a well-characterized laboratory strain.
Editor: Rich Boden

ABSTRACT

Autotrophic fixation of carbon dioxide into cellular carbon occurs via several pathways but quantitatively, the Calvin–Benson–Bassham cycle is the most important. CbbR regulates the expression of the *cbb* genes involved in CO_2 fixation via the Calvin–Benson–Bassham cycle in a number of autotrophic bacteria. A gene potentially encoding CbbR (*cbbR*^{AF}) has been predicted in the genome of the chemolithoautotrophic, extreme acidophile *Acidithiobacillus ferrooxidans*. However, this microorganism is recalcitrant to genetic manipulation impeding the experimental validation of bioinformatic predictions. Two novel functional assays were devised to advance our understanding of *cbbR*^{AF} function using the mutated facultative autotroph *Ralstonia eutropha* H14 Δ*cbbR* as a surrogate host to test gene function: (i) *cbbR*^{AF} was expressed in *R. eutropha* and was able to complement Δ*cbbR*; and (ii) CbbR^{AF} was able to regulate the *in vivo* activity of four *A. ferrooxidans cbb* operon promoters in *R. eutropha*. These results open up the use of *R. eutropha* as a surrogate host to explore *cbbR*^{AF} activity.

Keywords: *Acidithiobacillus ferrooxidans*; *Ralstonia eutropha*; autotroph; CbbR; RubisCO; CO_2 fixation

INTRODUCTION

The genus *Acidithiobacillus* contains acidophilic, obligate chemolithoautotrophic species that are proposed to form a new class called the *Acidithiobacillia* within the *Proteobacteria* (Williams and Kelly 2013). *Acidithiobacillus ferrooxidans* is a model species for acidophiles and its genome has been sequenced, leading to reconstructions of many aspects of its growth and metabolism (Valdes *et al.* 2008). *Acidithiobacillus ferrooxidans* thrives at extremely low pH (pH 1–3), forms biofilms on sulfide mineral surfaces (Vera *et al.* 2009), gains energy and reducing power from the aerobic oxidation of ferrous iron, inorganic sulfur compounds (ISCs), elemental sulfur and hydrogen (Bonnefoy

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and Holmes 2012; Dopson and Johnson 2012; Hedrich and Johnson 2013), and grows anaerobically via sulfur oxidation coupled to reduction of ferric iron (Osorio *et al.* 2013). *Acidithiobacillus ferrooxidans* is an important member of a consortium of microorganisms used for the industrial recovery of copper (biomining) where it is suggested to be a major contributor of fixed carbon (Nancucheo and Johnson 2010; Liu *et al.* 2011). Thus, it is important to evaluate the role of *A. ferrooxidans* in CO_2 fixation in both industrial and natural microbial communities (Bryan *et al.* 2012).

Acidithiobacillus ferrooxidans has been shown to have five *cbb* operons that potentially encode all the enzymes required for CO_2 assimilation via the Calvin–Benson–Bassham cycle (CBB cycle; also termed the 'reductive pentose phosphate cycle'). Four of these operons (*cbbL1*, *cbbL2*, *cbb3* and *cbb4*) are regulated by promoters that bind CbbR (hereafter referred to as $CbbR^{AF}$) (Esparza *et al.* 2010). The four *cbb* operons are variably expressed under different growth conditions such as iron versus sulfur growth medium (Appia-Ayme *et al.* 2006) and at different concentrations of carbon dioxide derived by culturing in air for aerobic growth and sparging the culture with a 10% CO_2–90% N_2 gas mix for approximately 30 min a day during anaerobic growth (Osorio *et al.* 2013).

Acidithiobacillus spp. have been recalcitrant to genetic manipulation and it is only very recently that rudimentary genetic systems are becoming available (Van Zyl, Van Munster and Rawlings 2008; Wen *et al.* 2014; Yu *et al.* 2014). However, it is still not currently possible to assess gene function by complementation of *A. ferrooxidans* knockout mutants. As a result, direct proof of $CbbR^{AF}$ function is difficult to obtain and an autotrophic surrogate host was sought to advance our understanding of $CbbR^{AF}$ function.

Ralstonia eutropha (synonyms: *Alcaligenes eutrophus*, *Wautersia eutropha* and *Cupriavidus necator*) grows in both heterotrophic and autotrophic conditions. During autotrophic growth on either hydrogen plus carbon dioxide or formate, the *R. eutropha* CbbR protein ($CbbR^{RE}$) activates expression of the *cbb* genes, whereas during heterotrophic growth on pyruvate the $CbbR^{RE}$ acts as a repressor (Bowien and Kusian 2002). *Ralstonia eutropha* is a good candidate for a surrogate host as its genome sequence is available (Pohlmann *et al.* 2006); it is amenable to genetic manipulation (Brigham *et al.* 2012); its metabolism is well characterized (reviewed in Cramm (2009)) including its CO_2 fixation system (Jeffke *et al.* 1999; Bowien and Kusian 2002); and finally because of the availability of the HB14 strain that is a *cbbR* deletion (*cbbr*Δ) mutant (Jeffke *et al.* 1999).

In this study, $cbbR^{AF}$ was cloned into *R. eutropha* and shown to complement $cbbR^{RE}$ and that $CbbR^{AF}$ was able to bind to the *R. eutropha cbbL1* promoter to regulate expression of the cbb^{RE} genes. This shows that *R. eutropha* is a good candidate for the study of CO_2 fixation by *A. ferrooxidans*.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic	Source or reference
Bacterial strains		
Acidithiobacillus. ferrooxidans	Type strain	ATCC 23270
Ralstonia eutropha H16	Wild type (synonym *Cupriavidus necator* H16)	ATCC 17699
Ralstonia eutropha HB14	*cbbR*Δ, derivative of *R. eutropha* H16	Jeffke *et al.* (1999)
Escherichia coli S17–1 λpir	Sm^r Tp^r *mod1 res thi pro recA*; integrated RP4 (Tc::Mu-Km::Tn*7*)	Simon, Priefer and Pühler (1983)
Plasmids		
pGEMT	Amp^r promoter *lacZ*	Promega
pBK	Tc^r; double operon fusion plasmid with divergently oriented *lacZ* (β-galactosidase) and *gusA* (β-glucuronidase) as reporter genes	Kusian and Bowien (1995)
pUW7	Tcr; pVK101::1,031-bp *DdeI* fragment containing *cbbR* from *R. eutropha* H16	Windhovel and Bowien (1991)
$pGEMTcbbR^{AF}$	pGEMT:: 1.1-kb fragment contains *cbbR* from *A. ferrooxidans* ATCC 23270 expressed from own promoter	This study
$pBK\text{-}cbbR^{AF}$	pBK::1.1-kb fragment contains *cbbR* from *A. ferrooxidans* ATCC 23270 expressed from own promoter	This study
pBK-cbbL1	pBK::209 bp *XbaI-PstI* fragment contains *cbbL1* operon promoter from *A. ferrooxidans* ATCC 23270	This study
pBK-cbbL2	pBK::222 bp *XbaI-XbaI* fragment contains *cbbL2* operon promoter from *A. ferrooxidans* ATCC 23270	This study
pBK-cbb3	pBK::218 bp *XbaI-XbaI* fragment contains *cbb3* operon promoter from *A. ferrooxidans* ATCC 23270	This study
pBK-cbb4	pBK:: 218 bp *XbaI-PstI* fragment contains *cbb4* operon promoter from *A. ferrooxidans* ATCC 23270	This study
pBK-cbbL1R	$pBK\text{-}cbbR^{AF}$::209 bp *XbaI-PstI* fragment contains *cbbL1* operon promoter from *A. ferrooxidans* ATCC 23270	This study
pBK-cbbL2R	$pBK\text{-}cbbR^{AF}$:: 222 bp *XbaI-XbaI* fragment contains *cbbL2* operon promoter from *A. ferrooxidans* ATCC 23270	This study
pBK-cbb3R	$pBK\text{-}cbbR^{AF}$:: 218 bp *XbaI-XbaI* fragment contains *cbb3* operon promoter from *A. ferrooxidans* ATCC 23270	This study
pBK-cbb4R	$pBK\text{-}cbbR^{AF}$:: 218 bp *XbaI-PstI* fragment contains *cbb4* operon promoter from *A. ferrooxidans* ATCC 23270	This study

Abbreviations: ATCC, American Type Culture Collection; Amp^r, ampicillin resistance; Sm^r, streptomycin resistance; Tp^r, trimethoprim resistance; and Tc^r, tetracycline resistance.

Table 2. Primer sequences used for PCR amplification of DNA.

	Forward primer (5′ to 3′)		Reverse primer (5′ to 3′)
Cloning and expression of *cbbR*^{AF}			
cbbR^{AF}fw	TCTATCCGTCATGCAACCTTG	*cbb*^{AF}Rrev	GCGCCATTCCTTTTCACCATG
Complementation and β-galactosidase assays			
pcbbR^{AF}fw	CTAGACTTTTTTACGGCCATGCTT	*cbbR*^{AF}rev	CCATTACTTCATTCTTGAGCG
pcbbL1fw	CGGCAGTCTAGATCTTGAGTTGGTGC	pcbbL1rev	CTCCGGCTGCAGACTTTTTTACGG
pcbbL2fw	ACGACGGCGTCTAGAACCGCC	pcbbL2rev	CCGGTAATCCTCTAGACCCGCGTT
pcbb3fw	TCTAGAACAGGGTCAGCTCCTGG	pcbb3rev	TCTAGAGTCTTTGATCATGCGCC
pcbb4fw	TTTGGGGTGGCATCTAGAAGTCCT	pcbb4rev	GGAAACGGCTGCAGAGGTGAA

AF = *Acidithiobacillus ferrooxidans* and RE = *Ralstonia eutropha*.

MATERIALS AND METHODS

Bioinformatics

ClustalW was used for determining amino acid sequence similarity relations (Larkin *et al.* 2007) and PSI-PRED was used for protein secondary structure predictions (Bryson *et al.* 2005). Alignment of DNA sequences was carried out using Blast Two Sequences at http://blast.ncbi.nlm.nih.gov (Altschul *et al.* 1997).

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are shown in Table 1. *Acidithiobacillus ferrooxidans* ATCC 23270 cells were cultured in 9 K medium (without addition of ferrous iron and adjusted to pH 3.5 with H_2SO_4) containing 5 g L^{-1} elemental sulfur at 30°C under aerobic conditions on a rotary shaker at 150 rpm as described previously (Quatrini *et al.* 2007). *Acidithiobacillus ferrooxidans* cultures were centrifuged at 800 × *g* to remove solid sulfur precipitates prior to cell harvesting at 8000 × *g* for 10 min. The cell pellet was washed in 9 K salts solution (Quatrini *et al.* 2007).

Strains of *R. eutropha* were grown in a nutrient broth or mineral medium (MM) at 30°C as described previously (Windhovel and Bowien 1991). MM was routinely supplemented with 0.2% (wt/vol) fructose for heterotrophic cultures until reaching an optical density of 2 at 436 nm. The headspaces of chemoautotrophic cultures were gassed with a mixture of H_2, CO_2 and O_2 (8:1:1 [vol/vol/vol]). All MM and the gas mixture were sterilized by filtration with a 0.22-μm filter. Colonies were grown on agar plates containing MM with the respective antibiotics and cultured in gas jars with the same H_2, CO_2 and O_2 gas mixture as described above. Growth experiments were replicated three times and a representative result presented.

Escherichia coli was cultured in Luria–Bertani (LB) medium at 37°C or 30°C containing ampicillin (50 mg mL^{-1}) or tetracycline (15 mg mL^{-1} for *E. coli* or 20 mg mL^{-1} for *R. eutropha*) as required. Plasmids were conjugally transferred from *E. coli* S17–1 to strains of *R. eutropha* by biparental mating (Srivastava, Urban and Friedrich 1982).

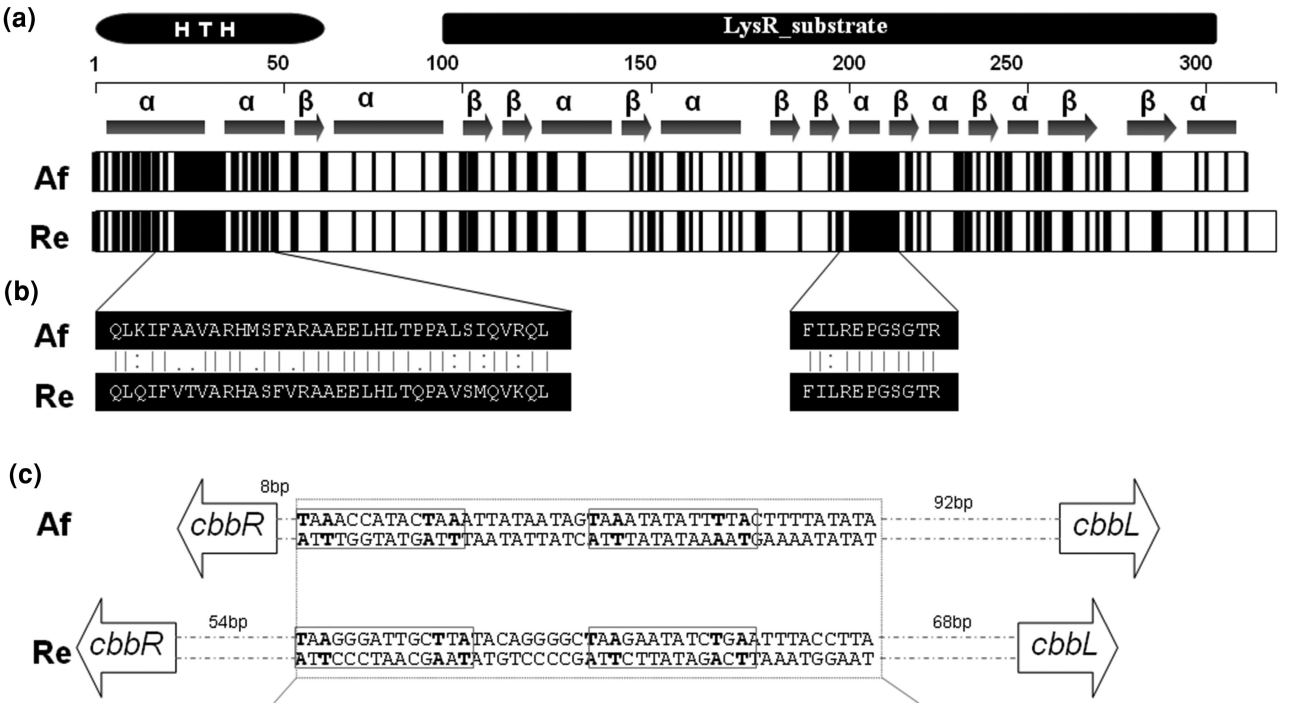


Figure 1. (**a**) *Acidithiobacillus ferrooxidans* and *R. eutropha* CbbR predicted protein secondary structure alignment; (**b**) protein alignment of the CbbR HTH and LysR domains; and (**c**) *cbbR* nucleotide similarity of the promoter regions.

General DNA techniques and DNA sequencing

Standard procedures (Sambrook, Fritsch and Maniatis 1989) were employed to isolate genomic DNA from bacteria and to transform plasmid DNA into *E. coli*. *Escherichia coli* plasmids were isolated as described previously (Holmes and Quigley 1981). Restriction endonucleases and DNA-modifying enzymes were used under the reaction conditions recommended by the manufacturers. Plasmid DNA from *R. eutropha* was prepared with the QIAprep Spin Mini-Kit (Qiagen). Polymerase chain reaction (PCR) products were amplified with *Taq* DNA polymerase (Fermentas) using the manufacturer's recommended reaction mix and were purified from agarose gels with the QiaEx DNA purification kit (Qiagen). PCR conditions were initial denaturing step at 95°C for 5 min followed by 30 amplification cycles (denaturation at 95°C for 30 s, annealing at the appropriate temperature for the specific primer pairs for 20 s and elongation at 72°C) and a final elongation step at 72°C for 10 min.

Plasmid constructions

A DNA fragment containing *cbb*R with its own promoter (cbbR^{AF}) was generated by PCR from *A. ferrooxidans* ATCC 23270 genomic DNA using primers cbbR^{AF}Fw and cbbR^{AF}Rrev (Table 2). After purification by agarose gel electrophoresis, the fragment was cloned into pGEMT easy vector (Promega) at the multiple cloning site resulting in pGEMTcbbR^{AF}. Conformation of the resulting recombinant construction was obtained by DNA sequencing (Göttingen Genomics Laboratory, Germany; data not shown). *cbbR*^{AF} was excised with *Eco*RI from pGEMTcbbR^{AF} and inserted into the *Eco*RI sites of pBK and pBK2241, resulting in plasmids pBKcbbR^{AF} and pBK2241cbbR^{AF}, respectively.

Four DNA fragments containing the predicted regions of promoters cbbL1, cbbL2, cbb3 and cbb4 respectively were generated by PCR from *A. ferrooxidans* ATCC 23270 genomic DNA using primers that include the XbaI–PstI restricted site (Table 2). After purification by agarose gel electrophoresis, each fragment was cloned into the XbaI–PstI site of pBK and pBKcbbR^{AF}, previously digested with the enzymes XbaI and PstI, resulting in plasmids pBK-cbbL1, pBK-cbbL2, pBK-cbb3, pBK-cbb4 and pBK-cbbL1R, pBK-cbbL2R, pBK-cbb3R, pBK-cbb4R (Table 1).

β-Galactosidase assays

β-Galactosidase activity in *R. eutropha* strains HB14 cell extracts was assayed colorimetrically (Miller 1972). β-Galactosidase-specific activity = activity (1 unit equals 1 μmol product/min) per mg protein used.

Cultures of the strains were grown to an optical density of 2.5 at 436 nm, cells were harvested, resuspended in β-galactosidase buffer and disrupted by sonication as previously described (Jeffke *et al.* 1999). Cell extracts were obtained after centrifugation at 14 000 × g for 20 min to remove unbroken cells and cell debris. Protein concentrations in the extracts were estimated by the Bradford method (Bradford 1976). All data are presented as means ± standard deviations (number of replicates (n) = 3).

RESULTS AND DISCUSSION

Bioinformatic comparison of CbbR^{AF} and CbbR^{RE}

To search for a suitable expression system to study the *A. ferrooxidans* CbbR (CbbR^{AF}), the protein sequence was used to search for similar sequences in the NCBI database using BlastP (Altschul *et al.* 1997). Extensive similarity of the amino acid sequences of CbbR^{AF} with CbbR^{RE} was observed (data not shown). Predicted secondary structure alignments of the two CbbRs (Fig. 1a) and a comparison of the sequence similarity of the respective *cbbR* promoters (Fig. 1b) also demonstrated substantial similarity suggesting that *R. eutropha* might be a suitable surrogate species to explore the function of *cbbR*^{AF}. This possibility warranted further exploration because *R. eutropha* is a facultative autotroph (Cramm 2009) potentially allowing exploration of autotrophic functions of *A. ferrooxidans* and, most importantly, it can be genetically manipulated (Brigham *et al.* 2012) permitting the use of genetic analysis of cloned *A. ferrooxidans* genes.

Complementation of *R. eutropha* HB14 ΔcbbR with *A. ferrooxidans* cbbR^{AF}

Triplicate growth experiments of the transconjugants containing *cbbR*^{AF} cloned into the plasmid pBK (Fig. 2) on MM incubated under autotrophic conditions (CO_2 gas was the only source of carbon) indicated that *cbbR*^{AF} was able to complement the *R. eutropha* HB14Δ*cbbR* (Fig. 2a). In contrast, no colonies were formed under the same conditions for *R. eutropha* HB14 harboring only the vector pBK (Fig. 2b). The size and rate of colony development

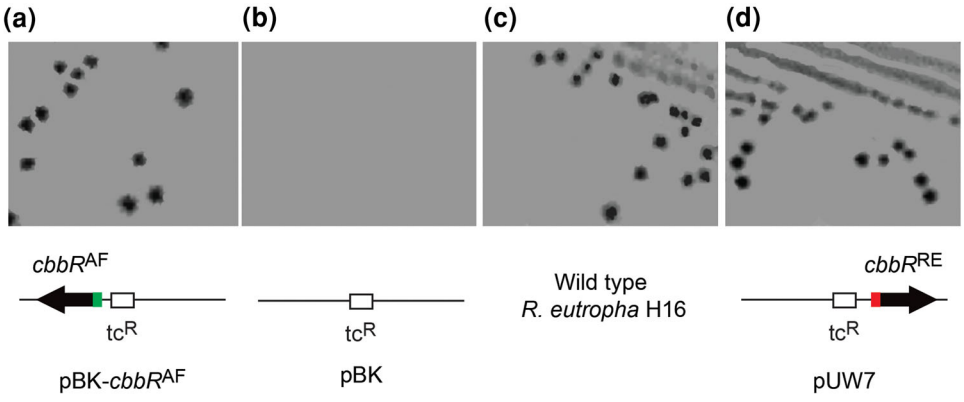


Figure 2. Complementation of the *R. eutropha* HB14 Δ*cbbR* with *cbbR*^{AF} grown under autotrophic conditions (upper panel) and the plasmid constructions used (lower panel) where (**a**) *R. eutropha* HB14 Δ*cbbR* without *cbbR*^{AF}; (**b**) *R. eutropha* HB14 Δ*cbbR*^{RE} complemented with *cbbR*^{AF}; (**c**) wild-type *R. eutropha* HB16; and (**d**) *R. eutropha* HB14 Δ*cbbR* complemented with *cbbR*^{RE} in the plasmid pUW7. The experiment was carried out in triplicate and representative images shown. Only the relevant genetic markers are represented in the plasmid drawings; for example, pBR contains a promoterless *lacZ* that is not shown here. Green box = *cbbR*^{AF} and red box = T7 promoter.

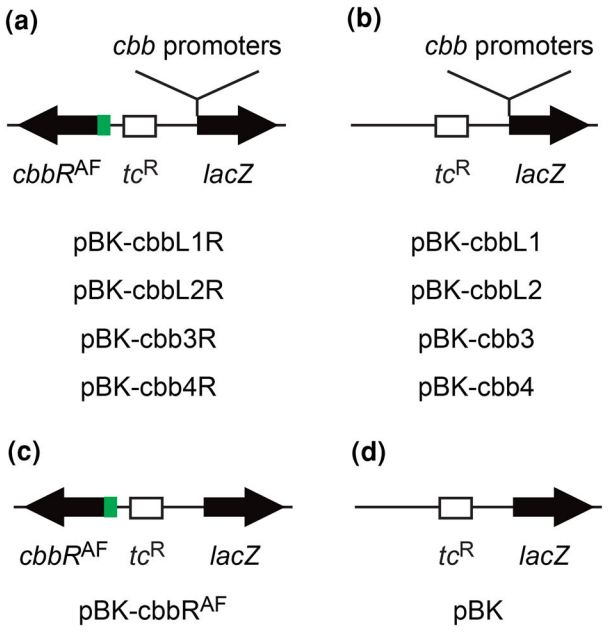
after complementation with *cbbR*^{AF} was comparable to wild-type *R. eutropha* HB16 (Fig. 2c) and by *R. eutropha* HB14 complemented with pUW7 carrying a copy of *cbbR*^{RE} (Fig. 2d). This demonstrated that *cbbR*^{AF}, functioning from its own promoter, was sufficient to restore the wild-type phenotype of *R. eutropha* HB14 under autotrophic growth conditions.

Activity of the *cbbL1* promoter from *R. eutropha* in the presence of CbbR^{AF}

Since CbbR^{AF} complemented the activity of CbbR^{RE}, the ability of CbbR^{AF} to control the expression of four *A. ferrooxidans cbbR* operons (*cbbL1*, *cbbL2*, *cbb3* and *cbb4*) within the surrogate host *R. eutropha* HB14 Δ*cbbR* strain was evaluated under autotrophic growth conditions (Fig. 3). Figure 3a–d shows the plasmid constructions that were conjugally transferred into *R. eutropha* HB14 Δ*cbbR* or into the wild-type *R. eutropha* H16 in order to test this hypothesis. The basic plasmid construction contains *cbbR*^{AF} whose expression is driven by its own promoter as described above, linked to a promoterless *lacZ* encoding β-galactosidase. Four versions of this plasmid were constructed in which the region labeled 'cbb promoters' (Fig. 3a) was occupied by one of four *A. ferrooxidans cbbR* operon promoters; the latter were placed in a position where they could potentially drive the expression of *lacZ* if activated by the binding of *cbbR*^{AF}. The specific activity of β-galactosidase detected in *R. eutropha* HB14 Δ*cbbR* after the conjugal transfer of each of these four constructions is shown in Fig. 3e. Activity was observed in all four constructions consistent with the hypothesis that CbbR^{AF} was capable of driving the expression of the *cbb* genes via its interaction with the respective cbbR promoter. Three sets of control plasmids were constructed: (i) a series containing each of the four *cbb* promoters but lacking *cbbR*^{AF} (Fig. 3b) and no growth of *R. eutropha* HB14 Δ*cbbR* was observed (Fig. 3e). This supports the earlier observation that *cbbR*^{AF} is required to support growth of *R. eutropha* HB14 Δ*cbbR*; (ii) a plasmid construction containing *cbbR*^{AF} and *lacZ* but no *cbb* promoters (Fig. 3c) and (iii) a plasmid construction containing *lacZ* but neither *cbbR*^{AF} nor the *cbb* promoters (Fig. 3d). The latter two constructions were conjugated into the wild-type *R. eutropha* HB14 resulting in the production of only 5.3 and 5.1 units of β-gal activity, respectively, supporting the idea that both the presence of the individual four cbb promoters from *A. ferrooxidans* and *cbbR*^{AF} are required to obtain the levels of β-gal activities observed in Fig. 3a.

This work establishes *R. eutropha* as a heterologous expression and analysis system for investigating the function of the CBB genetic regulator CbbR^{AF} from *A. ferrooxidans*. Since *R. eutropha* is amenable to genetic manipulation, a system has been developed that will provide an opportunity to test hypotheses regarding the functions of *A. ferrooxidans* genes involved in CO_2 acquisition and fixation and aspects of their genetic regulation. Since *R. eutropha* can oxidize hydrogen (Cramm 2009), it could also potentially serve as a surrogate host for exploring the function of genes predicted to be involved in hydrogen oxidation in *A. ferrooxidans* (Valdes *et al.* 2008).

It is now feasible to undertake mutational analyses to examine, in more detail, the function of CbbR^{AF} and the promoter regions to which it binds. Since *R. eutropha* is a facultative autotroph, it may be possible to evaluate whether metabolites such as NADPH and phosphoenolpyruvate that activate and repress the activity of CbbR^{RE}, respectively (Grzeszik *et al.* 2000; Bowien and Kusian 2002) can also regulate the function of CbbR^{AF}. Such studies would not only help advance our understanding of the function of CbbR^{AF}, but could also shed light on the features of CbbR and its regulation that respond to heterotrophic versus autotrophic metabolic signals. In addition, studies suggest that CbbR^{RE} activity in *R. eutropha* is regulated by the global two-component signal transduction RegBA system (Bowien and Kusian 2002). In *A. ferrooxidans*, RegBA has been shown to be involved in redox sensing affecting iron and sulfur energy metabolism (Moinier *et al.* 2013). Shedding light on how redox sensing could couple energy metabolism with the regulation of the energetically expensive fixation of CO_2 is of paramount importance for understanding the metabolism of *A. ferrooxidans* and its role in bioleaching.



(e)

R. eutropha host	Plasmid	β-gal sp. activity*
HB14ΔcbbR	pBK-cbbL1R	46 ± 8
	pBK-cbbL2R	208 ± 41
	pBK-cbb3R	611 ± 32
	pBK-cbb4R	1760 ± 118
	pBK-cbbL1	No growth
	pBK-cbbL2	No growth
	pBK-cbb3	No growth
	pBK-cbb4	No growth
Wild type H16	pBK-cbbR^{AF}	5.3± 0.9
	pBK	5.1± 0.8

Figure 3. (**a–d**) plasmid constructions used to test the hypothesis that CbbR^{AF} can activate the expression of the β-galactosidase gene under the control of four *A. ferrooxidans cbb* operon promoters (cbbL1, cbbL2, cbb3 and cbb4). The green colored box indicates the native *cbbR*^{RE} promoter. Details of the plasmids are provided in the text. Expression of the β-galactosidase gene after introduction of the respective plasmids into either *R. eutropha* HB14 Δ*cbbR*^{RE} or the wild-type *R. eutropha* H16. *β-Galactosidase-specific activity = activity (1 unit equals 1 μmol product/min) per mg protein used. The mean and standard deviation of three independent determinations per experiment is shown.

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Conflict of interest. None declared.

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