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

Running head: Expression of carbonic anhydrase in *Acinetobacter*

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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. *Acinetobacter* sp. Tol 5 shows high adhesiveness via its cell-surface protein AtaA. We previously demonstrated its application to bacterial immobilization and gas-phase bioproduction. Here, we developed Tol 5 cells expressing carbonic anhydrase and evaluated $CO_2$ conversion ability as whole-cell biocatalysts. A codon-optimized carbonic anhydrase from *Sulfurihydrogenibium yellowstonense* (SyCA) was produced in the cytoplasm, but the cells showed little activity as a whole-cell biocatalyst. To enhance activity, we fused six signal peptides (SPs) to SyCA for periplasmic expression. The Omp38-SP fusion of SyCA was properly processed to the mature size, yielding higher whole-cell activity. By contrast, the other constructs were either undetectable or remained unprocessed, resulting in lower activities. These results show that periplasmic expression of SyCA is important for efficient $CO_2$ hydration in Tol 5 cells as whole-cell biocatalysts.

**Keywords**

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

## 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 (Yurak and Fedorov, 2025). A variety of strategies, including physical absorption, chemical solvent scrubbing, membrane separation, and physical and chemical adsorption, have been explored to mitigate $CO_2$ emissions (Hanifa et al., 2023; Kammerer et al., 2023; Nagireddi et al., 2023). Among them, biocatalytic and bio-integrated approaches that operate under ambient temperature and pressure without organic solvents are methods currently attracting significant attention because of their sustainability (Carceller et al., 2023; Marra et al., 2024; Sieborg et al., 2024).

Carbonic anhydrase (EC 4.2.1.1) is a zinc-dependent metalloenzyme that accelerates the reversible hydration of $CO_2$ to bicarbonate ($HCO_3^-$). Solubilized bicarbonate can be captured as calcium carbonate or utilized by microorganisms as a metabolic substrate (Liu et al., 2020; Talekar et al., 2022). The catalytic efficiency of carbonic anhydrase is very high, with a $k_{cat}$ value of $10^6$ $s^{-1}$, thereby enhancing $CO_2$ solubility by several orders of magnitude (Khalifah, 1971; Talekar et al., 2022; Shao et al., 2024). Many studies have shown that purified carbonic anhydrase markedly accelerates $CO_2$ capture (Talekar et al., 2022); however, the scale required for industrial carbon capture makes the use of purified enzymes economically impractical. Consequently, whole-cell catalysts that do not require a costly purification process are gaining attention as a cost-effective alternative (Moon et al., 2020; Ting et al., 2022; Zhu et al., 2022; Baidya et al., 2024).

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 (Ishikawa et al., 2012). 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 (Ishikawa et al., 2014; Hori et al., 2015). 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 (Ishikawa et al., 2014). Furthermore, cells immobilized via AtaA can be placed directly in the gas phase, enabling efficient reactions with gaseous substrates (Yoshimoto et al., 2017; Usami et al., 2020). 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.

## Materials and methods

### 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 (Ishikawa and Hori, 2024; Inoue et al., 2025). *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.

### Plasmid construction

84 The oligonucleotides and synthetic DNA used in this study are listed in Table S1. An
85 *Acinetobacter* codon-optimized gene encoding SyCA (carbonic anhydrase from *S.*
86 *yellowstonense*) amino acid residues 21–246 was synthesized with a ribosome-binding
87 site immediately upstream and a C-terminal hexahistidine tag followed by a stop codon
88 and the T7 terminator. The synthesized gene was inserted into the EcoRI and XbaI sites
89 of pARP3, generating pARP3-SyCA. To construct a plasmid expressing SyCA fused to
90 a signal peptide (SP), the SyCA coding region was amplified by PCR from pARP3-
91 SyCA using primers SyCA-F and SyCA-R, producing Fragment-1. For PelB, TorA, and
92 Omp38, complementary oligonucleotides (SP-F and SP-R) were annealed to yield the
93 double-stranded fragment (Fragment-2). For Tat-1, Tat-2, or Tat-3, complementary
94 oligonucleotides (SP-F1 and SP-R1) were annealed, and a DNA fragment was amplified
95 by PCR using primers SP-F2 and SP-R2 with the double-stranded oligonucleotides as a
96 template, yielding Fragment-2′. The backbone of pARP3-SyCA was linearized with
97 EcoRI and XbaI to obtain Fragment-3. Fragments 1, 2 (or 2′), and 3 were assembled
98 using HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, United
99 Kingdom), generating the expression plasmid for SP-fused SyCA. The constructed
100 plasmid was introduced into Tol $5_{REK}$ cells by electroporation as previously
101 described(Ishikawa and Hori, 2024).
102
103 **Protein expression**
104 One milliliter of the overnight culture of Tol $5_{REK}$ harboring the expression plasmid was
105 inoculated into 100 ml of LB medium supplemented with gentamicin (10 µg/mL), 0.5%
106 arabinose, and 0.5 mM zinc sulfate and cultured at 28°C for 18 h at 115 rpm. Cells were
107 harvested by centrifugation at 5,000 × g for 5 min at 4°C. The cell pellet was

108 resuspended in SDS-sample buffer (5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.02%
109 (w/v) bromophenol blue, 62.5 mM Tris-HCl, pH 6.8) and subjected to SDS-PAGE
110 followed by western blotting using an anti-His-tag antibody to verify expression.
111

112 **Wilbur-Anderson unit (WAU) assay**

113 $CO_2$ hydration activity was evaluated using the Wilbur and Anderson unit (WAU) assay
114 (Wilbur and Anderson, 1948) with slight modifications. Cultured cells were harvested
115 by centrifugation at 5,000 × g for 5 min at 4°C, and the cell pellet was resuspended in
116 pure water at an optical density of 1.0 at 660 nm. Eight milliliters of 20 mM Tris-HCl
117 (pH 8.3) and 1 ml of the cell suspension were added to a 30-ml beaker on ice.
118 Immediately after 6 ml of chilled $CO_2$-saturated water was added to the beaker, the pH
119 drop was recorded from 8.3 to 6.3 with stirring on ice using a pH sensor (PH-208;
120 SATOTECH, Kanagawa, Japan) equipped with a logger (MJ-LOG2; SATOTEC). The
121 obtained times for the cell suspension sample and blank (water) were designated T and
122 $T_0$, respectively. The WAU was defined as $(T_0 - T)/T$. Differences among the samples
123 were evaluated using the Brunner–Munzel test with Holm's step-down adjustment.
124

125 **Results**

126 **Cytoplasmic expression of SyCA**

127 The α-type carbonic anhydrase from *Sulfurihydrogenibium yellowstonense* (SyCA)
128 (Capasso et al., 2012) was chosen as the model enzyme because of its high catalytic
129 efficiency and remarkable thermostability. A synthetic SyCA gene, codon-optimized for
130 *Acinetobacter*, was cloned into the *Acinetobacter* expression plasmid pARP3 under the
131 control of the arabinose-inducible promoter, with a C-terminal His-tag for detection.

132 The constructed plasmid was introduced into Tol $5_{REK}$, a restriction-modification system
133 and *ataA*-deficient strain of Tol 5, for accurate enzymatic activity measurement due to
134 its non-agglutination nature. SDS-PAGE of whole cell samples followed by western
135 blotting with an anti-His antibody revealed a clear band corresponding to SyCA,
136 confirming successful cytoplasmic expression (Fig. 1A).
137 Whole-cell carbonic anhydrase activity was assessed with the Wilbur–
138 Anderson unit (WAU) assay (Wilbur and Anderson, 1948), which measures the rate of
139 $CO_2$ hydration to bicarbonate via the associated pH drop in $CO_2$-saturated water. A
140 representative pH–time profile is shown in Fig. 1B. Cells expressing cytoplasmic SyCA
141 produced a similar pH–time curve as the blank, which was water instead of cell
142 suspension, and the time required to reach pH 6.3 was almost the same. These results
143 indicate that the cytoplasmic SyCA exhibited little activity as a whole-cell biocatalyst
144 under the conditions.
145
146 **Selection of signal peptides for the periplasmic expression of SyCA**
147 A previous study has shown that cytoplasmic expression of carbonic anhydrase leads to
148 poor enzymatic activity because the cell membrane limits $CO_2$ access and can even
149 cause intracellular toxicity, presumably via pH imbalance (Jo et al., 2013). Therefore,
150 periplasmic expression is regarded as an alternative strategy for efficient heterologous
151 expression of carbonic anhydrase. We thus attempted to express SyCA in the periplasm
152 of Tol $5_{REK}$.
153 Proteins that need to be secreted to the periplasm are mediated by either the
154 general secretion (Sec) pathway or the twin-arginine translocation (Tat) pathway
155 (Mergulhao et al., 2005; Natale et al., 2008). In the Sec pathway, the unfolded

156 polypeptide is secreted from the cytoplasm to the periplasm through the SecYEG
157 channel. A signal peptidase then cuts off its N-terminal signal peptide (SP), and the
158 protein folds in the periplasm. In the Tat system, polypeptides that have already folded
159 are secreted from the cytoplasm to the periplasm through the TatABC complex, and
160 then their SPs are cleaved by a signal peptidase. To identify suitable signal peptides
161 (SPs) for exporting SyCA, we first selected two well-established *E. coli* SPs(Matos et
162 al., 2012; Jo et al., 2013): PelB for the Sec pathway and TorA for the twin-arginine
163 translocation (Tat) pathway, as candidates. The Sec-type SP of Omp38 (Ishikawa et al.,
164 2016), which is an abundant OM protein known as OmpA (Confer and Ayalew, 2013),
165 was also selected as a native Sec-type SP from Tol 5. Because no Tat-type SPs have
166 been characterized in Tol 5, open reading frames of the Tol 5 genome were screened
167 with SignalP (Teufel et al., 2022), yielding three non-lipoproteins (BCX74450,
168 BCX75992, and BCX75163), two lipoproteins (BCX75681 and BCX73201), and one
169 protein of uncertain classification (BCX72454). We selected one representative from
170 each of the three groups possessing putative Tat-type SPs, resulting in three candidates:
171 BCX72454, BCX75163, and BCX75681, which we named Tat-1, Tat-2, and Tat-3,
172 respectively. In total, six SPs were selected (Table 1). Each SP sequence, spanning from
173 the start methionine to its predicted cleavage site, was fused to the N-terminus of SyCA
174 through a short triglycine (GGG) linker (Fig. 2A). The six fusion genes were cloned into
175 the pARP3 vector, with a C-terminal His-tag, and were introduced into Tol $5_{REK}$.
176

177 **Expression of SyCA fused to each signal peptide**

178 Tol $5_{REK}$ cells harboring the expression plasmid were cultured at 28 °C for 18 h with
179 0.5 % arabinose to induce the expression of SyCA. After cell lysis, proteins were

180 analyzed by western blotting with an anti-His antibody. SyCA was detected in every
181 construct except when fused to the SP of PelB (Fig. 2B). The constructs fused to SPs
182 from TorA, Tat-1, Tat-2, and Tat-3 exhibited slightly higher molecular weights than the
183 cytoplasmically expressed SyCA, suggesting that their SPs were not removed. In
184 contrast, the SyCA fused to the SP of Omp38 showed a band at the same size as the
185 cytoplasmic SyCA, indicating proper cleavage of its SP and secretion into the
186 periplasm. These results suggest that the Omp38-SP is the most suitable for periplasmic
187 expression of SyCA in Tol $5_{REK}$.
188
189 **Catalytic activity of cells expressing SyCA fused to each signal peptide**
190 We evaluated whether fusion with the SPs improved whole-cell carbonic anhydrase
191 activity. All SyCA-producing constructs, except the one fused to PelB-SP, were tested
192 using the WAU assay. Figure 3A shows the representative pH-time profile: cells
193 expressing SyCA fused to each SP lowered the pH faster than the blank, which was
194 water instead of a cell suspension. This suggests that these cells exhibit carbonic
195 anhydrase activity as a whole-cell biocatalyst. The WAU values calculated from three
196 independent measurements were summarized in Figure 3B. The Omp38-SP, which
197 follows the Sec pathway, yielded the highest activity. Tat-type SPs from either *E. coli*
198 (TorA) or Tol 5 gave lower activity. The Brunner–Munzel test was applied with Omp38
199 as the reference. After Holm adjustment for four comparisons, Omp38 showed
200 significantly higher WAU than TorA (adjusted $p < 0.01$), Tat-2 (adjusted $p < 0.01$), Tat-3
201 (adjusted $p < 0.01$), and Tat-1 (adjusted $p = 0.013$). These results indicate that
202 periplasmic expression with a suitable SP is important for achieving effective whole-cell
203 biocatalysis of carbonic anhydrase in Tol 5.

204

205 **Discussion**

206 In this study, we investigated the expression of the carbonic anhydrase SyCA in Tol
207 $5_{REK}$ and its $CO_2$ conversion activity as a whole-cell biocatalyst. Fusing SyCA with the
208 SP of Tol 5 Omp38 led to efficient processing of its SP, resulting in the highest WAU
209 value (Figs. 2, 3). These results highlight that periplasmic expression and the selection
210 of an appropriate SP are important for the catalytic performance of carbonic anhydrase
211 as a whole-cell biocatalyst. To the best of our knowledge, this is the first report on the
212 heterologous expression of carbonic anhydrase in *Acinetobacter* for carbon utilization.

213 Several studies have explored the periplasmic expression of carbonic anhydrase
214 in *E. coli*. Jo *et al.* expressed NgCA using the PelB (Sec) and TorA (Tat) signal peptides
215 and found that the TorA construct yielded higher catalytic activity (Jo et al., 2013). Patel
216 *et al.* expressed the Cab and Cam with PelB (Sec) and gIII (Sec) signals, reporting that
217 PelB conferred slightly higher activity for both enzymes (Patel et al., 2013). More
218 recently, Jo *et al.* showed that the periplasmic expression of TdCA with the PelB (Sec)
219 signal peptide enhanced enzyme activity (Jo and Hwang, 2020). Collectively, these
220 findings indicate that the optimal signal peptide for periplasmic expression appears to
221 be specific to each carbonic anhydrase even in *E. coli*. In Tol 5, the *E. coli* PelB-SP did
222 not yield detectable SyCA production (Fig. 2), and the *E. coli* TorA-SP produced only
223 weak activity (Fig. 3). These results suggest that SPs from *E. coli* do not match the
224 secretion machinery of Tol 5 and are consistent with previous reports that variations in
225 signal peptide sequences influence secretion efficiency and that some signal peptides
226 exhibit species specificity (Blaudeck et al., 2001; Freudl, 2018). The three Tat-type SPs
227 from Tol 5 were not fully cleaved and also yielded lower activity (Figs. 2, 3). In

228 contrast, the native Sec-type Omp38-SP from Tol 5 was cleaved efficiently and gave the
229 best activity, demonstrating its suitability for expressing SyCA in this host.
230 Tol 5 cells can be immobilized on many materials through AtaA and can be
231 applied to gas-phase reactions (Yoshimoto et al., 2017; Usami et al., 2020). Combining
232 the bacterial cell immobilization with the periplasmic expression of SyCA may lead to
233 stable, reusable catalysts for gas-phase $CO_2$ hydration or for supplying bicarbonate to
234 downstream biosynthetic steps. Such systems could contribute to efficient biological
235 carbon capture and conversion technologies.
236
237 **Conclusion**
238 In this study, we demonstrated that periplasmic expression and selection of an
239 appropriate SP are important for the catalytic performance of carbonic anhydrase in Tol
240 5 as a whole-cell biocatalyst. Integrating the adhesive properties of Tol 5 with
241 engineered periplasmic expression of SyCA could lead to efficient $CO_2$ capture and
242 conversion processes.
243
244 **Author contribution**
245 **Shogo Yoshimoto**: Conceptualization, Validation, Writing - Original Draft. **Hiroya**
246 **Oka**: Investigation. **Yuki Ohara**: Investigation. **Yan-Yu Chen**: Investigation. **Masahito**
247 **Ishikawa**: Investigation. **Katsutoshi Hori**: Conceptualization, Writing - Review &
248 Editing, Funding Acquisition
249
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**Disclosure statement**

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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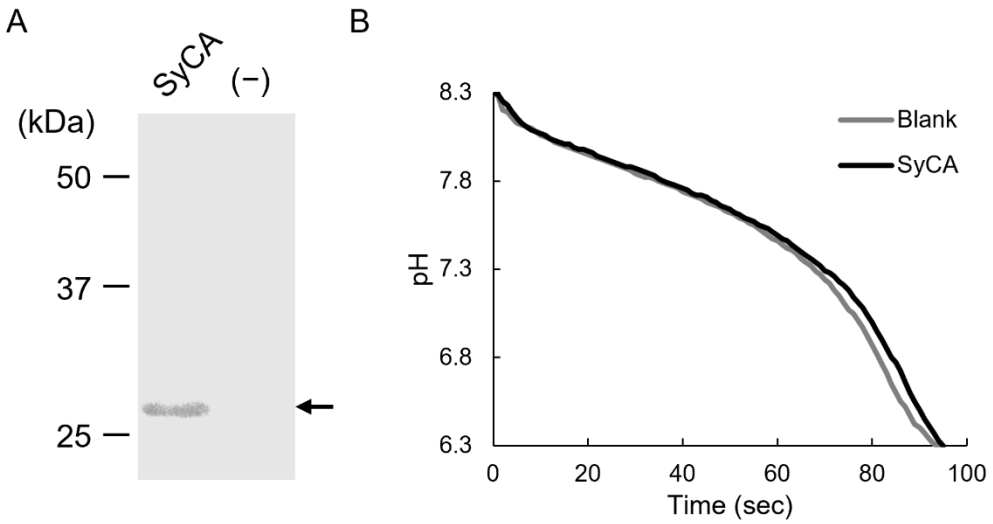
370 **Figure captions**

371




372

373 **Figure 1. Cytoplasmic expression of SyCA.** (A) Western blotting of whole-cell lysates

374 from Tol $5_{REK}$ cells carrying the SyCA expression plasmid (+) or carrying no plasmid

375 (–). Proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and

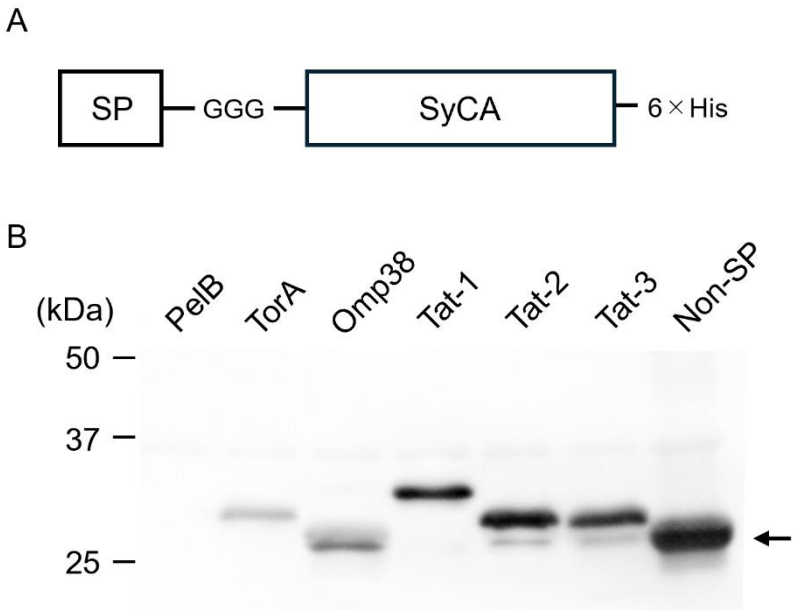
376 probed with an anti-His antibody; the arrowhead marks the band corresponding to

377 SyCA. (B) Time course of the pH changes during the WAU assay. Carbonic anhydrase

378 activity of cells expressing cytoplasmic SyCA or blank (water only, no cells) was

379 measured.

380




381

382

383 **Figure 2. Expression of SyCA variants fused to each signal sequence.** (A) Schematic

384 representation of SyCA fused to a signal peptide (SP). (B) Protein expression was

385 detected by western blotting using an anti-His antibody. The arrowhead indicates the

386 band corresponding to the cytoplasmically expressed SyCA (Non-SP).

387

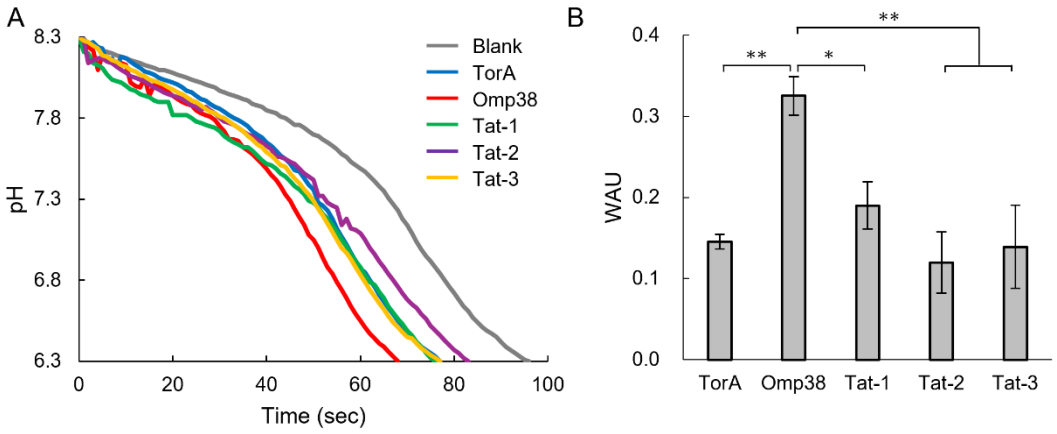



**Figure 3. Carbonic anhydrase activity of cells expressing SyCA variants fused to each signal sequence.** (A) Time course of the pH changes during the WAU assay of cells expressing SyCA variants fused to each signal sequence. (B) The WAU values calculated from three independent measurements. Data are expressed as mean ± SEM (n = 3). Statistical significance, *adjusted $p = 0.013$, **adjusted $p < 0.01$.

395 **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 |

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