J. Fish. Sci. Technol. 12(1), 24-28, 2009

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

Cloning and Characterization of the Tetracycline Resistant Gene, *tetB*, from *Vibrio parahaemolyticus*

Min-Seung Kang, Kunbawui Park[1], Hye-Jin Hwang, Hyang-Nam Bae, Keun-Sik Lim, Sung-Hwan Eom and Young-Mog Kim*

Department of Food Science and Technology, Pukyong National University, Busan 608-737, Korea

[1]South Sea Fisheries Research Institute, National Fisheries Research & Development Institute, Yeosu 556-823, Korea

A tetracycline resistant *Vibrio parahaemolyticus*, capable of growing on TCBS medium containing tetracycline, was isolated from cultivated fishes. A gene responsible for the tetracycline resistance was cloned from chromosomal DNA of the *V. parahaemolyticus* strain using *Escherichia coli* KAM3, which lacks major multi-drug efflux pumps *(△acrB)* as host cells. The nucleotide sequence and homology analysis revealed an open reading frame (ORF) for tetracycline resistance protein (TetB). In order to characterize the antibiotic resistance of TetB originated from the *V. parahaemolyticus* strain, the gene was subcloned into plasmid pSTV28. The resulting plasmid was designated as pSTVTetB and transformated into *E. coli* KAM3. *E. coli* KAM3 cells harboring the recombinant plasmid pSTVTetB are able to grow on plates containing tetracycline and oxytetracycline but not doxycycline, indicating that the *tetB* gene confers the tetracycline- and oxytetracycline-resistance to the host cell.

Key words: Antibiotic resistance, TetB, Tetracycline resistance, *Vibrio parahaemolyticus*

Introduction

Antibiotics are widely used in human, veterinary medicine and fish farming for increasing production or keeping them free from pathogens. As a result, the emergence and spreading of drug resistance bacteria has become a serious clinical issue in many countries. Furthermore, drug contamination of aquatic environments has also become a growing concern due to increasing drug-resistant genes which may affect overall human health via the food chain (Kim et al., 2007).

Tetracycline use in human and aquatic animals over the last 50 yrs has influenced the appearance of tetracycline resistance bacteria (Anderson and Sandra, 1994). In bacteria, there are three mechanisms of tetracycline resistance which have been known to be mediated by over 38 different tetracycline resistance genes; ribosomal protection, efflux pump and inactivating enzymes, (Pratt and Korolik, 2005; Roberts, 2005). Numerous studies have examined the distribution of different *tet* genes encoding efflux pumps in various aquaculture environments (Depaola et al., 1993; DePaola and Reberts, 1995; Schmidt et al., 2001; Nonaka and Suzuki 2002; Teo et al., 2002; Jun et al., 2007). However, there have been no unambiguous reports about the mechanism of the tetracycline resistant. Therefore, studies related to in the function of antibiotic resistant gene(s) or protein(s) are urgently necessary to elucidate the mechanism of tetracycline resistant at a molecular level.

Vibrio parahaemolyticus is one of the major food poisoning bacteria in Korea (Kim, 2001). It has been recently reported that over 97% of *V. parahaemolyticus* strains isolated from aquaculture farms or cultivated fishes exhibit some form of antibiotic resistance (Kim, 2001). In this paper, we report on the cloning and characteristics of a tetracycline resistant gene from *V. parahaemolyticus*.

Materials and Methods

Isolation of tetracycline resistant bacteria

Tetracycline resistant *Vibrio* sp. was isolated to culture on thiosulfate citrate bile salts sucrose (TCBS; Difco, Sparks, MD, USA) containing 20 μg/mL of

*Corresponding author: ymkim@pknu.ac.kr

tetracycline. Isolates were further identified by an API 20E kit (BioMerieux SA, Mqrcy-l'Etoile, France).

Antibiotic sensitivity assay

Antibiotic-sensitivity of isolates was evaluated by an agar diffusion assay on Muller Hinton (MH; Difco, Sparks, MD, USA) agar with disc containing 10 μg ampicillin (AM), 5 μg tetracycline (TC), 23.75 μg trimethoprim-sulfamethoxazole (SXT), 1.25 μg trimethoprim (TMP), 10 μg streptomycin (S), 5 μg chloramphenicol (C), 5 μg rifampin (RA), and 5 μg nalidixic acid (NA). Zones of growth inhibition were evaluated after overnight incubation according to NCCLS guidelines (the National Committee for Clinical Laboratory Standards, 2002).

Measurement of minimum inhibitory concentration

Measurement of minimum inhibitory concentration (MIC) of antibiotics against the isolates was determined by the two-fold serial dilution method in MH broth as described by the National Committee for Clinical Laboratory Standards (2002). MIC was defined as the lowest concentration of antibiotics that inhibited visual growth after incubation at 37°C for 24 h and was performed in triplicates.

Cloning of a gene responsible for tetracycline resistance

The isolated cells were cultured for 18 h at 30°C in Tryptic Soy Broth (TSB; Difco, Sparks, MD, USA) supplemented with 1% (w/v NaCl). Then, the culture was collected and chromosomal DNA was prepared using *Accu-Prep* Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). The DNA was partially digested with the restriction enzyme *Sau3A*I. DNA fragments of about 2.0 kbp were separated and then ligated into the *BamH*I site of pSTV28 (Takara, Shiga, Japan). Competent cells of *Escherichia coli* KAM3 (Δ*acrB*) were transformed with the ligated recombinant plasmids and then spread onto Luria-Bertani (LB) agar plates containing chloramphenicol (15 μg/mL) and tetracycline (20 μg/mL). The plates were incubated at 37°C for 3 days. One colony was archived and plasmid was isolated from the candidate. Plasmid DNA was used for restriction mapping and sequencing. The nucleotide sequence of the inserted DNA was determined by the dideoxy chain termination method (Sanger et al., 1977).

In order to sub-clone a gene encoding tetracycline resistant protein (TetB), PCR was carried out using two synthetic oligonucleotides based on the current study to generate a *BamH*I restriction enzyme site: (Forward) 5'-CGGGATCCCGTTTACCACTCCCTAT CAGTG-3', and (Reverse) 5'-CGGGATCCCGCGG AATAACATCATTTGGTG-3'. DNA was amplified through 30 cycles of denaturation (94°C, 30 sec), annealing (50°C, 30 sec), and polymerization (72°C, 60 sec). The PCR product was digested by *BamH*I restriction enzyme and then ligated into pSTV28, which had been digested with *BamH*I. The resulting plasmid was designated as pSTVTetB and transformed into *E. coli* KAM3 cells.

Results and Discussion

Isolation of tetracycline resistant *V. parahaemoliticus*

The tetracycline resistant *V. parahaemoliticus*, capable of growing on TCBS medium containing tetracycline, was isolated from cultivated fishes and identified (Table 1). Antibiotic resistance of each isolated strain was evaluated as described in Materials and Methods. As shown in Table 2, all isolates exhibited tetracycline resistance. Among them, *V. parahaemolyticus* 0854 showed antibiotic resistance against not only tetracycline but also rifampin, indicating that isolate 0854 may be a multi-drug resistance bacteria (Table 2). The MIC of tetracycline resistant isolates against tetracycline was over 64 μg/mL (Table 3).

Cloning of a gene responsible for tetracycline resistance

A gene responsible for tetracycline resistance was cloned from the chromosomal DNA of *V. parahaemolyticus* 0854 using *E. coli* KAM3, which lacks major multi-drug efflux pumps (△*acrB*) as a host cell. The obtained candidate recombinant plasmid enabled *E. coli* KAM3 cells to grow in the presence of tetracycline. The 2,018 bp DNA sequence analysis revealed that it contained an open reading frame (ORF) for tetracycline resistant protein TetB (Fig. 1). DNA sequences was translated in the reading frame (Fig. 1) and the putative product was compared, using BLAST algorithm, with all publicly available protein sequences contained in the nonredundant database. Comparison of the deduced primary structure of TetB with those of proteins present in the GenBank database indicated that the greatest similarity was with bacterial TetBs (Table 4). The putative TetB of *Photobacterium* sp. TC21 (Furushita et al., 2003) showed the highest similarity (99% identity) throughout the entire sequence (Table 4). Many of the

Table 1. Biochemical characteristics of tetracycline resistant *Vibrio* sp. isolates

Characteristics			Results			
Tests	Active ingredients	Reaction/enzymes	Strain 0854	Strain 1020	Strain 1021	Strain 9220
ONPG	2-nitrophenyl-βD-galactopyranoside	β-galactosidase (ortho nitrophenyl-βD-galactopyranosidase)	-	-	-	-
ADH	L-arginine	arginine dihydrolade	-	-	-	-
LDC	L-lysine	lysine decarboxylase	+	+	+	+
ODC	L-omithine	omithine decarboxylase	+	+	+	+
CIT	trisodium citrate	citrate utilization	-	-	-	-
H_2S	sodium thiosulfate	H_2S produstion	-	-	-	-
URE	urea	urease	-	-	-	+
TDA	L-tryptophane	tryptophane deAminase	-	-	+	-
IND	L-tryptophane	indole production	+	+	+	+
VP	sodium pyruvate	acetoin production (Voges Proskauer)	-	-	-	-
GEL	Gelatin (bovine origin)	gelatinase	+	-	-	-
GLU	D-glucose	fermentation/ oxidation (glucose)	+	+	+	+
MAN	D-mannitol	fermentation/ oxidation (mannitol)	+	+	+	-
INO	inositol	fermentation/ oxidation (inositol)	-	+	-	-
SOR	D-sorbitol	fermentation/ oxidation (sorbitol)	-	-	-	-
RHA	L-rhamnose	fermentation/ oxidation (rhamnose)	-	-	-	-
SAC	D-sucrose	fermentation/ oxidation (sucrose)	-	-	-	-
MEL	D-melibiose	fermentation/ oxidation (melibiose)	-	-	-	-
AMY	amygdalin	fermentation/ oxidation (amygdalin)	-	-	-	-
ARA	L-arabinose	fermentation/ oxidation (amygdalin)	+	+	+	+
OX	oxidase test	cytochrome-oxidase	+	+	+	+
NO_2	potassium nitrate	NO_2 prodution	+	+	-	+
N_2	potassium nitrate	reduction N_2 gas	-	-	-	-
MOB	microscope	motility	+	+	+	+
McC	MacConkey medium	growth	+	+	+	+
OF-F	glucose (API OF Medium)	fermentation : under mineral oil	+	+	+	+
OF-O	glucose (API OF Medium)	oxidation : exposed to the air	+	+	+	+
Identification (%) with *Vibrio parahaemolyticus*			99.9	99.9	99.9	99.8

Tetracycline resistant *Vibrio* sp. was isolated on thiosulfate citrate bile salts sucrose (TCBS) containing 20 μg/mL of tetracycline. Isolates were identified by an API 20E kit. +, positive result; -, negative result.

Table 2. Antibiotic resistance pattern of tetracycline resistant *Vibrio parahaemolyticus* isolates

Strains	AM	TC	SXT	TMP	S	C	RA	NA
0854	28	AG	26	18	14	22	AG	28
1020	10	AG	28	22	14	22	22	30
1021	30	AG	30	18	14	22	22	30
9220	AG	AG	22	18	12	16	20	28

Antibiotic-sensitivity of isolates was evaluated by an agar diffusion assay on Muller Hinton agar with disc containing each antibiotic. The numbers indicate the diameter of clear zone (mm). AG, no inhibition; AM, ampicillin (10 μg); TC, tetracycline (5 μg); SXT, trimethoprim-sulfamethoxazole (23.75 μg); TMP, trimethoprim (1.25 μg); S, streptomycin (10 μg); C, chloramphenicol (5 μg); RA, rifampin (5 μg); NA, nalidixic acid (5 μg).

Table 3. Minimum inhibitory concentration of tetracycline resistant *Vibrio parahaemolyticus* isolates

Strains	Tetracycline (μg/mL)
0854	64
1020	128
1021	128
9220	64

bacterial TetB registered in the GenBank database also showed similar levels of identity (99%) to the TetB of *V. parahaemolyticus* 0854 (Table 4).

Characterization of the cloned *tetB* gene

In order to elucidate the mechanism of antibiotic resistance caused by TetB, it was sub-cloned into plasmid pSTV28. The *tetB* gene was amplified by PCR using the VP-tetBF and VP-tetBR primer set and the amplified PCR product was expected to be

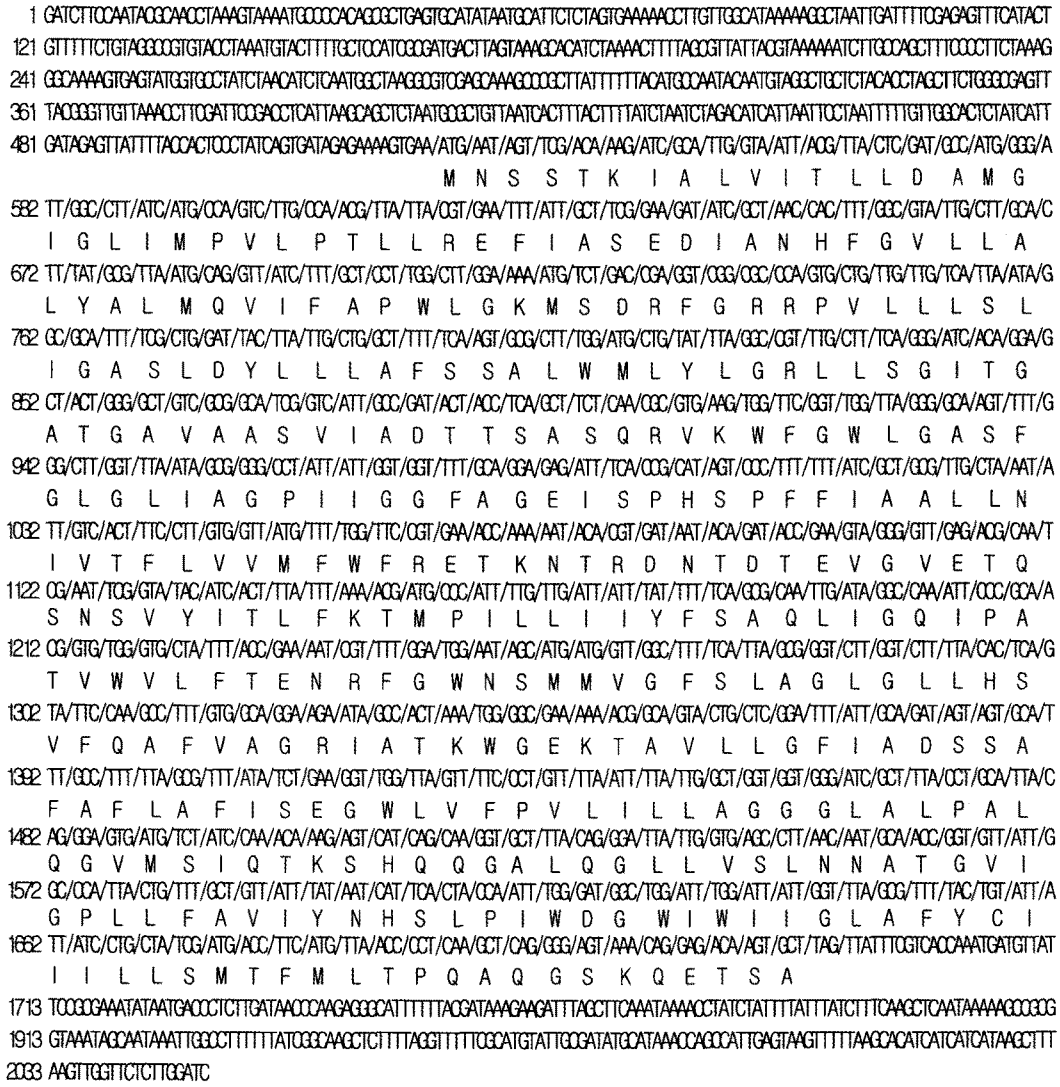

Fig. 1. Nucleotide sequences of the inserted DNA containing a gene responsible for tetracycline resistance. The deduced amino acid sequences of the tetracycline resistant gene, *tetB*, are indicated under the nucleotide sequences. The DNA sequence is numbered on the left.

Table 4. Homology analysis of the tetracycline resistance protein (TetB) of *Vibrio parahaemolyticus* 0854

Strains	Protein	Homology (%)	Accession No.
Photobacterium sp. TC21	tetracycline resistance protein	99	BAC67134
Photobacterium sp. TC33	tetracycline resistance protein	99	BAC67137
Vibrio sp. TC68	tetracycline resistance protein	99	BAC67141
Serratia marcescens	tetracycline resistance protein	99	NP_941291
Escherichia coli	tetracycline resistance protein	99	YP_001096450
Haemophilus parasuis	tetracycline resistance protein	99	YP_195816

Table 5. Antibiotic resistance profiles of the tetracycline resistance protein (TetB)

Strains	Minimum inhibitory concentration (μg/mL)		
	Tetracycline	Oxytetracycline	Doxycycline
Vibrio parahaemolyticus 0854	128	32	16
Escherichia coli KAM3/pSTV28	<4	<4	<4
E. coli KAM3/pSTVTetB	64	32	<4

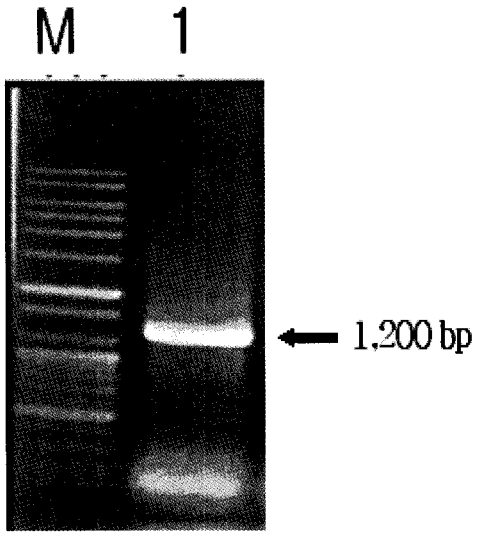

Fig. 2. PCR products of the *tetB* gene containing *BamH I* endonuclease sites. M, 100 bp plus DNA ladder (Bioneer, Korea); lane 1, *tetB* of *Vibrio parahaemolyticus* 0854.

about 1,200 bp (Fig. 2). The amplified DNA was digested with *BamHI* and then ligated into pSTV28 as described in Materials and Methods. The resulting plasmid was designated as pSTVTetB and transformated into *E. coli* KAM3 cells. The *E. coli* KAM3 cells harboring recombinant plasmid pSTVTetB are able to grow in MH medium containing tetracycline and oxytetracycline but not doxycycline, indicating that the TetB gene originated from *V. parahaemolyticus* 0854 confers its tetracycline- and oxytetracycline-resistance to host cells (Table 5).

Acknowledgments

This work was supported by a grant from the Korea Research Foundation (KRF-2006-331-F00046) funded by the Korean Government (MOEHRD). We also thank the Brain Busan 21 Project in 2008 for graduate support.

References

Andersen, S.R. and R.A. Sandra. 1994. Distribution of tetracycline resistance determinants among gram-negative bacteria isolated from polluted and unpolluted marine sediments. Appl. Environ. Microbiol., 60, 908-912

Depaola, A. and M.C. Roberts. 1995. Class D and E tetracycline resistance determinants in gram-negative bacteria from catfish ponds. Mol. Cell Probes., 9, 311-313.

Depaola, A., W.E. Hill and F.M. Harrell. 1993. Oligonucleotide probe determination of tetracycline resistant bacteria isolated from catfish ponds. Mol. Cell Probes., 7, 345-348.

Furushita, M., T. Shiba, T. Maeda, M. Yahata, A. Kaneoka, Y. Takahashi, K. Torii, T. Hasegawa and M. Ohta. 2003. Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. Appl. Environ. Microbiol., 69, 5336-5342.

Jeanette, W., P. Teo, M.C. Tan Theresa and L.P. Chit. 2002. Genetic determinants of tetracycline resistance in *Vibrio harveyi.* Antimicro. Agents Chemother., 46, 1038-1045.

Jun, L.J., J.B. Jeong, J.H. Kim, C.H. Lee, E.H. Kim and H.D. Jeong. 2007. Distribution of *tet* genes in the tetracycline resistant microorganisms isolated from marin-aquatic environments of Korea. J. Fish Pathol., 20, 169-178.

Kim, Y.B. 2001. Virulence factors and genotyping of *Vibrio parahaemolyticus* in sea water and clinical isolates. J. Bacteriol. Virol., 31, 229-238.

Kim, Y.H., L.J. Jun, S.H. Park, S.H. Yoon and J.K. Chung. 2007. Prevalence of *tet*(B) and *tet*(M) genes among tetracycline-resistant *Vibrio* spp. in the aquatic environments of Korea. Dis. Aquat. Org., 75, 209-216.

National Committee for Clinical Laboratory Standards. 2002. Performance standards for antimicrobial disk susceptibility test. National Committee for Clinical Laboratory Standards. Wayne, PA.

Nonaka, L. and S. Suzuki. 2002. New Mg^{2+} -dependent oxytetracycline resistance determinant *tet*34 in *Vibrio* isolates from marine fish intestinal contents. Antimicrob. Agents Chemother., 476, 1550-1552.

Pratt, A. and V. Korolik. 2005. Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolstes. J. Antimicrob. Agents Chemother., 55, 452-460.

Roberts, M.C. 2005. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev., 19, 1-24.

Sanger, F., S. Nicklen and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA., 74, 5463-5467.

Schmidt, A.S., M.S. Bruun, I. Dalsgaard and J.L. Larsen. 2001. Incidence, distribution and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. Appl. Environ. Microbiol., 67, 5675-5682.

Teo, J.W.P., T.M.C. Tan and C.L. Poh. 2002. Genetic determinants of tetracycline resistance in *Vibrio harveyi.* antimicrob. Agents Chemother., 46, 1038-1045.

(Received 14 January 2009; Reviced 1 February 2009; Accepted 20 March 2009)