

Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: https://www.tandfonline.com/loi/ienz20

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To cite this article: Ayşenur Eminoğlu, Daniela Vullo, Aycan Aşık, Dilşat Nigar Çolak, Claudiu T. Supuran, Sabriye Çanakçı & Ali Osman Beldüz (2016) Cloning, expression and biochemical characterization of a β-carbonic anhydrase from the soil bacterium *Enterobacter* sp. B13, Journal of Enzyme Inhibition and Medicinal Chemistry, 31:6, 1111-1118, DOI: 10.3109/14756366.2015.1100176

To link to this article: https://doi.org/10.3109/14756366.2015.1100176

Published online: 26 Oct 2015.

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Journal of Enzyme Inhibition and Medicinal Chemistry

www.tandfonline.com/ienz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, 2016; 31(6): 1111–1118
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RESEARCH ARTICLE

Cloning, expression and biochemical characterization of a β-carbonic anhydrase from the soil bacterium *Enterobacter* sp. B13

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Abstract

A recombinant carbonic anhydrase (CA, EC 4.2.1.1) from the soil-dwelling bacterium *Enterobacter* sp. B13 was cloned and purified by Co^{2+} affinity chromatography. Bioinformatic analysis showed that the new enzyme (denominated here B13-CA) belongs to the β-class CAs and to possess 95% homology with the ortholog enzyme from *Escherichia coli* encoded by the *can* gene, whereas its sequence homology with the other such enzyme from *E. coli* (encoded by the cynT gene) was of 33%. B13-CA was characterized kinetically as a catalyst for carbon dioxide hydration to bicarbonate and protons. The enzyme shows a significant catalytic activity, with the following kinetic parameters at 20 °C and pH of 8.3: k_{cat} of $4.8 \times 10^5\ s^{-1}$ and k_{cat}/K_m of $5.6 \times 10^7\ M^{-1} \times s^{-1}$. This activity was potently inhibited by acetazolamide which showed a K_I of 78.9 nM. Although only this compound was investigated for the moment as B13-CA inhibitor, further studies may reveal new classes of inhibitors/activators of this enzyme which may show biomedical or environmental applications, considering the posssible role of this enzyme in $CaCO_3$ biomineralization processes.

Keywords

β-Carbonic anhydrase, *Enterobacter* sp., kinetics

History

Received 26 August 2015
Revised 21 September 2015
Accepted 22 September 2015
Published online 22 October 2015

Introduction

Carbonic anhydrases (CAs) [EC 4.2.1.1] are Zn(II)-dependent metalloenzymes which catalyze the reversible hydration of CO_2 to HCO_3^- and H^{+1}. CAs have important functions in the eukaryotes as well as in prokaryotes, such as pH regulation, biosynthetic reactions, respiration, CO_2 transport, photosynthesis, among others[2], and are known to be among the fastest enzymes described so far. Bacterial CAs have an essential role in the life cycle of these organisms, as well as in the regulation of calcium carbonate mineralization, at least in some bacterial species. Indeed, $CaCO_3$ mineralization by bacteria is crucial for soil and ground water remediation, sequestration and capture of atmospheric CO_2, as well as sand and soil strengthening and consolidation[3].

CAs are encoded by six evalutionary unrelated families, the α, β, γ, δ, ζ[4] and η-CAs[5], which are present in organisms all over the phylogenetic tree. Among them, the α-, β-, γ- and δ-CAs are broadly distributed in many microorganisms[1], whereas the η-CAs were described only in protozoa for the moment[5].

To date, two different β-CAs have been reported in *Escherichia coli*, the most investigated bacterium for the molecular biology viewpoint. One such enzymes is encoded by the *cynT* gene and was the first β–CA identified in this bacterium, as a product of the *cyn* operon, being shown to possess sequence homology with higher plants β-CAs and to function in the process of cyanate utilization as a nitrogen source by this bacterium[6,7]. The second one is *can* (previously called *yadF*), which has 30% amino acid sequence homology with *CynT*[8]. It was reported that *can* is essential for the growth of *E. coli* under atmospheric CO_2 conditions[2]. It has been also shown that expression of *can* is susceptible to the increase of cell density and to the rise in the medium temperature. In *E. coli*, CA activity is normally provided by the expression of both these enzymes, although detailed data regarding their localization within the bacterium are missing for the moment. In fact, bicarbonate/CO_2 are necessary during the normal growth of this bacterium and for the biosynthesis of several biomolecules involved in vital functions. It has been suggested that *can* is conserved throughout *Enterobacteriaceae* genera (the type of bacteria to which *E. coli* also belongs)[9]. However, only four β–CA genes have been identified in *Enterobacteriaceae* so far, i.e. *cynT*, *can* (previously *yadF*), *cah* and *mig-5*[9]. Recently, β–CAs from two *Enterobacter* species were phylogenetically compared with the *E. coli* CAs[10], but only one of these enzymes has been investigated in detail for the moment, i.e. the one encoded by the *cynT* gene, for which the X-ray crystal structure was reported by Cronk et al.[8]

Although ultimately there are many studies on various bacterial β–CAs, the investigation of *Enterobacteriaceae* is

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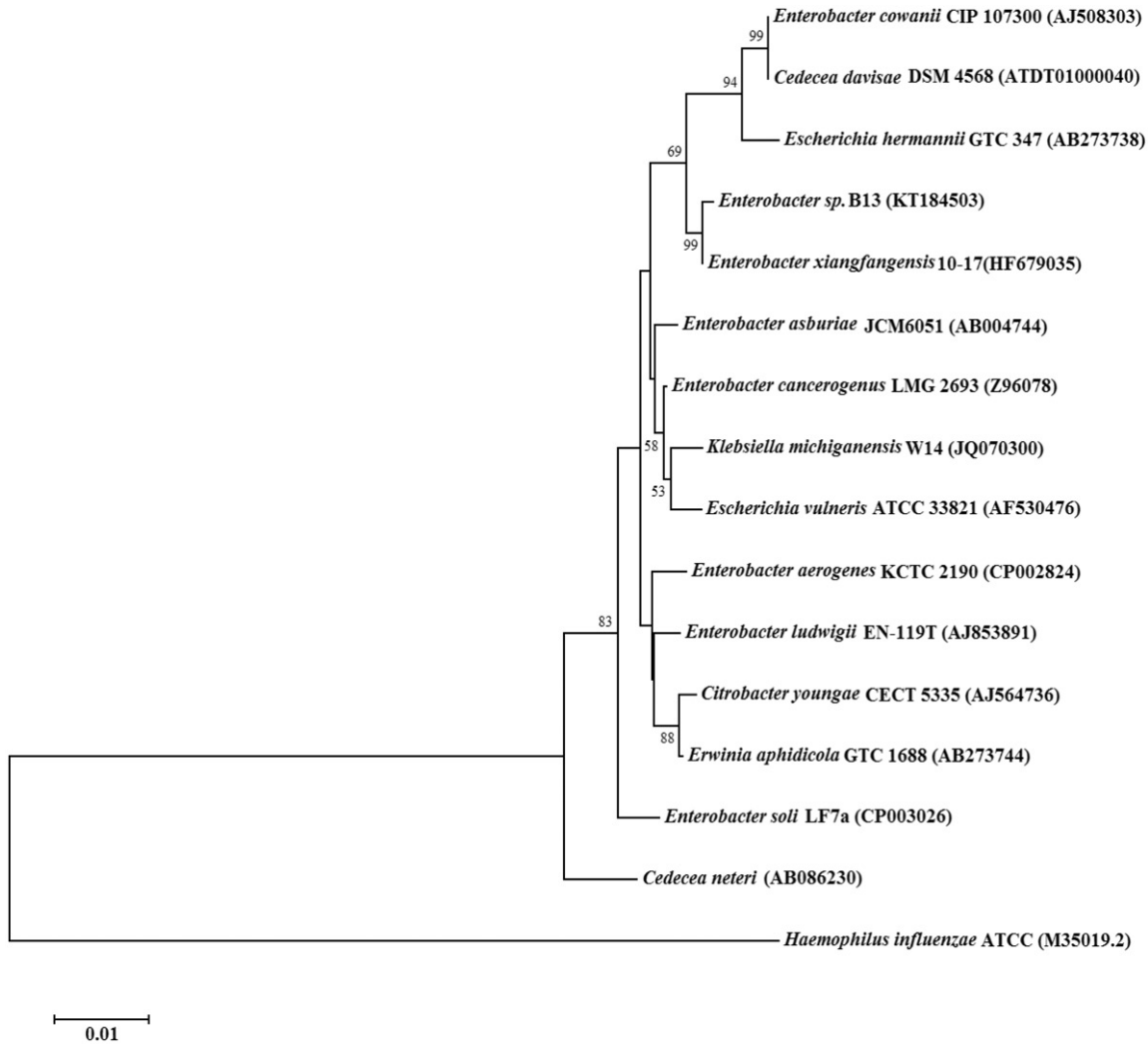


Figure 1. Phylogenetic analysis of 16S rDNA similarities of *Enterobacter* sp. B13 based on the BLAST result using the neighbor-joining method. Scale bar represents 0.01 substitutions per nucleotide position. The organisms and GeneBank accession numbers of analyzed sequences are given in parenthesis.

rather limited with only one *E. coli* enzyme being investigated in some detail, as mentioned above. Here, we describe a new β–CA from a bacterium belonging to this bacteria family, more precisely from an *Enterobacter* sp. B13 recently isolated from soil in Northern Turkey.

Materials and methods

Sampling site, culture conditions and microorganisms

The bacterial specimens were isolated from soil and mud samples which were collected from a riverside nearby Trabzon, Sürmene, Turkey. The samples were diluted with dH_2O and filtrated. The filtrate inoculated in TSA plates and incubated at different temperatures. Selected colonies which grew at 37 °C were then inoculated to modified B-4 agar plates to monitor the $CaCO_3$ decomposition properties of the grown bacteria. Among these isolates, clone B13 showed CO_2 hydratase activity[11] and was subsequently used as a source of genomic DNA in this study.

Identification of the bacterial strain by 16S rRNA analysis

For identification of the strain, the 16S rRNA gene was amplified via PCR from genomic DNA with eubacterial universal primers and subsequently sequenced (Macrogen Inc., Seoul, Korea).

Cloning of *Enterobacter* sp. B13 *can* and construction of the recombinant expression vector

The genomic DNA of *Enterobacter* sp. was obtained with Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and was used as a template for the CA gene. The amplification process was performed by PCR with two primers in order to amplify the *Enterobacter* sp. B13 *can* with *Nde*I (Biolab) and *BamHI* (Thermo Scientific, Waltham, MA) recognition sites (CA*Nde*I; 5′-CCA TAT GAA CGA CAT AGA TAC-3″ and *BamHI* CAR5′-CGG ATC CTT ATT TAT GGT TNA CGT GC-3′) with the following conditions: initial denaturation steps at 95 °C for 3 min, denaturation at 95 °C for 45 s, followed by annealing at 51 °C for 1 min and primer extension at 72 °C for 1 min 20 s, followed by a step at 72 °C for 5 min, for 35 cycles. The PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced (Macrogen Inc., Seoul, Korea). After the sequence verification, both the positive clone and the expression vector pET-15b (Novagen) were digested with *Nde*I-*BamHI*, then excised from the agarose gel (Qiaquick Gel Extraction Kit, Qiagen, Venlo, The Netherlands) and ligated (Thermo Scientific T4 Ligase, Thermo Scientific, Waltham, MA).

Construction and cloning of the recombinant plasmid was carried out in *E. coli* JM101 (Novagen, Podenzano, Italy) as described in the literature[12]. Transformants were checked by enzymatic digestion.

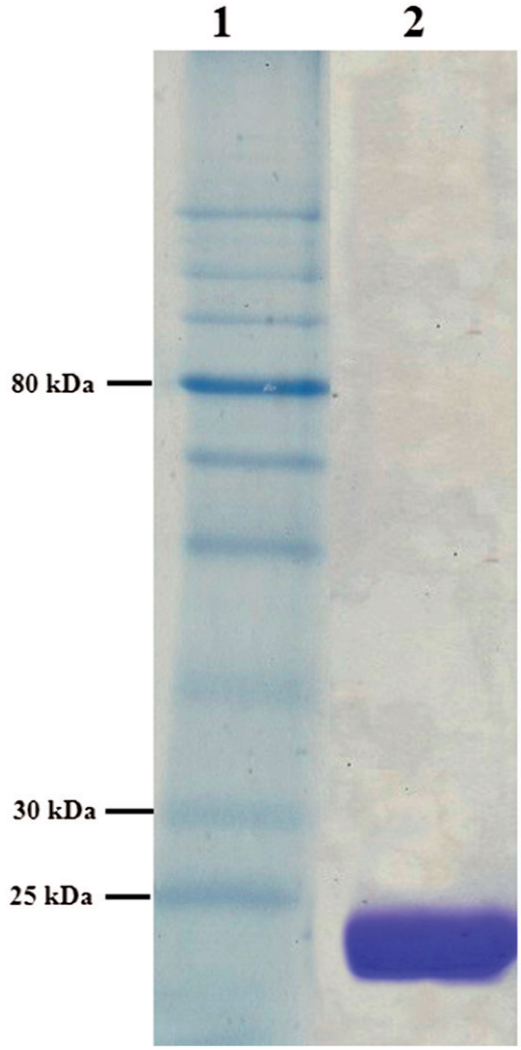


Figure 2. SDS–PAGE analysis (15%) of *Enterobacter* sp. B13-CA (stained with coomassie brilliant blue). Lane 1, protein molecular weight markers (NEB, P7710S), lane 2, purified *Enterobacter* sp. B13-CA.

Overexpression in *E. coli* BL21 (DE3) *pLysS* and purification of the β-CA

The recombinant vector designed as pB13 *can* was transformed into *E. coli* BL21 (DE3)*pLysS* (Novagen) for overexpression and sequenced for correctness. A single colony, harboring the recombinant plasmid was chosen and cultured overnight in Luria Broth (LB) medium containing 50 μg/ml ampicillin. This culture was used to inoculate 200 ml LB-ampicillin medium and incubated at 37 °C with vigorous shaking. When OD_{600} reached the mid-log phase, ~0.6–0.8, 0.5 mM $ZnSO_4$ were added to the culture and expression was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 24 h at 22 °C, for the production of recombinant B13-CA. Cells were then collected by centrifugation at 9000 rpm for 10 min at 4 °C. The pellet was resuspended in the lysis buffer (30 mM Tris–SO_4 (pH 8.0), 0.1% Triton X-100, 200 mM NaCl, 20 mM imidazole and 0.2 mg/ml lysozyme) and sonicated. After centrifugation at 7000 rpm for 20 min at 4 °C, the supernatant containing *N*-terminally His-tagged protein was purified by Co^{2+} (TALON Metal Affinity Resin, Clontech), pre-equilibrated with the wash buffer (50 mM NaH_2PO_4 (pH 8.0), 0.1% Triton X-100, 300 mM NaCl and 20 mM imidazole). After transferring, the lysate was incubated in the His beads in 15 ml falcon tubes overnight at +4 °C. Following the two washing steps at pH 8.0 and one at pH 7.0 by gentle shaking at +4 °C, the CA was eluted with 250 mM imidazole in the same buffer (pH 7.0). SDS-PAGE (15%) was run to evaluate the purity of the protein. Purified B13-CA was dialyzed overnight in dialyis buffer (30 mM Tris–SO_4, pH 8.0, 0.5 mM $ZnSO_4$, 1 mM dithiothreitol-DTT) at +4 °C. After dialysis, the concentration of the protein was measured with both NanoDrop Spectrophotometer 2000 and at 595 nm, with bovine serum albumin (BSA) as a standard.

Zymography

Zymography analysis of the CA activity was performed as described in the literature[13]. To determine the esterase activity, zymogram staining was performed with minor modifications as described previously[14] at two different temperatures (+4 °C and also at RT) on SDS-PAGE (15%). In brief, followed by electrophoresis the gels were incubated in 100 mM Tris–HCl (pH 7.5) including 0.5% Triton X-100, for 6 h at +4 °C and for 4 h at RT. After incubation, the gels were rinsed in 100 mM Tris–HCl, containing 100 mM α-naphthyl acetate (α-NAc), dissolved in 2 ml acetone, and 100 mM Tris–HCl (pH 7.5) buffer containing 20 mg Fast Red Salt (two solutions were added to the gels at the same time) until the bands become visible.

Bioinformatic analysis

The nucleotide sequence of *Enterobacter* sp. B13 *can* was submitted to GeneBank under accession number KT184504. The identification of 16S rDNA sequence was determined by the EzTaxon identification tool (http://www.ezbiocloud.net/eztaxon) against 16S rRNA sequences found in the database, i.e. strains with validly published prokaryotic names[15]. *Haemophilus influenzae* ATCC was selected as outgroup member. Aamino acid resemblance was determined by using the DELTA-BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For predicting the estimated molecular weight and to perform the multiple amino acid sequence alignment, Compute pI/Mw tool of ExPASy[16] and ClustalW[17] were used, respectively. Protein family and conserved domains were identified by Conserved Domain Search Database (http://www.ncbi.nlm.nih.gov/cdd). Determination of evolutionary familiarities was performed by Mega 6[18].

CO_2 hydrase activity assay of B13-CA

An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity[19]. Phenol red (at 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm with 10 mM TRIS (pH 8.3) as buffer and 0.1 M $NaClO_4$ (for maintaining constant ionic strength), at 20 °C, following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s (the uncatalyzed reaction needs around 60–100 s in the assay conditions, whereas the catalyzed ones are of around 6–10 s). The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Enzyme concentrations in the assay system were about 16 nM for all the enzymes considered in the present study.

Results and discussion

16S rDNAsequence analysis and characterization of the bacteria

The result of the microbial growth on the B-4 agar plates led to the observation that the B13 bacterial isolate was effectively

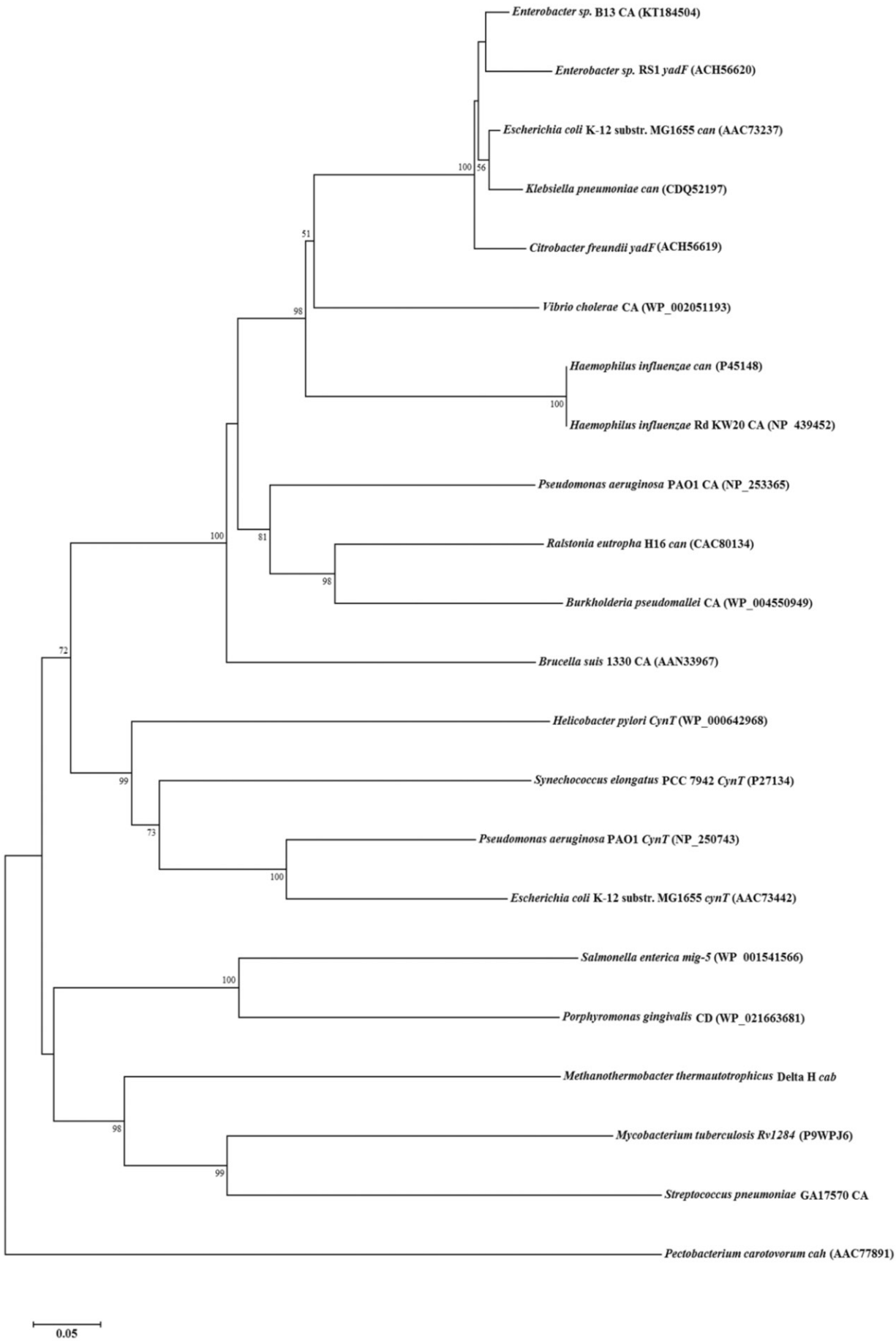


Figure 3. Evolutionary relationship analysis, by the neighbor-joining method, of 21 known CAs from prokaryotes, in comparison to *Enterobacter* sp. B13-CA (accession numbers are given in parenthesis). Scale bar represents 0.05 substitutions per nucleotide position.

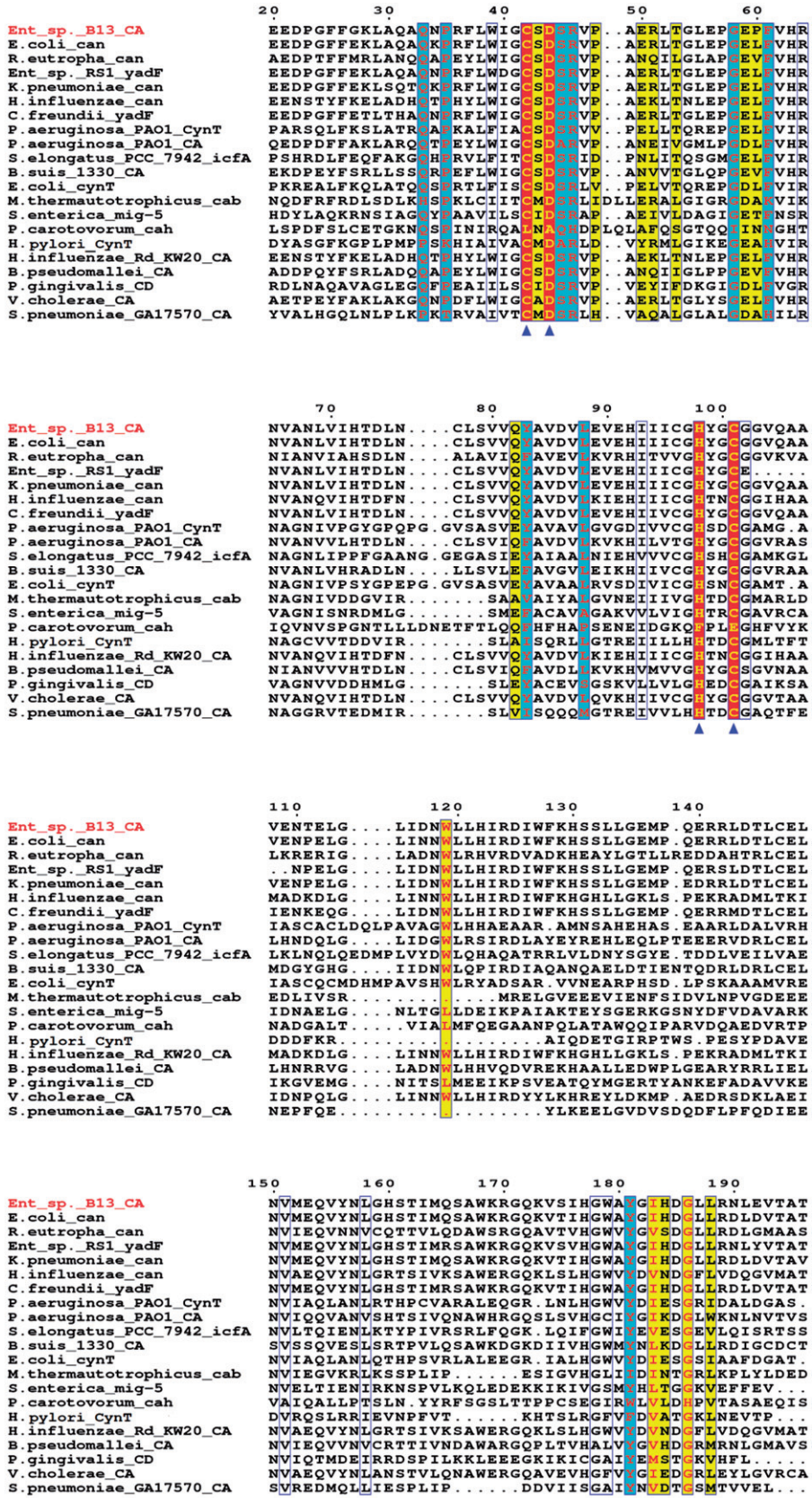


Figure 4. Multiple amino acid sequence alignment of 21 known β-class CAs: *Enterobacter* sp. B13-CA, *E. coli* str. K-12 substr. MG1655can, *Ralstonia eutropha* can, *Enterobacter* sp. RS1 *yadF*, *Klebsiella pneumoniae* can, *H. influenzae can*, *C. freundii* yadF, *P. aeruginosa* PAO1 cynT, *P. aeruginosa* PAO1 CA, *Synechococcus elongates* PCC 7942 *icfA*, *B. suis* 1330 CA, *E. coli* str. K-12 substr. MG1655 *cynT*, *M. thermautotrophicus cab*, *S. enterica mig-5*, *P. carotovorum cah*, *H. pylori cynT*, *H. influenzae* Rd KW20 CA, *Burkholderia pseudomallei* CA, *P. gingivalis* CD, *V. cholerae* CA, *S. pneumoniae* GA17570 CA and *M. tuberculosis Rv1284*. Triangles indicates the zinc ion-binding residues. Other active side conserved residues were shaded in blue (red letters). The figure was drawn with ESPript.

promoting $CaCO_3$ precipitation, presumably due to the CA activity present in the bacterium (data not shown). The EzTaxon identification results of 16S rDNA sequence of the B13 strain showed that it possesses a high percentage similarity with bacteria belonging to *Enterobacteriaceae*. Among various such bacteria, strain B13 was 99.04% identical to *Enterobacter xiangfangensis* strain 10–17, 98.63% to *Enterobacter asburiae* strain JCM6051, 98.56% to *Enterobacter cloacae* strain ATCC 13047, 98.45% to *Enterobacter cowanii* CIP 107300, 98.41% to *Klebsiella michiganensis* W14, 98.23% to *Escherichia hermannii* GTC 347, 97.90% to *Escherichia vulneris* ATCC 33821, to 97.89% *Citrobacter youngae* CECT 5335, to 97.77% *Enterobacter*

Table 1. Kinetic parameters for the CO_2 hydration reaction catalyzed by the human cytosolic isozymes hCA I and II (α-class CAs)[48] and the bacterial β-CAs: PgiCAb (from *P. gingivalis*)[28], HpyCA (from *H. pylori*)[35], LpCA1 and LpCA2 (from *L. pneumophila*)[24] and B13-CA (from *Enterobacter* sp. B13).

Enzyme	Class	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \times s^{-1}$)	K_I (acetazolamide) (nM)
hCA I	α	2.0×10^5	5.0×10^7	250
hCA II	α	1.4×10^6	1.5×10^8	12
PgiCAb	β	2.8×10^5	1.5×10^7	214
HpyCA	β	7.1×10^5	4.8×10^7	40
LpCA1	β	3.4×10^5	4.7×10^7	76.8
LpCA2	β	8.3×10^5	8.5×10^7	72.1
B13-CA	β	4.8×10^5	5.6×10^7	78.9

All the measurements have been made at 20 °C, pH 8.3 by a stopped flow CO_2 hydrase assay method[19]. Acetazolamide (AAZ) inhibition data are also provided.

ludwigii EN-119T, 97.73% to *Enterobacter soli* LF7a, 97.67% to *Cedecea davisae* DSM 4568, 97.63% *Cedecea neteri*, 97.60% to *Erwinia aphidicola* GTC 1688, 97.59% to *Enterobacter aerogenes* KCTC 2190 (Figure 1).

Purification of *Enterobacter* sp. B13-CA

The recombinant B13-CA was purified in concentrations ranging between 0.04 and 2.4 mg/ml from 250 ml cultures. The purified enzyme was subjected to SDS-PAGE analysis (Figure 2) which led to the observation of a distinct single band, with the predicted molecular mass of 24.95 kDa (deduced from the amino acid sequence). Data of the SDS-PAGE (Figure 2, lane 2) showed that the purified B13-CA had indeed a molecular weight (for the monomer) of 25 kDa.

Biochemical characterization of *Enterobacter* sp. B13-CA

According to the zymogram analysis, *Enterobacter* sp. B13 CA did not reveal any esterase activity. Furthermore, no esterase activity was observed by using a spectrophotometric assay with α-naphthyl acetate as substrate (data not shown). This is to be expected, since esterase activity was not reported for any β-class CAs, although many putative activated esters have been investigated as possible substrates[20]. In fact only the α-[20,21] and η-CAs[22] possess esterase activity with activated esters (such as 4-nitrophenyl- or α-naphthyl acetate) as substrates.

Bioinformatic analysis

Considering the sequence of the *can* gene of *Enterobacter* sp. B13, which contains 663 bp, it encodes a protein of 220 amino acid residues. Based on DELTA-BLAST results of this amino acid sequence, *Enterobacter* sp. B13 *can* shows 33% homology with *E. coli cynT* and 95% with *can* (from the same bacterium, i.e. *E. coli*). Phylogenetic analysis of amino acid resemblance revealed that B13-CA is a member of the β-CA class and has a close relationship with *can* genes identified in other bacteria belonging to *Enterobacteriaceae* (Figure 3). A conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) search of *Enterobacter* sp. B13 *can* sequences showed that the active and ion binding site residues of B13-CA are well conserved, as in all β-CAs investigated in detail, such as among others the enzymes from *H. influenzae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Synechococcus elongatus*, *Brucella suis*, *E. coli*, *Methanothermobacter thermautotrophicus*, *Salmonella enterica*, *Pectobacterium carotovorum*, *Helicobacter pylori*, *Porphyromonas gingivalis*, *Vibrio cholerae*, *Streptococcus pneumoniae*, *S. mutans* and *Mycobacterium tuberculosis Rv1284*[23–42] (Figure 4).

Catalytic activity of B13-CA

In fact there are two types of β-CAs in organisms all over the phylogenetic tree, which are defined by their pH–catalytic activity profile as well as active site structural configuration[41–52]. Type I β-CAs display catalytic activity over a broad pH range (6.5–9.0) with the active site zinc tetrahedrally coordinated by three amino acid residues (two Cys and one His) and a hydroxide/water. In contrast, type II β-CAs are catalytically active only at a pH 8 and higher where they adopt a functional active site configuration like that of type I. In fact, below pH 8 they are conformationally self-inactivated by the addition of a fourth amino acid (an Asp residue) which coordinates to the Zn(II) ion as the fourth ligand, and displacing the zinc bound solvent[39,52]. At pH >8, the Asp copordinated to Zn(II) makes a salt pair interaction with a conserved Arg residue, which ''opens'' the active site in the sense that a water molecule/hydroxide ion takes the place of the Asp residue, generating thus the nucleophile responsible for the catalytic activity of these enzymes[39,48–52].

As seen from Figure 4, where an alignment of the amino acid sequence of B13-CA with that of other 20 bacterial β-CAs is presented, the new enzyme described here has all the features of a catalytically effective β-CA: (i) the putative Zn(II) ligands, Cys42, Asp44 (in case B13-CA is a type II β-CA), His 98 and Cys101; (ii) The catalytic dyad involved in activation of the zinc-coordinated water molecule/hydroxide ion for catalysis[53], represented by the Asp44–Arg46 residues, which resembles in a way the activation of the water molecule in aspartic proteases[53].

As seen from Table 1, in which we compared the catalytic activity of the new enzyme reported here, B13-CA with that of other α- and β-class CAs from various organisms, B13-CA shows a significant activity as catalyst for the hydration of CO_2 with formation of protons and bicarbonate. Indeed, B13-CA possesses the following kinetic paramaters at 20 °C and pH of 8.3: k_{cat} of 4.8×10^5 s^{-1} and k_{cat}/K_m of 5.6×10^7 $M^{-1} \times s^{-1}$. In fact the catalytic activity of the new enzyme is in the same range as those of other β-CAs, such as those from *P. gingivalis* (PgiCAb)[28], *H. pylori* (HpyCA)[35], or *Legionella pneumophila* (LpCA1 and LpCA2)[24], recently characterized by one of our groups. Furthermore, the enzyme was inhibited by the clinically used[54–57] sulfonamide CA inhibitor (CAI) acetazolamide (AAZ) with an inhibition constant of 78.9 nM, in the same range as the human (h) hCA I isoform, HpyCA, LpCA1 and LpCA2 (which were inhibited with inhibition constants ranging between 40 and 250 nM). Only the physiologically dominant isoform hCA II was better inhibited by this drug, with a K_I of 12 nM (Table 1).

Conclusions

A recombinant CA (EC 4.2.1.1) from the soil-dwelling bacterium *Enterobacter* sp. B13 was cloned and purified by Co^{2+} affinity chromatography. Bioinformatic analysis showed the new enzyme (denominated here B13-CA) to belong to the β- CA class and to possess 95% homology with the ortholog enzyme from *E. coli* encoded by the can gene, whereas its sequence homology with the other such enzyme from *E. coli* (encoded by the *cynT* gene) was of 33%. B13-CA was characterized kinetically as a catalyst for carbon dioxide hydration to bicarbonate and protons. The enzyme showed a significant catalytic activity, with the following kinetic paramaters at 20 °C and pH of 8.3: k_{cat} of 4.8×10^5 s^{-1} and k_{cat}/K_m of 5.6×10^7 $M^{-1} \times s^{-1}$. This activity was potently inhibited by acetazolamide which showed a K_I of 78.9 nM. Although only this compound was investigated for the moment as

B13-CAI, further studies may reveal new classes of inhibitors/activators of this enzyme[58–60], which may show biomedical or environmental applications.

Declaration of interest

The authors do not have any conflicts of interest.

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