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Cloning and nucleotide sequence analysis of the chloramphenicol resistance gene on conjugative R plasmids from the fish pathogen *Photobacterium damselae* subsp. *piscicida*

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ABSTRACT: Transferable resistance to various drugs was investigated in *Photobacterium damselae* subsp. *piscicida* from Japan. Drug resistances were transferred via plasmids of 100, 50, and 40 kb. Resistance to chloramphenicol (Cm^r) was transferred on plasmids of all 3 sizes. The Cm^r gene (*cat*) was cloned from the 50 kb plasmids pPDP8511 and pPDP9106 transferred from *P. damselae* subsp. *piscicida* strains isolated in different years and places in Japan. Subcloning localized the *cat* to within 1.5 kb *Hin*dIII-*Hin*cII (or *Pst*I) fragments. Nucleotide sequences of the coding and flanking region of the *cat* were determined as 1607 bp (*Hin*dIII-*Hin*cII fragment) in pPDP8511 and 1568 bp (*Hin*dIII-*Pst*I fragment) in pPDP9106, which corresponded with the sequence from nucleotides 40 to 1607 in pPDP8511. The nucleotide sequences identified an open reading frame (ORF) encoding 213 amino acid residues with a calculated molecular mass of about 24.8 kDa, a size consistent with the molecular mass of known *cat* gene products, and the ORF had maximum homology (99.5%) with a Type II CAT variant from *Haemophilus influenzae*.

KEY WORDS: Cloning · Nucleotide sequence · Chloramphenicol resistance gene · Transferable R plasmid · *Photobacterium damselae* subsp. *piscicida* · Yellowtail

INTRODUCTION

Photobacterium damselae subsp. *piscicida* (formerly, *Pasteurella piscicida*) is well known as the causative agent of pseudotuberculosis in cultured yellowtail *Seriola quinqueradiata* in Japan. This disease has become of considerable economic importance, causing significant losses in farmed yellowtail. Since its initial recognition in yellowtail during the summer of 1969 (Kubota et al. 1970), the disease appears to have spread to other fish species, including red sea bream (Yasunaga et al. 1983) and black sea bream (Muroga et al. 1977, Ohnishi et al. 1982). Various antimicrobial compounds have been used to prevent this infection by chemoprophylaxis and chemotherapy. However, the efficacy of these drugs has been hindered by an increase of multiple drug resistance in *P. damselae* subsp. *piscicida*. Chloramphenicol (Cm) is an efficacious therapeutic agent that has been widely used in fish farms. However, since 1983 the use of Cm in Japanese fish farms against bacterial infection of cultured fish has been restricted (Kim & Aoki 1993a). Nevertheless, Cm resistant (Cm^r) strains of fish pathogenic bacteria including *P. damselae* subsp. *piscicida* are still prevalent (Kim & Aoki 1993a).

Bacterial resistance to Cm, an inhibitor of the peptidyltransferase activity of prokaryotic ribosomes, is commonly conferred by the enzyme chloramphenicol acetyltransferase (CAT). CAT variants have been isolated from numerous bacterial genera (Shaw 1983). Three classes of variants have been characterized among Gram-negative bacteria, and designated

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Types I, II, and III (Shaw 1983). CAT variants encoded on transferable R plasmids in fish pathogenic bacteria differ depending upon the bacterial genera and the year of isolation of the bacteria (Aoki 1988, 1989). A CAT variant of *Photobacterium damselae* subsp. *piscicida* was classified as Type I (Kim & Aoki 1993b). In this study, Cm^r determinants on transferable R plasmids from *P. damselae* subsp. *piscicida* strains, which were isolated from yellowtail in different years and places in Japan, were cloned, and nucleotide sequences of the Cm^r determinants were obtained and compared with those previously reported.

MATERIALS AND METHODS

Bacteria and plasmids. A total of 183 isolates of *Photobacterium damselae* subsp. *piscicida* were isolated from diseased yellowtail *Seriola quinqueradiata* in various areas of Kyushu (Nagasaki, Saga, Oita, Kumamoto, and Kagasima prefectures), Japan, between 1984 and 1994. The bacterial strains were normally recovered from fish cultured in different net pens. The strains PP8511 and PP9106, isolated in 1985 and 1991 in Nagasaki and Kumamoto prefectures, respectively, were used for cloning or nucleotide sequence analysis of Cm^r determinants. The strains carried resistances to ampicillin, Cm, kanamycin, nalidixic acid, sulfamonomethoxine, and tetracycline (Ap^r, Cm^r, Km^r, Na^r, Su^r, and Tc^r, respectively) in PP8511, and Ap^r, Cm^r, resistance to erythromycin (Em^r), Km^r, Na^r, Su^r, Tc^r, and resistance to trimethoprim (Tmp^r) in PP9106 (see Tables 1 & 2). *Escherichia coli* K-12 χ1037 Rp^r mutant strain (a rifampicin-resistant mutant strain of χ1037 [*galK2 galT22 hsdR lacY1 metB1 relA supE44*]; Iyobe et al. 1981, 1994) was used as the recipient for conjugal transfer of drug resistance. *E. coli* JM83 streptomycin-resistant (Sm^r) strain [F^-, thi, ara, del (lac-pro), strA (fi80, lacZ, del M15)] was used to obtain competent cells for transformation.

The R plasmids pPDP8511 and pPDP9106 from strains PP8511 and PP9106, respectively, were used as the source of the Cm^r determinants and the vectors pUC119 (Ap^r, M13IG, lacZ) or pUC19 (Ap^r, lacZ) were used for cloning or nucleotide sequence analysis of the determinants.

Media and growth conditions. *Photobacterium damselae* subsp. *piscicida* was incubated in brain heart infusion (BHI) broth (Difco, Becton-Dickinson) containing 2% NaCl at 28°C and *Escherichia coli* in Luria-Bertani (LB) broth (1% bacto tryptone [Difco], 0.5% bacto yeast extract [Difco], 1% NaCl, pH 7.5) at 37°C. Mueller-Hinton medium (Difco) containing 2% NaCl and 1.5% agar was used for the drug susceptibility test. Bromothymol blue (BTB)-lactose nutrient agar (1% bacto peptone [Difco], 1% beef extract [Difco], 1% lactose, 0.0045% BTB, 0.5% NaCl, 1.5% agar, pH 7.5) was used for the mating assay.

Drug susceptibility test. The drug susceptibility test was performed using Mueller-Hinton agar containing individual drugs that were prepared by a serial 2-fold dilution method, recommended by the Japanese Society of Chemotherapy (1981). The culture was grown overnight in Mueller-Hinton broth, and diluted to about 10^6 cells ml^{-1} using buffered saline with gelatin (BSG) solution (0.85% NaCl, 0.03% KH_2PO_4, 0.06% Na_2HPO_4, 0.01% gelatin) in accordance with the method of the Japanese Society of Chemotherapy (1981). The diluted culture was inoculated on Mueller-Hinton agar using a microplanter (3 mm in diameter and 4 µl for 1 microplanter, Toyo sokki). The minimal inhibitory concentrations (MICs) were read as the lowest concentration resulting in complete inhibition of growth after 24 to 48 h incubations at 28°C. The resistance to each antimicrobial compound was defined by the distribution of the MICs (see Fig. 1). The following antibiotics and chemotherapeutics were employed for the drug susceptibility test: Ap, cephalexin (Cex), Cm, Em, furazolidone (Nf), Km, Na, Su, Sm, Tc, and Tmp.

Conjugal transfer assay. Each donor strain was incubated in BHI broth under shaking conditions for 9 h and *Escherichia coli* recipient strain in LB broth for 8 h. Aliquots (0.5 ml each) of donor and recipient cultures were mixed in a 50 ml Erlenmeyer flask and 4 ml of equal volumes each of BHI and LB broths were added to the flask. Mating was performed at 28°C for 2 h. Of 10-fold serial dilutions of mating mixture, 0.1 ml was spread on BTB-lactose agar with Rp 50 µg ml^{-1} and each selected drug: Ap 100 µg ml^{-1}; Cm 25 µg ml^{-1}; Em 100 µg ml^{-1}; Km 100 µg ml^{-1}; Na 200 µg ml^{-1}; Su 3200 µg ml^{-1}; Tc 25 µg ml^{-1}; and Tmp 400 µg ml^{-1}. In addition, the *E. coli* recipient strain expressed MIC values as follows: Ap 3.13 µg ml^{-1}; Cm 12.5 µg ml^{-1}; Em 25 µg ml^{-1}; Km 12.5 µg ml^{-1}; Na 50 µg ml^{-1}; Su 3200 µg ml^{-1}; Tc 3.13 µg ml^{-1}; and Tmp 12.5 µg ml^{-1}. Colonies growing in this double-inhibitor-supplemented medium after 24 to 48 h of incubation at 37°C were scored as presumptive transconjugants, and the frequency of transfer was calculated as the number of transconjugants per initial number of donors. Ten or more transconjugants from mating were picked and tested for antibiotic resistance according to the methods mentioned above.

The transconjugants from mating were also used for isolation of R plasmids, which were further used for cloning and nucleotide sequence analysis of the Cm^r determinants.

Plasmid manipulation and characterization. R plasmids of the transconjugants obtained by conjugal

transfer assay were extracted using the method of Kado & Liu (1981) and electrophoresed on a 0.7% agarose gel. Plasmid DNA was prepared by rapid alkaline lysis and caesium chloride/ethidium bromide density gradient purification. Restriction endonuclease digested DNA was electrophoresed on a 1.0% low-melting-temperature agarose gel (Grosa et al. 1994) for separation of the DNA fragments. Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo and used according to the manufacturer's instructions.

Approximate molecular sizes of plasmids were calculated using reference plasmids R27 (167 kb), R100-1 (100 kb), RP4 (56 kb), pLA2917 (22 kb), pMSG-cat (8.4 kb), pBR322 (4.361 kb), pUC119 (3.162 kb), and pHSG398 (2.227 kb). Molecular sizes of restriction fragments were calculated using λDNA digested with *Sty* I (Nippon Gene).

Cloning of Cm^r determinant. The DNA of plasmids pPDP8511 and pPDP9106 was completely digested with the restriction endonuclease *Hin*dIII. The resulting fragments were cloned into the *Hin*dIII site of the vector pUC119. Competent *Escherichia coli* JM83 cells, which were prepared according to a slightly modified method of Hanahan (1983), were transformed with the recombinant plasmid DNA by a modified procedure of Lederberg & Cohen (1974). Clones carrying the Cm^r determinant were selected on LB agar plates containing either only Ap (100 µg ml^{-1}) or Ap (100 µg ml^{-1}) in combination with Cm (25 µg ml^{-1}) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Takara) and IPTG (isopropyl-FI-D-thio-galactopyranoside). The recombinant DNA insert was double digested with *Hin*dIII and *Hin*cII (or *Pst*I) and was subcloned into the *Hin*dIII- *Hin*cII (or -*Pst*I) site of the vector pUC19. Recombinant plasmids containing the Cm^r determinant were used as the source of the determinant and its adjacent sequences.

Nucleotide sequencing and analysis. Subcloned fragments containing the Cm^r determinant were sequenced by the dideoxy chain termination method using a BigDye Terminator Cycle Sequencing Kit and an ABI 377 DNA sequencer (Applied Biosystems). DNA sequencing was performed using M13 forward and reverse primers. Internal sequencing primers (primer sequences 5′-AATGCGGATTCAGCCTGAC-3′ and 5′-GACTGTCGCAGCAGTCTTT-3′) were designed based on the sequence of the subcloned fragments to complete the sequence walk in both directions. The nucleotide sequence of the Cm^r determinant was analyzed using DNASIS-Mac Ver 3.6 (Hitachi Software Engineering), and compared with DNA sequences in GenBank. Multiple sequence alignment of the deduced peptide sequence was carried out using CLUSTAL W.

RESULTS

MIC distribution of antimicrobial compounds

The distribution of MICs of 8 antimicrobial compounds showing resistance of 183 strains of *Photobacterium damselae* subsp. *piscicida* is shown in Fig. 1. The MIC distribution of Tmp exhibited 3 groups which were susceptible, intermediate-level resistant, and high-level resistant (the 3-group classification was also proposed by Aoki et al. 1990). The MIC distributions of Ap, Cm, Em, Km, Na, and Tc revealed 2 groups which were susceptible and resistant (the 2-group classification was also proposed by Aoki & Kitao 1985, Takashima et al. 1985, and Kim & Aoki 1993a). The MIC distributions of Cex, Nf, Sm, and Su showed a single group in which all the tested strains were susceptible to Cex, Nf, and Sm whilst resistant to Su (the MIC values of the susceptible strains in *P. damselae* subsp. *piscicida* were below 25 µg ml^{-1}, which is in accordance with previous reports, Aoki & Kitao 1985, Takashima et al. 1985, Kim & Aoki 1993a).

Conjugal transfer of drug resistance

The drug resistance patterns (different combinations of drug resistance markers) of 150 strains of *Photobacterium damselae* subsp. *piscicida* that carried Cm^r and drug resistances transferred from donor *P. damselae* subsp. *piscicida* strains to recipient *E. coli* K-12 χ1037 Rp^r strain are shown in Table 1. The different resistance patterns of the 150 strains are numbered from 1 to 13. All strains were resistant to between 3 and 8 drugs. A high percentage of the strains classified showed resistance patterns of Su Km Tc Cm/Ap/Na/Tmp (Patterns 3, 5, 10, and 12).

Ap^r, Cm^r, Em^r, Km^r, Su^r, Tc^r, and Tmp^r were transferred from the donors to the recipient. However, the transferability of drug resistances differed with the pattern and level of drug resistances. The transfer of Tmp^r was restricted to strains carrying high-level resistance (Pattern 9). Ap^r was transferred only from donors containing Na^r Tc^r or Na^r Tmp^r as part of their multiresistance (Patterns 10 to 13). However, Ap^r could not transfer in all the strains containing Na^r Tc^r (Pattern 10). Strains exhibiting intermediate-level resistance to Na and Tmp did not transfer these resistances. None of the drug resistances transferred from those donors that did not contain Tc^r, Na^r Tc^r, or Na^r Tmp^r (Pattern 1).

Transferability of drug resistance

In most cases, the resistance of transconjugants was the same as the donors (Table 1). Exceptions to this

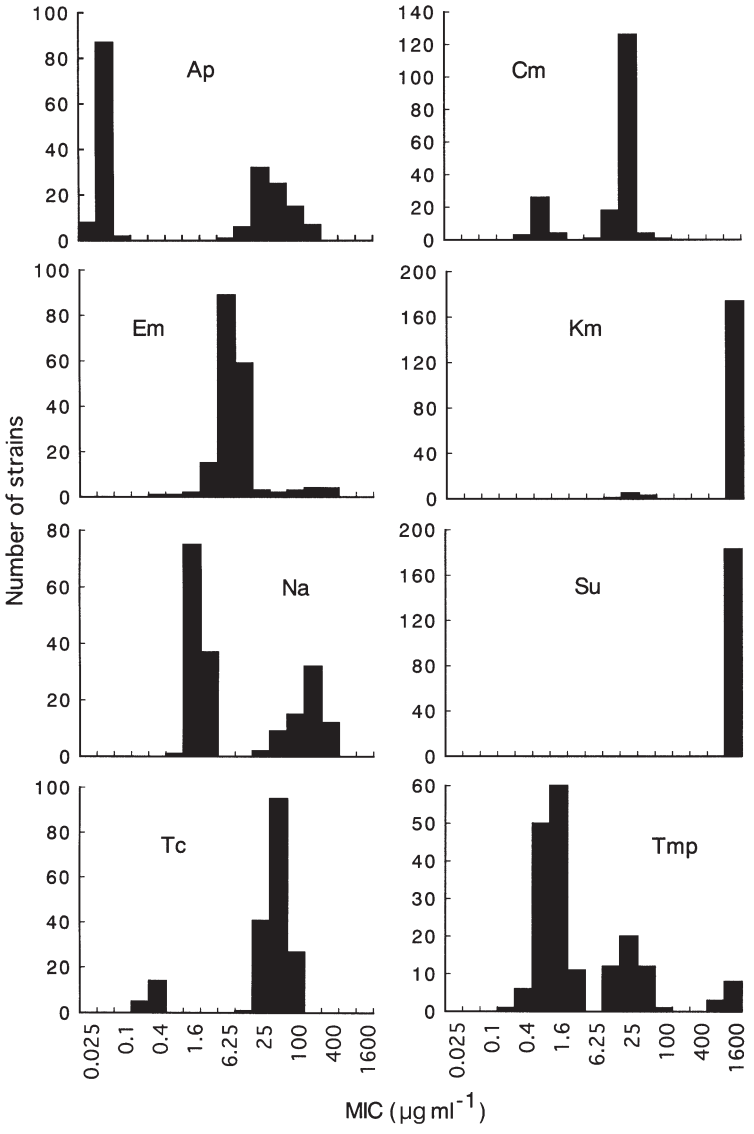

Fig. 1. Distribution of minimal inhibitory concentrations (MIC) of 8 antimicrobial compounds which exhibited resistance to 183 strains of *Photobacterium damselae* subsp. *piscicida* isolated from cultured yellowtail. Ap: ampicillin; Cm: chloramphenicol; Em: erythromycin; Km: kanamycin; Na: nalidixic acid; Su: sulfamonomethoxine; Tc: tetracycline; Tmp: trimethoprim

were as follows. Resistance to Na was never transferred and is probably chromosomally mediated. Resistance to Ap was not transferred from Strains 5 and 8 and from 5 of the strains with resistance pattern 10. Ap resistance was transferred from 10 strains with resistance pattern 10 and all strains with resistance patterns 11 to 13. Tmp resistance was not transferred from strains of Patterns 11 to 13, but was transferred from the strains with Pattern 9.

The plasmid content and frequency of transfer varied (Table 2). The experiment was performed for all the tested strains and the results are presented for 1 strain in each resistance pattern since the results were similar among strains in each pattern. Patterns 2 to 9 seemed to contain a single plasmid of 100 kb, transferred at frequencies of 10^{-3} to 10^{-4}. Strains with resistance patterns 10 to 13 seemed to contain 2 or 3 plasmids: 1 or 2 of 100 kb plus one of 50 or 40 kb, which transferred at different frequencies. The transfer frequency of the 100 kb plasmid was higher than that of the 40 or 50 kb plasmids, but transfer frequency could not be determined when more than 1 plasmid was transferred.

Cloning of DNA fragment carrying Cm^r gene

In plasmids pPDP8511 and pPDP9106, cloned *Hin*dIII fragments carrying Cm^r determinants were approximately 4.7 and 4.3 kb and the subcloned *Hin*dIII-*Hin*cII or -*Pst*I fragment with the determinant was approximately 1.5 kb. The subclones expressed Cm^r without induction with IPTG.

Nucleotide sequence analysis

The DNA sequence of the *Hin*dIII-*Hin*cII fragment of plasmid pPDP8511 has been submitted to the DNA Data Bank of Japan (DDBJ) under accession no. AB082569. The subcloned *Hin*dIII-*Hin*cII fragment of pPDP8511 and the *Hin*dIII-*Pst*I fragment of pPDP9106 were determined as 1607 and 1568 bp, respectively, and the nucleotide sequence from nucleotides 40 to 1607 in the former corresponded with those from nucleotide 1 to 1568 in the latter. The Cm^r gene open reading frame (ORF) was identified from nucleotides 316 to 954 in the subcloned pPDP8511 DNA fragment and from 277 to 915 in the subcloned pPDP9106 DNA fragment. The Cm^r genes were predicted to contain 213 amino acid residues and have been estimated to have a molecular mass of 24.8 kDa. The first base of the hexanucleotide AGAAGG, which represents the consensus prokaryotic ribosome binding site, occurs 13 nucleotides upstream of the ATG translational start codon. Nucleotide sequences within the 5′ and 3′ non-coding regions represent consensus motifs for the initiation and termination of transcription; the –35 sequence (5′-TTGAGA-3′ from nucleotides 63 to 58 upstream of the ATG) and –10 (Pribnow box) sequence (5′-TATTAAT-3′ from nucleotides 37 to 31 upstream of the ATG), respectively. The 3′ non-coding sequence includes an inverted repeat

Table 1. Drug resistance pattern of *Photobacterium damselae* subsp. *piscicida* strains that carried Cm^r and drug resistance transferred from donor *P. damselae* subsp. *piscicida* strains to recipient *Escherichia coli* K-12 χ1037 Rp^r strain. See Fig. 1 for abbreviations

Donor			Recipient	
No.	Drug resistance pattern	No. of strains	Drug resistance transferred	No. of strains
1	Su Km Cm	1	Not transferred	1
2	Su Tc Cm	1	Su, Tc, Cm	1
3	Su Km Tc Cm	39	Su, Km, Tc, Cm	39
4	Su Km Tc Cm Na	11	Su, Km, Tc, Cm	11
5	Su Km Tc Cm Ap	37	Su, Km, Tc, Cm	37
6	Su Km Tc Cm Em	2	Su, Km, Tc, Cm, Em	2
7	Su Km Tc Cm Na Tmp	1	Su, Km, Tc, Cm	1
8	Su Km Tc Cm Ap Tmp	2	Su, Km, Tc, Cm	2
9	Su Km Tc Cm Em Tmp	9	Su, Km, Tc, Cm, Em, Tmp	9
10	Su Km Tc Cm Ap Na	15	Su, Km, Tc, Cm, Ap	10
			Su, Km, Tc, Cm	5
11	Su Km Cm Ap Na Tmp	1	Su, Km, Cm, Ap	1
12	Su Km Tc Cm Ap Na Tmp	29	Su, Km, Tc, Cm, Ap	29
13	Su Km Tc Cm Ap Em Na Tmp	2	Su, Km, Tc, Cm, Ap, Em	2
Total		150	Total	150

Table 2. Drug resistance of transconjugants from mating between the donor strains that showed different resistance patterns and the recipient strain, plasmid content of the transconjugants, and transfer frequency of the resistances. +: resistant; –: susceptible; /: not determined. See Fig. 1 for abbreviations

Drug resistance pattern	Donor strain studied	Drugs used in transconjugant selection	Drug resistance of respective transconjugants							Plasmid content of respective transconjugants (kb)	Transfer frequency of respective resistances
			Su	Km	Tc	Cm	Ap	Em	Tmp		
2	PP8401	Su, Tc, Cm	+	/	+	+	/	/	/	100	10^{-4}
3	PP8702	Su, Km, Tc, Cm	+	+	+	+	/	/	/	100	10^{-3}
4	PP9214	Su, Km, Tc, Cm	+	+	+	+	/	/	/	100	10^{-4}
5	PP8808	Su, Km, Tc, Cm	+	+	+	+	–	/	/	100	10^{-3}
6	PP9202	Su, Km, Tc, Cm, Em	+	+	+	+	/	+	/	100	10^{-4}
7	PP8906	Su, Km, Tc, Cm	+	+	+	+	/	/	–	100	10^{-4}
8	PP8836	Su, Km, Tc, Cm	+	+	+	+	–	/	–	100	10^{-4}
9	PP9401	Su, Km, Tc, Cm, Em, Tmp	+	+	+	+	/	+	+	100	10^{-4}
10	PP8511	Su, Km, Tc	+	+	+	–	–	/	/	100	10^{-4}
		Cm, Ap	–	–	–	+	+	/	/	50	10^{-6}
		Cm, Ap	+	+	+	+	+	/	/	100 + 50	/
		Cm, Ap	+	+	+	+	+	/	/	100	/
	PP8608	Su, Km, Tc, Cm	+	+	+	+	–	/	/	100	10^{-4}
11	PP8912	Su, Km	+	+	/	–	–	/	–	100	10^{-4}
		Cm, Ap	–	–	/	+	+	/	–	50	10^{-6}
		Cm, Ap	+	+	/	+	+	/	–	100 + 50	/
12	PP9010	Su, Km	+	+	–	–	–	/	–	100	10^{-4}
		Tc, Cm, Ap	–	–	+	+	+	/	–	50	10^{-6}
		Tc, Cm, Ap	+	+	+	+	+	/	–	100 + 50	/
		Tc, Cm, Ap	+	+	+	+	+	/	–	100	/
13	PP9106	Su, Km	+	+	–	–	–	–	–	100	10^{-4}
		Tc, Cm, Ap	–	–	+	+	+	–	–	50	10^{-6}
		Tc, Cm, Ap	+	+	+	+	+	–	–	100 + 50	/
		Cm, Em	–	–	–	+	–	+	–	40	10^{-6}
		Cm, Em	+	+	–	+	–	+	–	100 + 40	/

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AB082569    -----MNFTRIDLNTWNRREHFALYRQQIKCGFSLTTKLDITALRTALAETDYKFYPVMI
X53797      -----MNFTRIDLNTWNRREHFALYRQQIKCGFSLTTKLDITAFRTALAETDYKFYPVMI
X53796      -----MNFTRIDLNTWNRREHFALYRQQIKCGFSLTTKLDITALRTALAETGYKFYPLMI
X07848      -----MNYTKFDVKNWVRREHFEFYRHRLPCGFSLTSKIDITTLKKSLDDSAYKFYPVMI
D16171      MEKKITGYTTVDISQWHRKEHFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKFYPAFI
A00566      MEKKITGYTTVDISQWHRKEHFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKFYPAFI
                       *   * * * *        *    *   ***           * **

AB082569    YLISRVVNQFPEFRMAMKD-NALIYWDQTDPVFTVFHKETETFSALFCRYCPDISEFMAG
X53797      YLISRVVNQFPEFRMAMKD-NALIYWDQTDPVFTVFHKETETFSALFCRYCPDISEFMAG
X53796      YLISRAVNQFPEFRMALKD-NELIYWDQSDPVFTVFHKETETFSALSCRYFPDLSEFMAG
X07848      YLIAQAVNQFDELRMAIKD-DELIVWDSVDPQFTVFHQETETFSALSCPYSSDIDQFMVN
D16171      HILARLMNAHPEFRMAMKD-GELVIWDSVHPCYTVFHEQTETFSSLWSEYHDDFRQFLHI
A00566      HILARLMNAHPEFRMAMKD-GELVIWDSVHPCYTVFHEQHETFSSLWSEYHDDFRQFLHI
                   *   * **   *          *  * *    * **     *  *   *

AB082569    YNAVMAEYQHNTALFPQGALPENHLNISSLPWVSFDGFNLNITGNDDYFAPVFTMAKFQQ
X53797      YNAVMAEYQHNTALFPQGALPENHLNISSLPWVSFDGFNLNITGNDDYFAPVFTMAKFQQ
X53796      YNAVTAEYQHDTRLFPQGNLPENHLNISSLPWVSFDGFNLNITGNDDYFAPVFTMAKFQQ
X07848      YLSVMERYKSDTKLFPQGVTPENHLNISALPWVNFDSFNLNVANFTDYFAPIITMAKYQQ
D16171      YSQDIACYGENLAYFPKG-FIENMFFVSANPWVSFTSFDLNVANMDNFFAPVFTMGKYYT
A00566      YSQDVACYGENLAYFPKG-FIENMFFVSANPWVSFTSFDLNVANMDNFFAPVFTMGKYYT
            *      *              *    *  **  *  * **         *  *

AB082569    EDNRVLLPVSVQVHHAVCDGFHAARFINTLQMMCDNILK------
X53797      EDNRVLLPVSVQVHHAVCDGFHAARFINTLQMMCDNILK------
X53796      EGDRVLLPVSVQVHHAVCDGFHAARFINTLQLMCDNILK------
X07848      EGDRLLLPLSVQVHHAVCDGFHVARFINRLQELCNSKLK------
D16171      QGDKVLMPLAIQVHHAVCDGFHVGRMLNELQQYCDEWQGGA----
A00566      QGDKVLMPLAIQVHHAVCDGFHVGRMLNELQQYCDEWQGGA----
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Fig. 2. Alignment of the predicted amino acid sequences of the *Photobacterium damselae* subsp. *piscicida* CAT and the sequences of CAT variants from other bacteria. Asterisks indicate amino acids conserved in all CAT monomers. The sequences are indicated by their accession numbers. AB082569, CAT encoded by the plasmids pPDP8511 and pPDP9106 from *P. damselae* subsp. *piscicida* strains PP8511 and PP9106, respectively (this study); X53797, CAT from *Haemophilus influenzae* (Type II CAT variant); X53796, CAT from *Escherichia coli* (Type II CAT); X07848, CAT from Enterobacteriaceae (Type III CAT); D16171, CAT encoded by the plasmid pSP9351 from *P. damselae* subsp. *piscicida* strain; A00566, CAT from *E. coli* (Type I CAT)

region (5′-AAGCCAC-3′ and 5′-GTGGCTT-3′ from nucleotides 20 to 26 and from 44 to 50 downstream of the TAA translational stop codon, respectively) followed by several thymidine residues characteristic of the proposed secondary structure unit required for rho-factor-independent termination of transcription.

Alignments of the amino acid sequences of the *Photobacterium damselae* subsp. *piscicida* CAT variant identified in this study and CAT variants from other bacteria, i.e. CAT variants representative of the Type and the *P. damselae* subsp. *piscicida* CAT variant reported previously, are shown in Fig. 2. The pPDP8511 and pPDP9106 Cm^r gene ORFs showed 92.5 and 99.5% amino acid identity with the Type II CAT variants found in *Escherichia coli* (accession no. X53796) and *Haemophilus influenzae* (X53797), respectively; that is, the Cm^r determinants from pPDP8511 and pPDP9106 are conferred by the enzyme CAT. In fact, the CAT amino acid sequence in the *P. damselae* subsp. *piscicida* plasmids had only 1 substitution, Leu-39 for Phe-39, compared to the sequence encoded by *H. influenzae*. However, the CAT amino acid sequences encoded by pPDP8511 and pPDP9106 revealed 45.2 and 64.3% identity with the Type I and III CAT variants found in *E. coli* (A00566) and Enterobacteriaceae (X07848) respectively, and 45.7% identity with the CAT variant found in *P. damselae* subsp. *piscicida* (D16171). Moreover, the CAT amino acid residues encoded by pPDP8511 and pPDP9106 lack the first 5 and 2 residues present at the N- and C-terminals of the CAT variant, respectively, found in *P. damselae* subsp. *piscicida* (D16171).

DISCUSSION

The deduced amino acid sequences of the Cm^r gene ORFs of pPDP8511 and pPDP9106 showed 99.5% identity with Type II CAT variant found in *Haemophilus influenzae* (accession no. X53797). Therefore, CAT variants from *Photobacterium damselae* subsp. *piscicida* strains are classified as a Type II variant, since the regions typical for Type II variants were conserved in the CAT variants.

cat genes encoded on transferable R plasmids in fish pathogenic bacteria differ between bacterial genera. The Cm^r determinants on transferable R plasmids are classified as Type I variant for *Photobacterium damselae* subsp. *piscicida*, Type II variants for *Edwardsiella tarda* and *Aeromonas salmonicida*, and Type II (isolated after 1980) and the other variants (isolated before 1977) for *Vibrio anguillarum* (Aoki 1988, 1989, Zhao & Aoki 1992, Kim & Aoki 1993b). However, in this study Cm^r determinants on transferable R plasmids were classified as Type II variants for *P. damselae* subsp. *piscicida* that were sampled from different years and places in Japan. As only 2 strains have been examined, the prevalence of Type II CAT variants in Japanese fish farms is unknown at present.

Cm^r determinants on transferable R plasmids are classified as Type I variant for *Photobacterium damselae* subsp. *piscicida* that was isolated in 1993 from Ehime prefecture in Japan (Kim & Aoki 1993b) and as Type II variant for the same bacteria that was isolated in 1985 and 1991 from Nagasaki and Kumamoto in Japan (this study). Moreover, CAT variants encoded on transferable R plasmids in other fish pathogenic bacteria have been different depending upon the bacterial genera and the year of the bacterial isolation as described above. The reason for the difference in *cat* gene type in *P. damselae* subsp. *piscicida* and other fish pathogenic bacteria is difficult to understand, as many complex environmental factors have contributed to the acquisition of drug resistance in bacteria. The genetic pool in Ehime prefecture may be different from that in Nagasaki and Kumamoto, whereas it may be quite similar for Nagasaki and Kumamoto, because they are closely located on the Kyushu island in Japan, whereas Ehime prefecture is located on the Shikoku island in Japan and is quite far from Nagasaki and Kumamoto prefectures. However, future research needs to be done in order to gain further insight into the CAT gene classification.

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