Heterologous expression of carbonic anhydrase in *Acinetobacter* sp. Tol 5 for whole-cell biocatalysis

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Abstract

Carbonic anhydrase accelerates the hydration of carbon dioxide (CO_2) and is an attractive biocatalyst for carbon capture and utilization processes. *Acinetobacter* sp. Tol 5 shows high adhesiveness to materials due to its cell surface protein AtaA. Previously, we developed a method for immobilizing bacteria using AtaA and demonstrated its application to gas-phase bioproduction. In this study, we aimed to develop Tol 5 cells expressing carbonic anhydrase and to evaluate CO_2 conversion ability as a whole-cell biocatalyst. A codon-optimized carbonic anhydrase from *Sulfurihydrogenibium yellowstonense* (SyCA) was first produced in the cytoplasm. Western blotting confirmed expression, but the Wilbur–Anderson unit assay showed little carbonic anhydrase activity as a whole-cell biocatalyst. To enhance activity, we fused six signal peptides (SPs) to SyCA for periplasmic expression: PelB and TorA from *Escherichia coli*, the Sec-type SP of Tol 5 outer-membrane protein Omp38, and three putative Tat-type SPs from Tol 5. All constructs except the one fused to the SP of PelB produced SyCA protein. The Omp38-SP fused SyCA was correctly processed to the expected molecular size and exhibited increased whole-cell carbonic anhydrase activity, whereas other SPs were not processed and showed smaller activities. These results show that periplasmic expression of SyCA with a suitable SP is important for efficient CO_2 hydration in Tol 5. The integration of the adhesive properties of Tol 5 with engineered periplasmic expression of SyCA will lead to a simple platform for whole-cell biocatalysts in gas-phase carbon capture and utilization applications.

Keywords

carbonic anhydrase, whole-cell biocatalyst, periplasmic expression, *Acinetobacter*

1. Introduction

The amount of carbon dioxide (CO_2) emissions as a result of human activity increased from 24.9 to 41 billion tons per year in the period from 1990 to 2022 emphasizing the urgent need for efficient, scalable carbon capture and utilization technologies [1]. Biocatalytic approaches that operate under ambient temperature and pressure without organic solvents have attracted particular attention for their sustainability. Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc-dependent metalloenzyme that accelerates the reversible hydration of CO_2 to bicarbonate (HCO_3^-) with catalytic efficiencies approaching 10^6 s^{-1}, thereby enhancing CO_2 solubility by several orders of magnitude [2,3]. Solubilized bicarbonate (HCO_3^-) can be captured as calcium carbonate or utilized by microorganisms as a metabolic substrate [2,4].

The gram-negative bacterium *Acinetobacter* sp. Tol 5 exhibits high adhesiveness to various material surfaces, including hydrophobic plastics, hydrophilic glass, and metals, through its fibrous cell surface protein AtaA [5]. AtaA is a member of the trimeric autotransporter adhesin (TAA) family [6], and polypeptide chains of AtaA consisting of 3630 amino acids form a large homotrimeric fibrous structure that is over 1 MDa in molecular weight and 260 nm in length. TAAs, including AtaA, are secreted to the outer membrane (OM) by the type Vc secretion system [7]. We previously developed a method for immobilizing bacterial cells using AtaA; bacterial cells transformed with the *ataA* gene exhibit nonspecific high adhesiveness, and large amounts of growing and resting cells can be quickly and firmly immobilized onto various material surfaces [8,9]. The immobilized cells directly on surfaces through AtaA show better tolerance and increased chemical reaction rates and can be repeatedly used in reactions without inactivation [8]. Furthermore, cells immobilized via AtaA can be

placed directly in the gas phase, enabling efficient reactions with gaseous substrates [10,11]. Therefore, Tol 5 is expected to be useful for CO_2 utilization. However, to the best of our knowledge, there have been no reports of expressing a heterologous carbonic anhydrase in *Acinetobacter*.

In this study, we aimed to develop Tol 5 expressing carbonic anhydrase and to evaluate CO_2 conversion ability as a whole-cell biocatalyst.

2. Materials and methods

2.1. Bacterial strains

The restriction-modification system and *ataA*-deficient strain of *Acinetobacter* sp. Tol 5 REK123Δ*ataA* (Tol 5_{REK}) was used as the host strain [12,13]. *Escherichia coli* DH5α was used for plasmid construction. Tol 5_{REK} was cultured in LB medium at 28°C, and DH5α was cultured in LB medium at 37°C.

2.2. Plasmid construction

The oligonucleotides and synthetic DNA used in this study are listed in Table S1. An *Acinetobacter* codon-optimized gene encoding SyCA (carbonic anhydrase from *S. yellowstonense*) amino acid residues 21–246 was synthesized with a ribosome-binding site immediately upstream and a C-terminal hexahistidine tag followed by a stop codon and the T7 terminator. The synthesized gene was inserted into the EcoRI and XbaI sites of pARP3, generating pARP3-SyCA. To construct a plasmid expressing SyCA fused to a signal peptide (SP), the SyCA coding region was amplified by PCR from pARP3-SyCA using primers SyCA-F and SyCA-R, producing Fragment-1. For PelB, TorA, and Omp38, complementary oligonucleotides (SP-F and SP-R) were annealed to yield the

90 double-stranded fragment (Fragment-2). For Tat-1, Tat-2, or Tat-3, complementary
91 oligonucleotides (SP-F1 and SP-R1) were annealed, and a DNA fragment was amplified
92 by PCR using primers SP-F2 and SP-R2 with the double-stranded oligonucleotides as a
93 template, yielding Fragment-2′. The backbone of pARP3-SyCA was linearized with
94 EcoRI and XbaI to obtain Fragment-3. Fragments 1, 2 (or 2′), and 3 were assembled
95 using HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, United
96 Kingdom), generating the expression plasmid for SP-fused SyCA. The constructed
97 plasmid was introduced into Tol 5_{REK} cells by electroporation as previously described[13].
98

99 **2.3. Protein expression**

100 One milliliter of the overnight culture of Tol 5_{REK} harboring the expression plasmid was
101 inoculated into 100 ml of LB medium supplemented with gentamicin (10 μg/mL), 0.5%
102 arabinose, and 0.5 mM zinc sulfate and cultured at 28°C for 18 h at 115 rpm. Cells were
103 harvested by centrifugation at 5,000 × g for 5 min at 4°C. The cell pellet was
104 resuspended in SDS-sample buffer (5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.02%
105 (w/v) bromophenol blue, 62.5 mM Tris-HCl, pH 6.8) and subjected to SDS-PAGE
106 followed by western blotting using an anti-His-tag antibody to verify expression.
107

108 **2.4. Wilbur-Anderson unit (WAU) assay**

109 CO_2 hydration activity was evaluated using the Wilbur-Anderson unit (WAU) assay [14]
110 with slight modifications. Cultured cells were harvested by centrifugation at 5,000 × g
111 for 5 min at 4°C, and the cell pellet was resuspended in pure water at an optical density
112 of 1.0 at 660 nm. Eight milliliters of 20 mM Tris-HCl (pH 8.3) and 1 ml of the cell
113 suspension were added to a 30-ml beaker on ice. Immediately after 6 ml of chilled CO_2-

114 saturated water was added to the beaker, the pH drop was recorded from 8.3 to 6.3 with
115 stirring on ice using a pH sensor (PH-208; SATOTECH, Kanagawa, Japan) equipped
116 with a logger (MJ-LOG2; SATOTEC). The obtained times for the cell suspension
117 sample and blank (water) were designated T and T_0, respectively. The WAU was defined
118 as (T_0 - T)/T. Differences among the samples were evaluated using the Brunner–Munzel
119 test with Holm's step-down adjustment.
120

121 **3. Results**

122 **3.1. Cytoplasmic expression of SyCA**

123 The α-type carbonic anhydrase from *Sulfurihydrogenibium yellowstonense* (SyCA) [15]
124 was chosen as the model enzyme because of its high catalytic efficiency and remarkable
125 thermostability. A synthetic SyCA gene, codon-optimized for *Acinetobacter*, was cloned
126 into the *Acinetobacter* expression plasmid pARP3 under the control of the arabinose-
127 inducible promoter, with a C-terminal His-tag for detection. The constructed plasmid
128 was introduced into Tol 5_{REK}, a restriction-modification system and *ataA*-deficient strain
129 of Tol 5, for accurate enzymatic activity measurement due to its non-agglutination
130 nature. SDS-PAGE of whole cell samples followed by western blotting with an anti-His
131 antibody revealed a clear band corresponding to SyCA, confirming successful
132 cytoplasmic expression (Fig. 1A).

133 Whole-cell carbonic anhydrase activity was assessed with the Wilbur–
134 Anderson unit (WAU) assay [14], which measures the rate of CO_2 hydration to bicarbonate
135 via the associated pH drop in CO_2-saturated water. A representative pH–time profile is
136 shown in Fig. 1B. Cells expressing cytoplasmic SyCA produced a similar pH–time
137 curve as the blank, which was water instead of cell suspension, and the time required to

138 reach pH 6.3 was almost the same. These results indicate that the cytoplasmic SyCA
139 exhibited little activity as a whole-cell biocatalyst under the conditions.
140

141 **3.2. Selection of signal peptides for the periplasmic expression of SyCA**

142 A previous study has shown that cytoplasmic expression of carbonic anhydrase leads to
143 poor enzymatic activity because the cell membrane limits CO_2 access and can even
144 cause intracellular toxicity, presumably via pH imbalance [16]. Therefore, periplasmic
145 expression is regarded as an alternative strategy for efficient heterologous expression of
146 carbonic anhydrase. We thus attempted to express SyCA in the periplasm of Tol 5_{REK}.
147 Proteins that need to be secreted to the periplasm are mediated by either the
148 general secretion (Sec) pathway or the twin-arginine translocation (Tat) pathway [17,18]. In
149 the Sec pathway, the unfolded polypeptide is secreted from the cytoplasm to the
150 periplasm through the SecYEG channel. A signal peptidase then cuts off its N-terminal
151 signal peptide (SP), and the protein folds in the periplasm. In the Tat system,
152 polypeptides that have already folded are secreted from the cytoplasm to the periplasm
153 through the TatABC complex, and then their SPs are cleaved by a signal peptidase. To
154 identify suitable signal peptides (SPs) for exporting SyCA, we first selected two well-
155 established *E. coli* SPs[16,19]: PelB for the Sec pathway and TorA for the twin-arginine
156 translocation (Tat) pathway, as candidates. The Sec-type SP of Omp38 [20], which is an
157 abundant OM protein known as OmpA [21], was also selected as a native Sec-type SP
158 from Tol 5. Because no Tat-type SPs have been characterized in Tol 5, open reading
159 frames of the Tol 5 genome were screened with SignalP [22], yielding three non-
160 lipoproteins (BCX74450, BCX75992, and BCX75163), two lipoproteins (BCX75681
161 and BCX73201), and one protein of uncertain classification (BCX72454). We selected

162 one representative from each of the three groups possessing putative Tat-type SPs,
163 resulting in three candidates: BCX72454, BCX75163, and BCX75681, which we named
164 Tat-1, Tat-2, and Tat-3, respectively. In total, six SPs were selected (Table 1). Each SP
165 sequence, spanning from the start methionine to its predicted cleavage site, was fused to
166 the N-terminus of SyCA through a short triglycine (GGG) linker (Fig. 2A). The six
167 fusion genes were cloned into the pARP3 vector, with a C-terminal His-tag, and were
168 introduced into Tol 5_{REK}.
169

170 **3.3. Expression of SyCA fused to each signal peptide**

171 Tol 5_{REK} cells harboring the expression plasmid were cultured at 28 °C for 18 h with
172 0.5 % arabinose to induce the expression of SyCA. After cell lysis, proteins were
173 analyzed by western blotting with an anti-His antibody. SyCA was detected in every
174 construct except when fused to the SP of PelB (Fig. 2B). The constructs fused to SPs
175 from TorA, Tat-1, Tat-2, and Tat-3 exhibited slightly higher molecular weights than the
176 cytoplasmically expressed SyCA, suggesting that their SPs were not removed. In
177 contrast, the SyCA fused to the SP of Omp38 showed a band at the same size as the
178 cytoplasmic SyCA, indicating proper cleavage of its SP and secretion into the
179 periplasm. These results suggest that the Omp38-SP is the most suitable for periplasmic
180 expression of SyCA in Tol 5_{REK}.
181

182 **3.4. Catalytic activity of cells expressing SyCA fused to each signal peptide**

183 We evaluated whether fusion with the SPs improved whole-cell carbonic anhydrase
184 activity. All SyCA-producing constructs, except the one fused to PelB-SP, were tested
185 using the WAU assay. Figure 3A shows the representative pH-time profile: cells

186 expressing SyCA fused to each SP lowered the pH faster than the blank, which was
187 water instead of a cell suspension. This suggests that these cells exhibit carbonic
188 anhydrase activity as a whole-cell biocatalyst. The WAU values calculated from three
189 independent measurements were summarized in Figure 3B. The Omp38-SP, which
190 follows the Sec pathway, yielded the highest activity. Tat-type SPs from either *E. coli*
191 (TorA) or Tol 5 gave lower activity. The Brunner–Munzel test was applied with Omp38
192 as the reference. After Holm adjustment for four comparisons, Omp38 showed
193 significantly higher WAU than TorA (adjusted $p < 0.01$), Tat-2 (adjusted $p < 0.01$), Tat-3
194 (adjusted $p < 0.01$), and Tat-1 (adjusted $p = 0.013$). These results indicate that
195 periplasmic expression with a suitable SP is important for achieving effective whole-cell
196 biocatalysis of carbonic anhydrase in Tol 5.
197
198 **4. Discussion**
199 In this study, we investigated the expression of the carbonic anhydrase SyCA in Tol
200 5_{REK} and its CO_2 conversion activity as a whole-cell biocatalyst. Fusing SyCA with the
201 SP of Tol 5 Omp38 led to efficient processing of its SP, resulting in the highest WAU
202 value (Figs. 2, 3). These results highlight that periplasmic expression and the selection
203 of an appropriate SP are important for the catalytic performance of carbonic anhydrase
204 as a whole-cell biocatalyst. To the best of our knowledge, this is the first report on the
205 heterologous expression of carbonic anhydrase in *Acinetobacter* for carbon utilization.
206 Several studies have explored the periplasmic expression of carbonic anhydrase
207 in *E. coli*. Jo *et al.* expressed NgCA using the PelB (Sec) and TorA (Tat) signal peptides
208 and found that the TorA construct yielded higher catalytic activity [16]. Patel *et al.*
209 expressed the Cab and Cam with PelB (Sec) and gIII (Sec) signals, reporting that PelB

210 conferred slightly higher activity for both enzymes [23]. More recently, Jo *et al.* showed
211 that the periplasmic expression of TdCA with the PelB (Sec) signal peptide enhanced
212 enzyme activity [24]. Collectively, these findings indicate that the optimal signal peptide
213 for periplasmic expression appears to be specific to each carbonic anhydrase even in *E.*
214 *coli*. In Tol 5, the *E. coli* PelB-SP did not yield detectable SyCA production (Fig. 2), and
215 the *E. coli* TorA-SP produced only weak activity (Fig. 3). These results suggest that SPs
216 from *E. coli* do not match the secretion machinery of Tol 5 and are consistent with
217 previous reports that variations in signal peptide sequences influence secretion
218 efficiency and that some signal peptides exhibit species specificity [25,26]. The three Tat-
219 type SPs from Tol 5 were not fully cleaved and also yielded lower activity (Figs. 2, 3).
220 In contrast, the native Sec-type Omp38-SP from Tol 5 was cleaved efficiently and gave
221 the best activity, demonstrating its suitability for expressing SyCA in this host.
222 Tol 5 cells can be immobilized on many materials through AtaA and can be
223 applied to gas-phase reactions [10,11]. Combining the bacterial cell immobilization with
224 the periplasmic expression of SyCA may lead to stable, reusable catalysts for gas-phase
225 CO_2 hydration or for supplying bicarbonate to downstream biosynthetic steps. Such
226 systems could contribute to efficient biological carbon capture and conversion
227 technologies.
228

229 **5. Conclusion**

230 In this study, we demonstrated that periplasmic expression and selection of an
231 appropriate SP are important for the catalytic performance of carbonic anhydrase in Tol
232 5 as a whole-cell biocatalyst. Integrating the adhesive properties of Tol 5 with
233 engineered periplasmic expression of SyCA could lead to efficient CO_2 capture and

conversion processes.

CRediT authorship contribution statement

Shogo Yoshimoto: Conceptualization, Validation, Writing - Original Draft. **Hiroya Oka**: Investigation. **Yuki Ohara**: Investigation. **Yan-Yu Chen**: Investigation. **Masahito Ishikawa**: Investigation. **Katsutoshi Hori**: Conceptualization, Writing - Review & Editing, Funding Acquisition

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Declaration of competing interest

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary materials.

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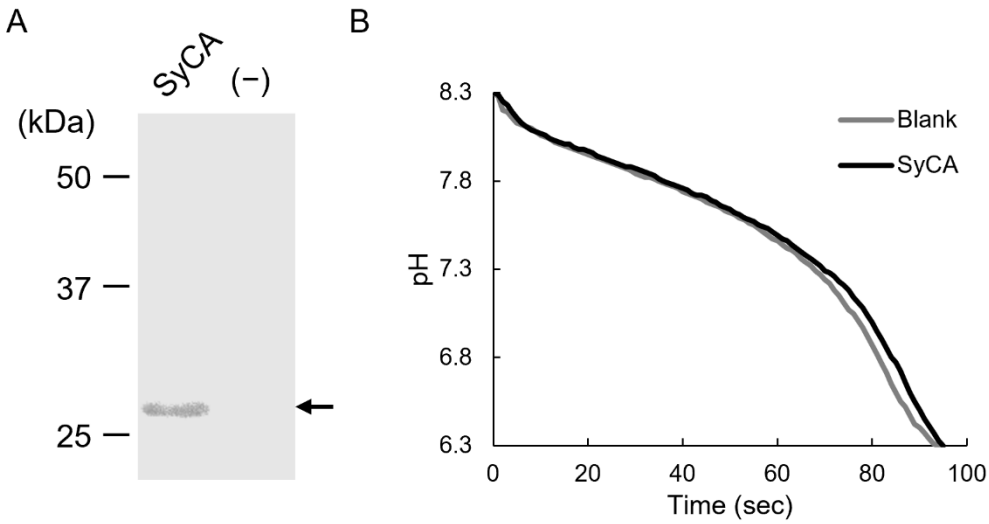
331 **Figure captions**

332



333

334 **Figure 1. Cytoplasmic expression of SyCA.** (A) Western blotting of whole-cell lysates
335 from Tol 5_{REK} cells carrying the SyCA expression plasmid (+) or carrying no plasmid
336 (–). Proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and
337 probed with an anti-His antibody; the arrowhead marks the band corresponding to
338 SyCA. (B) Time course of the pH changes during the WAU assay. Carbonic anhydrase
339 activity of cells expressing cytoplasmic SyCA or blank (water only, no cells) was
340 measured.

341

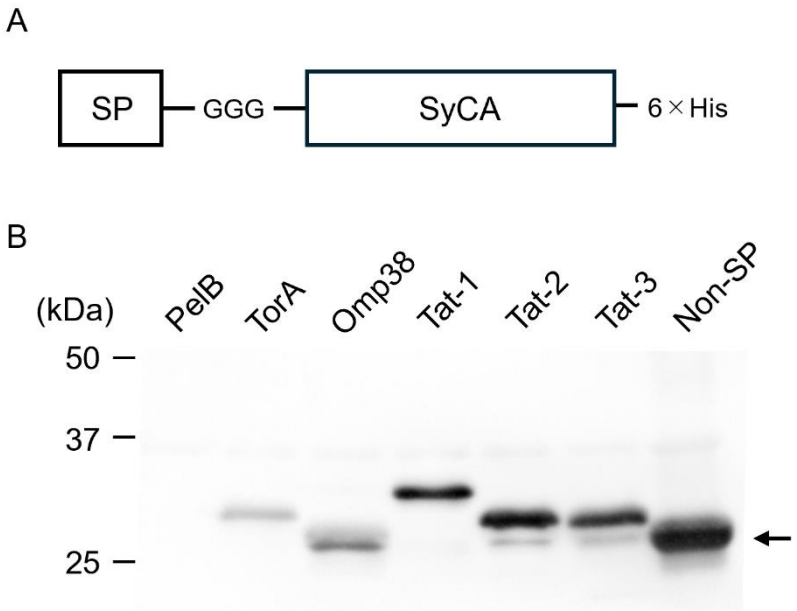
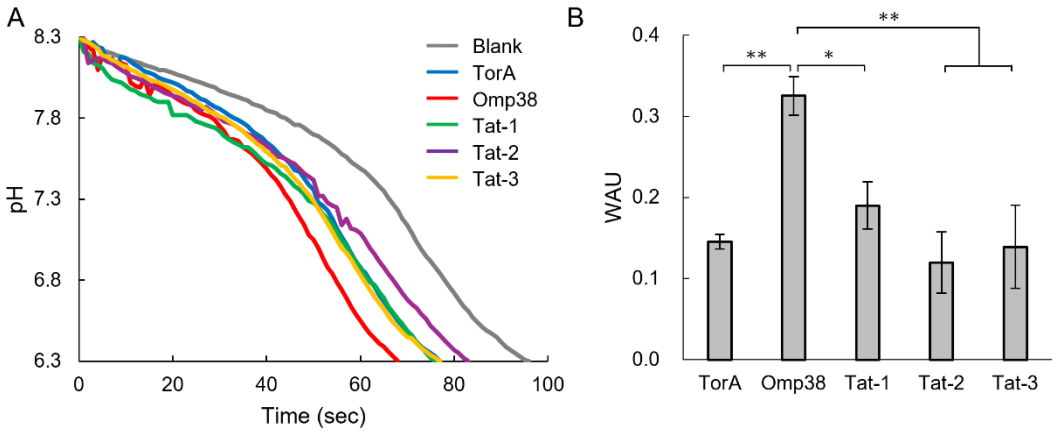


Figure 2. Expression of SyCA variants fused to each signal peptide. (A) Schematic representation of SyCA fused to a signal peptide (SP). (B) Protein expression was detected by western blotting using an anti-His antibody. The arrowhead indicates the band corresponding to the cytoplasmically expressed SyCA (Non-SP).



349

350 **Figure 3. Carbonic anhydrase activity of cells expressing SyCA variants fused to**

351 **each signal peptide.** (A) Time course of the pH changes during the WAU assay of cells

352 expressing SyCA variants fused to each signal peptide. (B) The WAU values calculated

353 from three independent measurements. Data are expressed as mean ± SEM (n = 3).

354 Statistical significance, *adjusted $p = 0.013$, **adjusted $p < 0.01$.

355

356 **Table 1. Signal peptides used in this study.**

Source	Gene	Sec/Tat	Sequence
E. coli	*pelB*	Sec	MKYLLPTAAAGLLLLAAQPAMA
E. coli	*torA*	Tat	MNNNDLFQASRRRFLAQLGGLTVAGMLG PSLLTPRRATA
Tol 5	*omp38*	Sec	MKLSRIALAMLVAAPLAAANAG
Tol 5	BCX72454 (Tat-1)	Tat	MTDLTPYHEDQELDNNTSNNIHFRDILEQ RISRRSLITKTASGAAALALASSLTG
Tol 5	BCX75163 (Tat-2)	Tat	MNKKVDFDPAKRDFLKTMGSLAAFGAVT TWMPVQSAKA
Tol 5	BCX75681 (Tat-3)	Tat	MTNSANQHTRREVLTWLASIPFLPLGAM ATSATLAG

357

358