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Short Communication

A novel family of intrinsic chloramphenicol acetyltransferase CATC in *Vibrio parahaemolyticus*: Naturally occurring variants reveal diverse resistance levels against chloramphenicol

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ABSTRACT

Intrinsic resistance of bacteria to antibiotics plays an increasingly significant role in antibiotic resistance. However, the breadth of intrinsic resistance has not been fully elucidated. Here we identified a novel class of chloramphenicol acetyltransferase (type C CAT or CATC) in *Vibrio parahaemolyticus* and its closely related species *V. alginolyticus, V. antiquarius*, and *V. diabolicus*. The *catC* genes encoding the CATC clade are distributed among the four *Vibrio* species and are consistently found in the same conserved genomic regions. Based on their prevalence, these genes are considered to be intrinsic in *V. parahaemolyticus, V. alginolyticus, V. antiquarius*, and *V. diabolicus*. We also demonstrated that naturally occurring variants of CATC can confer diverse resistance levels against chloramphenicol in *Escherichia coli*. Furthermore, the enzyme kinetics of CATC variant proteins supported the diversity of their resistance phenotypes. This work provides insights into the distribution and resistance phenotypes of a novel class of intrinsic resistance genes in bacteria.

1. Introduction

Intrinsic resistance is currently attracting more and more attention, along with the full understanding of antibiotic resistance. The aminoglycoside-modifying enzymes encoded by *aphA6* and *aac(6′)-Ih* are intrinsic in *Acinetobacter guillouiae* and *A. gyllenbergii*, respectively. However, these genes have been acquired by *A. baumannii* via insertion sequence elements [1,2], suggesting that horizontal gene transfer also applies to intrinsic resistance genes. Furthermore, our previous study revealed that intrinsic gene *ant(3")-II* can be horizontally transferred among *Acinetobacter* spp. by homologous recombination [3].

Despite the potential adverse effects of chloramphenicol, it remains a hot research topic due to excellent tissue penetration and can serve as an alternative drug for epidemic meningitis treatment [4,5]. With the advent of antibiotic resistance era, chloramphenicol may play a role in treating intra-abdominal and respiratory tract infections [5]. The dominant resistance mechanism of chloramphenicol is enzymatic inactivation by chloramphenicol acetyltransferase (CAT) [6], which acetylates the C-3 hydroxyl of chloramphenicol with an acetyl group derived from acetyl-CoA [7].

Hitherto, there have been two types of CATs in bacteria: type A and B (CATA and CATB), which can be divided into 16 and five groups, respectively. Both types of CAT proteins, within each of their corresponding subgroups, display more than 80% amino acid identity. Most CATs are located on mobile elements, while others are located on chromosomes [8]. The representative of CATA is CATIII from big plasmid R387 of *Shigella flexneri*, and the first CATB described is CATB1 from the chromosome of *Agrobacterium tumefaciens* [9,10]. They share only 12% identity with each other. Both enzymes are homotrimers with 209–212 amino acid residues [7,8].

Some reports demonstrated that naturally occurring variants of antibiotic-inactivating enzymes can confer diverse resistance phenotypes. For instance, a spontaneous point mutation (S83L) in AAC(6′)-Ib of *Pseudomonas fluorescens* resulted in resistance to gentamicin but not amikacin, while the wild-type confers resistance to amikacin but not gentamicin [11]. In addition, the naturally occurring variant of AAC(6′)-Ib with two amino acid substitutions (W102R and D179Y) reduced the susceptibility to ciprofloxacin in clinical bacterial isolates [12]. Furthermore, variant (D179Y) of

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AAC(6′)-Ib displayed resistance to gemifloxacin [13]. OXY-2-15, a new OXY-2 allele variant, showed increased ceftazidime hydrolytic activity [14].

In the present study, we searched bacterial genomes of *Vibrio* spp. and found a new class of CAT, CATC enzyme, which is extensively distributed in the conserved chromosome regions of *Vibrio parahaemolyticus, V. alginolyticus, V. antiquarius*, and *V. diabolicus*. Furthermore, the diverse resistance phenotypes of the naturally occurring variants of intrinsic CATC from different *V. parahaemolyticus* isolates were also investigated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Escherichia coli DH5α was used to express putative transferase resistance genes in vitro. The genomic DNA of *V. parahaemolyticus* was used as the template for polymerase chain reaction (PCR). Strains were grown on Luria–Bertani (LB) broth or plate at 37°C.

2.2. Bioinformatic methods and phylogenetic analysis

In order to search new antibiotic-modified transferases, two databases were selected. One database composed of amino acid sequences of known antibiotic-modified transferases, including aminoglycoside, macrolide, and chloramphenicol transferases obtained from the Antibiotic Resistance Genes Database (ARDB; http://ardb.cbcb.umd.edu/index.html), and CARD (Comprehensive Antibiotic Resistance Database; https://card.mcmaster.ca/?q=CARD/ontology/36484). The other was the complete and draft genome sequences of the genus *Vibrio* from NCBI (ftp://ftp.ncbi.nlm.nih.gov/) in spring 2017. The antibiotic-modified transferases were searched by alignment of the two databases using TBLASTN. The protein sequences sharing greater than 30% identity and 50% coverage with known antibiotic transferases were acquired for expression experiments.

We acquired putative CATC protein sequences from all complete and draft genomes of *V. parahaemolyticus, V. alginolyticus, V. antiquarius*, and *V. diabolicus* available in the NCBI database. All type A and B protein sequences described by Schwarz et al. [8] were downloaded from NCBI. The accession numbers for all these protein sequences were listed in Supplementary Table S1. These relevant sequences were aligned using the CLUSTALW with standard parameters [15]. Cladograms were constructed using the Neighbor-Joining (NJ) method in MEGA 6 software [16]. The partial deletion parameter was set at 80% and 1000 bootstrap replicates. Other parameters were set to default levels.

2.3. Construction of recombinant plasmids carrying putative transferase resistance genes

Twenty-eight open reading frames (ORF) of the putative CATC were chosen from *V. parahaemolyticus, V. alginolyticus, V. antiquarius*, and *V. diabolicus*. These ORF sequences were synthesized by GENEWIZ Biotech (Suzhou, China). The synthesized DNAs carrying EcoRI at the 5′-end and SalI at the 3′-end were cloned into multiple cloning sites of pUC18 vector, resulting in recombinant plasmids harboring different *catC* genes. These resultant plasmids were transformed into *E. coli* DH5α, and were selected on LB plates supplemented with 100 μg/mL ampicillin. The transformants carrying the inserted fragments were verified by restriction enzyme digestion (EcoRI and SalI) and sequencing. The resistance of transformants to chloramphenicol was assayed using the microdilution method, with induction by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), as recommended by the CLSI (http://www.clsi.org/). The accession numbers of bacterial strains used for experiments are listed in Supplementary Table S2.

3. Results and discussion

3.1. Putative chloramphenicol acetyltransferases are widespread in V. parahaemolyticus, V. alginolyticus, V. antiquarius, and V. diabolicus

By comparing the amino acid sequences of known aminoglycoside, macrolide, and chloramphenicol transferases to the complete and draft genomes of *Vibrio* spp., we identified 45 protein sequences that shared more than 30% amino acid identity with known antibiotic resistance genes, and less than 80% amino acid identity with one another. Among them, there was one protein sequence shared the highest amino acid sequence identity (55%) with CATB8 (Supplementary Table S3). The putative protein was only located in conserved chromosomal regions of four *Vibrio* species including *V. parahaemolyticus, V. alginolyticus, V. antiquarius*, and *V. diabolicus*.

We generated a phylogenetic tree to assess the relationship between the putative CATs and all known CATA and CATB [8] (Fig. 1). In the phylogenetic tree, all protein sequences from four species of *Vibrio* spp. are distinct from any known CATA and CATB enzymes, forming a highly supported monophyletic clade (with 100% bootstrap confidence), designated type C CAT (or CATC). These CATC protein sequences share 95–99% amino acid identity with each other, and 47–55% and less than 12% amino acid identity with CATB and CATA, respectively. The core-genome tree showed that *V. parahaemolyticus, V. alginolyticus, V. antiquarius*, and *V. diabolicus* are closely phylogenetic, clustering a monophyletic clade (Supplementary Fig. S1), which was consistent with the previous report [17]. Most *catA* and *catB* genes were derived from mobile elements, such as plasmids, integrons, and transposons [8]. *catA* genes were distributed in Gram-negative and -positive bacteria, and *catB* genes were only found in Gram-negative bacteria. In contrast, *catC* genes exist in *V. parahaemolyticus* and its close species.

3.2. Functional characterization of chloramphenicol acetyltransferases from Vibrio spp

The putative *cat* genes from *V. parahaemolyticus* VPA-67, *V. parahaemolyticus* ATCC 17802, *V. alginolyticus* 40B, *V. antiquarius* Ex25_939, and *V. diabolicus* CNCM I-1629 were functionally expressed in *E. coli*. These candidate genes enhanced chloramphenicol resistance of *E. coli* by two- to 16-fold compared to *E. coli* harboring the null vector pUC18 (Table 1). Interestingly, the expression of CAT_{VPA-67} and $CAT_{ATCC\ 17802}$ resulted in significant difference in chloramphenicol resistance (128 vs 16 μg/mL). However, their amino acid sequences share 99.5% identity. The resistance level of *E. coli* containing CAT_{40B} was 32 μg/mL and the protein shares 98.6% identity with CAT_{VPA-67}. These results suggested that natural variants in *V. parahaemolyticus* could confer different resistant phenotypes.

To characterize the products of enzymatic modification, CATC from *V. parahaemolyticus* VPA-67 was over-expressed in *E. coli* BL21 (DE3) and purified. The putative molecular weight of CATC carrying the His tag was 27 kDa (Supplementary Fig. S2). We used liquid chromatography–mass spectrometry (LC-MS) to detect the enzyme activity. Chloramphenicol (retention time 13.3 min, *m/z* 323.0 $[M+H]^+$) served as a substrate, the reaction product with an $[M+H]^+$ ion at *m/z* 365.1 (retention time 15.3 min) was detected in the reaction system catalysed by CATC (Supplementary Fig. S3A–D), indicating a single acetyl group was added to chloramphenicol.

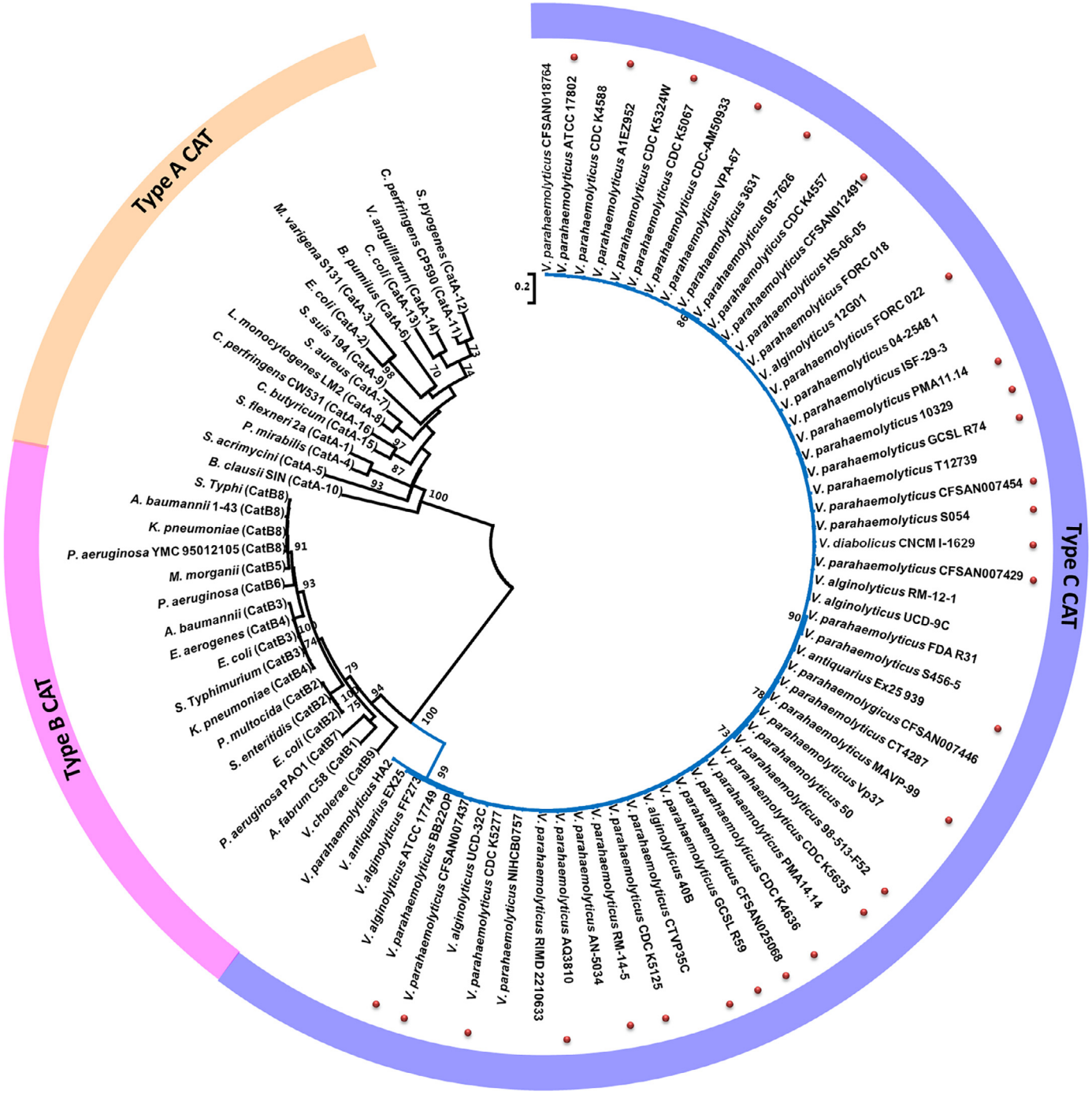

Fig. 1. Phylogenetic neighbor-joining tree of chloramphenicol acetyltransferases (CATs). Numbers above each node are the percentages of tree configurations that occurred during 1000 bootstrap replicates. Only values greater than 70% are shown. Species of bacteria are abreviated as follows: *S. pyogenes, Steptococcus pyogenes; C. perfringens, Clostridium perfringens; V. anguillarum, Vibrio anguillarum; C. coli, Campylobacter coli; B. pumilus, Bacillus pumilus; M. varigena, Mannheimia varigena; E. coli, Escherichia coli; S. suis, Streptococcus suis, S. aureus, Staphylococcus aureus; L. monocytogenes, Listeria monocytogenes; C. butyricum, Clostridium butyricum; S. flexneri, Shigella flexneri; P. mirabilis, Proteus mirabilis; S. acrimycini, Streptomyces acrimycini; B. clausii, Bacillus clausii; S. Typhi, Salmonella Typhi; A. baumannii, Acinetobacter baumannii; K. pneumoniae, Klebsiella pneumoniae; P. aeruginosa, Pseudomonas aeruginosa; M. morganii, Morganella morganii; E. aerogenes, Enterobacter aerogenes; S. Typhimurium; Salmonella Typhimurium; P. multocida, Pasteurella multocida; S. enteritidis, Salmonella enteritidis; A. fabrum, Agrobacterium fabrum; V. cholerae, Vibrio cholerae*. Red dots represent the type C CAT from *Vibrio* species expressed in *E. coli*.

Comparative analysis of LC-MS/MS fragmentation patterns of the reaction product with that of the substrate further confirmed the acetylation of the substrate (Supplementary Fig. S3E,F). This result illustrated that CATC of *V. parahaemolyticus* can acetylate chloramphenicol and therefore validate itself as a chloramphenicol acetyltransferase.

3.3. *catC* is intrinsic in *V. parahaemolyticus*, *V. alginolyticus*, *V. antiquarius*, and *V. diabolicus*

We found that *catC* gene in *V. parahaemolyticus* isolates was located at the same chromosomal site, which contains a >20-kb-long highly conserved region among most strains (Supplementary

Table 1
Functional analysis of putative chloramphenicol acetyltransferases from four *Vibrio* species expressed in *Escherichia coli*.

Original strain	Protein ID	Expression host	MIC (μg/mL) Chloramphenicol
		E. coli DH5α	8
V. parahaemolyticus VPA-67	KGT34314	*E. coli* DH5α	128
V. parahaemolyticus ATCC 17802	KKI10712	*E. coli* DH5α	16
V. antiquarius Ex25_939	OKQ16303	*E. coli* DH5α	128
V. diabolicus CNCM I-1629	CDT86443	*E. coli* DH5α	64
V. alginolyticus 40B	EEZ84567	*E. coli* DH5α	32

MIC, minimum inhibitory concentration.

Table 2
Functional analysis of putative chloramphenicol acetyltransferase (CAT) variants from *Vibrio parahaemolyticus* isolates expressed in *Escherichia coli*.

*Construct	Protein ID	#Variation	MIC (μg/ml) Chloramphenicol
pUC18		–	8
pUC18-CAT_{VPA-67}	KGT34314	–	128
pUC18-$CAT_{98-513-F52}$	OQS98533	A97T, G108D, A149T, K175N, T179M	128
pUC18-$CAT_{MAVP-99}$	OXD28463	A97T, S173N, T179M, E191G	128
pUC18-CAT_{GCSL_R59}	ODX29627	A97T, A149T, G206D	128
pUC18-$CAT_{CFSAN025068}$	KZW78074	A97T, A149T, G206C	128
pUC18-CAT_{CDC_K4636}	ODZ02573	A97T, A149T, A196V	128
pUC18-CAT_{BB22OP}	AGB12970	V44A, A97T, G108D	128
pUC18-CAT_{CDC_K5125}	OEA11544	A97T, A149T	128
pUC18-$CAT_{CFSAN007429}$	OQT56383	A97T, S173G	128
pUC18-$CAT_{CFSAN007437}$	OQU06623	A97T, (220-222: YCR)	128
pUC18-CAT_{CDC_K5277}	OEA28381	A97T	128
pUC18-CAT_{S054}	WP_025559383	G25D, A97T, S173G	64
pUC18-CAT_{CDC_K5067}	ODZ99433	I92T	64
pUC18-$CAT_{CFSAN007454}$	OAR36810	V24I, D63N, A97T, S173G, S203N	32
pUC18-$CAT_{08-7626}$	KJR23262	D63N, A207T, A209V	32
pUC18-$CAT_{CFSAN012491}$	OAR63147	D63N, G89S	16
pUC18-$CAT_{ATCC\ 17802}$	KKI10712	A143T	16
pUC18-$CAT_{A1EZ952}$	KYZ60169	R169H	16
pUC18-CAT_{AQ3810}	EDM59266	A43P, A97T, A149T	16
pUC18-CAT_{CDC_K5635}	OEB86923	A97T, S129N, A149T, S173N	8
pUC18-$CAT_{RM-14-5}$	KON50976	A43T, A97T, A149T	8
pUC18-$CAT_{PMA11.14}$	OOX50286	S64N, G108R, V120A	8
pUC18-CAT_{GCSL_R74}	ODX43549	G108R, V160E	8
pUC18-CAT_{10329}	EGF40477	G108R	8
pUC18-$CAT_{FORC\ 022}$	APC90608	S38I, S173G	8
pUC18-CAT_{S38I}		S38I	8
pUC18-CAT_{S173G}		S173G	128
pUC18-CAT_{H90A}		H90A	16

MIC, minimum inhibitory concentration.
* represents *E. coli* DH5α host harboring the relevant recombinant plasmid.
# Indicates the amino acid mutations in the corresponding CAT protein.

Fig. S4), and no mobile elements were involved in the vicinity of the gene. Furthermore, the gene was present in 837 of 844 *V. parahaemolyticus* genomes. Hence, *catC* is suggested to be intrinsic due to the conserved chromosomal location and high occurrence rate among *V. parahaemolyticus* isolates. Similarly, *catC* in *V. alginolyticus, V. diabolicus*, and *V. antiquarius* is also located in conserved chromosomal areas, respectively (Supplementary Figs. S5–7). The genes were present in all *V. alginolyticus, V. antiquarius, V. diabolicus* strains, indicating that *catC* is intrinsic in these corresponding species. Moreover, *catC* and its surrounding sequences are highly conserved in *V. parahaemolyticus, V. alginolyticus, V. antiquarius*, and *V. diabolicus* (Supplementary Fig. S8). Consequently, *catC* gene is intrinsic in *V. parahaemolyticus* and its closely related species *V. alginolyticus, V. antiquarius*, and *V. diabolicus*.

3.4. Naturally occurring variants of CATC are responsible for diverse chloramphenicol resistance phenotypes

We selected 25 CATC variants as representatives (Supplementary Fig. S9), which made up 79% of the total *V. parahaemolyticus* isolates from NCBI. We expressed these representatives in *E. coli* and found that they had diverse resistance levels. As shown in Table 2, 15 of 25 variants showed the high resistance levels (32–128 μg/mL), four of 25 displayed low resistance levels (16 μg/mL), and six out of 25 exhibited no resistance (8 μg/mL) compared with the control. Most of intrinsic genes are species-specific and have plenty of variants in the same species. However, these variants usually do not have significant phenotypic changes. Our study is the first report for the identification of naturally occurring variants having distinct resistance phenotypes for intrinsic genes.

These mutation sites are distributed throughout CATC enzymes (Supplementary Fig. S10). In comparison with CAT_{VPA-67}, the mutations occurring in those variants with minimum inhibitory concentration (MIC) of 128 μg/mL do not influence resistance levels. Therefore, these mutations were neglected while we considered mutations responsible for the alterations in the resistance levels of CATC variants. Based on this principle, three single mutations (A143T, R169H, and A43P) were involved in the decrease from 128 to 16 μg/mL; four single mutations (S129N, A43T, G108R, and S38I) dominated the resistance change from 128 to 8 μg/mL.

To illustrate whether these site mutations locate at the active site of CATC, we performed homology modeling using CATB7 (PDB: 1XAT) as the template. CATB7 has the highest identity (55%) with CATC. The results showed that residues Ser38, Ala43, Gly108, and S129 are located at the interface between subunits of CATC (Supplementary Fig. S11). An interface at CATB and CATA enzymes can be regarded as the area of active sites [18,19]. Hence, we speculated that these single mutations in the interface belong to active sites and could explain the drop in chloramphenicol resistance. CATC shares conserved residues with CATB, such as Ser38, A43, G108, S129, A143, and R169. There are no relevant conserved sites with CATA. The CATA and CATB enzymes have an evident catalytic site (histidine) located in position 195 and 79, respectively [7,18]. The histidine site mutation resulted in a remarkable decrease in chloramphenicol resistance [20]. For CATC, the corresponding conserved amino acid residue histidine is situated in position 90. The substitution of this site with alanine (H90A) in CAT_{VPA-67} led to a significant resistance decrease against chloramphenicol (eight-fold), suggesting that His 90 was a catalytic site in CATC.

3.5. Enzyme kinetics of type C CAT for chloramphenicol

In order to further investigate the characteristics of CATC variants, we selected variants with different resistance levels to perform enzyme kinetic characterizations. As shown in Supplementary Table S4, variants that conferred high-level resistance (CAT_{VPA-67} and $CAT_{CFSAN007454}$) exhibited lower K_m values than those of variants that conferred low-level resistance ($CAT_{ATCC\ 17802}$ and CAT_{10329}). This suggested that high-level resistance variants had a stronger binding affinity for chloramphenicol. The k_{cat}/K_m value of CAT_{10329} was lower than that of CAT_{VPA-67}, $CAT_{CFSAN007454}$, and $CAT_{ATCC\ 17802}$, which indicated that the variations of catalytic efficiency were basically consistent with the resistance changes of variants.

4. Conclusions

In the present study, we discovered a new family of chloramphenicol acetyltransferase, CATC in *V. parahaemolyticus* and its closely related species. We also revealed that a variety of naturally occurring variants of CATC were extensively distributed in different strains of *V. parahaemolyticus*, which was potentially dangerous to humans when these encoding genes were disseminated in pathogenic bacteria. Moreover, these findings further improved our understanding of the intrinsic resistance and displayed the importance of intrinsic resistance. Bacterial antibiotic-modified enzymes have the potential to evolve high-level resistances, which may be underestimated for intrinsic resistance genes, and should attract greater attention in the future.

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Competing Interests

The authors declare no conflicts of interest.

Ethical Approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.03.012.

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