Cloning, Expression and Characterization of Two Beta Carbonic Anhydrases from a Newly Isolated CO₂ Fixer, *Serratia marcescens* Wy064

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Abstract Bacterial strains from karst landform soil were enriched via chemostat culture in the presence of sodium bicarbonate. Two chemolithotrophic strains were isolated and identified as *Serratia marcescens* Wy064 and *Bacillus* sp. Wy065. Both strains could grow using sodium bicarbonate as the sole carbon source. Furthermore, the supplement of the medium with three electron donors (Na₂S, NaNO₂, and Na₂S₂O₃) improved the growth of both strains. The activities of carbonic anhydrase (CA) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) could be detected in the crude enzyme of strain Wy064, implying that the strain Wy064 might employ Calvin cycle to fix CO₂. *S. marcescens* genome mining revealed four potential CA genes designated CA1–CA4. The proteins encoded by genes CA1–3 were cloned and expressed in *Escherichia coli*. The purified recombinant enzymes of CA1 and CA3 exhibited CO₂ hydration activities, whereas enzyme CA2

was expressed in inclusion bodies. A CO₂ hydration assay demonstrated that the specific activity of CA3 was significantly higher than that of CA1. The maximum CO₂ hydration activities for CA1 and CA3 were observed at pH 7.5 and 40 °C. The activities of CA1 and CA3 were significantly enhanced by several metal ions, especially Zn²⁺, which resulted in 21.1-fold and 26.1-fold increases of CO₂ hydration activities, respectively. The apparent *K_m* and *V_{max}* for CO₂ as substrate were 27 mM and 179 WAU/mg for CA1, and 14 mM and 247 WAU/mg for CA3, respectively. Structure modeling combined with sequence analysis indicated that CA1 and CA3 should belong to the Type II β-CA.

Keywords *Serratia marcescens* · CO₂ fixation · Carbonic anhydrase · Expression · Enzymatic properties

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Introduction

Global warming is a serious environmental issue and carbon dioxide (CO₂) is a greenhouse gas that plays a major role in global warming [1]. Accordingly, the absorption and reuse of CO₂ have received a great deal of attention in recent years [2]. One of the most effective strategies to alleviate increasing CO₂ levels is microbial CO₂ fixation mediated through ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) or carboxylating enzymes by algae, cyanobacteria and chemolithotrophic bacteria [3]. Algae and hydrogen-oxidizing bacteria have been shown to have the potential for efficient CO₂ fixation in previous studies [4, 5]. However, a few disadvantages such as light requirements for algae during culture and the need for hydrogen gas supply for hydrogen-oxidizing bacteria have limited their application for CO₂ fixation [6, 7].

Chemolithotrophic bacteria are regarded as a promising candidate for CO₂ fixation because they can fix CO₂ via oxidation of inorganic compounds [8, 9]. Therefore, it is useful to isolate CO₂ fixing bacteria without the requirement of light or hydrogen as an energy donor during culture.

Carbonic anhydrase (CA) has been shown to be one of the key enzymes involved in CO₂ sequestration by bacteria during microbial CO₂ fixation [10–12]. Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reversible conversion between CO₂ and bicarbonate involved in Zn²⁺-bound OH[−] nucleophilic attack and the active site regeneration via Zn²⁺-bound H₂O [12, 13]. The CA enzyme can accelerate CO₂ hydration into bicarbonate ion, which is transferred on the bacterial cell surface by bicarbonate ion transporters. Bicarbonate enters into the cytosol of CO₂ fixing bacteria through its specific channel, after which bicarbonate ion in the cytosolic pool enters the carboxysomes, where CO₂ produced via the reversible dehydration of bicarbonate ion by CA enzymes is sequestered by RuBisCO [14, 15]. To date, five convergently evolved categories of CA (α , β , γ , δ , ξ) have been classified and characterized based on their structure and origins [16–19]. The most thoroughly studied class of CA is the α -form, which is generally found in mammals, and α -CA always exists as a monomer containing a Zn²⁺ ion that is coordinated to three His residues [20]. The β class is comprised of enzymes found in the chloroplasts of plants and algae, as well as in bacteria and archaea, that presents as dimers, tetramers, hexamers, and octamers [21, 22]. Conversely, γ -CA always exists as a trimer, is mainly found in archaea and exhibits higher CO₂ hydration activity by using iron or cobalt instead of zinc [23, 24]. Finally, δ - and ξ -CA with Cd as the metal ion catalyst were extracted from marine diatoms [25, 26].

In this study, we isolated two CO₂ fixing strains from the soil of karst landform through enrichment culture in the presence of sodium bicarbonate. Combined with physiological and biochemical assay and 16S rDNA commercial sequencing, two newly isolated bacteria were identified and designated as *S. marcescens* Wy064 and *Bacillus* sp. Wy065. Furthermore, two carbonic anhydrases from *S. marcescens* Wy064 were identified and expressed in *E. coli* and characterized for their enzymatic properties. The sources of the newly isolated strains and the recombinant CAs provided a potential application for CO₂ sequestration and fixation.

Materials and Methods

Enrichment and Isolation of CO₂ Fixing Strain

Soil samples were collected from the karst landform region of Anshun, Guizhou Province, China, and suspended in sterile distilled water (1:10, w/v). The suspension as inocula was used for bacteria enrichment by chemostatic culture in mineral salt medium (MSM). The MSM was composed of (per liter): Na₂HPO₄·2H₂O 7.8 g, KH₂PO₄ 6.8 g, Na₂S·9H₂O 0.187 g, MgSO₄ 0.2 g, Ca(NO₃)₂·4H₂O 0.05 g, NaNO₃ 0.085 g, FeSO₄·7H₂O 0.4 g, 50 mM NaHCO₃, and 1 mL trace element (MnCl₂·4H₂O 2.0 g, H₃BO₃ 0.06 g, CoCl₂·6H₂O 0.47 g, CuCl₂·2H₂O 0.17 g, FeCl₃·6H₂O 5.4 g, Na₂MoO₄·2H₂O 0.005 g, ZnCl₂·6H₂O 0.68 g). The enrichment culture was serially diluted in physiological saline and spread onto MSM agar plates that were subsequently incubated at 30 °C for 24 h. The relatively large colonies were selected and purified twice to obtain pure cultures. The isolated strains were then identified based on their physiological and biochemical characteristics, as well as their 16S rDNA sequences [24].

Effects of Three Electron Donors on the Growth of Isolated Strain

Three electron donors, Na₂S, Na₂S₂O₃, and NaNO₂, were selected to evaluate the effects on the growth of newly isolated CO₂-fixation strains. The electron donors in the range of 0–1.2 g/L were supplemented into the MSM medium. For seed preparation, the isolated strains were inoculated into LB medium and cultivated on a rotary shaker for 12 h at 150 rpm and 30 °C. The bacterial cells were obtained by centrifugation and then washed twice to remove the LB medium. Subsequently, the harvested cells were suspended and diluted to 0.5 OD₆₀₀ as bacterial seeds, which were inoculated into the MSM medium with/without the electron donors. All culture processes were performed in triplicate for 78 h at 150 rpm and 30 °C. The strain growth was determined at the desired intervals based on the optical density at 600 nm using a spectrophotometer (UV-1800, MAPADA).

Cloning and Expression of CA Genes

The sequences encoding the *CAI–3* genes were amplified by PCR using the primers with *Eco*RI and *Hind*III restriction sites (Table 1). The genomic DNA of newly isolated *S. marcescens* Wy064 was used as a template. The PCR-amplification product of *CAI–3* was subjected to double digestion, then ligated into the pET28a vector to generate the recombinant plasmids pET-*CAI*, pET-*CA2*,

Table 1 Bacterial strains, plasmids, and primers used in this study

Strains, plasmids, or primers	Genotype, properties or sequences	Source
Strains		
<i>Serratia marcescens</i> Wy064	Wild-type, isolated from karst landform soil	This study
<i>Bacillus</i> sp. Wy065	Wild-type, isolated from karst landform soil	This study
<i>Escherichia coli</i> DH5 α	F [−] , ϕ 80d/ <i>lacZ</i> ΔM15, Δ(<i>lacZYA-argF</i>)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk [−] mk ⁺), <i>phoA</i> , <i>supE44</i> , λ [−] , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Tiagen Biotech
<i>Escherichia coli</i> BL21 (DE3)	F [−] , ompT, hsdSB(rB-mB [−]), gal(λ c I 857, ind1, Sam7, nin5, lacUV-T7 gene1), dcm(DE3)	Tiagen Biotech
Plasmids		
pET28a	Km ^r ; expression vector	Laboratory stock
pET-CA1	Km ^r ; the gene of carbonic anhydrase 1 in pET28a	This study
pET-CA2	Km ^r ; the gene of carbonic anhydrase 2 in pET28a	This study
pET-CA3	Km ^r ; the gene of carbonic anhydrase 3 in pET28a	This study
Primers		
CA1F	TCCGAATTCATGCAACATATCATTGAAGG	<i>EcoRI</i>
CA2R	GACAAGCTTTTATTCGAAATAGACGTCGG	<i>HindIII</i>
CA2F	TCCGAATTCATGAAAGAGGTAATTGAGGG	<i>EcoRI</i>
CA2R	GACAAGCTTTTGGCTCCTTTTGTGGCTT	<i>HindIII</i>
CA3F	TCCGAATTCATGAAAGAAATCGAAGAGCT	<i>EcoRI</i>
CA3R	GACAAGCTTTTATACGCCTTTATCCTGCT	<i>HindIII</i>

and pET-CA3. Three recombinant plasmids were respectively transformed into *E. coli* BL21 (DE3) for commercial sequencing and protein expression.

Analysis and alignment of the CA1–3 amino acid sequences were conducted using the BioEdit software. All the analyzed sequences were obtained from GenBank database. Additionally, the InterProScan web server (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) was used to search the conserved domain of the CA enzymes. The Modeller 9.10 software (<https://salilab.org/modeller/9.10/release.html>) was used to conduct structure modeling of the CA enzymes.

Preparation and Purification of the CA Enzymes

The recombinant strains harboring pET-CA1, pET-CA2 and pET-CA3 were cultured in 250 mL flasks containing 30 mL LB medium amended with 50 μ g/mL kanamycin at 37 °C overnight. The cultures (1%, v/v) were subsequently inoculated into 30 mL fresh LB medium with 50 μ g/mL kanamycin. After 2.5 h of cultivation at 37 °C, isopropyl-beta-D-thiogalactopyranoside (IPTG) as an inducer was supplemented into the culture at a final concentration of 0.5 mM, and the cells continued to grow at 30 °C for 6 h for protein expression. SDS-PAGE was used to analyze the expression level of the recombinant proteins. For

purification of the recombinant CA enzymes, the induced cells were harvested by centrifugation at 12,000 $\times$ g for 10 min at 4 °C. The cell pellets were then suspended in lysis buffer (20 mM phosphate, 500 mM NaCl, and 20 mM imidazole, pH 7.8). Cell disruption was performed by sonication in an ice bath for 5 min. The resulting lysate was centrifuged at 12,000 $\times$ g and 4 °C for 10 min to remove the insoluble cell debris. The crude enzyme extract was collected and purified using a Ni-NTA His-Bind Resin according to the purification protocol (Sangon, Shanghai). The binding enzyme was eluted with elution buffer (20 mM phosphate, 500 mM NaCl, and 100 mM imidazole, pH 7.8), and subsequently ultra-filtered for desalting using Amicon® Ultra-15 10 kD centrifugal filter device (Merck Millipore Ltd, Ireland). The concentrations of the purified CA proteins were determined using the Bradford method.

RuBisCO Enzyme Assay

RuBisCO assay was measured using RuBP-dependent oxidation of NADH with coupling enzymes glyceraldehyde-3-phosphate dehydrogenase (GPDH) and phosphoglycerate kinase (PGK), which were obtained from Sigma-Aldrich (Shanghai, China) [27, 28]. The reaction were performed in 1.0 mL of the reaction mixture containing

Tris–HCl buffer (200 mM, pH 7.8) with 1 mM MgCl_2 , 132 mM NaHCO_3 , 10 mM DTT, 5 mM ATP, 5 Unit GPDH and 5 Unit PGK, 2 mM NADH, 1.25 mM D-Ribulose 1,5-diphosphate (RuBP) and 100 μL crude enzyme. 4 μmol of NADH is oxidized for 1 μmol RuBP utilized. One unit of RuBisCO activity was defined as the amount of enzyme required to oxidation 1 μmol of NADH per min.

CO₂ Hydration Assay

The CO₂ hydration activity of CA was measured as previously described. Briefly, a mixture containing 1.02 mL ice-cold Tris-SO₄ buffer (pH 8.3, 20 mM) with 1 mM ZnSO_4 , 20 μL enzyme, 60 μL phenol red (0.05%), and 400 μL ice-cold CO₂-saturated solution (70 mM) was used to initiate the hydration reaction. pH values from pH 8.3 to 6.2 were calculated from the plot of absorbance versus time using phenol red as an indicator. The CA activity was calculated and expressed using Wilbur-Anderson Units per mg of the enzyme as follows: $1 \text{ WAU} = (t_0 - t_c)/t_c$. The time interval (t_0 and t_c) represented the time required for the pH value to decline from 8.3 to 6.2 in the reaction mixture without or with CA, respectively [29, 30]. Increasing concentrations of CO₂ were used to determine their kinetic constants. Different CO₂ concentrations from 10 to 63 mM were made by serially diluting ice-cold CO₂-saturated solution (70 mM) using ice-cold CO₂ non-saturated solution. The CA activities at different CO₂ concentrations were determined, and all the reactions were performed in triplicate. The kinetic parameters of K_m and $V_{\max}$ were obtained by nonlinear fitting with the Michaelis–Menten equation.

Results and Discussion

Isolation and Identification of CO₂-Fixation Strains

The bacteria from the sample were enriched in MSM containing 50 mM Na_2HCO_3 as a sole carbon source for 7 days. The enrichment culture was then serially diluted and spread onto MSM agar plates. Most colonies were red, while only a few were white. Therefore, two colonies were selected at random according to colony color and cultured. Physiological characteristics analysis and biochemical tests showed that one of the two isolates, Wy064, was gram-negative, rod-shaped, motile, catalase and nitrate reductase positive, VP-positive, indole and methyl red negative, oxidase-negative, and produced red pigment. Another isolate, Wy065, was gram-positive, rod-shaped, spore-forming, VP-positive, and catalase and oxidase positive. The 16S rDNA of the two isolates was amplified by PCR using

the universal primers and identified by commercial sequencing. Sequence alignment using the NCBI BLASTn program indicated that the 16S rDNA sequence of strain Wy064 shared 99% identity with *S. marcescens* WW4, while that of strain Wy065 exhibited the identity of 98% with *Bacillus* sp. Therefore, the isolates were identified as *S. marcescens* Wy064 and *Bacillus* sp. Wy065, respectively. As previously reported, *Serratia* and *Bacillus* are non-photosynthetic CO₂-fixation strains that were able to produce several products, including biosurfactant and lipids by sequestering CO₂ [15, 28].

Effects of Electron Donors on the Growth of Strain Wy064 and Wy065

Microbial CO₂ fixation requires energy and electron sources such as light and hydrogen. For non-photosynthetic CO₂ fixing strains, some inorganic compounds could act as electron donors and allow CO₂ fixation instead of light and hydrogen [31]. Herein, three electron donors including Na_2S , $\text{Na}_2\text{S}_2\text{O}_3$, and NaNO_2 at different concentrations were used to evaluate the effects on CO₂ fixation efficiencies of two newly isolated strains. As shown in Fig. 1, three electron donors obviously promoted the growth of two strains in response to supplementation with electron donors. The optimal concentrations of Na_2S , $\text{Na}_2\text{S}_2\text{O}_3$, and NaNO_2 for Wy064 growth were 0.3, 1.2, and 0.6 g/L, which resulted in increases in biomass of 59.5, 51.4, and 59.4%, respectively, when compared to the control without electron donors. For strain Wy065, maximum biomass increases with 33.3%, 17.1%, and 34.8% were obtained when the concentrations of Na_2S , $\text{Na}_2\text{S}_2\text{O}_3$, and NaNO_2 were 0.6, 0.3, and 0.9 g/L, respectively. These results showed that the two newly isolated non-photosynthetic strains could use electron donors to release energy and electrons to fix CO₂ via oxidation of inorganic compounds when NaHCO_3 was used as the sole carbon source. In previous studies, use of Na_2S , $\text{Na}_2\text{S}_2\text{O}_3$, and NaNO_2 as electron donors instead of hydrogen enabled the improved efficiency of CO₂ fixing by non-photosynthetic members of the microbial community [31, 32].

Furthermore, the activities of CA and RuBisCO enzymes from both strains were assayed and compared. The CA activity in the crude enzyme of *S. marcescens* Wy064 exhibited higher activity (9.11 WAU/mg) of CO₂ hydration, and a weak CA activity (0.39 WAU/mg) could be determined by *Bacillus* sp. Wy065. The RuBisCO activity in the crude enzyme of the Wy064 strain was 6.05 nmol CO₂/mg enzyme/min, whereas no RuBisCO activity was detected in the crude enzyme of the strain Wy065. These results suggested that the strain *S. marcescens* Wy064 might employ Calvin cycle to fix CO₂ due to the existence of CA and RuBisCO enzymes.

Fig. 1 Effects of Na₂S (a, d), Na₂S₂O₃ (b, e) and NaNO₂ (c, f) as electron donors on CO₂ fixation efficiencies of the newly isolated Wy064 (a–c) and Wy065 (d–f) strains

Identification of Putative *S. marcescens* Wy064 CA Genes

The enzyme extract of the newly isolated *S. marcescens* Wy064 strain exhibited higher CA hydration activity when compared with that of *Bacillus* sp. Wy065. Here, we explored the related CA genes based on gene annotation of the *S. marcescens* WW4 genome and sequence alignment using the NCBI BLASTn program. In silico analysis revealed that four putative CA genes, designated CA1–4, were present in the *S. marcescens* genome sequence. The full-length sequences of the putative genes from the *S. marcescens* WW4 genome were 636, 654, 657, and 561 bp for CA1–4, respectively. PCR was conducted using the *S. marcescens* Wy064 genomic DNA as a template and the primer pairs designed according to the putative gene sequences (Table 1). The sequencing results indicated that the amplified product of CA1–4 shared 95, 97, 98, and 95% homology, respectively, with those from *S. marcescens* WW4. The deduced amino acids were used to perform sequence analysis and alignment with the NCBI BLASTp and BioEdit software. The results showed that CA1, CA2, and CA3 were members of the beta carbonic anhydrase superfamily and CA4 belonged to the gamma carbonic anhydrase superfamily, which was characterized in a previous study [24]. The gamma class of CA is generally reported in archae and methanogens. In silico sequence analysis showed that gamma CA widely distributed in Enterobacteriaceae, Pseudomonadaceae, and Burkholderiaceae members based on the CA4 amino acid sequence from the genus of *Serratia* [24]. The role of four CAs

simultaneously existed in *S. marcescens* Wy064 still remain to be clarified during the CO₂ fixation process. Using InterProScan web server (<http://www.ebi.ac.uk/Tools/pfa/ipscan/>) to search for the conserved domain of the CA1 enzymes revealed the presence of two conserved sequences for zinc binding site (39C, 41D, 98H, 101C) and active site clefts (30Q, 32P, 39C, 41D, 42S, 43R, 55G, 58F, 83Y, 88L, 98H, 101C, 182Y) in the sequences of CA1 enzymes (Fig. S1). Similarly, two conserved sequences were also observed in CA2 and CA3 (Fig. S1). The Cys₂-His/Asp motif for the zinc binding site represented a classic feature in the beta CA superfamily [21, 33]. The beta CA class could furthermore be divided into two different subclasses including type I beta CA and type II beta CA. A classic Cys₂His(X) motif could be observed in the type I beta CA structure, and X represented an alternative ligand (acetate or water molecule) used as the fourth ligand to Zn²⁺. For the type II beta CA, Asp in the Cys₂HisAsp motif replaced acetate or water molecule in the Cys₂-His(X) motif of the type I beta CA to interact with Zn²⁺ [21].

Expression and Purification of CA Enzymes

The genes of CA1, CA2, and CA3 from *S. marcescens* Wy064 were ligated into the expression plasmid pET28a and protein expression was conducted in *E. coli* BL21 (DE3) using IPTG (0.5 mM) as inducer. Expression analysis of the whole-cell protein by SDS-PAGE revealed the presence of over-expressed protein, indicating that the target enzymes were successfully expressed in *E. coli*

Fig. 2 Expression and purification of the CA1, CA2, and CA3 protein. M: protein marker (120, 100, 70, 50, 40, 30, 25, 14 kDa); 1,5,9: *E. coli* harboring pET28a (control); 2,6,10: *E. coli* harboring pET-CA1, pET-CA2 and pET-CA3 induced with 0.5 mM IPTG; 3,7,11: soluble fraction of *E. coli* harboring pET-CA1, pET-CA2 and pET-CA3 induced with 0.5 mM IPTG; 4,8,12: purified CA1, CA2, and CA3

(Fig. 2). However, the recombinant CA2 protein was expressed in inclusion. The purified CA1 and CA3 were given in Fig. 2. The specific CO₂ hydration activity of the purified CA1 with 1 mM Zn²⁺ was determined to be 165 WAU/mg lower than the CA3 activity of 234 WAU/mg. These findings implied that some differences might exist in the structure of CA1 and CA3. The comparative enzyme activities among CA1, CA3, and other reported CAs in previous studies were shown in Table 2.

Effects of Temperature and pH on the Activities of CA1 and CA3

The effects of temperature on the activities of CA1 and CA3 were determined in the range of 30–80 °C. The results

presented in Fig. 3a, b showed that the optimal temperature for CO₂ hydration activities of CA1 and CA3 was 40 °C. A rapid decline in CO₂ hydration activities of CA1 and CA3 was observed in the range of 40 °C–80 °C. Less than 30% of the maximum CA1 and CA3 activities were retained beyond 60 °C. The pH effects on CO₂ hydration activities of CA1 and CA3 were measured in the range of 4.5–10.0 at 40 °C using 100 mM citrate phosphate buffer (4.5–5.0), phosphate buffer (6.0–8.0), or glycine–NaOH buffer (9.0–10.0). As shown in Fig. 3c, d, the maximum CO₂ hydration activities of CA1 and CA3 were observed at pH 7.5. More than 50% of the residual activity for CA3 could be retained in the pH range of 4–10, implying that the CA3 enzyme had good pH stability. In contrast, the CA1 stability was limited narrow pH range from 6 to 8, which retained more than 60% of the residual CO₂ hydration activity.

Effects of Metal Ions on the Activities of CA1 and CA3

The effects of metal ions including Na⁺, Mn²⁺, Fe³⁺, Zn²⁺, Hg²⁺, Co²⁺, Ca²⁺, and Mg²⁺ on CO₂ hydration activities of CA1 and CA3 were investigated at the concentration of 1 mM. As shown in Fig. 4, all tested metal ions could activate and enhance CO₂ hydration activity of CA1 and CA3, especially Zn²⁺, which significantly increased CO₂ hydration activity to 2210% and 2710%, respectively when compared to that of no ion control. These results indicated that Zn²⁺ was the most effective cation for the activation of CA1 and CA3 from *S. marcescens* Wy064.

Table 2 Comparison of the carbonic anhydrase (CA) hydration activities among CA1, CA3, and other reported CA enzymes

Species	Category of CA	CO ₂ hydration activity (WAU/mg)	References
<i>Chlamydomonas</i> sp. ICE-L	α-CA	0.4	[34]
<i>Hahella chejuensis</i>	α-CA	478	[30]
<i>Persephonella marina</i> EX-H1	α-CA	376	[35]
<i>Lactobacillus delbrueckii</i> CGMCC8137	α-CA	468	[36]
<i>Pseudomonas fragi</i>	Unknown	70.6	[17]
<i>Micrococcus lylae</i>	Unknown	66.5	[17]
<i>Micrococcus luteus</i>	Unknown	61.0	[17]
<i>Bacillus halodurans</i>	α-CA	3425	[37]
<i>Chlamydomonas reinhardtii</i> (CAH7)	β-CA	3.1	[38]
<i>Chlamydomonas reinhardtii</i> (CAH8)	β-CA	4.2	[38]
<i>Serratia marcescens</i> Wy064 (CA1)	β-CA	165	This study
<i>Serratia marcescens</i> Wy064 (CA3)	β-CA	234	This study

Fig. 3 Effects of temperature and pH on the hydration activities of CA1 (a, c) and CA3 (b, d). a and b relative activity at different temperatures; c and d: relative activity at different pH conditions

Fig. 4 Effects of metal ions on CO₂ hydration activities of CA1 and CA3

Kinetics Parameter Assays

To determine the kinetic parameters, the reaction mixtures were prepared with the substrate concentration of CO₂ varied from 10 to 63 mM. Non-linear regression fitting with the Michaelis–Menten equation for the hydration of CO₂ to HCO₃[−] indicated that the K_m and V_{max} values were 27 mM and 179 WAU/mg for CA1, and 14 mM and 247 WAU/mg for CA3, respectively.

Overall Structure Description of CA1 and CA3

The three-dimensional structures of CA1 and CA3 were built by homology modeling by employing the X-ray crystallographic structure of CAs (5SWC and 1I6P) from *Synechocystis* sp. PCC 6803 and *Escherichia coli* as templates, respectively [33, 39]. As shown in Fig. S2a and S2b, the CA1 was composed of six subunits and appeared to be a hexamer, whereas four subunits were assembled into a tetramer by CA3. The active site zinc ion in CA1 and CA3 is coordinated to four ligands: Cys39(42), Asp41(44), His98 and Cys101 (Fig. S2c and S2d). No other zinc ligands and a broken Arg41(44)-Arg43(46) dyad, which represented two special features in the type II beta CA, were found in the structures of CA1 and CA3, implying that both types of CA should belong to the type II beta CA. In the type I beta CA, Zn²⁺ in the active site interacts with a classic Cys₂His(X) motif, and X as an alternative ligand (acetate or water) forms a hydrogen bond with the side chain of Asp from an Asp-Arg dyad and binds directly to zinc ion [21]. In addition, a distinct difference in the two type beta CAs is a Trp-Arg-Tyr triad, which is present or absent in the substrate binding pocket of CAs [33, 39]. The residues that form bicarbonate binding triad (Trp39-Arg64-Trp181) could be found in the CA3 structure, which was absent in the CA1 structure. The catalytic site in CA1 and CA3 is located on the dimerization interface and forms a narrow, hydrophobic active site cleft including Phe58, Ala63(Val66), and Tyr83. The main difference between

CA1 and CA3 is that Gln30 in CA1 may form a hydrogen bond with a bicarbonate ion, whereas the Gln33 in CA3 (Gln30 in CA1) is out of the catalytic site region. The CA1 and CA3 protein with these characteristics from newly isolated *S. marcescens* Wy064 were similar to CAs from *Synechocystis* sp. PCC 6803 and *Escherichia coli*, which represented a classic type II beta subclass, and could be assigned to the type II beta CA [33, 39].

Conclusion

In the current study, two chemolithotrophic strains (*S. marcescens* Wy064 and *Bacillus* sp. Wy065) for CO₂-fixation were isolated and identified. Three electron donors (Na₂S, NaNO₂, and Na₂S₂O₃) could efficiently improve CO₂ fixation efficiencies by the two newly isolated chemolithotrophic strains. The *S. marcescens* Wy064 strain might employ Calvin cycle to perform CO₂-fixation based on its detectable CA and RuBisCO activities. In addition, three CA genes from *S. marcescens* Wy064 were cloned and identified. The hydration of CO₂ to HCO₃[−] indicated that the *K_m* and *V_{max}* values were 27 mM and 179 WAU/mg for CA1, and 14 mM and 247 WAU/mg for CA3, respectively. The sources of the newly isolated strains and the recombinant CAs provided a potential application for CO₂ sequestration and fixation.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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