



# Regulation of multiple carbon monoxide consumption pathways in anaerobic bacteria

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Carbon monoxide (CO), well known as a toxic gas, is increasingly recognized as a key metabolite and signaling molecule. Microbial utilization of CO is quite common, evidenced by the rapid escalation in description of new species of CO-utilizing bacteria and archaea. Carbon monoxide dehydrogenase (CODH), the protein complex that enables anaerobic CO-utilization, has been well-characterized from an increasing number of microorganisms, however the regulation of multiple CO-related gene clusters in single isolates remains unexplored. Many species are extraordinarily resistant to high CO concentrations, thriving under pure CO at more than one atmosphere. We hypothesized that, in strains that can grow exclusively on CO, both carbon acquisition via the CODH/acetyl CoA synthase complex and energy conservation via a CODH-linked hydrogenase must be differentially regulated in response to the availability of CO. The CO-sensing transcriptional activator, CooA is present in most CO-oxidizing bacteria. Here we present a genomic and phylogenetic survey of CODH operons and *cooA* genes found in CooA-containing bacteria. Two distinct groups of CooA homologs were found: one clade (CooA-1) is found in the majority of CooA-containing bacteria, whereas the other clade (CooA-2) is found only in genomes that encode multiple CODH clusters, suggesting that the CooA-2 might be important for cross-regulation of competing CODH operons. Recombinant CooA-1 and CooA-2 regulators from the prototypical CO-utilizing bacterium *Carboxydothemus hydrogenoformans* were purified, and promoter binding analyses revealed that CooA-1 specifically regulates the hydrogenase-linked CODH, whereas CooA-2 is able to regulate both the hydrogenase-linked CODH and the CODH/ACS operons. These studies point to the ability of dual CooA homologs to partition CO into divergent CO-utilizing pathways resulting in efficient consumption of a single limiting growth substrate available across a wide range of concentrations.

**Keywords:** carbon monoxide, thermophiles, hydrogenogens, carboxydrotrophs, *Carboxydothemus hydrogenoformans*, carbon monoxide dehydrogenase, CooA

## INTRODUCTION

Anaerobic microbial CO-utilization, which was first characterized by Uffen (1976) in a mesophilic  $\alpha$ -proteobacterium, *Rhodospseudomonas* spp., is now recognized as a widespread metabolic capacity (King and Weber, 2007; Techtmann et al., 2009). Carbon monoxide is ubiquitous in geothermal environments and surprisingly common in aerobic microbial ecosystems, albeit in low concentrations (Dunfield and King, 2005; Techtmann et al., 2009). In both aerobic and anaerobic species, optimal conditions for growth span a wide range of CO concentrations. The aerobic utilization of CO via *cox* genes is extremely common and has been thoroughly studied (Meyer and Schlegel, 1983; Kraut et al., 1989; Hugendieck and Meyer, 1992; Meyer et al., 1993; Schubel et al., 1995; King, 2003a,b; King and Weber, 2007; Wu et al., 2009). The pathways to CO oxidation in aerobes via the *cox* genes are distinct from the anaerobic CO oxidation mechanisms which use variants of the enzyme CO dehydrogenase encoded by *cooS* genes (Morton

et al., 1991; Ferry, 1995; Ragsdale, 2008). These anaerobes use CO variously as a carbon and/or energy source. Energy conservation is achieved by coupling the oxidation of CO to the reduction of water or various metals. Hydrogenogenic carboxydrotrophs utilize the electrons generated from the oxidation of CO for generation of hydrogen via a membrane bound hydrogenase. This physiology is based on the water gas shift reaction  $\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2$  (Svetlitchnyi et al., 1991; Kerby et al., 1992, 1995; Sokolova et al., 2004b). Other species shunt the electrons generated from CO oxidation to the reduction of various metals, such as iron or manganese (Slobodkin et al., 1997; Sokolova et al., 2004a).

The extreme thermophile *Carboxydothemus hydrogenoformans* is the prototype thermophilic hydrogenogenic carboxydrotrophic, providing the first genome sequence of a microorganism growing on CO as sole carbon and energy source (Wu et al., 2005). Its genome is highly specialized for CO metabolism, encoding five CO dehydrogenases in different genomic

contexts, representing pathways drawing from a common pool of CO (Wu et al., 2005). Coordination of these multiple pathways must be key to the survival of CO-utilizers and is evidently effective in *C. hydrogenoformans*, which shows extraordinary adaptability, growing from very low CO concentrations up to greater than 1 atm (Svetlitchnyi et al., 1991).

The regulation of anaerobic CO metabolism is usually controlled by the positive gene regulator, CooA (Shelver et al., 1995; Fox et al., 1996; He et al., 1996, 1999; Roberts et al., 2001, 2005; Ibrahim et al., 2007). This heme protein is a member of the CRP family of positive gene regulators, represented by the cAMP binding protein (CAP) that controls hierarchical catabolite utilization in *E. coli*. Like the CAP protein, CooA binds to promoter sites upstream from the -35 box and activates transcription when bound by the activator ligand, which in the case of CooA is CO (Shelver et al., 1995, 1997). CooA is a dimeric, symmetrical protein containing two heme prosthetic groups that bind CO tightly, replacing the cAMP binding site of CAP. CO-binding induces an allosteric conformational change that results in promoter binding to a specific target sequence resulting in positive gene regulation. In many genomes multiple CODH operons coincide with the occurrence of dual CooA homologs. Here we present a genomic and phylogenetic survey of the number of CODH operons and *cooA* genes found in CooA-containing bacteria. To further our study of these genomes, the CO-dependent gene regulators were cloned and expressed from the prototype carboxydophilic *C. hydrogenoformans* to establish the basis for differential regulation of multiple CODHs in response to CO “feast” and “famine” conditions.

## MATERIALS AND METHODS

### EVOLUTIONARY ANALYSIS

CooA homologs were identified using criteria similar to those used previously (Youn et al., 2004). Multiple sequence alignment of various *cooA* genes was performed using the Muscle multiple sequence alignment program (Edgar, 2004a,b). Maximum likelihood trees were constructed using the PhyML 3.0 program (Guindon et al., 2003). Tree topology was verified by bootstrap analysis using 100 iterations.

### BACTERIAL STRAINS AND PLASMIDS

*Carboxydotherrmus hydrogenoformans* Z-2901 was isolated from Stolbovskii Spring on Kunashir Island in the Kuril Islands of Russia as described previously (Svetlitchnyi et al., 1991). *C. hydrogenoformans* Z-2901 was a gift from Vitali Svetlitchnyi (Bayreuth University, Germany) in 1995, after being serially grown in culture since its original isolation in 1990. *C. hydrogenoformans* DSMZ was purchased from the DSMZ GmbH (Braunschweig, Germany). *C. hydrogenoformans* was grown under conditions previously described in Wu et al. (2005). Standard recombinant technique was used and unless otherwise noted, *E. coli* DH5 $\alpha$ , BL21 (DE3), and VJS6737 were cultivated in liquid or solidified (15 g/l Bacto-Agar) Luria Broth. *E. coli* VJS6737 was a gift from Gary Roberts, University of Wisconsin.

### EXPRESSION AND PURIFICATION OF CooA-1 AND CooA-2

*E. coli* VJS6737 carrying a CooA-1 expression construct and purified CooA-1 were a gift from Gary Roberts (U. Wisconsin –

Madison). CooA-1 was prepared as previously described (Youn et al., 2004; Clark et al., 2006). In summary, *E. coli* VJS6737 carrying an expression plasmid encoding the *cooA-1* gene was cultivated in liquid LB supplemented with appropriate antibiotic and 9.8 mg/l ferric citrate (Sigma F3388). Cultures of the expression strain were grown in stoppered-flasks to an OD<sub>600</sub> of 0.4 and expression was induced with 0.5 mM IPTG. Expression was allowed to proceed for 3 h. Cells were harvested by centrifugation at 6000  $\times$  g for 10 min at 4°C. The cells were resuspended in Buffer A (25 mM MOPS, pH 7.4, 100 mM NaCl, 5% glycerol). The cells were lysed using a French pressure cell. The lysate was centrifuged at 20,000  $\times$  g for 20 min at 4°C. The soluble fraction was collected and loaded onto a Protino Ni-IMAC column (Macherey-Nagel, Bethlehem, PA, USA). The column was washed with 10 column volumes of Buffer A. This was followed by two washes, one with 10 ml of Buffer A plus 10 mM imidazole and another with 10 ml of 20 mM imidazole. The column was then washed with 10 ml each of higher imidazole concentrations (50, 150, and 250 mM). Fractions were screened for the presence of CooA-1 by absorbance at 425 nm and by SDS-PAGE. After the Ni-IMAC step, CooA-1 was precipitated with 50% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and resuspended in 25 mM MOPS, 0.5 M KCl, pH 7.4.

CooA-2 was purified according to a similar protocol with the following changes. After the Ni-IMAC step, CooA-2 containing fractions were pooled and loaded onto a Bio-Scale Mini Macro-Prep High S column (BioRad, Hercules, CA, USA) equilibrated with Buffer A. CooA-2 was eluted via a linear gradient from 100 to 800 mM NaCl over five column volumes at a flow rate of 1 ml/min. CooA-2 eluted around 300 mM NaCl. Presence and purity of CooA-2 were determined by SDS-PAGE. Protein concentration was determined using the Bradford assay (Bradford, 1976) using a standard curve constructed from various concentrations of BSA.

Visible spectra for both CooA-1 and CooA-2 were recorded in a Beckman DU60 spectrophotometer. CooA spectra were obtained in 25 mM MOPS, 100 mM NaCl, pH 7.4 under oxic conditions, reduced conditions (N<sub>2</sub> sparged buffer with 5 mM DTT), and reduced conditions with 1 atm CO.

### PROMOTER FRAGMENTS FOR USE IN DNA-BINDING ASSAYS

The promoter upstream of the CODH-linked hydrogenase (henceforth referred to as *pcooC<sup>hyd</sup>*) and the promoter upstream of the CODH/ACS (to be referred to as *pacs*) were examined for specific CooA binding. We identified probable regions *pcooC<sup>hyd</sup>* and *pacs* by examining the sequences 500 bp upstream of the start codon of the first gene in both the CODH-linked hydrogenase operon (*cooC<sup>hyd</sup>*) and the ACS operon (*acs*) from the genome sequence of *C. hydrogenoformans*. These sequences were run through the bprom program (<http://softberry.com>) to predict the putative -10 and -35 sites. From this prediction, primers were designed to create a product that started at the putative -150 site and stretched to start codon. The fragments were the following sizes: *pcooC<sup>hyd</sup>* was 217 bp and the *pacs* was 381 bp. These fragments were used in all of the DNA-binding experiments described below.

### DNA-BINDING ASSAYS USING SURFACE PLASMON RESONANCE

Surface plasmon resonance (SPR) experiments were carried out on the Biacore T100 at 25°C. 5'-biotinylated promoter regions were

PCR amplified. The purified biotin labeled DNA was loaded onto a streptavidin sensor chip (Biacore Sensor Chip SA). The *ccoC<sup>hyd</sup>* promoter was immobilized on flow cell 2 until the response reached 300 response units (RU). RU is the unit used to measure the amount of ligand bound to the target molecule attached to the sensor chip. Typically one RU is equivalent to 1 pg of ligand bound per mm<sup>2</sup> of the sensor chip surface. The *acs* promoter was immobilized on flow cell 4 until the response reached 500 RU. Flow cells 1 and 3 and any free streptavidin on flow cells 2 and 4 were blocked with free biotin. Flow cells 1 and 3 were used as controls to subtract out any non-specific binding.

All of the experiments were done using buffer HBS-P (10 mM HEPES, pH 7.4, 0.15 M NaCl, 10 mM MgCl<sub>2</sub>, 0.05% P20; Biacore) supplemented with 5% glycerol and 5 mM DTT. Kinetics tests were performed with either CoaA-1 or CoaA-2 at concentrations ranging from 8 to 200 nM. These CoaA solutions were prepared in anaerobic CO-saturated HBS-P supplemented with 5% glycerol and 5 mM DTT. The kinetic measurements were performed at a flow rate of 30  $\mu$ l/min with 120-s injections. Dissociation data were collected for 300 s.

#### DNA-BINDING ASSAY USING ELECTROPHORETIC MOBILITY SHIFT ASSAYS

The two promoter fragments were end-labeled as follows: *pccoC<sup>hyd</sup>* (15 pmol) and *pacS* (12 pmol); 5  $\mu$ l [ $\gamma$ <sup>32</sup>-P] ATP (10 mCi/ml; Perkin Elmer); 2  $\mu$ l 10 $\times$  polynucleotide kinase buffer (500 mM tris-HCl, pH 7.6, 100 mM MgCl<sub>2</sub> and 100  $\mu$ M  $\beta$ -mercaptoethanol); 2  $\mu$ l T4 polynucleotide kinase (10 U) were incubated at 37°C for 30 min, followed by 15 min at 70°C. End-labeled fragments were purified from unincorporated radionucleotide using the Qiaquick Nucleotide Purification Kit (Qiagen) according to the manufacturers instructions. Fragments were eluted from the filters into 100  $\mu$ l of water. The efficiency of labeling and recovery was about 20%, determined through scintillation counting. Scintillation counting revealed that the *pacS* fragment was 126,000 cpm/ $\mu$ l and the *pccoC<sup>hyd</sup>* fragment was 120,000 cpm/ $\mu$ l.

For electrophoretic gel shift analysis, 0.3  $\mu$ l of BSA (5 mg/ml), 0.15  $\mu$ l MgCl<sub>2</sub> (0.5 M), 11.5  $\mu$ l HBS-P (10 mM HEPES, pH 7.4, 0.15 M NaCl, 10 mM MgCl<sub>2</sub>, 0.005% P20; Biacore) supplemented with 5% glycerol and 5 mM DTT, and 0.5  $\mu$ g of sonicated salmon sperm DNA were mixed with 1  $\mu$ l of radiolabeled promoter fragment. To this reaction 1  $\mu$ l of a CoaA-1 or CoaA-2 protein solution was added and allowed to bind at 25°C for 30 min. Various concentrations of CoaA were used (as described in the legends of **Figure 4**). One microliter of loading dye was added to each reaction and the reactions were run on an 8% polyacrylamide gel at 150 V for 1.5 h in TBE. The gel was dried on a vacuum blotter at 60°C for 2 h and subsequently exposed to a phosphor screen overnight. The screens were imaged on a Typhoon scanner (GE healthcare).

#### CO-BINDING ASSAYS

CO-binding was determined by examining the changes in the visible absorbance spectrum for CoaA between 400 and 600 nm (Beckman DU60). Absorbance changes in the Soret wavelength

region were used to determine fractional saturation (*Y*). A stoppered glass cuvette was filled with a 2.2  $\mu$ M CoaA-1 or CoaA-2 solution in anaerobic buffer (25 mM MOPS, 100 mM NaCl, pH 7.4). The buffer was made anaerobic by repeatedly evacuating and sparging the buffer with argon. Two stock solutions were prepared, both with 5 mM DTT. One of the stock solutions was purged with argon, and the other was purged with CO. The pressure in the bottles was equilibrated to 1 atm. The concentration of the dissolved CO in the CO-purged bottle was calculated to be 1000  $\mu$ M. This was determined by using Henry's law with a Henry's law constant appropriate for 25°C of  $9.9 \times 10^{-4}$  mol CO/liter/atm CO (Lide and Frederikse, 1995) and a headspace CO partial pressure of 1 atm as described previously (Puranik et al., 2004). Using a Gastight Hamilton syringe, a 2.2  $\mu$ M solution of anaerobic CoaA was titrated with the 1000  $\mu$ M CO solution. After each addition of CO the cuvette was gently mixed and the visible spectrum was recorded. The CO solution was added until there was no change in the visible spectrum of CoaA. The maximum saturation of CoaA was determined by sparging the CoaA solution with CO for 5 min then recording the visible spectrum.

Because CO binds tightly to CoaA, the concentration of CO was corrected for the bound fraction as follows:  $[CO]_{free} = [CO]_{total} - (Y \times [CoaA]_{total})$ . Fractional saturation (*Y*) was determined experimentally by using the change in absorbance of the Soret peak relative to the no-CO spectrum

**Table 1 | Occurrence of CoaA-2 homologs and CODH/ACS operons in CO-oxidizing species.**

Species	Number of CODHs	CODH/ ACS	CoaA-2
<i>Desulfitobacterium hafniense</i> DCB-2	4	Yes	Yes
<i>Desulfitobacterium hafniense</i> Y51	4	Yes	Yes
<i>Carboxydotherrnus hydrogenoformans</i> Z-2901	5	Yes	Yes
<i>Thermosinus carboxydivorans</i> Nor1	3	No	No
<i>Carboxydotherrnus ferrireducens</i>	4	Yes	Yes
<i>Geobacter bemidjensis</i> Bem	2	No	No
<i>Thermincola</i> sp. JR	5	Yes	No
<i>Azotobacter vinelandii</i> DJ	1	No	No
<i>Rhodospirillum rubrum</i> ATCC 11170	1	No	No
<i>Rhodospseudomonas palustris</i> BisB18	1	No	No
<i>Desulfovibrio desulfuricans</i> G20	1	No	No
<i>Desulfohalobium retbaense</i> DSM 5692	1	No	No
<i>Desulfovibrio vulgaris vulgaris</i> Hildenborough	1	No	No
<i>Desulfovibrio vulgaris</i> RCH1	1	No	No
<i>Desulfovibrio vulgaris</i> DP4	1	No	No
<i>Desulfovibrio magneticus</i> RS-1	1	No	No
<i>Desulfomicrobium baculatum</i> DSM 4028	1	No	No
<i>Desulfovibrio aespoeensis</i> Aspo-2	1	No	No
<i>Desulfovibrio salexigens</i> DSM 2638	1	No	No
<i>Desulfovibrio desulfuricans</i> desulfuricans ATCC 27774	1	No	No
<i>Bacterium</i> S5	1	No	No

divided by the maximum change in absorbance ( $Y = \Delta A / \Delta A_{\max}$ ). Fractional saturation was graphed versus  $[\text{CO}]_{\text{free}}$ . The data was fitted to the Hill equation using Kaleidagraph (Synergy Software).

$$Y = \frac{[\text{CO}]^n}{K_H + [\text{CO}]^n}$$

$K_H$  is the Hill equilibrium constant. In our experiments it was the disassociation constant, proportional to the affinity of CO-binding to CooA. Here we report  $P_{50}$ , which is defined as the concentration of CO needed to occupy half of the CO-binding sites in CooA.  $n$  is the Hill coefficient, which describes the cooperativity of the binding of CO by CooA. An  $n = 1$  is indicative of a binding reaction that is non-cooperative, whereas an  $n = 2$  for a dimer is a binding reaction that is fully cooperative.

## RESULTS

### CO-OCCURRENCE OF CooA-2 WITH THE CODH/ACS

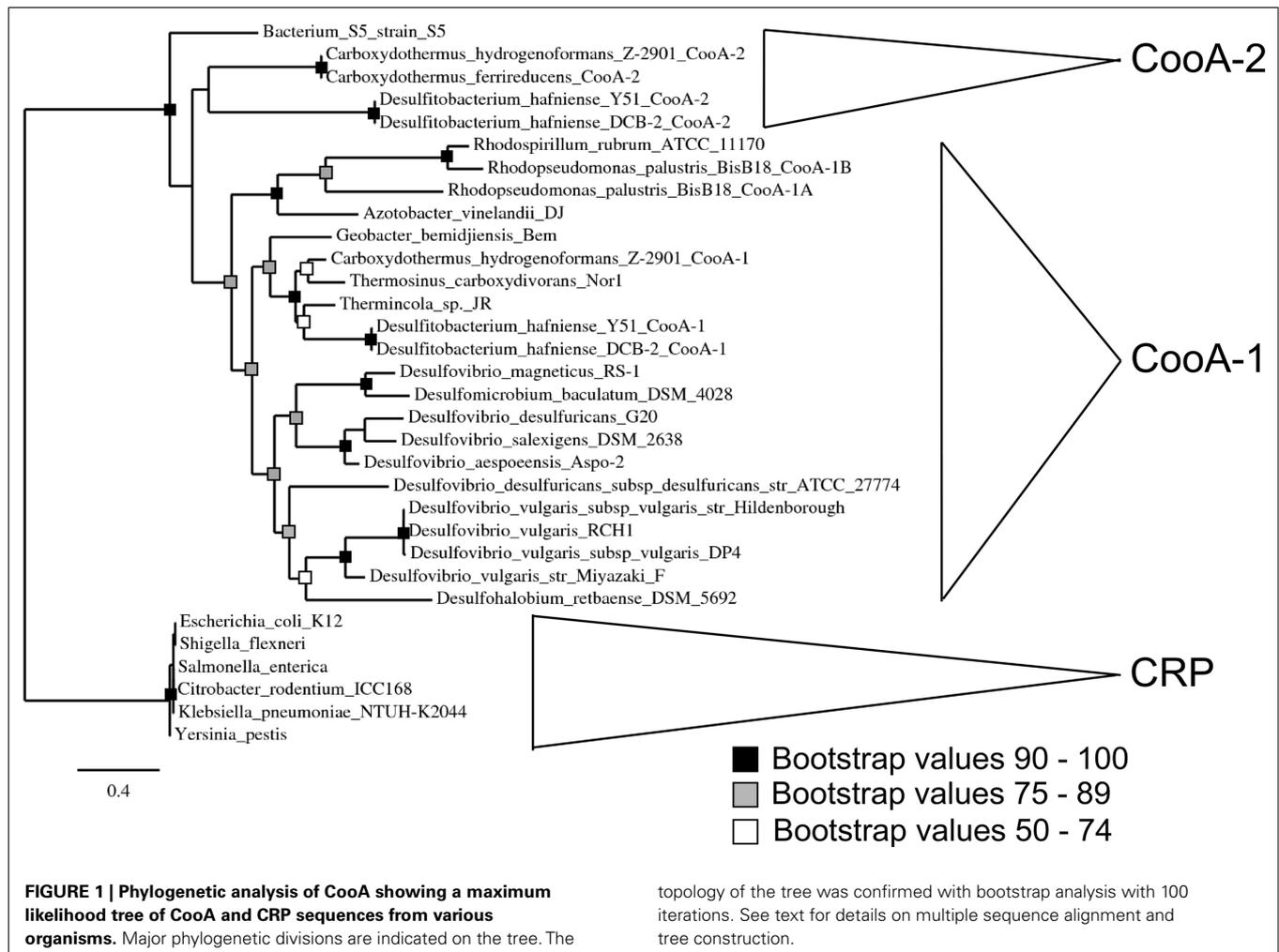
Phylogenetic analysis of the CooA regulators using a maximum likelihood algorithm revealed the presence of two distinct clades of CooAs (CooA-1 and CooA-2 in **Figure 1**). The larger clade with 21 members contains the well-characterized CooA-1 from *Rhodospirillum rubrum* and CooAs from diverse species spanning divergent bacterial phyla. In general, the CooA-1 homologs are

distributed in subclades that follow evolutionary divisions defined by 16S rDNA phylogeny (i.e., CooAs from Firmicute bacteria cluster with other Firmicute CooAs). The CooA-2 clade contains four homologs and is deeply branching relative to all CooA-1 protein sequences. The CooA-2 genes found to date are all found in the genomes of Firmicute bacteria that also encode multiple CODH operons.

**Table 1** details the copy number of CODHs contained in the genomes of *cooA* encoding bacteria. With the exception of *Thermincola* sp. JR, the genomes that encode CooA-2 also encode CODH/ACS clusters. However, the CODH/ACS cluster may also occur in species without CooA, e.g., *Moorella thermoacetica* and all the homoacetogens. *C. hydrogenoformans* was chosen as a model system to explore the role of CooA-2 in regulating the CODH/ACS since it contains all the genetic elements in the CO “toolkit.”

### IDENTIFICATION OF KEY FRAMESHIFT MUTATIONS

Further evidence supporting a role for CooA-2 in regulating the CODH/ACS was uncovered upon analysis of the *C. hydrogenoformans* genome. The strain of *C. hydrogenoformans* that was used for sequencing was *C. hydrogenoformans* Z-2901. This strain was grown serially in our laboratory over the course of several years. The standard growth medium was supplemented with pyruvate in



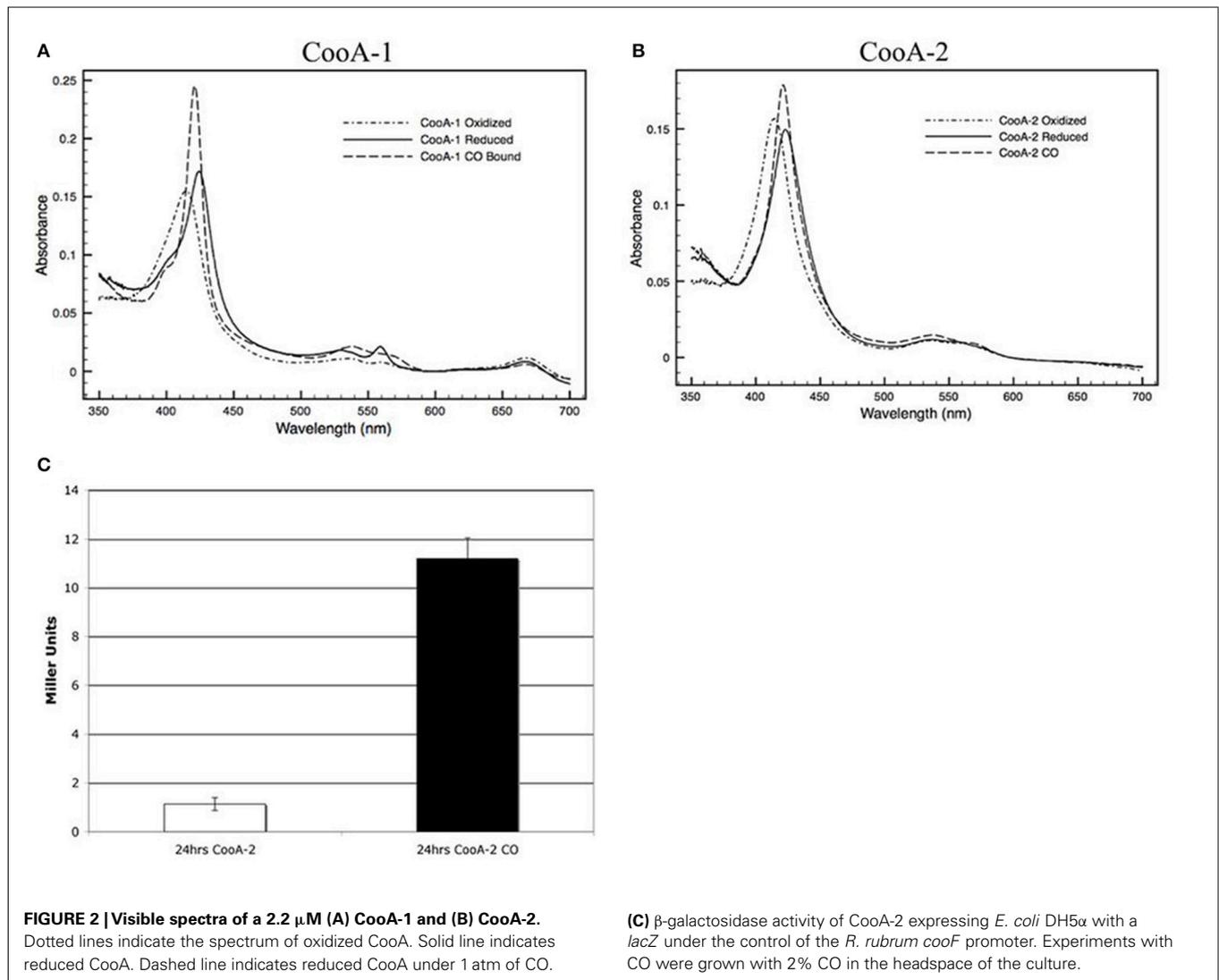
order to promote high growth yields. Manual annotation of the genome identified multiple frameshifts (Wu et al., 2005), many of which were in genes associated with proper Coenzyme A processing. Two mutations were detected in genes related to carbon monoxide oxidation. One frameshift was found in the catalytic subunit of the CODH/ACS operon (*cooS-III*; Wu et al., 2005). The *cooS-III* gene was amplified and re-sequenced from strain Z-2901, confirming that this frameshift was not a sequencing error. Additionally *cooS-III* was sequenced from the type strain of *C. hydrogenoformans*, which was deposited in the DSMZ culture collection shortly after isolation by Svetlitchnyi et al. (1991). This strain is referred to as *C. hydrogenoformans* DSMZ 6008, and maintained under non-growing conditions since 1991. The strain thus archived contained an intact *cooS-III* gene.

Upon further analysis of the *cooA* genes reported by Wu et al. (2005), it became evident that the *cooA-2* annotated in the published genome also contained a frameshift mutation, which was absent in the DSMZ strain. It appears likely that serially culturing *C. hydrogenoformans* under lab conditions (high CO and pyruvate)

resulted in mutations in the *cooS-III* and *cooA-2* genes. Concerted mutation of these genes under the same conditions suggested a possible role for CooA-2 in regulating carbon fixation via the CODH/ACS. This led to the expression and characterization of the CooA proteins in this present study.

#### EXPRESSION AND CHARACTERIZATION OF CooA-2

*E. coli* overexpressing wild type CooA-2 acquired a red color similar to the color of CooA-1 expressing *E. coli* as described in Youn et al. (2004) due to the accumulation of the heme containing protein (Figure 2D). The purified CooA-2 exhibited typical heme spectral features similar to CooA-1 with a strong Soret peak at 425 nm (Figures 2A,B). Upon addition of CO the Soret peak increased in height and was blue-shifted to 421 nm. This response is characteristic of other CO-binding heme proteins, such as hemoglobin, and has been demonstrated in all of the characterized CooA homologs (Youn et al., 2004). As an indicator of CooA regulator activity, an *E. coli* reporter strain was constructed with genomic integration of a *lacZ* gene under the



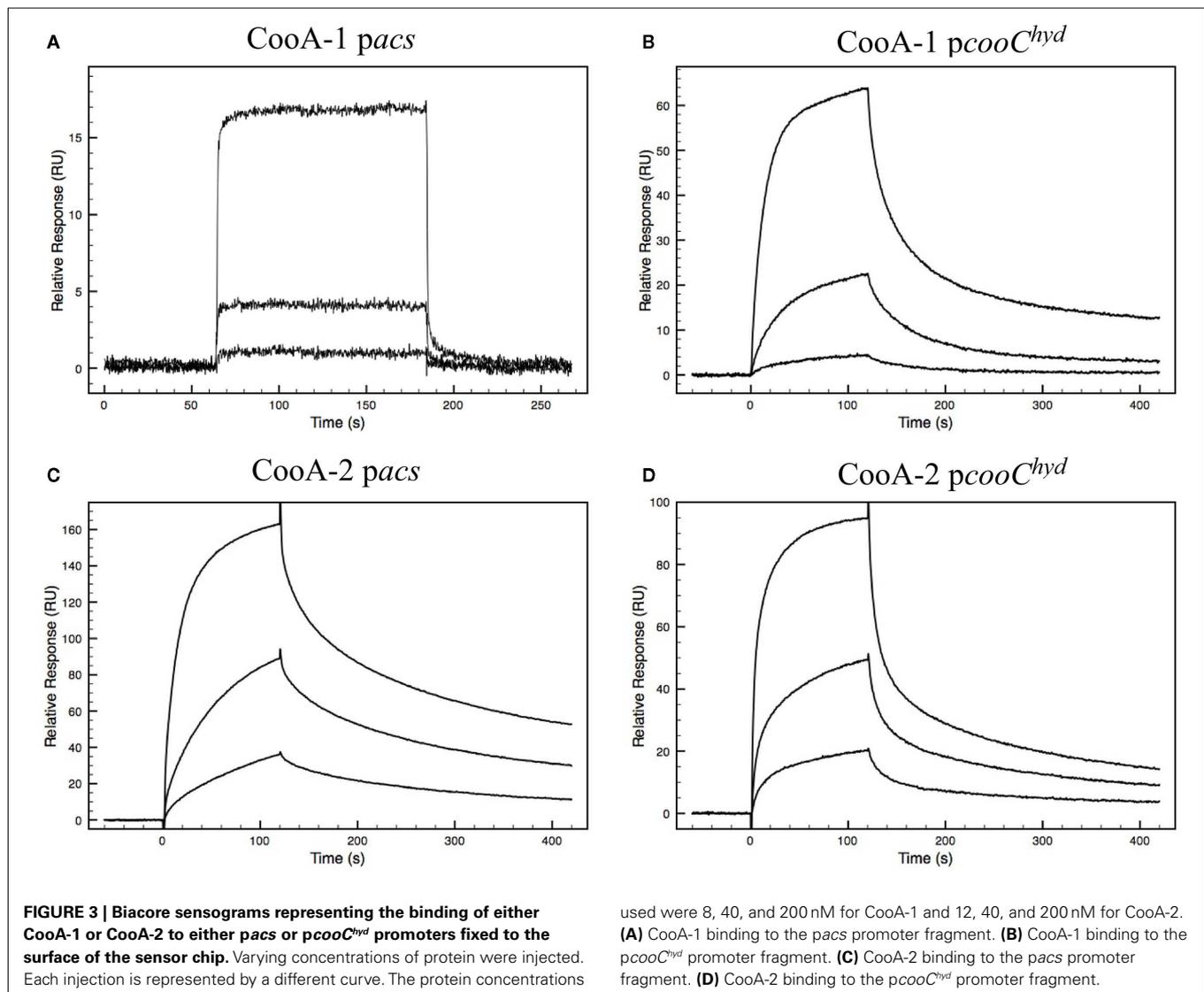
control of a CooA-responsive promoter (*R. rubrum cooF* promoter). This strain has been used to identify functional CooAs from various organisms (Youn et al., 2004). As Youn et al. (2004) have previously shown, CooA-1 is able to activate *lacZ* expression in a CO-responsive manner. CooA-2, like other CooAs, is able to activate expression of LacZ in response to CO, confirming that it is a CO-dependent transcriptional activator (Figure 2C).

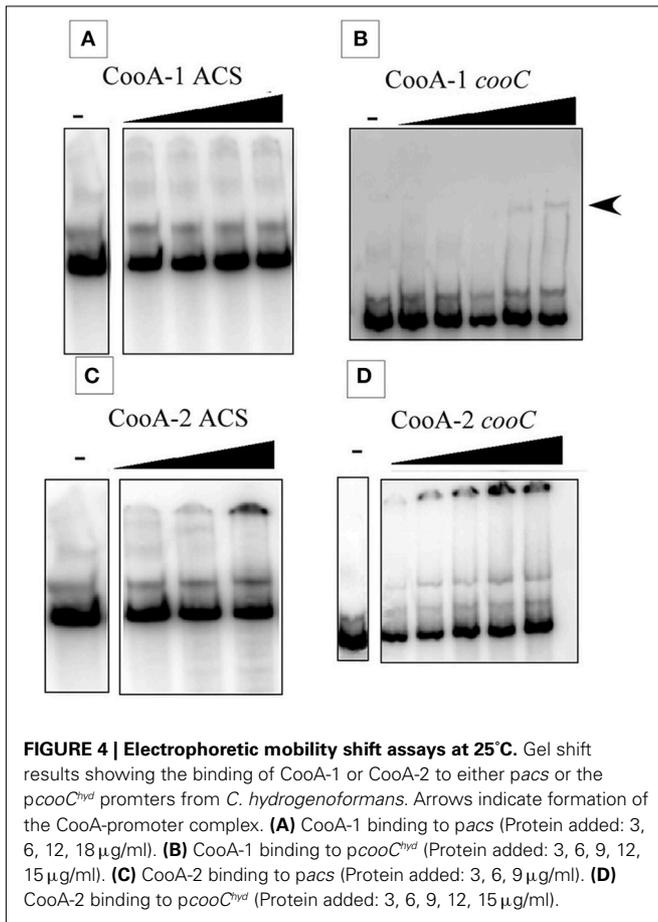
#### IDENTIFICATION OF DIFFERENTIAL PROMOTER BINDING

At first glance, the occurrence of dual CO-sensing transcriptional activators in one species appears to be a functional redundancy. To further define their physiological roles the CooA proteins were expressed, purified and tested as follows. SPR was used to measure promoter affinity and kinetic constants. The SPR binding curves for CooA-1 binding to the *cooC<sup>hyd</sup>* promoter exhibited a binding curve typically associated with specific high-affinity binding of a protein with DNA. CooA-1 accumulated on the DNA during the injection and then slowly disassociated after the injection was stopped (Figure 3B). However the binding of CooA-1 to the *acs*

promoter demonstrated a binding curve typical of non-specific low-affinity binding. In this case the overall RU were much lower than with CooA-1 binding to the hydrogenase promoter and the signal dropped instantaneously upon termination of the injection (Figure 3A). The binding of CooA-2 to both promoters tested is typical of specific high-affinity binding (Figures 3C,D). Classical electrophoretic mobility shift assays (EMSA; Figure 4) confirmed the affinity order and lack of binding of CooA-1 to *acs* promoter. Affinity constants were determined by fitting the SPR binding data to a two-state binding model (Figure 3). The use of this model was supported by the results of a linked-reactions test. CooA-1 bound with extremely low-affinity to *pacs*, and did not fit a binding model in a meaningful way typical of the non-specific binding of regulator proteins to DNA sequences unrelated to their cognate target motifs.

The fitted SPR data confirms that CooA-1 binds to the hydrogenase promoter with a  $K_D$  of 82.8 nM. CooA-2 bound to both of these promoters with higher affinity (*pcooC<sup>hyd</sup>*:  $K_D$  = 30.7 nM; *pacs*:  $K_D$  = 16.8 nM; Figure 3; Table 2). These constants resemble





**FIGURE 4 | Electrophoretic mobility shift assays at 25°C.** Gel shift results showing the binding of CooA-1 or CooA-2 to either *pacs* or the *pccoC<sup>hyd</sup>* promoters from *C. hydrogenoformans*. Arrows indicate formation of the CooA-promoter complex. **(A)** CooA-1 binding to *pacs* (Protein added: 3, 6, 12, 18 µg/ml). **(B)** CooA-1 binding to *pccoC<sup>hyd</sup>* (Protein added: 3, 6, 9, 12, 15 µg/ml). **(C)** CooA-2 binding to *pacs* (Protein added: 3, 6, 9 µg/ml). **(D)** CooA-2 binding to *pccoC<sup>hyd</sup>* (Protein added: 3, 6, 9, 12, 15 µg/ml).

**Table 2 | Biacore kinetic and affinity data for the binding of CooA-1 and CooA-2 to the *cooC<sup>hyd</sup>* and the *acs* promoter regions.**

Parameter	CooA-1 <i>cooC</i>	CooA-2 <i>cooC</i>	CooA-2 <i>acs</i>
$k_a^1$ (1/Ms)	$3.186 \times 10^5$	$2.823 \times 10^6$	$7.460 \times 10^5$
$k_d^1$ (1/s)	0.06273	0.2061	0.04123
$k_a^2$ (1/s)	0.004339	0.005667	0.008221
$k_d^2$ (1/s)	0.033120	0.004122	0.003576
$K_D$ (nM)	82.8	30.7	16.8
$R_{max}$	110.4	112.3	178.7
$\chi^2$	0.536	3.4	5.07

$k_a^1$  and  $k_d^1$  are the kinetic constants for the first binding event.  $k_a^2$  and  $k_d^2$  are the kinetic constants for the second binding event.  $R_{max}$  is a measurement of the stoichiometry of the binding of the CooA protein to the DNA. These  $R_{max}$  values are consistent with one binding site per promoter.  $\chi^2$  is a statistical measurement for how well the fit matches the experimental data. These  $\chi^2$  values indicate that this model fits the data relatively well.

the semi-quantitative data obtained in the EMSA experiment, but with greater precision and control of CO concentration (Figure 4). Table 2 describes both the kinetic and affinity data for CooA-1 and CooA-2 binding.

## DETERMINATION OF CO-BINDING CONSTANTS

The variable binding affinities of the CooA proteins for promoters suggested that they might regulate the CODH operons of *C. hydrogenoformans* differentially in high and low CO concentrations. To detect CO-binding, CooA-1 was titrated with CO at pH 7.4 at 25 and 70°C while monitoring the visible spectrum (Figures 5A,B). Upon addition of CO to CooA-1 the Soret peak increased in amplitude until saturation. By measuring the changes in peak height in relation to [CO], both binding constants and cooperativity were determined. Spectral changes in the Soret peak were used to determine the fractional saturation ( $Y$ ), which in turn was used to determine  $P_{50}$  as described in Section “Materials and Methods” (Figures 5 and 6).  $P_{50}$  is the concentration of CO at which half of the CO-binding sites are bound by CO. The value of  $P_{50}$  for CooA-1 at 25°C was determined to be  $17.4 \pm 0.5 \mu\text{M}$ , whereas at 70°C it was determined to be  $5.6 \pm 0.2 \mu\text{M}$ . CooA-2 was titrated with CO at 25 and 55°C (Figures 6A,B). For CooA-2, the  $P_{50}$  at 25°C was  $6.2 \pm 0.2 \mu\text{M}$  and at 55°C the  $P_{50}$  is  $1.9 \pm 0.1 \mu\text{M}$  (Figures 6C,D). The temperature used for CooA-2 analysis was 55°C because CooA-2 was unstable at 70°C. For comparison the  $P_{50}$  for *R. rubrum* CooA-1 to CO at 25°C is  $2.2 \mu\text{M}$  (Puranik et al., 2004).

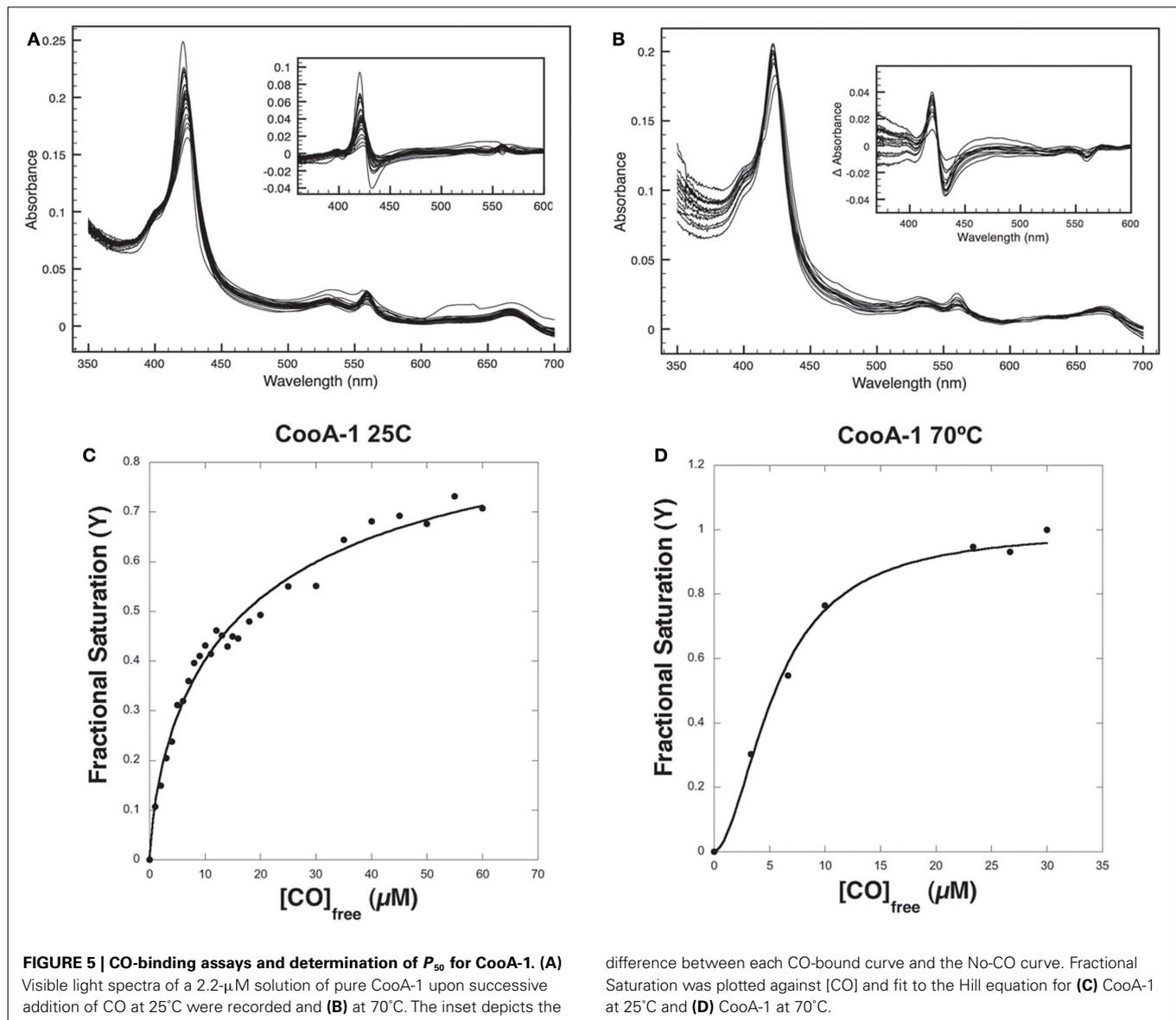
## COOPERATIVITY OF CO-BINDING DIFFERS BETWEEN THE TWO REGULATORS

Multisubunit heme proteins may bind to their ligands with cooperative kinetics following the classic model for allosteric intersubunit interactions first described by Hill (1910) for hemoglobin. The method for determining cooperative kinetics is by application of the Hill equation as described in Section “Materials and Methods” (Hill, 1910). Table 3 summarizes the CO-binding data for both CooA-1 and CooA-2. From the Hill equation it was determined that at 25°C CooA-1 had  $n = 0.73 \pm 0.03$  indicating non-cooperative binding or negative cooperativity, whereas CooA-2 had an  $n$  value of  $1.8 \pm 0.11$  (Figures 5C and 6C) indicating a significantly cooperative process. Cooperativity of CO-binding by CooA-1 was different at a higher temperature corresponding to the normal growth conditions. At 70°C the  $n$  value for CooA-1 increased to approximately  $1.8 \pm 0.2$  (Figure 5D). The  $n$  value for CO-binding to CooA-2 at 55°C does not change significantly from 25°C with  $n = 1.6 \pm 0.1$  (Figure 6D).

## DISCUSSION

The ability of an organism to use CO as an electron source for divergent cellular processes may be essential for growth on CO as a sole carbon and energy source. Our work points to a new paradigm for efficient CO-utilization in bacteria. This analysis uses both genomic and biochemical techniques to demonstrate the importance of multiple CO-utilization pathways within *C. hydrogenoformans*.

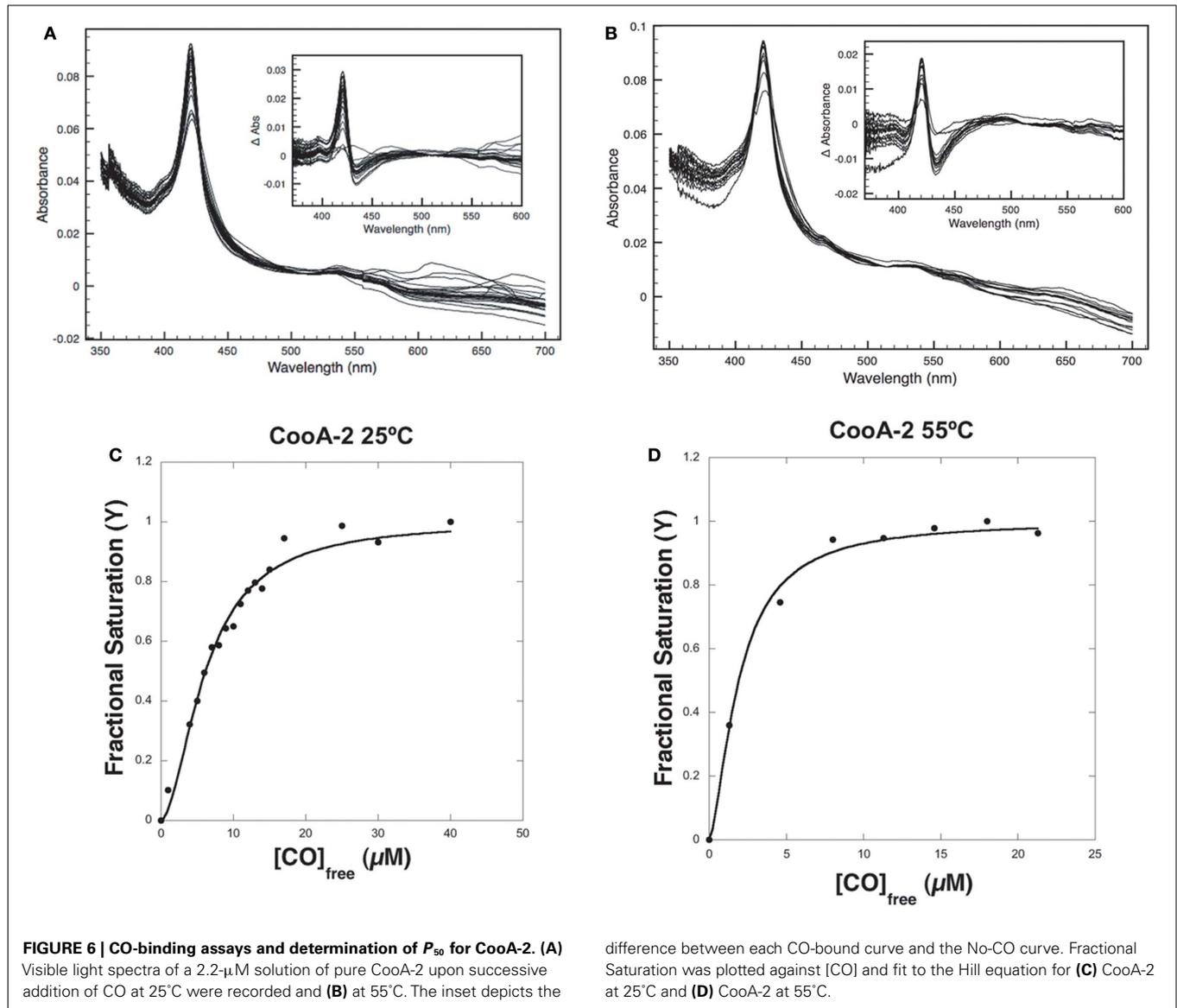
Many species that encode a CooA homolog embody a primary physiology that does not include CO, but their possession of *cooS* and *cooA* genes imply underlying or backup CO-dependent physiologies. As an example, *C. ferrireducens* was first isolated as a primary metal reducer growing on fumarate and originally named *Thermoterrabacterium ferrireducens* (Slobodkin et al., 1997). Its identical 16S rRNA sequence to *C. hydrogenoformans* led to its



renaming as *C. ferrireducens* (Slobodkin et al., 2006). In collaboration with the Joint Genome Institute, we are currently sequencing the genome of *C. ferrireducens*. This draft sequence revealed that *C. ferrireducens* retains four of the five CODH operons found in *C. hydrogenoformans* and is only lacking the hydrogenase-linked CODH (CODH-I) of *C. hydrogenoformans*. Additionally *C. ferrireducens* is missing the *cooA-1* but not *cooA-2*. Furthermore, physiological studies revealed the ability of *C. ferrireducens* to grow on CO in the presence of an alternative electron acceptor (Henstra and Stams, 2004). The lack of a CODH-I and a CooA-1 in *C. ferrireducens* further supports the link between the CooA-1 clade of CooAs and the hydrogenase-linked CODH operon. Furthermore, *C. hydrogenoformans* is an interesting case where CooA-2 is the primary CO-responsive regulator, suggesting perhaps that CooA-2 is able to efficiently regulate the all of the remaining CODH operons.

Monofunctional CODHs are capable of driving many processes from the same substrate, and it is not surprising that they occur in many prokaryotic lineages capable of diverse metabolism. The CODH-linked hydrogenase module is the signature gene cluster predicting hydrogenogenic carboxydotorophy. This complex has been found in bacteria ranging from thermophilic Firmicutes to mesophilic photosynthetic purple non-sulfur bacteria such as *R. rubrum*, an  $\alpha$ -Proteobacterium. The bacterial lineages encoding this complex have recently been joined by archaea in the form of three *Thermococcus* sp. (*Thermococcus* sp. AM4, Sokolova et al., 2004b; *Thermococcus onnurineus*, Lee et al., 2008; Kim et al., 2010; and *Thermococcus barophilus*, Kim et al., 2010).

**Table 1** shows the occurrence of multiple CODH operons in *cooA*-containing carboxydotorophs. The range of organisms that encode multiple CODH operons had not been fully



**Table 3 | Disassociation constants and cooperativity data for CO-binding to CooA-1 or CooA-2 as determined from fitting the changes in fractional saturation to the Hill equation.**

Constant	$P_{50}$	$n$	$R^2$
CooA-1 (25°C)	17.4 $\mu\text{M}$ ( $\pm 0.5$ )	0.73 ( $\pm 0.03$ )	0.9912
CooA-2 (25°C)	6.2 $\mu\text{M}$ ( $\pm 0.2$ )	1.8 ( $\pm 0.1$ )	0.99193
CooA-1 (70°C)	5.6 $\mu\text{M}$ ( $\pm 0.2$ )	1.8 ( $\pm 0.2$ )	0.99741
CooA-2 (55°C)	1.9 $\mu\text{M}$ ( $\pm 0.1$ )	1.6 ( $\pm 0.1$ )	0.99706
<i>R. rubrum</i> CooA	2.2 $\mu\text{M}$	1.4	–

appreciated until now and includes both bacteria and archaea. This is prevalent in the *Peptococcaceae* family, including species such as *C. hydrogeniformans*, *Thermincola* sp. JR, and the *Desulfitobacterium* genus. Five species encode either four or five CODH operons and *C. hydrogeniformans* and *Thermincola*

sp. JR 1 have five CODH operons apiece (Wu et al., 2005; Byrne-Bailey et al., 2010).

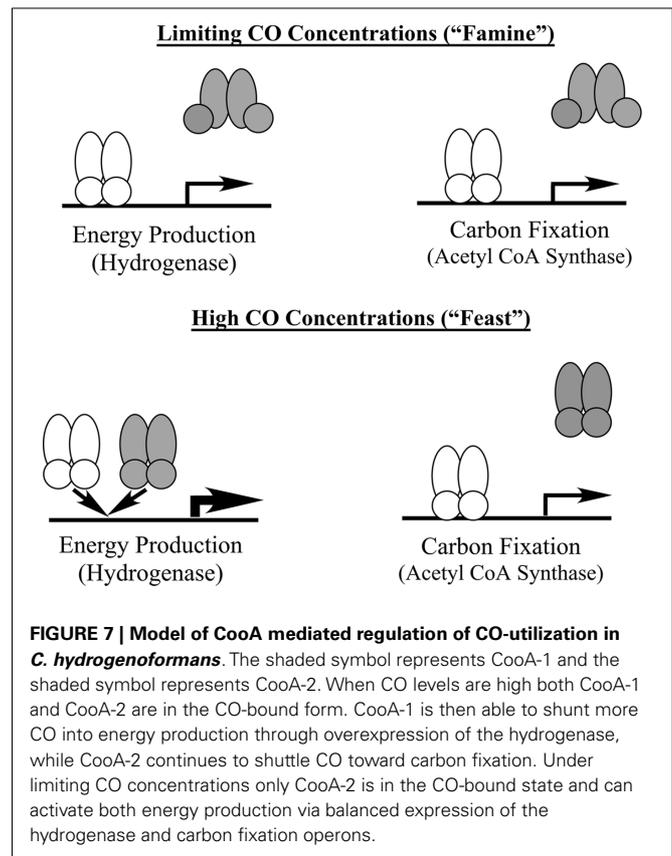
The pathways encoded by these operons constitute different and competing destinations for a single feedstock, CO, and we considered it probable that coordinated regulation of the transcription and activity of the CODHs may have evolved to allow for efficient CO-utilization. The CO-sensing transcriptional activator – CooA – is the most well studied CO-responsive regulator. Therefore, it is important to understand the responses of the CooA dependent regulation to different levels of CO. Interestingly, bacteria encoding multiple CODH operons also encode multiple CooA homologs that fall into two distinct clades (**Figure 1**). In this context, our original finding that CooA-2 and CooA-1 both regulated the *cooChyd* promoter linked to a *lacZ* gene in *E. coli* (**Figure 2C**) was an unexpected and seemingly anomalous result.

In an effort to understand the functional difference between members of the two subclasses of CooA, the CooA-1 and CooA-2 proteins from *C. hydrogeniformans* were expressed, purified, and

characterized. Initial work by the Roberts group at the University of Wisconsin characterized CooA in *R. rubrum*. In these elegant studies, He et al. (1996, 1999) demonstrated that the *R. rubrum* CooA acted as a positive regulator of the hydrogenase-linked CODH in *R. rubrum*. Further work by Youn et al. (2004) described the ability of *C. hydrogenoformans* CooA-1 to activate *lacZ* expression in a CO-responsive manner in an *E. coli* reporter strain. Our work expands on this previous work by examining the promoter binding of CooA-1. Here we demonstrated that CooA-1 is a specialized regulator specific for the monofunctional CODH-linked to the CO-tolerant hydrogenase found in CO-dependent hydrogenogens. Based on phylogenetic analysis, the *C. hydrogenoformans* CooA-1 and the *R. rubrum* CooA are members of the CooA-1 clade whereas *C. hydrogenoformans* CooA-2 is a member of the CooA-2 clade. Therefore, the finding that *C. hydrogenoformans* CooA-1 regulates the CODH/hydrogenase operon like *R. rubrum* CooA is not surprising. The inability of *C. hydrogenoformans* CooA-1 to bind to the promoter of the CODH/ACS operon was unexpected.

The CooA-2 from *C. hydrogenoformans* is a bifunctional regulator and is capable of activating the hydrogenase operon as well as the CODH/ACS operon at low CO partial pressures. CooA-2 was also shown to be able to bind CO cooperatively with a  $P_{50}$  value of  $1.9 \mu\text{M}$ , about three-fold lower than CooA-1 ( $6.2 \mu\text{M}$ ). These data indicate that the functional difference between the CooA-1 and CooA-2 in *C. hydrogenoformans* is not only their promoter specificity, but also their threshold concentration for CO activation.

Our working model for the interplay between these regulatory elements is shown in Figure 7. Under “feast” conditions represented by an intracellular concentration of  $\text{CO} > 6 \mu\text{M}$ , both CooA-1 and CooA-2 are fully activated and hydrogen production and acetyl CoA production are fully induced, corresponding to exponential growth in *ad libitum* CO. As CO becomes limiting (approaching famine, less than  $2 \mu\text{M}$  CO in the cytoplasm), hydrogen production and energy conservation are limited in favor of acetyl CoA production and C-fixation. This mechanism allows *C. hydrogenoformans*, and likely the other species encoding CooA-2 homologs, to modulate expression of the CODH operons in ratios appropriate to direct the CO flux for optimal growth and survival across a wide range of CO concentrations. As we have described previously, *C. hydrogenoformans* can grow under a headspace of 1–2 atm of pure CO, which is lethal to most microbial species (Techtmann et al., 2009). The ability to “convert” excess CO to  $\text{H}_2$  under toxic CO stress conditions would benefit CO-sensitive neighbor species in the geothermal mat community. *C. hydrogenoformans* has been the subject of extensive work characterizing its ability to grow synergistically with CO-sensitive sulfate reducing bacteria. In these co-cultures *C. hydrogenoformans* acts as a safety valve to disperse CO, producing hydrogen which is an essential substrate for sulfate reducers (Parshina et al., 2005). Thus the ability to consume CO and produce  $\text{H}_2$  is potentially a community asset, which would explain the relatively high counts ( $10^{4-6}$  cells/ml) of carboxydrotrophic bacteria recorded in hot spring effluent mats (Slepova et al., 2007), as well as the relative ease of isolation of CO oxidizer strains from widespread geothermal sites (Robb et al., 2005; Techtmann et al., 2009). In a recent review we have described how a typical geothermal mat community might use



CO as a “currency” metabolite that can be exchanged for other key metabolites such as  $\text{H}_2$ ,  $\text{CH}_4$ , and  $\text{H}_2\text{S}$  (Techtmann et al., 2009).

This study revealed the concerted laboratory evolution resulting in a null mutation of the CODH/ACS operon and its cognate CooA-2 regulator in response to the relaxation of selective pressure to use CO as a sole carbon source. Paralogous classes of CooA regulators were shown to be capable of regulating different CODH operons in trans. This accentuates the dynamic genome evolution probably taking place in geothermal communities, which represents the majority of CO-utilizing microorganisms isolated to date. Finally, we have characterized the kinetics of CO-binding in the CooA-1 and CooA-2 proteins and can assign them to coarse and fine control ranges that allow the efficient distribution of a single metabolic feedstock into carbon and energy metabolism. So far, little has been published concerning the metabolic crosstalk involving CO/ $\text{H}_2$  interconversion in microbial mats and biofilms, which may be very significant given the key roles that hydrogen and CO may play as energy sources in geothermal microbial systems (Spear et al., 2005; Shock et al., 2010).

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- that could be construed as a potential conflict of interest.

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