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Biochemistry and Physiology of the β Class Carbonic Anhydrase (Cpb) from *Clostridium perfringens* Strain 13

R. Siva Sai Kumar,[a] William Hendrick,[b] Jared B. Correll,[c] Andrew D. Patterson,[c] Stephen B. Melville,[b] James G. Ferry[a]

Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania, USA[a]; Department of Biological Sciences, Virginia Tech, Blacksburg, Virginia, USA[b]; Department of Veterinary and Biomedical Science, Pennsylvania State University, University Park, Pennsylvania, USA[c]

The carbonic anhydrase (Cpb) from *Clostridium perfringens* strain 13, the only carbonic anhydrase encoded in the genome, was characterized both biochemically and physiologically. Heterologously produced and purified Cpb was shown to belong to the type I subclass of the β class, the first β class enzyme investigated from a strictly anaerobic species of the domain *Bacteria*. Kinetic analyses revealed a two-step, ping-pong, zinc-hydroxide mechanism of catalysis with K_m and k_{cat}/K_m values of 3.1 mM CO_2 and 4.8 × 10^6 s^{-1} M^{-1}, respectively. Analyses of a *cpb* deletion mutant of *C. perfringens* strain HN13 showed that Cpb is strictly required for growth when cultured in semidefined medium and an atmosphere without CO_2. The growth of the mutant was the same as that of the parent wild-type strain when cultured in nutrient-rich media with or without CO_2 in the atmosphere, although elimination of glucose resulted in decreased production of acetate, propionate, and butyrate. The results suggest a role for Cpb in anaplerotic CO_2 fixation reactions by supplying bicarbonate to carboxylases. Potential roles in competitive fitness are discussed.

Carbonic anhydrase (CA) is a metalloenzyme catalyzing the reversible interconversion of CO_2 and bicarbonate ($CO_2 + H_2O = HCO_3^- + H^+$). To date, five independently evolved classes (αβγζδ) are known (1). The β class is abundant in higher plants and photosynthetic algae of the domain *Eukarya* (2). Several β class CAs from the domain *Bacteria* have been described, including those from photosynthetic (3–5) and nonphotosynthetic species of which several are pathogenic (6–10). The β class CAs of photosynthetic species function to concentrate atmospheric CO_2, whereas it is proposed that nonphotosynthetic species utilize β class CAs to retain intracellular CO_2 by conversion to bicarbonate for anaplerotic carboxylation reactions (11–14). The only characterized β class CA from the domain *Archaea* (Cab) is that from *Methanothermobacter thermoautotrophicus* (formerly *Methanobacterium thermoautotrophicum* strain ΔH) (15–18), a microbe that obtains energy for growth by reducing CO_2 to methane. It is proposed that Cab functions to concentrate CO_2 for both methanogenesis and anaplerotic carboxylation reactions.

CAs are important for survival and growth of several pathogens and, therefore, a potential target for development of novel antimicrobial agents (19–22). *Clostridium perfringens* strains are ubiquitous, strictly anaerobic pathogens that inhabit many environmental niches, including soil, sediments, and the intestinal tracts of mammals (23). In humans, different strains of *C. perfringens* cause gas gangrene (myonecrosis), acute food poisoning, and necrotic enteritis. The *CPE0413* gene of strain 13 is annotated as encoding a probable CA (Comprehensive Microbial Resource [http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi]) that has yet to be investigated for enzymatic activity or physiological function. Here, we report the results of a phylogenetic, biochemical, and physiological characterization of the protein encoded by *CPE0413*. The results show that the protein is a type I β class CA, the first biochemical and physiological investigation of a CA from the genus *Clostridium*. The results further reveal that Cpb is essential for growth in semidefined media with no added CO_2, suggesting that Cpb functions in nature to retain intracellular levels of CO_2 and supply bicarbonate for anaplerotic reactions.

MATERIALS AND METHODS

Bacterial strains, growth conditions. Bacterial strains, plasmids, and primers used in this study are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani (LB) medium supplemented with antibiotics as needed: 400 μg/ml erythromycin, 100 μg/ml ampicillin, or 20 μg/ml chloramphenicol. *Clostridium perfringens* was grown in a Coy anaerobic chamber at 37°C. Four different media were used for growth of *C. perfringens*: brain heart infusion (BHI) medium (Difco), TY medium (3% tryptone, 2% yeast extract, 0.1% sodium thioglycolate), PGY medium (3% proteose peptone 3, 2% glucose, 1% yeast extract, 0.1% sodium thioglycolate) and semidefined media (0.1% yeast extract, 50 mM glucose, 50 mM $NaKPO_4$ [pH 7.0], 19.79 mg/liter $MnCl_2 \cdot 4H_2O$, 122 mg/liter $MgCl_2 \cdot 6H_2O$, 29.4 mg/liter $CaCl_2 \cdot 2H_2O$, 11.5 mg/liter $ZnSO_4 \cdot 7H_2O$, 0.025 mg/liter $CuSO_4 \cdot 5H_2O$). Strains were first inoculated onto plates containing BHI medium (with chloramphenicol for growth of the complemented strain) incubated in a 100% nitrogen atmosphere and then inoculated onto two identical agar plates containing semidefined media, one incubated in the anaerobic chamber (10% CO_2) and the other in an anaerobic jar containing 100% nitrogen.

In-frame deletion of gene *CPE0413* (*cpb*). Mutagenesis of *C. perfringens* HN13, a derivative of *C. perfringens* strain 13, was performed as previously described (24). Briefly, overlap extension PCR using primers OWH9, OWH10, OWH11, and OWH12 was used to amplify ~900-bp regions flanking *CPE0413* (*cpb*). The resulting DNA fragment was cloned into pGEM-T Easy (pWH5) and then pCM-GALK (24), yielding pWH6. In-frame deletion mutagenesis was then performed using this construct (24), resulting in a deletion of the region from 18 to 555 bp out of the 570-bp *cpb* gene reading frame; this strain was named WH1. The muta-

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Address correspondence to James G. Ferry, jgf3@psu.edu.
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TABLE 1 Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s) or sequence (5′ to 3′)	Source or reference
Strains		
E. coli DH10B	F$^-$ *mcrA*Δ *mrr-hsdRMS mcrBC* ϕ80d*lacZ*ΔM15 *lacX74 deoR recA1 araD139*Δ *ara leu7697 galU galK* Δ*rpsL endA1 nupG*	Gibco/BRL
C. perfringens		
HN13	Strain 13; Δ*galKT*	24
WH1	Strain HN13 with deletion of the region from bp 18 to 555 in *cpb*	This study
Plasmids		
pGEM-T Easy	PCR cloning vector; ampicillin resistance	Promega
pKRAH1	Contains *bgaR*-P_{bgaL}; chloramphenicol resistance	25
pCM-GALK	Contains a *Clostridium beijerinckii galK* gene under the control of a ferredoxin promoter from *C. perfringens*	24
pET22b	Protein production vector	Novagen
pCPE0413	*cpb* gene in pET22b	This study
pWH5	pGEM-T Easy with overlapping PCR product of DNA flanking the *cpb* gene	This study
pWH6	pCM-GALK with *cpb* deletion fragment	This study
pWH7	pGEM-T Easy with *cpb* gene from HN13	This study
pWH8	pKRAH1 with *cpb* gene from HN13	This study
Primers		
CA forward	TTTTTTCATATGGAAAAGAATGTGAGAAGACTAGAAG	
CA reverse	TTTTTTAAGGTTATTCGTATCCATTTACTATAACCTTAAGTTCTCC	
OWH9	GTCGACTTCACAAATCAATAGAAGAAATTGAAG	
OWH10	GTTTTTTAATTTTTATTTTATTCGTATCCATTCACATTCTTTTCCATACTAAATACC	
OWH11	GGTATTTAGTATGGAAAAGAATGTGAATGGATACGAATAAAATAAAAATTAAAAAAC	
OWH12	GGATCCCTTTTTGTTTATTTTCAATTCCAAATATAC	
OWH13	AATGAAAAAGGAGATAAAACTCCAG	
OWH14	CCATATACAGGACTAATTGGTCC	
OWH15	GTCGACGTGGGTTTAAAGTAAAGACTTTAAATC	
OWH24		

tion was confirmed using PCR with flanking primers OWH13 and OWH14.

Complementation. To complement the *cpb* mutation in strain WH1, a plasmid was constructed by amplifying the *cpb* gene plus 134 bp upstream with primers OWH15 and OWH24, cloning the *cpb* gene into plasmid pGEM-T Easy (pWH7) and then into pKRAH1 (pWH8), which placed the gene under the control of the lactose-inducible promoter, P_{bgaL} (25). Plasmids were introduced into strain WH1 by electroporation (26).

Cloning and heterologous production of Cpb. The *CPE0413* gene encoding Cpb was amplified by PCR using *Clostridium perfringens* strain 13 genomic DNA as a template. The forward primer (CA forward) partially corresponds to nucleotides encoding amino acids 1 to 9, and the reverse primer (CA reverse) corresponds to nucleotides 536 to 572 downstream of the beginning of the gene (Table 1). NdeI and HindIII restriction sites were introduced in the forward and reverse primers, respectively. The PCR product was digested with NdeI and HindIII and cloned into the digested pET22b(+) vector (Novagen) to yield pCPE0413. This constructed plasmid was transformed into *E. coli* strain Rosetta (DE3) p*Lac*I (Novagen) which was used to inoculate Luria-Bertani broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The cells were grown at 37°C to an A_{600} of 0.6 to 0.8 and then induced with 1 mM isopropyl thiogalactopyranoside (IPTG) and 500 μM $ZnSO_4$ to overproduce Cpb. Induced cultures were allowed to continue growth for 4 to 5 h. The cells were harvested by centrifugation and stored frozen at −80°C until lysis.

Enzyme purification. Cell paste (5 g [wet weight]) was thawed in 30 ml of buffer A (50 mM potassium phosphate [pH 7.0]) containing 0.1 mM zinc and passed twice through a chilled French pressure cell at 138 MPa. The cell debris was removed by centrifuging the cell lysate at 29,000 × *g* for 30 min. After centrifugation, the supernatant was passed through a 0.45-μm filter before loading onto a Q-Sepharose (GE Healthcare) column equilibrated with buffer A. The column was developed with the same buffer to elute unbound protein. Bound proteins were then eluted with a linear gradient (0 to 1 M) of NaCl in buffer A. The fractions with CA activity were concentrated and chromatographed on a Sephadex G-150 superfine gel filtration column (GE Healthcare) using buffer B (50 mM potassium phosphate [pH 7.0]) containing 0.1 M NaCl. Fractions with CA activity were pooled and stored at −20°C. CA activity was measured at room temperature by using the electrometric method described previously (27).

Enzyme activity. Steady-state CO_2 hydration activity was measured by stopped-flow spectroscopy using the changing pH indicator method described previously (28). Saturated solutions of CO_2 (32.9 mM) were prepared by bubbling CO_2 into distilled, deionized water at 25°C. The CO_2 concentration was varied from 5 to 27 mM by mixing with an appropriate volume of N_2-saturated water. The buffer/indicator pairs (the wavelengths used shown in parentheses) were morpholineethanesulfonic acid (MES)/chlorophenol red (574 nm) at pH 5.5 to 6.8, morpholinepropanesulfonic acid (MOPS)/*p*-nitrophenol (400 nm) at pH 6.8 to 7.5, HEPES/phenol red (557 nm) at pH 7.5 to 8.0, and *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS)/*m*-cresol purple (578 nm) at pH 8.0 to 9.0. Buffer concentrations were 50 mM. The total ionic strength was adjusted to 150 mM with Na_2SO_4. The final pH indicator concentration was 50 μM. The initial 5 to 10% of the total absorbance change was used to calculate the initial steady-state rate with the mean of 5 to 10 reaction traces per experiment. The steady-state parameters k_{cat} and k_{cat}/K_m and their standard errors were determined by fitting the observed initial rates (corrected for the uncatalyzed reaction) to the Michaelis-Menten equation using the KaleidaGraph data analysis and graphing software. The pH-independent values of k_{cat} and pK_a for the CO_2 hydration reaction were determined by fitting the experimental pH-dependent

Michaelis-Menten parameters to equations 1 and 2 where k_{cat}^{obs} is the observed k_{cat} and K_m^{obs} is the observed K_m.

$$k_{cat}^{obs} = k_{cat}/(1 + 10^{pKa-pH}) \quad (1)$$

$$k_{cat}/K_m^{obs} = (k_{cat}/K_m)/(1 + 10^{pKa-pH}) \quad (2)$$

Esterase activity, using *p*-nitrophenylacetate as a substrate, was determined as previously described (29).

Protein, metal, and volatile fatty acid (VFA) analyses. Protein concentrations were estimated by measuring the absorbance of solutions (A_{280}), using a theoretical monomer extinction coefficient of 11,710 cm^{-1} M^{-1} and a calculated monomer molecular mass of 21,351 Da. For analysis of metals, protein concentrations were determined by the biuret method (30) using bovine serum albumin as the standard.

The homogeneity of the enzyme was determined by 12% SDS-PAGE (31). The gels were stained for protein with Coomassie brilliant blue. Markers in the molecular mass range from 20 to 250 kDa were used to estimate the molecular mass. The native molecular mass was determined by gel filtration chromatography using a Sephadex G-150 gel filtration column (GE Healthcare) equilibrated with buffer B and calibrated with lysozyme (14.7 kDa), bovine erythrocyte CA (29 kDa), bovine serum albumin (monomer, 66 kDa; dimer, 132 kDa), the β class CA (Cab) from *M. thermautotrophicus* (90 kDa), and urease (trimer, 272 kDa; hexamer, 545 kDa). The column was developed with a flow rate of 0.2 ml/min using buffer B.

A comprehensive metal analysis (20 elements) was conducted using inductively coupled plasma (ICP) atomic emission spectroscopy at the Chemical Analysis Laboratory, University of Georgia, Athens. All solutions were made metal free by treating with Chelex 100 (Sigma). Prior to metal ion analysis, samples were dialyzed for about 24 h at 4°C with 4 to 6 changes against 20 mM potassium phosphate (pH 7.0).

Lactate and formate were determined using commercial kits (Megazymes, Ireland). Cells were grown for ~15 h to stationary phase and then removed by centrifugation, and the supernatant was filtered through a 0.22-μm filter and stored at −80°C until analyzed.

Acetate, butyrate, and propionate were determined by liquid chromatography-mass spectrometry (LC-MS). Samples were analyzed using an Agilent 6890 N gas chromatograph (GC) with a Waters GCT classic mass spectrometer operated in electron ionization (EI) mode. Aliquots (2 μl) were injected using a split/splitless injector at 250°C with a 5:1 split onto a VF5 (5% diphenyl–95% dimethyl polysiloxane) capillary column (Varian) (20-m column with 150-μm internal diameter and 0.15-μm film thickness) using helium as the carrier gas at a flow rate of 1.4 ml/min. The column was held at an initial temperature of 50°C for 1 min and then heated at 10°C/min to 100°C where it was held for 2 min before heating at 30°C/min to a final temperature of 230°C. Following a 6-min solvent delay, EI mass spectra were acquired over the mass range of 45 to 400 kDa at 1 scan/second. Under these conditions, the pentafluorobenzyl (PFB) derivatives of acetic acid, propionic acid-2,2-d2, propionic acid (internal standard), and butyric acid had retention times of 7.05, 8.74, 8.78, and 9.69 min, respectively. The reconstructed ion chromatograms (RIC) for *m/z* 240, 256, 254, and 268 were generated, and the integrated areas were used for quantitation. A standard curve was prepared from a mixture of the three VFAs at a concentration of 240, 120, 60, 30, 15, 7.5, and 3.75 μg/ml containing 8 μg/ml of the internal standard. The amounts of each of the VFAs in the samples were calculated from the integrated area of the RIC, using the response factor for each VFA and normalized for the internal standard (IS) response.

RESULTS AND DISCUSSION

Purification and characterization of Cpb encoded by *CPE0413*. The protein encoded by the *CPE0413* locus of the *C. perfringens* genome is annotated as a probable CA (32). The gene was amplified by PCR from *C. perfringens* genomic DNA using primers shown in Table 1. The PCR product was cloned into pET22b(+) to yield the expression vector pCPE0413 that was transformed into *E. coli* strain Rosetta (DE3) p*Lac*I for overproduction of the encoded protein. The protein was purified to homogeneity (Fig. 1) from cell extract of IPTG-induced cells by ion-exchange and gel filtration chromatography. CA activity was monitored using the electrometric method (27). The purified protein showed CO_2 hydration activity comparable to the activities of kinetically characterized β class CAs (Table 2) validating the annotation; therefore, the gene is designated *cpb* (*Clostridium perfringens* beta class carbonic anhydrase). SDS-PAGE (Fig. 1) revealed a molecular mass of 23 kDa consistent with 21.3 kDa calculated from the deduced amino acid composition. The molecular mass of ~88 kDa, determined by gel filtration chromatography (data not shown), indicated that the native enzyme is a tetramer. A comprehensive metal analysis (20 elements) performed by plasma emission spectroscopy, using two independent enzyme preparations with similar specific activities, revealed 0.89 and 0.93 zinc per subunit, indicating that the enzyme contains one active site zinc per subunit. The kinetic constants and subunit composition of Cpb (Table 2) most resemble those of the β class CA (Cab) from *M. thermautotrophicus* of the domain *Archaea* (15–17). Both Cpb and Cab have lower K_m values for CO_2 compared to all other β class CAs (Table 2), a result consistent with a role for Cpb and Cab in capturing environmental CO_2 and retaining intracellular levels for anaplerotic CO_2 fixation reactions. Cpb and Cab belong to one of two clades identified in the phylogenetic analysis of prokaryotic β class CAs (Fig. 2) from species other than *Clostridium* species. A search of the nonredundant databases revealed 23 closely related homologs of *C. perfringens* strain 13 *cpb* belonging to the genus *Clostridium* represented by pathogenic species and nonpathogenic species of environmental and biotechnological importance (see Fig. S1 in the supplemental material).

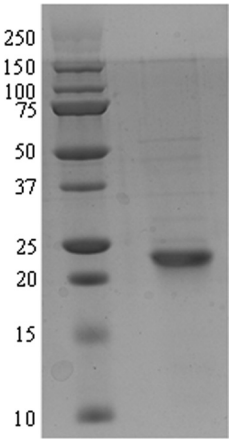

FIG 1 Denaturing polyacrylamide gel electrophoresis of Cpb. The right lane contains 10 μg of purified protein. The left lane contains molecular mass standards. The positions of molecular mass standards (in kilodaltons) are shown to the left of the gel. The gel was stained for protein with Coomassie blue R-250.

CAs characterized thus far employ a two-step, ping-pong, metal-hydroxide mechanism of catalysis (equations 3 and 4) where E represents enzyme residues, M is metal, and B is buffer.

$$E\text{-}M^{2+}\text{-}OH^- + CO_2 = E\text{-}M^{2+}\text{-}HCO_3^- \quad (3a)$$

$$E\text{-}M^{2+}\text{-}HCO_3^- + H_2O = E\text{-}M^{2+}\text{-}H_2O + HCO_3^- \quad (3b)$$

$$E\text{-}M^{2+}\text{-}H_2O = H^+\text{-}E\text{-}M^{2+}\text{-}OH^- \quad (4a)$$

TABLE 2 Subunit composition and kinetic parameters of characterized prokaryotic β class carbonic anhydrases

Enzyme source	Composition[a]		Kinetic parameters[a]			Reference(s)
	Subunit mass (kDa)	Holoenzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	
Clostridium perfringens strain 13	21.3	Tetramer	1.5×10^4	3.1	4.8×10^6	This study
Methanothermobacter thermautotrophicus	18.9	Tetramer	1.7×10^4	2.9	5.9×10^6	16
Mycobacterium tuberculosis (Rv1284)	18.2	Tetramer	3.9×10^5	10.5	3.7×10^7	9, 33
Mycobacterium tuberculosis (Rv3588c)	21.8	Dimer	NA	NA	NA	9
Brucella suis (bsCAI)	25	Dimer	6.4×10^5	16.4	3.9×10^7	6
Brucella suis (bsCAII)	25	NA	1.1×10^6	12.3	8.9×10^7	20
Helicobacter pylori	25.8	NA	7.1×10^5	14.7	4.8×10^7	7
Streptococcus pneumoniae	21.1	NA	7.4×10^5	11.3	6.5×10^7	34
Salmonella enterica (stCAI)	24.8	NA	1.0×10^6	12	8.3×10^7	35
Salmonella enterica (stCAII)	26.6	NA	7.9×10^5	15.2	5.2×10^7	35
Halothiobacillus neapolitanus	57.3	NA	8.9×10^5	3.2	2.8×10^7	36

[a] NA, not available.

$$H^+\text{-E-}M^{2+}\text{-}OH^- + B = \text{E-}M^{2+}\text{-}OH^- + BH^+ \quad (4b)$$

In the first step, a pair of electrons residing on the metal-bound hydroxide attack the carbon of CO_2 (equation 3a), producing metal-bound HCO_3^- that is next displaced by water (equation 3b). In the next step, a proton is extracted from the metal-bound water (equation 4a) and transferred to bulk solvent or buffer (equation 4b), regenerating the metal-bound hydroxide (37). The steady-state parameters of k_{cat} and k_{cat}/K_m for Cpb increased with increasing pH (Fig. 3A and B), suggesting that an unprotonated form is required for activity consistent with a metal-hydroxide mechanism of catalysis. Single ionizations with pK_a values of 6.9 ± 0.15 and 7.1 ± 0.2 were observed for k_{cat} and k_{cat}/K_m (Fig. 3A and B). The dependence of CO_2 hydration on the concentration of HEPES buffer was determined at pH 7.5. The k_{cat} was dependent on the concentration of buffer in a saturable manner with an apparent K_m of 4 mM for HEPES (Fig. 3C). This result indicates that buffer acts as the second substrate in a ping-pong mechanism accepting a proton from the enzyme during CO_2 hydration. The K_m parameter also increased with increasing buffer concentration, resulting in a k_{cat}/K_m that was independent of the buffer concentration (Fig. 3D). These results are consistent with the metal-hydroxide mechanism in which the interconversion of CO_2 and

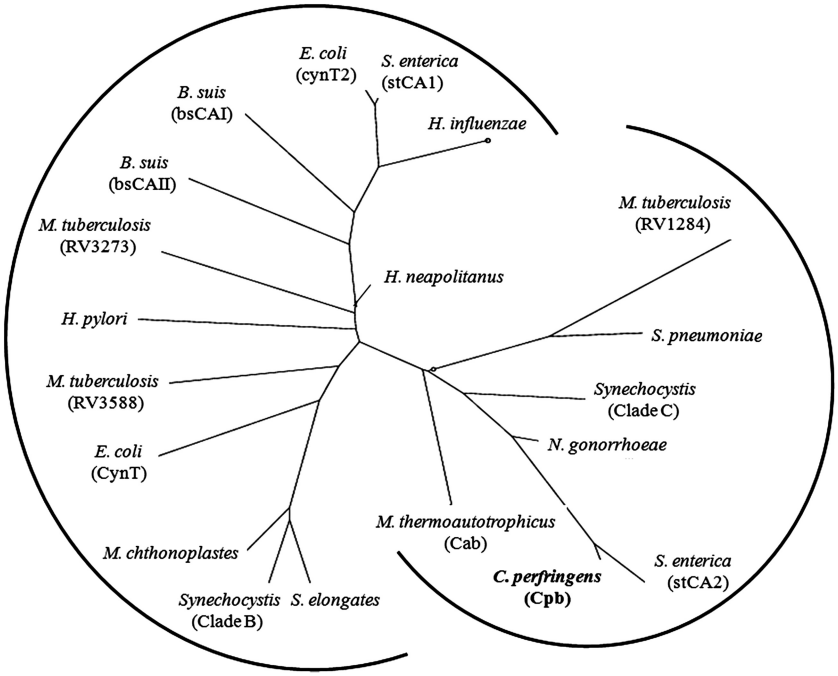

FIG 2 Phylogenetic analysis of prokaryotic β class carbonic anhydrases. The sequences were from the following species (gene identification numbers shown in parentheses or brackets): *Clostridium perfringens* strain 13 (gi|18309395), *Methanothermobacter thermautotrophicus* strain ΔH (gi|15679577), *Escherichia coli* (*cynT* [gi|386607709] and *cynT2* [gi|14277938]), *Brucella suis* (bsCAI [gi|260567947] and bsCAII [gi|260568899]), *Helicobacter pylori* (gi|188526816), *Mycobacterium tuberculosis* (strain RV1284 [gi|298524787], RV3588C [gi|15610724], and RV3273 [gi|15610409]), *Salmonella enterica* serovar Typhimurium (stCA2 [gi|378987335] and stCA1 [gi|16763561]), *Neisseria gonorrhoeae* (gi|59802380), *Streptococcus pneumoniae* (gi|307066706), *Haemophilus influenzae* (gi|99031788), *Synechocystis* (clade C [gi|1001130] and clade B [gi|1653251]), *Synechococcus elongates* (gi|56685078), *Microcoleus chthonoplastes* (gi|78057950), and *Halothiobacillus neapolitanus* (gi|88193005). The tree was constructed using the phylogeny/evolution analysis tools obtained at ExPASy (http://expasy.org/phylogeny_evolution). A distance-based method was used for the construction.

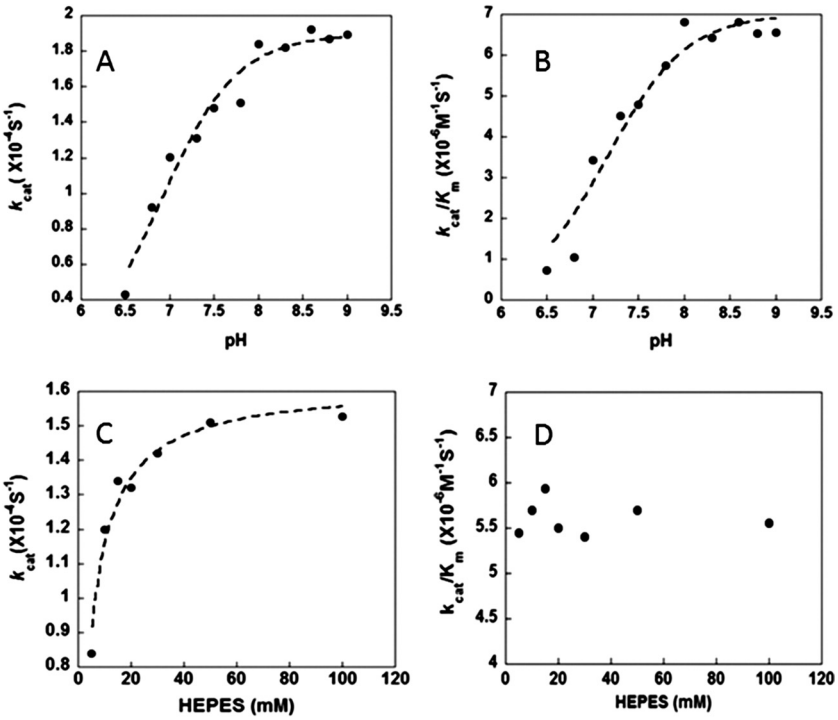

FIG 3 pH and buffer dependence of CO_2 hydration catalyzed by Cpb. (A) pH versus k_{cat}; (B) pH versus k_{cat}/K_m; (C) HEPES concentration versus k_{cat}; (D) HEPES concentration versus k_{cat}/K_m.

HCO_3^- (equation 3) reflected by k_{cat}/K_m is separate from the intermolecular proton transfer reflected by k_{cat} (equation 4).

There are two distinct subclasses of the β class (2). The type 1 subclass is characterized by ionizable water serving as a fourth ligand to zinc and pH rate profiles for CO_2 hydration featuring only one ionization (pK_a). In contrast, the type II subclass is characterized by Asp serving as the fourth ligand to zinc and a cooperative pH rate profile with multiple ionizations and only slight activity below pH 8 compared to type I. The type II subclass is further distinguished by a trio of residues (Trp, Tyr, and Arg) in a putative bicarbonate-binding allosteric site represented by the enzyme from *Haemophilus influenzae* (38). The Cpb CA does not contain the cognate Trp39 or Arg64 of the *H. influenzae* bicarbonate-binding allosteric site, and phylogenetic analyses place Cpb farthest from the *H. influenzae* enzyme (Fig. 2). In contrast, Cpb from *C. perfringens* is in the same clade (Fig. 2) and has substantial sequence identity with Cab from *M. thermautotrophicus* (Fig. 4), which is representative of the type I subclass. Furthermore, the pH profiles of Cpb revealed only one pK_a each with activity extending well below pH 8 (Fig. 3A and B). A distinctive feature of all β class

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C.p   ENKEYE-KYKATKKPEKKLVILSCMDTRLTELLPKAMNIKNGDAKIIKNAGATIMHPFGS  78
      EN+++  +  +  K   KL I++CMD+RL +LL +A+ I  GDAK+IKNAG  +
M.t.  ENQDFRFRDLSDLKHSPKLCIITCMDSRLIDLLERALGIGRGDAKVIKNAGNIVDD---G  65
                              3234 36
C.p.  IVRSILVAIYEFNAEDVLVVGHHGCGMSNLNSKDMISKMEDRGISEETILTIKHCGIDVE  138
      ++RS  VAIY     ++++VGH  CGM+ L+   ++S+M + G+ EE    I++  IDV
M.t.  VIRSAAVAIYALGVNEIIIVGHHTDCGMARLDEDLIVSRMRELGVEEE---VIENFSIDV-  121
                              87 90
C.p.  KWLHGFGCVEESVKESVTSLKNHPLMPSDVNVHGLVIDPHTGELK  183
        L+  G  EE+V E V  LK+ PL+P  + VHGL+ID +TG LK
M.t   --LNPVGDEEENVIEGVKRLKSSPLIPESIGVHGLIIDINTGRLK  164
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FIG 4 Alignment of the sequences for Cpb from *Clostridium perfringens* and Cab from *Methanothermobacter thermautotrophicus*. The deduced sequences were aligned by using ClustalX (50). Abbreviations and gene identification numbers: C.p., *C. perfringens*, gi|110799007; M.t., *M. thermautotrophicus*, gi|1272331. The numbering corresponds to the *M. thermautotrophicus* sequence. Residues 32, 87, and 90 are ligands to zinc. Residues 34 and 36 are the Asp/Arg pair important for the proton transfer step of catalysis.

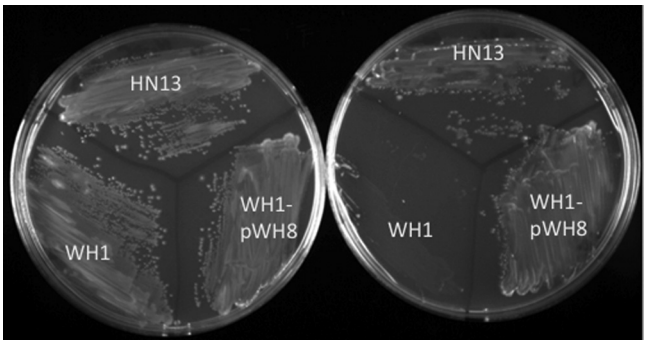

FIG 5 Growth of *C. perfringens* strains on solid semidefined medium. Wild-type (HN13), *cpb* deletion mutant (WH1), and complemented mutant (WH1-pWH8) strains were cultivated on solid agar containing semidefined medium with 10% CO_2 (left) and without CO_2 (right) added to the atmosphere (see Materials and Methods).

CAs is ligation of the active site zinc with sulfur atoms of two Cys residues and a nitrogen atom of His (39). Comparison of sequences in Fig. 4 indicates that zinc ligands in type I Cab are conserved in Cpb, in addition to the Asp/Arg pair essential for ensuring that water is the fourth ligand to zinc and playing a role in the proton transfer step in the mechanism of Cab (15, 18). Thus, the kinetic parameters, phylogeny, and sequence comparisons establish that Cpb of *C. perfringens* belongs to the type I subclass of β class CAs.

Although commercially available bovine α class CA showed an esterase activity of ~37 mol of *p*-nitrophenylacetate hydrolyzed min^{-1} mol^{-1} of enzyme, the *C. perfringens* CA (Cpb) had no detectable esterase activity (<0.05 mol of *p*-nitrophenylacetate hydrolyzed min^{-1} mol^{-1} of tetramer), also a property of Cab from *M. thermautotrophicus* (16, 17).

Physiological role of Cpb. Annotation of the genome of *C. perfringens* strain 13 indicated that a single CA-encoding gene, *CPE0413* (*cpb*), was present (40). To confirm that no other CAs are present, the deduced *cpb* gene product was entered into the BLASTP program and compared to the strain 13 genome sequence. No significant homology was seen to any other proteins, suggesting there is only a single CA in this strain. The same analysis was done with the other 8 *C. perfringens* strains with sequenced genomes, and only a single CA gene was detected in each, with 100 to 94% identity to the CA from strain 13 (blast.ncbi.nlm.nih.gov). The *cpb* gene of strain 13 is predicted to be monocistronic by the operon prediction software available in MicrobesOnline (http://www.microbesonline.org/cgi-bin/fetchLocus.cgi?locus=184876&disp=1). A rho-independent transcription terminator is predicted to lie in the 141-bp intergenic region between *cpb* and *CPE0414* using the ARNold software (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php).

CAs have been shown to mediate a wide variety of physiological effects on species from the domains *Bacteria* and *Eukarya* (11, 13, 21, 22, 41–47). Therefore, an in-frame deletion of the *cpb* gene was constructed in wild-type strain HN13, a derivative of *C. perfringens* strain 13 that has a deletion of the *galT-galK* genes (24). Introducing a heterologous *galK* gene into the chromosome by insertion of a nonreplicating plasmid (pWH6) at the *cpb* locus established a counterselectable marker strategy where the addition of galactose is toxic to the cell due to the accumulation of galactose-1-phosphate (Gal-1-P) (24). Cells that have undergone a second recombination event that leads to deletion of the *cpb* gene are resistant to galactose treatment. This strategy was used to produce mutant strain WH1 for evaluating the physiological role of Cpb. There were no differences in the growth rate or final A_{600} of the mutant strain and wild-type strain when cultured in nutrient-rich PY, BHI, or PGY liquid medium (not shown). However, *E. coli* and other prokaryotes lacking a functional CA have shown deficiencies in the ability to grow in an atmosphere largely devoid of CO_2 required for anaplerotic carboxylation reactions (11, 13, 21, 46). Therefore, we tested the ability of the *cpb* deletion mutant WH1 to grow on solid media in the absence of CO_2 by replacing the headspace gas in anaerobic jars with 100% N_2. As shown in Fig. 5, the *cpb* mutant strain WH1 was unable to grow in the absence of CO_2 on a semidefined medium comprised of 0.1% yeast extract, 50 mM glucose, and mineral salts. However, growth in semidefined medium was restored when a wild-type copy of the *cpb* gene was provided to strain WH1, yielding complemented strain WH1-pWH8 (Fig. 5). In contrast, growth was comparable for wild-type strain HN13 and mutant strain WH1 when cultured on solid nutrient-rich medium (BHI, TY, or PGY) in an atmosphere of either 100% N_2 or 80% N_2, 10% H_2, and 10% CO_2 (not shown). These results demonstrate a strict requirement for Cpb during growth of *C. perfringens* in semidefined medium.

The absence of growth for the *cpb* deletion mutant strain WH1 when cultured in a semidefined medium and an atmosphere devoid of CO_2 is likely due to low activity of anaplerotic CO_2-fixing

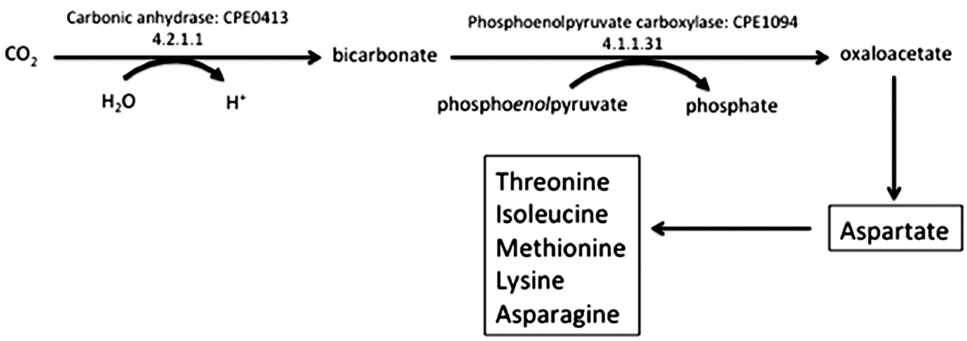

FIG 6 Proposed involvement of *C. perfringens* Cpb CA and PEP carboxylase leading to oxaloacetate and amino acid biosynthetic pathways. Carbonic anhydrase is necessary for bicarbonate formation, which is the substrate for the anaplerotic enzyme, PEP carboxylase, leading to oxaloacetate formation and amino acid biosynthesis (boxes).

TABLE 3 Volatile fatty acid production in three strains of *C. perfringens* cultured in PY medium

Trial	Total Ac/Pr/Bu[a] produced (μg/ml) [ratio] in strain:		
	HN13[b]	WH1[c]	WH1-pWH8[d]
1	1,310/460/280 [2.8/1/0.6]	300/110/63 [2.7/1/0.6]	970/415/240 [2.3/1/0.6]
2	1,230/430/280 [2.9/1/0.7]	311/110/62 [.8/1/0.6]	1,030/420/580 [2.5/1/1.4]

[a] Ac/Pr/Bu, acetate/propionate/butyrate.
[b] HN13 is the wild-type strain.
[c] WH1 is the *cpb* deletion mutant strain.
[d] WH1-pWH8 is the *cpb*-complemented mutant cultured with lactose present in PY medium.

reactions dependent on Cpb to supply HCO_3^- to carboxylases. One candidate anaplerotic enzyme is the phosphoenolpyruvate (PEP) carboxylase from *C. perfringens* (48) that produces oxaloacetate (Fig. 6). Oxaloacetate is the first intermediate in the reverse tricarboxylic acid (TCA) cycle that *C. perfringens* strains use to synthesize the carbon backbones of amino acids (KEGG database [http://www.genome.jp/kegg/kegg2.html]) that are most likely present in nutrient-rich media although in low concentrations or absent in semidefined media. The PEP carboxylase from *C. perfringens* utilizes HCO_3^- and is homologous in sequence (49) (Fig. 2) and similar in structure (48) to the PEP carboxylase of *M. thermautotrophicus* that also utilizes HCO_3^- (49). The *C. perfringens* archaeal-type PEP carboxylase is only one of three known in the domain *Bacteria* (49). The finding that Cpb has a relatively low K_m for CO_2 (Table 2) is, at least, consistent with a role for Cpb to supply HCO_3^- for anaplerotic reactions similar to that catalyzed by PEP carboxylase (Fig. 6).

There was no statistically significant difference in the wild-type strain (HN13) and the *cpb* deletion mutant strain (WH1) in L-lactate, D-lactate, or formate production grown in either low-carbohydrate (PY) medium or high-carbohydrate (PGY) medium containing 111 mM glucose (data not shown). There was also no statistically significant difference in either the total or ratio of the acetate, butyrate, and propionate VFAs produced when the strains were cultured in PGY medium (data not shown). In contrast, mutant strain WH1 was deficient in the amount of total VFAs produced in PY medium compared to wild-type strain HN13, although the ratios of VFAs and final A_{600} were unchanged (Table 3). Addition of the wild-type *cpb* gene under the control of the lactose-inducible promoter P_{bgaL} to mutant strain WH1 restored VFA production in the complemented strain (WH1-pWH8) to wild-type levels (Table 3). While *C. perfringens* can ferment lactose, the amount of lactose added for induction had no significant effects on VFA production by the wild-type strain HN13 cultured in PGY or PY medium (data not shown). Finally, there was no significant difference in the growth rate or final A_{600} between wild-type strain HN13 and the *cpb* deletion mutant strain WH1 (data not shown). These results establish that maximum production of VFAs in a low-carbohydrate medium is dependent on Cpb.

The finding that the *cpb* deletion mutant (WH1) showed no discernible defect in growth phenotype when cultured in nutrient-rich media diminishes the possibility that Cpb plays an important role in gas gangrene infections where *C. perfringens* is exposed to a surplus of nutrients by breaking down tissue and releasing soluble components. However, it remains to be determined whether Cpb has a more direct role in pathogenicity. Furthermore, although the results indicate that Cpb is necessary for maximum VFA production when carbohydrates are limiting, the effects of reduced VFA levels on pathogenicity have yet to be determined. Nonetheless, it appears likely that Cpb is valuable in soil environments where there is greater competition for nutrients and CO_2 levels are limiting. The results reported here encourage further research to determine whether Cpb homologs identified from other *Clostridium* species, isolated from diverse anaerobic environments, play important roles in the physiology and ecology of these organisms.

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