Type B Chloramphenicol Acetyltransferases Are Responsible for Chloramphenicol Resistance in Riemerella anatipestifer, China

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Riemerella anatipestifer causes serositis and septicaemia in domestic ducks, geese, and turkeys. Traditionally, the antibiotics were used to treat this disease. Currently, our understanding of \textit{R. anatipestifer} susceptibility to chloramphenicol and the underlying resistance mechanism is limited. In this study, the \textit{cat} gene was identified in 69/192 (36\%) \textit{R. anatipestifer} isolated from different regions in China, including \textit{R. anatipestifer} CH-2 that has been sequenced in previous study. Sequence analysis suggested that there are two copies of \textit{cat} gene in this strain. Only both two copies of the \textit{cat} mutant strain showed a significant decrease in resistance to chloramphenicol, exhibiting 4 \(\mu\)g/ml in the minimum inhibitory concentration for this antibiotic, but not for the single \textit{cat} gene deletion strains. Functional analysis of the \textit{cat} gene via expression in \textit{Escherichia coli} BL21 (DE3) cells and \textit{in vitro} site-directed mutagenesis indicated that His79 is the main catalytic residue of CAT in \textit{R. anatipestifer}. These results suggested that chloramphenicol resistance of \textit{R. anatipestifer} CH-2 is mediated by the \textit{cat} genes. Finally, homology analysis of types A and B CATs indicate that \textit{R. anatipestifer} comprises type B3 CATs.

\textbf{Keywords:} Riemerella anatipestifer, chloramphenicol acetyltransferase, antibiotics resistance, homologous recombination, site-directed mutagenesis

\section*{INTRODUCTION}

\textit{Riemerella anatipestifer} is a gram-negative, non-flagellar bacterium belonging to the \textit{Flavobacteriaceae} family of bacteroidetes that causes serositis and septicaemia in domestic ducks, geese, and turkeys. Currently, the fatality rate of \textit{R. anatipestifer}-infected ducks has reached up to 75\%, thereby resulting in significant economic losses in the duck industry (Ruiz and Sandhu, 2013).

The extensive use of antibiotics for the treatment and prevention of serositis and septicaemia has resulted in multi-drug resistance in \textit{R. anatipestifer} (Zhong et al., 2009). It was found that 40.5\% of \textit{R. anatipestifer} strains were resistant to chloramphenicol (Chen et al., 2010). Based on the reported whole genome sequence of \textit{R. anatipestifer} (GenBank accession number: CP004020) (Wang et al., 2014), we searched for resistance genes in \textit{R. anatipestifer} CH-2 in the Comprehensive Antibiotic Resistance Database (Aakra et al., 2010). We have identified two copies of the \textit{cat} gene in \textit{R. anatipestifer} CH-2, namely, G148_1769 and G148_1772.
The cat gene encodes chloramphenicol acetyltransferases (CATs) that inactivate the drugs chloramphenicol, thiamphenicol, and azidamfenicol by acetylation, which is the most common mechanism conferring chloramphenicol resistance in bacteria (Schwarz et al., 2004). However, CAT did not inactivate florfenicol because of the replacement of the hydroxyl group at C3 by a fluor residue, and the acceptor site of the acetyl groups was structurally altered in florfenicol (Schwarz et al., 2004). In addition to acetylation inactivation of chloramphenicol, other enzymatic inactivation mechanisms, such as O-phosphorylated (Mosher et al., 1995) and hydrolysis reaction have been identified (Mosher et al., 1990; Tao et al., 2012). Moreover, resistance to chloramphenicol may also be due to mutations/modifications of the target site (Montero et al., 2007), decreased outer membrane permeability (Burns et al., 1989), and the presence of efflux pumps that often act as multidrug extrusion transporters (Daniels and Ramos, 2009), thereby reducing the effective intracellular drug concentration.

In this study, the cat gene was identified in 69/192 (36%) R. anatipestifer isolated from different regions in China by PCR. In order to verify whether cat gene was responsible for chloramphenicol resistance in R. anatipestifer, we constructed the cat gene deletion strains, complement strains and assessed the protein enzyme activity.

### MATERIALS AND METHODS

#### Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids in this study are listed in Table 1. R. anatipestifer strains were grown at 37°C in tryptic soybean broth (TSB, Oxoid) or tryptic soy agar (TSA, Oxoid) in an atmosphere of 5% CO₂. Escherichia coli (E. coli) strains were grown on Luria-Bertani (LB, Oxoid) broth or agar at 37°C. When required, antibiotics were added at the following final concentrations (µg/ml): Chloramphenicol (Cm, Sigma), 25; cefotaxin (Cfx, Sigma), 1; kanamycin (Kan, Sigma), 100; ampicillin (Amp, Sigma), 100 or spectinomycin (Spc, Sigma), 70. Diaminopimelic acid (DAP, 50 µg/ml) to E. coli X7213αpir cultures (Edwards et al., 1998).

#### Detection of the cat Gene in R. anatipestifer Isolates

For this study, 192 R. anatipestifer isolates were collected from different regions of China. All isolates were identified using the Biolog Microbial Identification System (Biolog, Hayward, CA, USA), as well as PCR and biochemical analyses (data not shown). After lysing the bacteria in lysis buffer (0.5% NP-40, Sigma; 200 ng/ml protease K, Takara Biotechnology Co., Ltd. Dalian, China), the presence of the cat gene was determined by PCR analysis using primers cat-F1 and cat-R1 (Table 2).

#### Construction of R. anatipestifer CH-2 cat Deletion Mutants and Generation of catH79A Mutant

The cat genes were deleted by homologous recombination using a suicide vector pRE112 (Kong et al., 2011) as described previously (Luo et al., 2015). Briefly, the right flanking sequence (~620 bp) and the left flanking sequence (~620 bp) of the target genes G148_1769 and G148_1772 were amplified using primers 1769up-F and 1769up-R, 1769down-F and 1769down-R, 1772up-F and 1772up-R, and 1772down-F and 1772down-R, respectively (Table 2). The 1,145 bp SpcR cassette and the 1,192 bp CfxR cassette were amplified from plasmid pYES1 (Luo et al., 2015) and pLMF01 (Liu et al., 2016) using primers Spc-F, Spc-R and Cfx-F, Cfx-R, respectively (Table 2). The SpcR cassette and the CfxR cassette were used for deletion of G148_1769 and G148_1772, respectively. The PCR fragments were overlapped using the PCR method (Xiong et al., 2006). The fused PCR fragments were ligated to suicide plasmid pRE112, respectively, to produce pRE112: 1769USD (SpcR) and pRE112: 1772UCD (CfxR). Subsequently, the recombinant plasmids were introduced into R. anatipestifer CH-2 by conjugation as described previously (Liao et al., 2015). The transconjugants were selected on TSA plates supplemented with Spc (40 µg/ml) or Cfx (1 µg/ml). The gene-deletion mutant strains, which were designated as RA-CH2Δ1769, RA-CH2Δ1772, and RA-CH2Δ1769Δ1772, were identified by PCR analysis.

The catH79A mutant was constructed by in vitro site-directed mutagenesis. The upstream and downstream mutated regions of the cat gene of R. anatipestifer CH-2 amplified using primers MF1, MR1 and MF2, MR2 his, respectively (Table 2). The fragments were fused by overlap extension PCR to yield the mutant gene catH79A.

#### Construction of the Recombinant Vector for Complementation and Expression

Complete cat and catH79A genes were amplified by PCR from R. anatipestifer CH-2 chromosomal DNA and by in vitro site-directed mutagenesis using primers catF2 and catR2, catF2, catR2, MR1 and MF2, catR2 (Table 2), for complementation. Complete cat and catH79A genes were amplified by PCR from R. anatipestifer CH-2 chromosomal DNA and by in vitro site-directed mutagenesis using primers MF1 and MR2 his, MF1, MR1 and MF2, MR2 his, respectively (Table 2), for expression of CAT and CATH79A proteins. The complementation fragments were purified and digested with NcoI and XhoI, and ligated to the pLMF02 plasmid digested with NcoI and XhoI. The expression fragments were purified and digested with NdeI and XhoI, and ligated with the pET30a plasmid digested with corresponding restriction endonucleases. The ligation mixtures were introduced into CaCl₂-competent DH5α cells. Transformants were screened by PCR, and positive clones were sequenced.

#### Construction of R. anatipestifer ATCC 11845 Cat and CatH79A Complementary Strains

The plasmids, pLMF02, pLMF02:: cat, and pLMF02:: catH79A, were introduced into R. anatipestifer ATCC 11845, respectively, by the method described previously (Liao et al., 2015). The transconjugants were selected on TSA plates supplemented with Cfx (1 µg/ml) and Kan (40 µg/ml). The complementation strains, RA-ATCC11845 (pLMF02), RA-ATCC11845 (pLMF02::
TABLE 1 | Strains and plasmids used in this study.

| Strains                  | Description                        | Source or reference |
|--------------------------|------------------------------------|---------------------|
| *Riemerella anatipestifer* ATCC 11845 | Serotype 6                         | ATCC                |
| R. anatipestifer CH-2     | Serotype 2                          | Laboratory collection |
| RA-CH2a,1769              | RA-CH2a,1769, Spc^R                | This study          |
| RA-CH2a,1772              | RA-CH2a,1772, Cfx^R                | This study          |
| RA-CH2a,1769Δ1772         | RA-CH2a,1769Δ1772, Spc^R, Cfx^R    | This study          |
| RA-ATCC11845 (pLMF02)     | *R. anatipestifer* ATCC11845 carrying pLMF02, Amp^R, Cfx^R | This study          |
| RA-ATCC11845 (pLMF02:: cat) | *R. anatipestifer* ATCC11845 carrying pLMF02:: H79A, Amp^R, Cfx^R | This study          |
| RA-ATCC11845 (pLMF02:: cat^H79A) | *R. anatipestifer* ATCC11845 carrying pLMF02:: cat^H79A | This study          |

| *Escherichia coli* strains | Description                                                                 | Source or reference |
|---------------------------|-----------------------------------------------------------------------------|---------------------|
| X7232                     | endA1 hisdR17 (pC[m^M]_I) glnV44 thi-1 recA1 gyrA relA1Δ(lacZYA-argF)U169pir deoR (Φ80dΔlac Δ(lacZ)M15) | Roland et al., 1999 |
| X7232 (pRE112:: 1769USD)  | *E. coli* X7232 pRE112:: 1769USD, Spc^R Cm^R                                  | This study          |
| X7232 (pRE112:: 1772UCD)  | *E. coli* X7232 pRE112:: 1772UCD, Cfx^R Cm^R                                  | Roland et al., 1999 |
| X7213                     | thi-1 thr-1 leuB6 glnV44 fhuA21 lacY1 recA1 RP4-2-Tc:: Mu asdA4 Δzhf-2:: Tn10 | This study          |
| X7213 (pRE112:: 1769USD)  | *E. coli* X7213 pRE112:: 1769USD, DAP, Spc^R Cm^R                            | This study          |
| X7213 (pRE112:: 1772UCD)  | *E. coli* X7213 pRE112:: 1772UCD, DAP Cfx^R Cm^R                            | This study          |
| BL21 (DE3)                | *E. coli* BL21 (DE3), expressing host cell                                   | Laboratory collection |
| BL21 (DE3) (pET30a)       | *E. coli* BL21 (DE3) carrying pET30a, Kan^R                                 | This study          |
| BL21 (DE3) (pET30a:: cat-s) | *E. coli* BL21 (DE3) carrying pET30a:: cat-s, Kan^R                         | This study          |
| BL21 (DE3) (pET30a:: cat^H79A) | *E. coli* BL21 (DE3) carrying pET30a:: cat^H79A, Kan^R                  | This study          |
| S17-1                     | Thr-1 thr leu tonA Y supE recA::RP4-2-Tc:: Mu Kan^R                           | Miller and Mekalanos, 1988 |
| S17-1 (pLMF02)            | S17-1 carrying pLMF02, Amp^R Cfx^R                                           | This study          |
| S17-1 (pLMF02:: cat)      | S17-1 carrying pLMF02:: cat, Amp^R Cfx^R                                     | This study          |
| S17-1 (pLMF02:: cat^H79A)  | S17-1 carrying pLMF02:: cat^H79A, Amp^R Cfx^R                                | This study          |

| Plasmids                  | Description                                                                 | Source or reference |
|---------------------------|-----------------------------------------------------------------------------|---------------------|
| pET30a                    | pBR322 lacZ, IPTG-inducible promoter, Kan^R                                  | Laboratory collection |
| pET30a:: cat-s            | pET30a carrying cat adding his tag from *R. anatipestifer* CH-2, Kan^R       | This study          |
| pET30a:: cat^H79A-s       | pET30a carrying cat^H79A adding his tag from *R. anatipestifer* CH-2, Kan^R| This study          |
| pLMF02                    | shuttle vector transferred between *E. coli* and *R. anatipestifer* Amp^R, Kan^R | Liu et al., 2016    |
| pLMF02:: cat              | pLMF02 carrying cat from *R. anatipestifer* Amp^R, Cfx^R                   | This study          |
| pLMF02:: cat^H79A         | pLMF02 carrying cat^H79A, Amp^R Cfx^R                                       | This study          |
| pYES1new                  | YAC-BAC shuttle plasmid with Spc^R                                          | Laboratory collection |
| pRE12                     | sacB mobRP4 R6K ori Cm^R, pRE12-T-vector                                      | Laboratory collection |
| pRE12:: 1769USD           | pRE12 carrying 1769USD from *R. anatipestifer* CH-2 and plasmid pCP29, Cfx^R | This study          |
| pRE12:: 1772UCD           | pRE12 carrying 1772UCD from *R. anatipestifer* CH-2 and plasmid pCP29, Cfx^R | This study          |

**ATCC:** American Type Culture Collection.

*cat*), and RA-ATCC11845 (pLMF02:: cat^H79A), were identified by PCR analysis.

**Expression and Purification of CAT and CAT^H79A* His-Tagged Proteins**

Strains *E. coli* BL21 (DE3) (pET30a:: cat-s) and *E. coli* BL21 (DE3) (pET30a:: cat^H79A-s) were grown overnight in LB medium containing Kan (100 μg/ml). Stationary-phase cultures were diluted to an OD₆₀₀ of 0.05 in 500 ml of LB medium containing Kan (100 μg/ml) and incubated with shaking at 37°C until the culture density reached an OD₆₀₀ of 0.6. Cells were then induced with 0.4 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and reincubated at 37°C. The cells were harvested by centrifugation for 10 min at 8,000 rpm at 4°C, and then the pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0; 50 ml) and sonicated. The cell lysate was clarified.
by centrifugation to eliminate cell debris and then applied to a metal affinity resin column that was equilibrated with the same buffer. The column was successively washed with buffers containing 20 mM, 50 mM imidazole, and phosphate buffer (pH 4.4 and 5.0, respectively). Recombinant proteins were ultrafiltered with storage buffer (20 mM Tris-HCl, pH 7.8).

**Determination of mRNA Levels of the cat Gene by Real-Time PCR (RT-PCR) Analysis**

To assess whether the cat gene of *R. anatipestifer* was regulated by chloramphenicol, the wild-type strain was grown with TSB with or without 1 µg/ml of chloramphenicol. Total RNA was isolated from strains grown to log phase (OD_600_ ≈ 0.8–1.0) by using the RNAiso Plus kit (TaKaRa). DNA was removed using RNase-Free DNase. cDNA was generated by using the Senscript RT kit (TaKaRa), according to the manufacturer’s instructions. Real-time quantitative PCR (qPCR) was performed to measure cat mRNA levels using SYBR Premix EX Taq II (TaKaRa). The primers used in real-time qPCR analysis are listed in Table 2. The expression level of the cat gene was normalized to that of the recA gene, which was used as a reference. All PCR reactions were performed in triplicate. The efficiency of primer binding was determined by linear regression by plotting the cycle threshold (CT) value vs. the log of the cDNA dilution. Relative quantification of the transcript was determined using the comparative CT method (2^(-ΔΔCT)), calibrated to recA. The experiments were performed multiple times independently and generated comparable results. The findings are presented as fold-change relative to the mRNA expression levels of the control strains.

### Table 2 | Primers used in this study.

| Primers     | Description                                                                 | Source and reference |
|-------------|-----------------------------------------------------------------------------|----------------------|
| 1769up-F    | 5′-ATTCCAGTTTTTCAAAATCTCAATCTCCCTA-3′                                     | This study           |
| 1769up-R    | 5′-CTGTCCTGCTGTGATATTCAATTCATTTAAATTTAACATTA-3′                          | This study           |
| Spc-F       | 5′-ATATGTAAATACCGACGCCGCAAGAAATGCG-3′                                     | This study           |
| Spc-R       | 5′-CTCTTTTTTATTTTTGCGGACTACCTTGGTGA-3′                                    | This study           |
| 1769down-F  | 5′-CCGCAATAATAAAAAGGAGGTCCGGAAAAT-3′                                     | This study           |
| 1769down-R  | 5′-TTGATGGCATTTGCGCACTAT-3′                                               | This study           |
| 1769dent-F  | 5′-TTTCGGAAGAACCGGCTAA-3′                                                 | This study           |
| 1769dent-R  | 5′-CAAAGTGTTTCCGCGCGT-3′                                                  | This study           |
| 1769Big-F   | 5′-TACCTAACCGCGGATTTCGCA-3′                                               | This study           |
| 1769Big-R   | 5′-AAGGAGCCAGCCAAGGACTG-3′                                                | This study           |
| 1772up-F    | 5′-ATTCTCCAAGATCGGATCTGTTGAAATTTTATAATATATTTATGATTTTAATAAATTTAACATATTA-3′| This study           |
| 1772up-R    | 5′-GCTTGCGGCTCAATTATATATTATCATATATTATAACATATTTAACATATTTAACATT-3′          | This study           |
| Cfx-F       | 5′-ATATGTGTTAATATATGACCCCGGGAAGGCTG-3′                                   | This study           |
| Cfx-R       | 5′-GAACCTTTTATTTATTAAAGATTAAAAATTTACTGAAATTGCCATT-3′                      | This study           |
| 1772down-F  | 5′-TTCAATCGAAATTTTTAATGGAAGGAGGTCCGGAAAATC-3′                            | This study           |
| 1772down-R  | 5′-CTTATCAAACTGTTTTTGTGCGAC ACAACCTTAC-3′                                | This study           |
| 1772dent-F  | 5′-ATTTTGACGGATTTATTAGTTGTT-3′                                           | This study           |
| 1772dent-R  | 5′-TTCCGTATAAGCTTCTGCAATAATTTTTAAATTTAACATTA-3′                          | This study           |
| 1772Big-F   | 5′-AATTTTGACGTTAATACCGCGC-3′                                              | This study           |
| 1772Big-R   | 5′-ACTACGTCGACACATCGATGTTG-3′                                             | This study           |
| 16SrRNA-F   | 5′-CGAAAGTGAATAAGTTACCGCGGACCT-3′                                        | This study           |
| 16SrRNA-R   | 5′-GCAAGAACCCTTGAATAATGCGGCGCC-3′                                        | This study           |
| cat-F1/MF1  | 5′-GGGAAATTCCTATGGAATTTCCTGCAAAAGTC-3′                                   | This study           |
| cat-R1/MR2  | 5′-CCGCTCGAGTCAATGTGCGTGTGGTGTGGTGGTGTGGTGCATTATTTTCGAAACCTTAC-3′         | This study           |
| MR1         | 5′-ATATTTACCCGATTTGACGCGC-3′                                              | This study           |
| MF2         | 5′-GTAATCAAAGGCGTCAATATGATTG-3′                                           | This study           |
| cat-F2      | 5′-CATGCGCATGAGTGAATTTCTCTGCGAAATGTC-3′                                  | This study           |
| cat-R2      | 5′-CGGCTCGAGTCAATTTTCGTTTAAAAAACCTTAC-3′                                 | This study           |
CAT Activity Assay
CAT catalyzes the transfer of an acetyl group from acetyl-CoA to Cm, producing acetylated Cm and CoASH. The CATase activity was assayed based on the disappearance of acetyl-CoA during Cm acetylation (Kobayashi et al., 2015). The reaction mixture contained 0.25 ml of 0.2 M Tris-HCl (pH 7.8), 0.05 ml of 1 mM acetyl-CoA, 0.05 ml of 1 mM Cm, 0.05 ml of 10 mM DTNB [5,5-dithio-bis (2-nitrobenzoic acid)], and 0.1 ml enzyme extract. The reaction was initiated by the addition of Cm. An increase in absorbance at a wavelength of 412 nm, which arises from 5-thio-2-nitrobenzoic acid, was determined using its molar extinction coefficient at 412 nm (13,600 M$^{-1}$ cm$^{-1}$). The value was then used in the determination of the amount of CoASH produced during the reaction. One unit of enzyme activity is defined as the amount of activity catalyzing 1 µmol of acetyl transfer per min under the assay conditions.

Softwares
The changes of mRNAs were expressed as fold expression and calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method. The error bars represent the standard deviation of three independent experiments.

RESULTS
Identification and Sequence Analysis of the cat Gene in R. anatipestifer Isolates
The cat gene was identified in 69/192 (36%) R. anatipestifer isolates collected from China, thereby suggesting that the cat gene was widely distributed among R. anatipestifer strains. Sequence analysis found that there are two copies of the cat gene (G148_1769: 1854900...1855529 and G148_1772: 1858427...1859056) in R. anatipestifer CH-2. There is no similarity between the cat gene from R. anatipestifer CH-2 and type A cat genes previously reported. However, the cat genes share 99–100% identity in R. anatipestifer strains reported in NCBI.

MIC of Chloramphenicol for R. anatipestifer CH-2 and Other Strains
To verify whether the cat genes of R. anatipestifer CH-2 were responsible for chloramphenicol resistance, the deletion strains and complementation strains were constructed. Table 3 showed that the chloramphenicol MICs of R. anatipestifer CH-2 and RA-CH2Δ1769 were 32 and 64 µg/ml, respectively. Compared to the MIC of the wild-type strain, the MIC of RA-CH2Δ1769 increased (Table 3). Similarly, another signal cat gene deletion strain RA-CH2Δ1772 had no obviously decreased in resistance to chloramphenicol, exhibiting 32 µg/ml in the minimum inhibitory concentration for chloramphenicol (Table 3). Thus, we supposed that the two copies of cat gene in R. anatipestifer CH-2 were involved in chloramphenicol resistance. The two copies of the cat gene deletion strain RA-CH2Δ1769Δ1772 was constructed. The level of chloramphenicol resistance was determined to be significantly reduced, 4 µg/ml.

To further verify that the cat genes are related to chloramphenicol resistance in R. anatipestifer, shuttle plasmid pLMF02 with the cat gene was introduced into R. anatipestifer ATCC 11845, which is sensitive to chloramphenicol. It was restored the level of chloramphenicol resistance (Table 3). These results strongly suggested that the cat gene was responsible for chloramphenicol resistance in R. anatipestifer.

The Transcription of cat Gene Was Increased in RA-CH2Δ1769
According to the study described above, the minimum inhibitory concentrations for chloramphenicol between RA-CH2Δ1769 and RA-CH2Δ1772 are not same (Table 3). To explore whether the transcription of cat gene is affected by single deletion strain, RT-PCR analysis was performed. The result revealed that G148-1772 was upregulated 3.82-fold in the RA-CH2Δ1769 mutant (Figure 1). However, the mRNA level of G148-1769 in RA-CH2Δ1772 did not increased significantly. This information could explain why the resistance level of RA-CH2Δ1769 is greater than RA-CH2Δ1772 and wild-type
strain. The result showed that the cat genes do mediate the production of chloramphenicol resistance and the relationship of the two cat copies is complementary and cooperative in \textit{R. anatipestifer} CH-2.

### The Transcription of cat Gene Was Induced by Chloramphenicol

In order to study the cellular strategies used by \textit{R. anatipestifer} CH-2 and mutant strains in the presence of chloramphenicol, we decided to analyze transcriptional changes of cat gene in these strains growing in the presence of 1 \( \mu \text{g/ml} \) of this antibiotic. Chloramphenicol treatment had no bactericidal effect when sub-inhibitory concentration of chloramphenicol were applied (data not shown). We found that the mRNA level of cat genes was increased 11-fold, 13.94- and 18.31-fold in wild strain and mutant strains RA-CH2\(\Delta1\)1769, RA-CH2\(\Delta1\)1772, respectively (Figure 2). These results suggested that cat genes were regulated by chloramphenicol.

### Catalytic Activity of the CAT and CAT\(^{H79A}\) Proteins

In a previous study involving \textit{Pseudomonas aeruginosa}, His79 served as a major catalytic residue (Beaman et al., 1998).

The two amino acid sequences of cat from \textit{P. aeruginosa} and \textit{R. anatipestifer}, respectively, showed 86.95\% identity (Figure 3). To examine the main catalytic site of CAT from \textit{R. anatipestifer} CH-2, the cat gene and the cat\(^{H79A}\) gene were expressed in \textit{E. coli} cells. The gene products, which contained C-terminal His-tag, were purified by Ni-agarose affinity chromatography, yielding a distinct protein band in the SDS-PAGE gel, with an approximately molecular weight of \( \sim 25 \) kDa. The catalytic activities of CAT and CAT\(^{H79A}\) were analyzed at \( 37^\circ \text{C} \). The detailed information of the reaction mixtures is described in the Materials and Methods. The specific activities of CAT and CAT\(^{H79A}\) were 8.33 \( \pm 0.38 \) and 0 U\(\cdot \text{mg}^{-1}\), respectively (Table 4). Meanwhile, the MIC of ATCC 11845 harbored cat\(^{H79A}\) was significantly lower than that of ATCC 11845 carried the cat gene (Table 3). Thus, the H79A substitution had a significant effect on CAT activity.

### DISCUSSION

CATs inactivate chloramphenicol via acetylation, which is the most prevalent mechanism of resistance to chloramphenicol in bacteria (Shaw, 1983; Murray and Shaw, 1997; Schwarz et al., 2004). CATs have been described in both gram-positive and gram-negative bacteria. There are two defined types of CATs that distinctly differ in their structure: The classical CATs, which are referred to as type A CATs, and the novel CATs, which are also known as type B CATs (Schwarz et al., 2004). There are at least 16 distinct groups of cat\(^A\) genes (A1–A16) and at least 5 different groups of type B cat genes (B1–B5) (Schwarz et al., 2004). Types A and B CATs are both capable of acetylating the hydroxyl group at C\(_3\) of chloramphenicol.

In our case, there was two copies of the cat gene in \textit{R. anatipestifer} CH-2. Not surprisingly, the phenomenon of having 2 copies of the cat gene was found in other bacteria,

| Protein | Specific activity (U/mg) |
|---------|-------------------------|
| CAT     | 8.75 8.25 8.0 8.33 \( \pm 0.38 \) |
| CAT\(^{H79A}\) | 0 0 0 0 |

\[\text{TABLE 4 | The CAT and CAT}^{H79A}\text{ activity.}\]

![Figure 2](image2.png)

![Figure 3](image3.png)
for example *Clostridium sporogenes* (CP009225) (Zhang et al., 2015), *Chryseobacterium* sp. (AP014624) (Morohoshi et al., 2014), *M. odoratimimus* (CP013690) (Hu et al., 2016), and *Aliivibrio wodanis* (LN554847). Mutant strains were constructed. Only both two copies of the *cat* mutant strain showed a significant reduction in resistance to chloramphenicol, but not for the single *cat* gene deletion strains. ATCC 11845 is a *R. anatipestifer* strain that was isolated from ducklings in 1932, and genome analysis indicated that it does not harbor the *cat* gene and is sensitive to chloramphenicol. Complementation ATCC 11845 with the *cat* gene from *R. anatipestifer* CH-2 restored the level of chloramphenicol resistance. These results showed that the *cat* genes do mediate the production of chloramphenicol resistance and the relationship of the two *cat* copies is complementary and cooperative in *R. anatipestifer*.

To explore the function and the active site of the *cat* gene in *R. anatipestifer*, CAT and CAT<sup>H79A</sup> were expressed and purified. Enzymatic activity analysis of CAT and CAT<sup>H79A</sup> produced by *in vitro* site-directed mutagenesis indicated that CAT<sup>H79A</sup> had no catalytic activity, thereby suggesting that His79 is the main catalytic residue of CAT. In addition, the present study further demonstrated that the *cat* gene is involved in chloramphenicol resistance, thus supporting our hypothesis that the *cat* genes are chloramphenicol resistance determinant factors in *R. anatipestifer*.

Type B CATs can be further classified into at least five groups. We constructed a homology tree of types A and B CATs (Figure 4) based on their reported amino acid sequence (van Hoek et al., 2011; Roberts et al., 2012). Types A and B CATs showed 10% similarity. We also determined that the *R. anatipestifer* CAT forms a separate branch from the type B CATs. In addition, types A4 and A7 CATs were observed to be 100% similarity. Thus, the classification of CATs should be revisited. Two types of genes that encode CATs could be based on their structure, namely, types A and B, by using the criterion of ≥80% amino acid identity to define a subgroup (Roberts and Schwarz, 2009). The sequence information of types A and B CATs is listed in the Supplementary Table 1. A total of 15 distinct groups were identified, A1–A15 for type A CATs and five different groups for type B CATs, B1–B5. Types A4 and A7 share 100% identity and belong to a subclass that we designated as A4. Groups A8–A16 were renamed as A7–A15. Groups B2, B3, and B6 showed >80% homology. These three categories are classified as a subclass, namely, B2. The CAT of *R. anatipestifer* was designated as B3. The rest of the type B classifications remained the same.

It was reported that the *cat* genes identified in gram-positive bacteria *Bacillus* spp. and *Staphylococcus* were inductively expressed by chloramphenicol (Mongkolsuk et al., 1984; Bruckner and Matzura, 1985; Duvall et al., 1985). To verify whether the *cat* genes were induced by chloramphenicol in *R. anatipestifer* CH-2, RT-PCR was performed to determine the *cat* transcript level of the wild-type strain and mutant strains in the presence or absence of chloramphenicol at a concentration of 1 µg/ml. The results exhibited that the level of transcription of the *cat* gene increased in the presence of chloramphenicol. However, the inducing mechanism is not understood at this time in *R. anatipestifer*.

It has been demonstrated that *catA86* and *catA112* were regulated by a mechanism known as translation attenuation.

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**FIGURE 4** | Homology analysis of types A and B CATs based on amino acid identity using DNAMAN 8.0 (Lynnon-Biosoft, Ontario, Canada). The sequence information of types A and B CATs are listed in the Supplementary Table 1.
in the previous studies (Lovett, 1996). Later, translational attenuation has been proposed as the regulatory mechanism for the chloramphenicol-inducible catB1 gene of Agrobacterium tumefaciens (Rogers et al., 2002). Sequence analysis found that CAT from *R. anatipestifer* shared 65% identity with that of *A. tumefaciens*. It is unclear whether they have the same inducing mechanism. Further studies determining the regulatory mechanism underlying the cat gene in *R. anatipestifer* are warranted.

**AUTHOR CONTRIBUTIONS**

DZ and AC conceived and designed the project; LH and HY constructed the cat deletion mutant of *R. anatipestifer* and detected resistance; ML and XZ detected the mRNA levels of the *cat* gene by RT-PCR; LH and HY constructed ATCC 11845 *cat* and catH79A complementary strains. LH, RJ, and SC performed expression and purification of CAT and CATH79A His-tagged proteins; LH, QY, and YW performed CAT activity assay; MW, KS, and XC detected the cat gene in *R. anatipestifer* isolates; LH and DZ drafted and revised the manuscript. All authors have read and approved the final version manuscript.

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**SUPPLEMENTARY MATERIAL**

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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