Mutations in the chloramphenicol acetyltransferase (S61G, Y105C) increase accumulated amounts and resistance in *Pseudomonas aeruginosa*

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Abstract

A chloramphenicol acetyltransferase (*catB7*) gene containing two point mutations, 181A/G and 314A/G, has been recently reported to be a determinant for high-level chloramphenicol resistance phenotype in a *Pseudomonas aeruginosa* strain PAhcr1. The mutant CATB7 was further characterized in vitro and in vivo to elucidate the molecular basis of high-level resistance. CAT assay demonstrated that the mutant and wild-type recombinant CATB7 had similar specific activities. Dot blotting revealed that the accumulated amounts of CATB7 in *P. aeruginosa* strains PAO1 and PAhcr1 were proportionate to the respective anti-chloramphenicol level. Site-directed mutagenesis showed that G61S and Y105C contributed synergistically to the PAhcr1 resistance phenotype. It could be proposed that the mutant CATB7 was more structurally stable than catalytically efficient as a chloramphenicol resistance determinant in PAhcr1.

Keywords: *Pseudomonas aeruginosa*; Chloramphenicol acetyltransferase; CATB7 mutant; Structural modification

1. Introduction

Chloramphenicol acetyltransferase (*cat*) genes are widespread among most genera of gram-positive and gram-negative bacteria and represent the best-understood mechanism of microbial resistance to chloramphenicol (Cm) [1,2]. Chloramphenicol acetyltransferases (CATs) catalyze transfer of the acetyl moiety from acetyl CoA to a Cm molecule, resulting in inactive O-acetoxy derivative of Cm that no longer binds to the peptidyl transfer center on the microbial 50S ribosomal subunit [3,4]. All naturally occurring CAT variants reported up to date are in size range of 23–26 kDa, and the active form in solution are homotrimers [5].

CATs can be divided into two classes: the CATAs and the CATBs. The former, typified by CATA1 (previously designated CAT1) from transposon Tn9 [6], CATA2 (previously designated CAT11) from *Haemophilus influenza* [7] and CATA3 (previously designated CAT11) from the transmissible plasmid R387 [8], is often referred as classical CAT and has been extensively studied, in terms of structural and biochemical features, over three decades. CATB proteins, which also catalyze transfer of the acetyl from acetyl CoA to a Cm but show no significant similarity to the known CATAs, have been recently identified [9–14]. Relative to CATAs, CATBs have a low affinity (high $K_m$) for Cm and they cannot further acetylate 3-acetoxy-Cm to form 1,3-di-acetoxy-Cm. It was proposed that CATBs might use a diverse range of hydroxyl-containing molecules as acetyl acceptors and could be classified into the [LIV]-[GAED]-X$_2$-[SATV]-X hexapeptide-repeat motif-containing xenobiotic acetyltransferase (XATs) superfamily [5,15].

It was proposed that the *catB7* gene is present only in the chromosome of *Pseudomonas aeruginosa* strains and...
the catB7 gene from P. aeruginosa strain PAO222, when cloned into pUC18 or pMAQ50, conferred resistance to Cm in Escherichia coli at a level slightly lower than those of the same constructs of the catB1, catB3 and catB5 genes [13]. The open reading frame (ORF) PA0706 in the chromosome of P. aeruginosa strain PAO1 [16] encoding CATB7 sharing 100% and 98% amino acid sequence identities with the reported sequences of CATB7 from P. aeruginosa strains PAO222 and PA103, respectively. 

An N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) mutagenized PAO1-derived strain PAhcr1, resistant to high-level Cm, has been recently described [17]. It was proposed that this resistance phenotype is due to two point mutations, 181A/G and 314A/G, resulting in S61G, Y105C amino acid substitution, within the catB7 gene. The aim of the present work was to further elucidate why the mutated catB7 gene was responsible for the increased Cm resistance phenotype of PAhcr1.

2. Materials and methods

2.1. Materials

Strains and plasmids were listed in Table 1. LB medium was used as a rich medium for both E. coli and P. aeruginosa, while VBMM medium was used as a minimal medium for P. aeruginosa. Selection media were supplemented with required antibiotics. All antibiotics used in this work, including chloramphenicol, kanamycin and gentamicin, were from AMRESCO Inc. (Solon, OH, USA). Sucrose-resistant colonies were obtained by streaking P. aeruginosa merodiploids on VBMM medium containing 5% sucrose.

Restriction enzymes, T4 DNA ligase, and Pyrobest™ DNA polymerase were purchased from TaKaRa BIOTECH Co. (Dalian, China). pET expression system including pET28-(a–c) expression plasmids, E. coli strain BL21 (DE3) and Ni–NTA His-Bind® Resin was purchased from Novagen (Madison, WI, USA). Quick-Change™ Site-directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA, USA). Oligodeoxyribonucleotide used for plasmids construction and site-directed mutagenesis, which were synthesized in Sangon Biotech. Co. (Shanghai, China), were listed in Table 2. Acetyl-CoA and 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit anti-CAT serum was a generous gift from Dr. Xiaoping Dong (Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, China) [18]. Goat anti-Rabbit IgG-HRP was purchased from Sino-America Biotech. Co. (Luoyang, China). All other reagents and chemicals were of analytical grade.

Table 1
Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5 a</td>
<td>Sup E 44 ΔlacU169 φO80 lacZ Δ AM15) hsd R 17 recA 1 endA gyrA 96 thi-1 rel A 1</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>E. coli BL21(DE3)</td>
<td>F−omp T hsd S(r g m) gal dcm (DE3)</td>
<td>Novagen</td>
</tr>
<tr>
<td>E. coli SM10</td>
<td>thi−1 thr leu tonA lacY supE recA::RP4-2- Tc::Mu (KmR)</td>
<td>Stephen Lory’s Lab [23]</td>
</tr>
<tr>
<td>P. aeruginosa PA01</td>
<td>Wild-type prototroph</td>
<td>Stephen Lory’s Lab [23]</td>
</tr>
<tr>
<td>P. aeruginosa PAhcr1</td>
<td>An unmarked PA01-derived laboratory strain, high-level chloramphenicol resistant</td>
<td>Lab collection [17]</td>
</tr>
<tr>
<td>P. aeruginosa PAclr1</td>
<td>A PA01-derived strain, wild-type catB7 gene of which was replaced with mutant one (314A/G) through site-directed mutagenesis and genetic recombination</td>
<td>This work</td>
</tr>
<tr>
<td>P. aeruginosa PAcr314</td>
<td>A PA01-derived strain, wild-type catB7 gene of which was replaced with mutant one (314A/G) through site-directed mutagenesis and genetic recombination</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid pETPaCATw</td>
<td>Histidine-tagged wild-type catB7 from P. aeruginosa cloned as an Ndel-HindIII fragment in pET28a</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid pETPaCATm</td>
<td>Histidine-tagged mutant (G61S, Y105C) catB7 from P. aeruginosa cloned as an NdeI–HindIII fragment in pET28a</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid pETPaCATm181</td>
<td>Derived from pETPaCATw through site-directed mutagenesis containing 181A/G mutation in catB7 gene</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid pETPaCATm314</td>
<td>Derived from pETPaCATw through site-directed mutagenesis containing 314A/G mutation in catB7 gene</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid pEX18Gm</td>
<td>GmR; oriT+ sacB−; gene replacement vector with MCS from pUC18</td>
<td>Stephen Lory’s Lab [23]</td>
</tr>
<tr>
<td>Plasmid pEXPaCATw</td>
<td>A 2.7 kb fragment containing wild-type catB7 cloned into EcoRI–HindIII sites of pEX18Gm</td>
<td>Lab collection [17]</td>
</tr>
<tr>
<td>Plasmid pEXPaCATm181</td>
<td>Derived from pEXPaCATw through site-directed mutagenesis containing 181A/G mutation in catB7 gene</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid pEXPaCATm314</td>
<td>Derived from pEXPaCATw through site-directed mutagenesis containing 181A/G mutation in catB7 gene</td>
<td>This work</td>
</tr>
</tbody>
</table>
2.2. Minimal inhibitory concentration determination

Susceptibility testing of *P. aeruginosa* was done by minimal inhibitory concentration (MIC) determination as previously described [19]. The MIC value was defined as the minimal chloramphenicol concentration at which no growth had occurred after a 16-h incubation at 30 °C.

2.3. DNA manipulation

All routine molecular biology manipulations were performed using standard methods [20]. Genomic DNA, isolated from *P. aeruginosa* wild-type strain PAO1 and previously described mutant strain PAhcr1 [17], respectively, were used as the templates for polymerase chain reaction (PCR)-amplification of catB7 gene. The 639 bp *Nde*I–HindIII fragments were amplified with PyrobesiTM DNA Polymerase (TaKaRa BIOTECH Co. Dalian, China) and cloned into the same sites of expression vector, pET28a (Novagen, Madison, WI). The resultant plasmids were designated as pETPaCATw and pETPaCATm, respectively.

The plasmids encoding S61G or Y105C mutant of catB7, named as pETPaCATm181 and pETPaCATm314, respectively, were constructed using the PCR-based QuickChange™ site-directed mutagenesis kit according to the manufacturer’s protocol, using plasmid pETPaCATm as the template.

The plasmid pEXPaCATw was constructed as pPA-CATm [17] except that the 2.7-kb insert was PCR-amplified from PAO1 genomic DNA and used as the template for site-directed mutagenesis to derive pEXPaCATm181 or pEXPaCATm314 containing 181A/G or 314A/G mutation in catB7 gene, respectively.

All plasmids were sequenced on both strands using AmpliTaq DyeDeoxy Terminator Sequencing Kit (Perkin–Elmer Applied Biosystems Division (PE-ABI)) with an ABI 377 sequencer, to establish only expected mutations present, while absent from any PCR or cloning artifacts in all plasmids.

2.4. Expression, purification and refolding of wild-type and mutant recombinant CATB7 proteins

The expression plasmids, pETPaCATw, pETPaCATm, pETPaCATm181 and pETPaCATm314, were separately used to transform competent *E. coli* strain BL21 (DE3) (Novagen). Single colony from the four transformants was independently inoculated 20 ml of LB media plus 25 μg/ml kanamycin in a 150-ml flask and cultivated with vigorous shaking at 37 °C until the optical density at 600 nm reached 0.6–1.0. The culture was then incubated for a further 3 h in the presence of 1 mM isopropyl–β-D-thiogalactopyranoside (IPTG) and then harvested by centrifugation.

A purification strategy under denatured condition was used as the great majority of overexpressed CATB7 were found in insoluble inclusion bodies. The cell pellets were suspended in GLB (6 M GuHCl, 100 mM NaH2PO4 and 10 mM Tris–HCl, pH 8.0) and shaken at room temperature until suspension became clear. The samples were clarified by centrifugation at 12000 rpm for 30 min before loading onto Ni–NTA His-Bind® Resin (Novagen) pre-equilibrated with GLB. The Resin was extensively washed with UWB (8 M urea, 100 mM NaH2PO4 and 10 mM Tris–HCl, pH 6.3) until A280 fell below 0.01, and then eluted with Elution buffer (8 M urea, 100 mM NaH2PO4, 10 mM Tris–HCl, pH 8.0, and 250 mM imidazole). The peak fractions were identified by spotting 10 μl of each eluted fraction on Whatman™ filter paper and staining with 0.025% CBB in 25% isopropanol and 10% acetic acid, and then de-staining with 5% isopropanol and 10% acetic acid. The enzyme concentration was determined by dye-binding colorimetric assay with bovine serum albumin (BSA) as the protein standard [21].

The pooled peak fractions were dialyzed extensively overnight against basal refolding buffer (20 mM Tris–HCl, pH 7.8, 20 mM NaCl, 3% glycerol and 1 mM β-ME) with a 4 M urea linear gradient over at least 100 sample volumes. In the case of wild-type CATB7, S61G and Y105C CATB7 mutants, 0.2% Triton X-100 or 0.5% PEG3350 was added to improve refolding ratio.

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Table 2

<table>
<thead>
<tr>
<th>Oligos</th>
<th>Sequences</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>pCAT639f</td>
<td>5’AGGGACGCACGGTGATATGGGCAAC3’</td>
<td>For amplification of catB7 coding region</td>
</tr>
<tr>
<td>pCAT639r</td>
<td>5’CTTGGTATCTCCAgctTTGCAACTT3’</td>
<td></td>
</tr>
<tr>
<td>pCAT2.7f</td>
<td>5’ACTCCAG02288TGACTCATGTTG3’</td>
<td>Site-directed mutagenesis oligos, for 181A/G and 314A/G mutation, respectively</td>
</tr>
<tr>
<td>pCAT2.7r</td>
<td>5’GATCCGACATCTGACTCATGTTG3’</td>
<td></td>
</tr>
<tr>
<td>pCAT181f</td>
<td>5’TGGACTCTGGAACATCTGCAACTT3’</td>
<td></td>
</tr>
<tr>
<td>pCAT181r</td>
<td>5’GTTGAACGGCTGTCAGCCAGCG3’</td>
<td></td>
</tr>
<tr>
<td>pCAT314f</td>
<td>5’TCTGGAACGGCTGTCAGCCAGCG3’</td>
<td></td>
</tr>
<tr>
<td>pCAT314r</td>
<td>5’GTCTGGTGTTAAGCCGCTTCAC3’</td>
<td></td>
</tr>
</tbody>
</table>

* Letters in lower-case denote nt inserted to introduce an Ndel site, two HindIII sites and an EcoRI site (underlined), respectively.

b Letters in lower-case denote nt for site-directed mutagenesis, i.e. 181A/G and 314A/G mutation, respectively.
followed by gradient removal of urea. The refolded samples were clarified by centrifugation and the suspension fractions were dialyzed into reaction buffer (100 mM Tris–HCl, pH 7.8, and 0.1 mM EDTA) for immediate activity assay or into storage buffer (100 mM Tris–HCl, pH 7.8, 1 mM EDTA and 50% glycerol) for storage at –20 °C.

2.6. Gene replacement

The MICs of wild-type \( P. \) \( aeruginosa \) strain PAO1 and an MNNG mutagenized strain, PAhcr1, were previously determined to be about 85 and 340 \( \mu \)g/ml, respectively. It was proposed that the high-level Cm resistance phenotype of PAhcr1 was due to two point mutations, 181 A/G and 314 A/G, respectively; within the chloramphenicol acetyltransferase (\( catB7 \)) gene. The aim of our present investigation was to find out the reason for the mutant phenotype. Two \( P. \) \( aeruginosa \) strains, termed as PAc181 and PAc314, respectively, were derived from PAO1 through site-directed mutagenesis and homologous recombination. And the MICs were determined to be about 125 and 220 \( \mu \)g/ml, respectively (Table 3).

2.7. Western dot blotting

Wild-type and 181A/G, 314A/G mutations-containing \( catB7 \) gene was amplified from PAO1 and PAhcr1 genomic DNAs, respectively; and cloned in pET28a as Nde-I–HindIII fragments to produce histidine-tagged recombinant CATB7 proteins (wild-type CATB7 and S61G, Y105C CATB7 mutant). While S61G CATB7 mutant and Y105C CATB7 mutant were all obtained through site-directed mutagenesis, using expression plasmids pETPaCATw as the template. CATB7 proteins were successfully overexpressed in \( E. \) \( coli \) strain BL21 (DE3) under standard IPTG induction. As the majority (about 70% S61G, Y105C CATB7 mutant and above 95% wild-type CATB7, S61G CATB7 mutant and Y105C CATB7 mutant) of overexpressed CATB7 proteins were found to be aggregated into cytoplasmic inclusion bodies (Fig. 1(a)), the purification membrane (Amersham Biosciences, Uppsala, Sweden) blocked in PBS containing 1% BSA (PBSB), incubated with PBSB plus appropriate amounts of rabbit anti-CAT serum, washed three times with PBS containing 0.05% Tween 20 (PBST) and then incubated with PBSB plus horseradish root peroxidase (HRP)-conjugated goat anti-rabbit IgG, washed again three times with PBST and then once with PBS. The membrane was soaked in DAB solution until brown spots appeared.

3. Results

3.1. The different level of chloramphenicol resistance in PAO1 and its derived strains

Table 3

<table>
<thead>
<tr>
<th>Strains</th>
<th>PAO1</th>
<th>PAhcr1</th>
<th>PAc181</th>
<th>PAc314</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC (( \mu )g/ml)</td>
<td>85</td>
<td>340</td>
<td>125</td>
<td>220</td>
</tr>
</tbody>
</table>
strategy involving in one-step immobilized metal-ion affinity chromatograph (IMAC) under denatured condition was employed. Typical yields from 20 ml-induced culture were 0.7–1.1 mg of CATB7 proteins with purity greater than 95%.

Denatured CATB7 proteins were reactivated through linear gradient removal of urea with (in the case of wild-type CATB7, S61G CATB7 mutant and Y105C CATB7 mutant) or without (in the case of S61G, Y105C CATB7 mutant) the aid of refolding additives, 0.2% Triton X-100 or 0.5% PEG3350. About 80% S61G, Y105C CATB7 mutant and about 50% wild-type CATB7 could be effectively reactivated (Fig. 1(b)). The refolding yields of the two single-point CATB7 mutants were similar to that of wild-type CATB7 (data not shown).

The specific activities and Michaelis–Menton kinetics parameters for all the wild-type and mutant CATB7 proteins were determined using purified and reactivated recombinant proteins (Table 3). The specific activity of wild-type CATB7 was 260 U/mg, which was consistent with the published value of 262 U/mg [14]. The apparent kinetics parameters, including $K_m$ value for acetyl-CoA, $K_m$ value for Cm and maximum velocity ($V_{max}$), of wild-type CATB7 determined here also were in agreement with the corresponding previously determined values using crude cell extract of *E. coli* containing CATB7 expression plasmid as the assay substrate [13]. Unexpectedly, the corresponding values of specific activities and Michaelis–Menton kinetics parameters for all CATB7 mutants determined here were similar to those of wild-type CATB7 with almost neglectable variations (Table 4).

### 3.3. The accumulated amounts of CATB7 proteins in PAO1 and its derived strains

The doubt arose of why the CATB7 mutants with similar specific activities could confer the PAO1-derived *P. aeruginosa* strains, PAhcr1, PAcr181 and PAcr314,

### Table 4

<table>
<thead>
<tr>
<th>CATB7 proteins</th>
<th>Specific activity (U/mg)</th>
<th>$K_{cat}$ (µM)</th>
<th>$K_{cat}$ (µM)</th>
<th>$V_{max}$ (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>260 ± 5</td>
<td>798 ± 5</td>
<td>806 ± 13</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>G61S mutant</td>
<td>255 ± 8</td>
<td>811 ± 16</td>
<td>820 ± 9</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>Y105C mutant</td>
<td>257 ± 3</td>
<td>809 ± 11</td>
<td>797 ± 10</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>G61S, Y105C mutant</td>
<td>262 ± 11</td>
<td>795 ± 15</td>
<td>809 ± 8</td>
<td>4.1 ± 0.4</td>
</tr>
</tbody>
</table>

*a* Values were determined using purified recombinant CATB7 proteins and expressed as means ± SE for at least three independent assays.
with higher dosage of Cm resistance phenotype than PAO1 to variable degree. Therefore, the accumulated amounts of CATB7 proteins in PAO1 and its derived strains were determined with Western dot blotting using equal amounts of total cellular proteins as the starting samples. The dilution factor was adjusted to be 1.5-fold to reveal the delicate difference between the samples. The results showed that the relative accumulated amounts of CATB7 in the crude extracts of *P. aeruginosa* strains PAO1, PAcrl81, PAcrl314 and PAhcr1 increased proportionate to the individual anti-Cm level (Fig. 2).

### 4. Discussion

It was proposed that *catB7* gene is present only in the chromosome of *P. aeruginosa* strains, but CAT activity did not correlate with the Cm susceptibility of all *P. aeruginosa* strains, indicating *catB7* may not be the main determinant of intrinsic Cm resistance in all *P. aeruginosa* strains [13]. However, in a recent work, the mutations existed in the chromosome of *P. aeruginosa* PAO1-derived strain PAhcr1 were identified to be 181A/G and 314A/G substitutions within *catB7* gene, and the genetic complementation assay indicated that this mutant *catB7* gene was sufficient to restore PAO1 strain’s survival on LB plate containing 340 μg/ml Cm [17]. Therefore, it could be deduced that *catB7* was likely to be the major, if not the single, determinant for Cm resistance in PAO1 (The endeavors to identify the major determinants for different intrinsic antibiotics resistances in PAO1 are currently being under the way in our laboratory.). The aim of the present work was to elucidate the molecular mechanism for the high-level Cm resistance phenotype in PAhcr1.

The specific activities and apparent kinetics parameters for wild-type and the three mutated CATB7 proteins were determined to be unexpectedly similar considering that PAhcr1 strain was obtained by screening an MNNG mutagenized PAO1 library on LB plates containing high concentration of Cm. It was proposed that mutations induced by DNA-alkylating reagent MNNG are mainly non-randomly distributed multiple point mutations [25]. And microbial resistance to Cm is believed to be mediated mainly by inactivation of this antibiotics through enzymatic acetylation pathway [2]. A CATB7 mutant with higher specific activity or higher affinity for Cm (lower *Km*) would be a more efficient and direct response to the selective pressure after mutant reagent treatment. One plausible explanation was that neither S61G nor Y105C in CATB7 belong to the active site residues revealed from the 3.2 Å resolution X-ray crystallographic structure of CATB7 and its complex with Cm and the cofactor analogue desulfo-CoA [15], so that observed effects could be attributed to structural modifications. The observation that S61G, Y105C CATB7 mutant behaved differently from wild-type CATB7, S61G and Y105C CATB7 mutants in the process of denaturation–reactivation cycle was consistent with the above deduction. Additionally, this was in agreement with the general view that the number of sites amenable to amino acid substitutions that result in improved protein folding and/or structure stabilization is limited [26]. In the case of CATB7, neither S61G nor Y105C substitution could efficiently convert wild-type CATB7 from a protein difficult to refold into a refolding-feasible one, indicating that S61G and Y105C substitution contributed commonly to the delicate structural modification in the double substitution CATB7 mutant, which turned to be a protein feasible to refold.

The accumulated amounts of CATB7 proteins (wild-type and mutant ones) in respective strain, as revealed by relatively quantitative analysis of dot blotting, were determined to be proportionate to the respective anti-Cm level when the same concentration of Cm imposed to the four cultures. The context for all four *catB7* genes were identical as no additional mutations have been detected in our quest to find mutations within the chromosome of PAhcr1 [17], while PAcrl81 and PAcrl314 strains were directly derived from PAO1 through site-directed mutagenesis and homologous recombination. As a result, the mRNA levels and the amounts of synthesized CATB7 proteins would be identical among PAO1, PAhcr1, PAcrl81 and PAcrl314 strain under the same culture condition. It could be concluded that the different levels of the accumulated amounts of CATB7 proteins were resultant from the different degrees of proteolytic degradation on wild-type and mutant CATB7 proteins. And the ability of anti-proteolytic degradation for all four CATB7 proteins might be ranked in the following sequence, wild-type CATB7 < S61G CATB7 mutant < Y105C CATB7 mutant < S61G, Y105C CATB7 mutant, which correlated to the anti-Cm level of the four *P. aeruginosa* strains, PAO1 < PAcrl81 < PAcrl314 < PAhcr1.

Our observations showed that S61G and Y105C contributed synergistically to the anti-Cm effect in PAhcr1. Y105C, however, might be regarded as a major contributor compared with S61G. Considering that there are three Cys amino acid residues in wild-type CATB7, an additional Cys would form a disulfide bond with a certain intrinsic Cys in Y105C and S61G, Y105C CATB7 mutants. The molar ratio of free –SH group between wild-type CATB7 and S61G, Y105C CATB7 mutant was determined to be 3:1 (data not shown). And disulfide bonds are believed to an important structural contributor to proteins. The observation that the MIC of *P. aeruginosa* strain PAcrl314 was higher than that of PAcrl81 is consistent with this deduction.
The observation that S61G, Y105C CATB7 mutant would be more structurally stable than catalytically efficient mutant as the high-level Cm resistance determinant in PAhcr1 yields more questions than answers. On one hand, that the specific activities and apparent kinetics parameters for wild-type ATB7 and S61G, Y105C CATB7 mutant were similar leaves two open questions, (1) whether there are any other mutations, e.g., mutations resulted in CATB7 mutants with higher specific activities or higher affinity for Cm or acetyl CoA, even mutations involved in other anti-drug pathways in P. aeruginosa, existed in the chromosomes of the population of MNNG mutagenized PAO1 library; (2) whether there are any difference between the three-dimensional structures of wild-type ATB7 and S61G, Y105C CATB7 mutant. On the other hand, questions arose of whether more target sites of one or more of the 30 proteases in PAO1 and PAhcr1 strains were proportionate to the respective anti-Cm level, which might be resultant from similar protein expression levels but different proteolytic degradation degrees. Further work is in progress to address these questions.

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References


