Cloning and nucleotide sequence analysis of the chloramphenicol and erythromycin resistance genes on a transferable R plasmid from the fish pathogen *Photobacterium damselae* subsp. *piscicida*

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Transferable resistance to various drugs was investigated in a strain of *Photobacterium damselae* subsp. *piscicida* from Japan. Drug resistance was transferred via three plasmids of 100, 50, and 40 kirobases (kb). Resistances to chloramphenicol and erythromycin were transferred on the 40-kb plasmid pPDP9106b. The cloned *Pst* I fragment of pPDP9106b was determined as being 6377 base pairs (bp). The chloramphenicol resistance gene conferring cross-resistance to florfenicol has been designated *floR*, which was found to be part of the 4279-bp transposon and coded 404 amino acid residues with a calculated molecular mass of 42.6 kDa. The transposon consisted of the gene *floR*, a putative regulatory gene (101 amino acid residues), and the transposase gene *tnpA* (497 amino acid residues) and constituted a circular form. The erythromycin resistance gene designated as *ermM*, which coded 303 amino acid residues with a calculated molecular mass of 35.6 kDa, was suggested to be a novel 23S rRNA methyltransferase from the presence of nine conserved sequence motifs that are important in target sequence, catalysis, and *S*-adenosyl-L-methionine binding and very low homology (35 to 15%) between the ErmM protein of *P. damselae* and the 23S rRNA methyltransferases of nineteen other bacteria.

Key Words: chloramphenicol resistance gene, cloning, erythromycin resistance gene, nucleotide sequence, *Photobacterium damselae* subsp. *piscicida*, transferable R plasmid.

INTRODUCTION

Photobacterium damselae subsp. *piscicida* (formerly *Pasturella piscicida*; heaeafter *P. damselae*) is well known as the causative agent of pseudotuberculosis in cultured yellowtail *Seliola quinqueradiata* in Japan. Since its initial recognition in yellowtail during the summer of 1969,[1] the disease has been recognized in many other fish species. Various antimicrobial compounds have been used to prevent this infection by chemoprophylaxis and chemotherapy. The efficacy of these drugs, however, has been hindered by an increase in multiple drug resistance in *P. damselae*. Chloramphenicol (Cm) is an efficacious therapeutic agant 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.[2] Nevertheless, Cm resistant strains of fish pathogenic bacteria including *P. damselae* are still prevalent. On the other hand, erythromycin (Em) has been used to prevent streptococcal infection of fish but not to treat pseudotuberculosis in various fish species of the Order Perciformes (perch-like fishes) including cultured yellowtail in Japan. Em resistance of *P. damselae*, however, has been found in cultured yellowtail taken pseudotuberculosis in Japan.[3-5]

Resistance to Cm has been reported to be mainly due to the production of inactivating enzymes, the Cm acetyl transferases (CATs).[6] Cm resistance of *P. damselae* has also been classified as Type I [7] and II[4] CAT variants. However, we detected Cm resistance gene other than *cat* genes on a transferable R plasmid from *P. damselae* by the nucleotide hybridization assay. Nonenzymatic Cm resistance was described in the late 1970s and early 1980s for plasmids from gram-negative bacteria.[6, 8] The *cml* gene of plasmid R 26 conferring nonenzymatic Cm resistance was reported in 1986 by Dorman et al.[9] and codes for a putative efflux pump related to the CmlA protein of the In*4* integron of transposon Tn*1696*.[10] Nonenzymatic Cm resistance has gained importance with the spread of multidrug-resistant *Salmonella enterica* serovar Typhimurium DT104 world-

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wide epidemic strains, which harbor on their chromosome an antibiotic resistance gene cluster comprising a nonenzymatic Cm resiastance gene conferring cross-resistance to florfenicol (Ff)[11-14] that is a fluorinated analog of Cm. So far, three Ff resistance genes, *cfr*, *fexA*, and *floR*, which also mediate resistance to Cm, have been described.[15] While *cfr*[16] and *fexA*[17] have been found exclusively in staphylococci, *floR* has been reported to occur in various gram-negative bacteria including *P. damselae*.[18] The *floR* gene was detected also on a conjugative[19] or nonconjugative plasmid[20] other than the chromosome and has been found to be part of a transposon that produces circular intermediate forms.[21] In the present study we analysed the nonenzymatic Cm resistance gene conferred by a conjugative R plasmid from *P. damselae*, with particular attention to possible cross-resistance to Ff, to detect whether its gene is part of a transposon producing circular intermediate forms.

Macrolide, lincosamide, and streptogramin B (MLS) antibiotics include Em, clindamycin, and lincomycin are active against various bacteria, including gram-positive cocci and rods and gram-negative cocci. Four mechanisms of bacterial resistance to MLS antibiotics have been detected; they involve enzymatic modification of the antibiotic, active efflux from the bacterial cell, mutation of the ribosomal target site, or, most commonly, enzyme-mediated chemical alteration of the rRNA target.[22-24] The last mechanism is mediated by the synthesis of a 23S rRNA methyltransferase that is encoded by a gene designated *erm* (erythromycin-resistance methylase), which is responsible for the N^6-dimethylation of a specific adenine residue in the 23S rRNA molecule.[25] Methyltransferases are enzymes that methylate a wide variety of substrates; they use *S*-adenosyl-L-methionine (SAM) as the universal methyl donor and release *S*-adenosyl-L-homocysteine as the reaction product.[26] Comparative analysis of over 40 DNA amino-methyltransferases[27] revealed the presence of nine converted sequence motifs that are important in target sequence specificity, catalysis, and SAM binding.[27, 28] Here we investigated on the presence of a novel *erm* gene, designated as *ermM*, in the *P. damselae* by determining the presence of the nine converted sequence motifs in the *ermM* gene and homology between the ErmM protein and the 23S rRNA methyltransferases from other bacteria.

MATERIALS AND METHODS

Bacteria and plasmids

Photobacterium damselae subsp. *piscicida* strain PP9106 isolated from the cultured diseased yellowtail in Kumamoto, Japan was used for conjugative transfer assay, cloning, and nucleotide sequence analysis of Cm and Em resistance genes. The strain carried resistances to ampicillin (Ap), Cm, Em, kanamycin (Km), nalidixic acid (Na), sulfamonomethoxine (Su), tetracycline (Tc), and trimethoprim (Tmp). *Escherichia coli* K-12 χ1037 Rp^r mutant strain (a rifampicin-resistant mutant strain of χ1037 [*galK2 galT22 hsdR lacY1 metB1 relA supE44*] was used as the recipient for conjugal transfer of drug resistance. *E. coli* JM109 (Takara shuzo) was used to obtain competent cells for transformation.

The R plasmid pPDP9106b from strain PP9106 was used as the source of the Cm and Em resistant genes and the vector pUC119 (Ap resistance, M131G, lacZ; Takara Shuzo) was used for cloning and nucleotide sequence analysis of the genes.

Media and growth conditions

P. damselae was incubated in BHI broth (Difco, Sparks, Nevada) containing 2% NaCl at 28℃ and *E. coli* in Luria-Bertani (LB) broth (1% bacto triptone [Difco], 0.5% bacto yeast extract [Difco], and 1% NaCl; pH7.5) at 37℃ . Bromothymol blue (BTB)-lactose nutrient agar (1% bacto peptone [Difco], 1% beef extract [Difco], 1% lactose, 0.0045% BTB, 0.5% NaCl, and 1.5% agar; pH7.5) was used for the mating assay.

Conjugal transfer assay

P. damselae donor strain was incubated in BHI broth under shaking conditions for 9 h and *E. coli* recipient strain in LB broth for 8 h. Aliquots (0.5 mL each) of the donor and recipient cultures were mixed in a 50-mL Erlenmeyer flask and 4 mL of equal volume each of the BHI and LB broths were added to the flask. Mating was performed at 28℃ for 2 h. Of 10-fold serial dilutions of the mating mixture, 0.1 mL was spread on BTB-lactose agar with rifanpicin (50 μg/mL) and each selected drug; Ap (6.25 μg/mL), Cm (25 μg/mL), Em (100 μg/mL), Km (25 μg/mL), Na (100 μg/mL), Su (3,200 μg/mL), Tc (25 μg/mL), and Tmp (400 μg/mL). The *E. coli* recipient strain expressed MIC values as follows: Ap (3.13 μg/mL), Cm (12.5 μg/mL), Em (50 μg/mL), Km (12.5 μg/mL), Na (50 μg/mL), Su (1,600 μg/mL), Tc (6.25 μg/mL), and Tmp (12.5 μg/mL). Colonies growing in this double-inhibitor-supplemented medium after 24 to 48 h of incubation at 37℃ 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. The transconjugants were also used for isolation of R plasmids.

Pasmid manipuration and characterization

R plasmids were extracted by the method of Kado and Liu[29] and electrophoresed on a 0.7% agarose gel. Plasmid DNA was prepared by rapid alkaline lysis and cesium chloride-ethidium bromide density gradient purification. Restriction-endonuclease-digested DNA was electrophoresed on a 1% low-melting-temperature agarose gel[30] for separation of the DNA fragments. Restriction endonucleases and T4 DNA ligase (TaKaRa Suzou) were used according to the manufacturer's instructions.

The approximate molecular size of plasmids were calculated from reference R27 (167 kirobase [kb]), R100-1 (100 kb), RP4 (56 kb), and pLA2917 (22 kb). The molecular size of restriction fragments were calculated using λDNA digested with *Sty*I (Nippon Gene, Tokyo, Japan).

Nucleotide hybridization

The procedure for Southern blotting was taken from the instructions given in the digoxigenin application manual for filter hybridization (Roche Molecular Biochemicals, Mannheim, Germany), DIG Oligonucleotide 3'-End Labeling Kit, 2nd Generation except that the depurination was carried out for 10 min. Plasmid DNA from the gels was capillary blotted onto nylon hybridization membranes (Hybond-N+; Amersham, Piscataway, New Jersey) and fixed at 120℃ for 30 min. Oligonucleotides were designed on the base of the nucleotide sequence of CAT variants (Type I [accession number A00566], II [AB082569], and III [X07848]) and enzymatically labeled at their 3' end with terminal transferase by incorporation of a single digoxigenin-labeled dideoxyuridine triphosphate. Hybridization was performed at 55℃ for 4 h as prescribed by the manufacturer.

Cloning of the chloramphenicol and erythromycin resistance genes

Nucleic acid from the plasmid pPDP9106b (40 kb-plasmid of 100 kb, 50 kb, and 40 kb-plasmids harbored in the strain PP9106) was completely digested with the restriction endnuclease *Pst*I. The resulting fragments were cloned into the *Pst*I site of the vector pUC119. Competent *E. coli* JM109 cells prepared according to the method of Hanahan[31] were transformed with the recombinant plasmid DNA by the procedure of Lederberg and Cohen.[32] Clones harboring the Cm and/or Em resistances were selected on LB agar plates containing Ap alone (50 μg/mL), Cm (25 μg/mL) and/or Em (100 μg/mL) in combination with Ap (50 μg/mL) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopiranoside; Takara Shuzo) and isopropyl-FI-D-thio-galactopyranoside.

Nucleotide sequencing and analysis

The cloned fragment containing the Cm and Em resistance genes was sequenced by the dideoxy chain termination method using BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and an ABI PRISMTM 377 sequencer (Perkin Elmer Co., USA). Nucleic acid sequencing was performed using M13 forward and reverse primers. Internal sequencing primers were designed on the basis of the sequence of the cloned fragment to complete the sequence walk in both directions. The nucleotide sequence of the Cm and Em resistance genes was analyzed with version 3.6 of DANASIS-Mac software (Hitachi Software Engineering, Chiba, Japan). Sequence similarity and homology searching against the nucleotide and deduced protein database were carried out with the Fasta programs at the Europian Molecular Biology Laboratory, Europian Bioinformatics Institute (EMBL-EBI). Multiple-sequence alignment of the nucleotide and deduced peptide sequence was performed with the ClustalW2 program at EMBL-EBI. The types of various Em resistance genes were determined by Antibiotic Resistance Genes Database (ARDB).

RESULTS AND DISCUSSION

Plasmid phenotypes

The drug resistance of transconjugants obtained from matings between the donor *P. damselae* and the recipient *E. coli* strains as well as the incidence and plasmid content of the transconjugants, and the transfer frequency of the resistances are shown in Table 1; agarose gel electrophoretic profiles of the R plasmids in the transconjugants are shown in Fig. 1. Transconjugants from the transfer assay harbored a single plasmid of 100, 50, and 40 kb each or two plasmids of 100 and 50 or 40 kb (see lanes C, D, E, F, and G in Fig. 1, respectively), and the 100-kb, 50-kb, or 40-kb plasmid carried Km/Su, Ap/Cm/Tc, or Cm/Em resistances, respectively. Transconjugants that harbored both of the 100-kb and 50-kb or 40-kb plasmids occasionally possessed 110-kb plasmid (see lanes H and I in Fig. 1, respectively), which carried all of the drug resistances encoding on both the plasmids. The 110-kb plasmids were considered as the presumptive cointegrate (see reference 3 in detail) that both the plasmids might have fused with each other because they exhibited the resistances possessed by both the plasmids and moreover were not harbored by the *P. damselae* donor (see lane B in Fig. 1). No transconjugant conferring resistance to Na and Tmp was founded.

The transfer frequency of the 100-kb plasmid was higher than that of the 50-kb and 40-kb plasmids and moreover the frequencies of the 110-kb plasmids were almost the same as that of the 50-kb or 40-kb plasmids.

Table 1 Drug resistance of transconjugants from mating between the donor strain PP9106 and the recipient strain *Escherichia coli* k-12 χ 1037, incidence and plasmid content of the transconjugants, and transfer frequency of the resistances. Drugs used for the mating assay were ampicillin (Ap), chloramphenicol (Cm), erythromycin (Em), kanamycin (Km), sulfamonomethoxicine (Su), and tetracycline (Tc).

Transconjugant[a] selected	Drug resistance of transconjugants						No. of transconjugants/No. of colonies selected	Plasmid conten of transconjugants (Kb)	Transfer frequency of resistance
	Su	Km	Tc	Cm	Ap	Em			
Su	+	+	-	-	-	-	10/10	100	1.3×10^{-3}
Km	+	+	-	-	-	-	10/10	100	4.9×10^{-3}
Tc	-	-	+	+	+	-	6/10	50	1.2×10^{-6}
Tc	+	+	+	+	+	-	4/10	100 & 50	nd
Cm	-	-	+	+	+	-	2/10	50	1.6×10^{-6}
Cm	+	+	+	+	+	-	4/10	100 & 50	nd
Cm	-	-	-	+	-	+	1/10	40	1.6×10^{-6}
Cm	+	+	-	+	-	+	3/10	110	1.6×10^{-6}
Ap	-	-	+	+	+	-	3/10	50	5.5×10^{-6}
Ap	+	+	+	+	+	-	6/10	100 & 50	nd
Ap	+	+	+	+	+	-	1/10	110	5.5×10^{-6}
Em	-	-	-	+	-	+	1/10	40	1.2×10^{-6}
Em	+	+	-	+	-	+	1/10	100 & 40	nd
Em	+	+	-	+	-	+	8/10	110	1.2×10^{-6}

[a] Indicated by thr drug with which it was serecterd.

[b] Plus signs indicate resistant transconjugants and minus signs susceptible one; and nd = not determined.

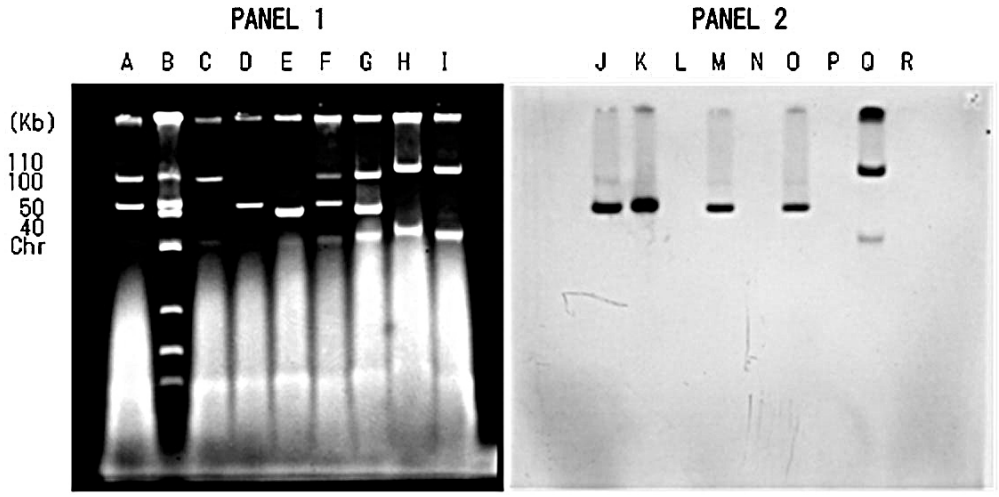

Fig.1 Southern blot of a hybridization of a digoxigenin-labeled Type II CAT variant gene (oligonucleotides) and R plasmids transferred from *Photobacterium damselae* subsp. *piscicida* strain PP9106. The Type II CAT variant was cloned from the 50-kb plasmid (see lane A) transferred from the *P. damselae* strain PP9106 (see reference 4). Panel 1 is an agarose gel electrophoretic profile of R plasmids in transconjugants selected with articular drugs in mating assay with the donor *P. damselae* strain PP9106 and the recipient *Escherichia coli* K-12 χ 1037 strain (lanes A and C-I) and R plasmids in the *P. damselae* donor (lane B). Panel 2 is an autoradiogram of a nitrocellulose filter blotted with the DNA in panel 1 and probed with a digoxigenin-labeled Type II CAT variant gene (oligonucleotides). Lanes B and K involve R plasmids harbored resistances to ampicillin (Ap^r), chloramphenicol (Cm^r), erythromycin (Em^r), kanamycin (Km^r), sulfamonometoxine (Su^r), and tetracycline (Tc^r); lanes A and J, F and O involve the 100- and 50-kb plasmids harbored Ap^r, Cm^r,, Km^r, Su^r, and Tc^r; lanes C and L involve the 100-kb plasmid harbored Km^r and Su^r; lanes D and M involve the 50-kb plasmid harbored Ap^r, Cm^r,, and Tc^r; lanes E and N involve the 40-kb plasmid harbored Cm^r and Em^r; lanes G and P involve the 100- and 40-kb plasmids harbored Cm^r, Em^r, Km^r,, and Su^r; lanes H and Q involve the 110-kb plasmid harbored Ap^r, Cm^r,, Km^r, Su^r, and Tc^r; lanes I and R involve the 110-kb plasmid harbored Cm^r, Em^r, Km^r, and Su^r.

CAT variant on the transferable R plasmid pPDP9106b

The 40-bp fragments from CAT variants (Type I, II, and III) were used to probes against the transferable R plasmids from the transconjugants. Hybridization with the probe from Type II CAT variant occurred at the 50-kb plasmids (see lanes A & J, B & K, D & M, and F & O in Fig. 1) and the 110-kb plasmid (see lanes H & Q in Fig. 1) that both the 100-kb and 50-kb plasmids might have fused with each other, but not at the 40-kb plasmids encoding Cm resistance (see lanes B & K, E & N, and G & P in Fig. 1) and the 110-kb plasmid (see lanes I & R in Fig. 1) that both the 100-kb and 40-kb plasmids might have fused with each other. The Type I and III CAT probes were not hybridized to the transferable R plasmids encoding Cm resistance.

Cm resistance gene on the transferable R plasmid pPDP9106b

The cloned *Pst*I fragment of pPDP9106b was determined as being 6377 base pairs (bp: DNA Databank of Japan [DDBJ] under accession number AB601890). Genetic organization of the *floR* transposon on the transferable R plasmid from *P. damselase* is shown in Fig. 2. Sequence

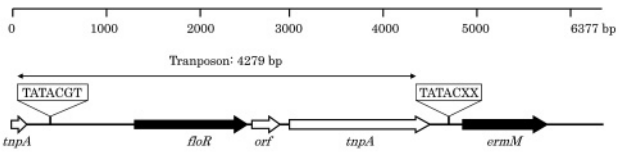

Fig.2 Genetic organization of the *floR* transposon and the erythromycin resistance gene *ermM* on a transferable R plasmid from *Photobacterium damselae* subsp. *piscicida*. The position and orientation of the genes are indicated by arrow. The 7-bp (or part of 7-bp) nucleotide sequences at the transposon junctions are shown in boxes. A distance scale in bp is given above the map.

analysis revealed the presence of a transposon-like element of 4279 bp. This element comprised the resistance gene *floR* (see below), which codes for a membrane-associated protein that exports Ff and Cm from bacterial cell,[10] was predicted to contain 404 amino acid residues and estimated to have a molecular mass of 42.6 kDa as having already been reported (see the Fasta programs). The first base of the tetranucleotide 5'-GAGG-3', which represents the consensus prokaryotic ribosome binding site, occurred nucleotides 15 to 12 upstream of the ATG translational start codon. Nucleotide sequences within the 5' and 3' noncoding regions represent consensus motifs for the initiation and termination of transcription, namely, the -35 sequence (5'-TTCATA-3' from nucleotides 164 to 159 upstream of the ATG) and -10 (Pribnow box) sequence (5'-TCAGAT-3' from nucleitides 140 to 135 upstream of the ATG), respectively. Further components of this transposon were an open reading frame (ORF) coding for a putative LysR-like transcriptional regulator of 101 amino acids (see below) and the gene *tnpA* coding for a putative transposase of 497 amino acid residues (see below). This transposon starts with a 7-bp sequence (5'-TATACGT-3') located nucleotides 903 to 897 upstream of the start codon of the *floR* gene, whereas nucleotides 227 to 231 downstream of the gene *tnpA* , the partial sequence (5'-TATACXX-3', a 5-bp sequence) of the 7-bp sequence was found and constitutes most likely the right-end junction of the transposon. In fact, the 7-bp and the 5-bp sequences located in the same position with the 7-bp sequences of the start and the end of the Tn*floR* transposon on plasmid from *E. coli*.[21] Moreover, the DNA sequence showed 97.2% homology between both the transposons (the start [the 7-bp sequence] to the end [the 5-bp sequence] of the transposons) in the *P. damselae* and the *E. coli*, but lost suddenly homology between the two sequence alignments after the end (the 5-bp sequence) of both the transposons.

The *floR* gene of *P. damselae* strain PP9106 revealed 100% amino acid identities to those of *floR* genes of various bacteria (data from the Fasta programs) including *E. coli*[21] but 89.6% amino acid identical to previously reported *floR* gene (*PP-flo* : 374 amino acid residues) on a transferable R plasmid from *P. damselae*.[18] The *floR* and *PP-flo* genes, however, showed 98.7% nucleotide identity. The ORFs coding for a putative LysR-like transcriptional regulator and a putative tnpA transposase that are the components of the transposon from *P. damselae* also showed 100% nucleotide identities to those of both the components of the transposon from *E. coli*.[21]

Em resistance gene on the transferable R plasmid pPDP9106b

The putative Em resistance gene ORF located 129-bp downstream of the end (the 5-bp sequence) of the transposon and was identified from nucleotides 4808 to 5719 in the cloned pPDP9106b DNA fragment (see Fig. 2). The Em resistance gene was predicted to contain 303 amino acid residues and estimated to have a molecular mass of 35.6 kDa. Nucleotide sequences within the 5' and 3' noncoding regions represent the -35 sequence (5'-TTCTTA-3' from nucleotides 36 to 31 upstream of the ATG) and -10 sequence (5'-TATAATTG-3' from nucleitides 14 to 7 upstream of the ATG).

The amino acid sequence alignments of the ErmM protein from *P. damselae* and 23S rRNA methyltransferases (ErmA to ErmY) of nineteen other bacteria (Fig. 3) revealed the presence of nine conserved sequence motifs that are important in target sequence speficity, catalysis, and SAM binding.[27, 28] Motif I forms a secondary structure known as the G loop that binds to the methionine moiety

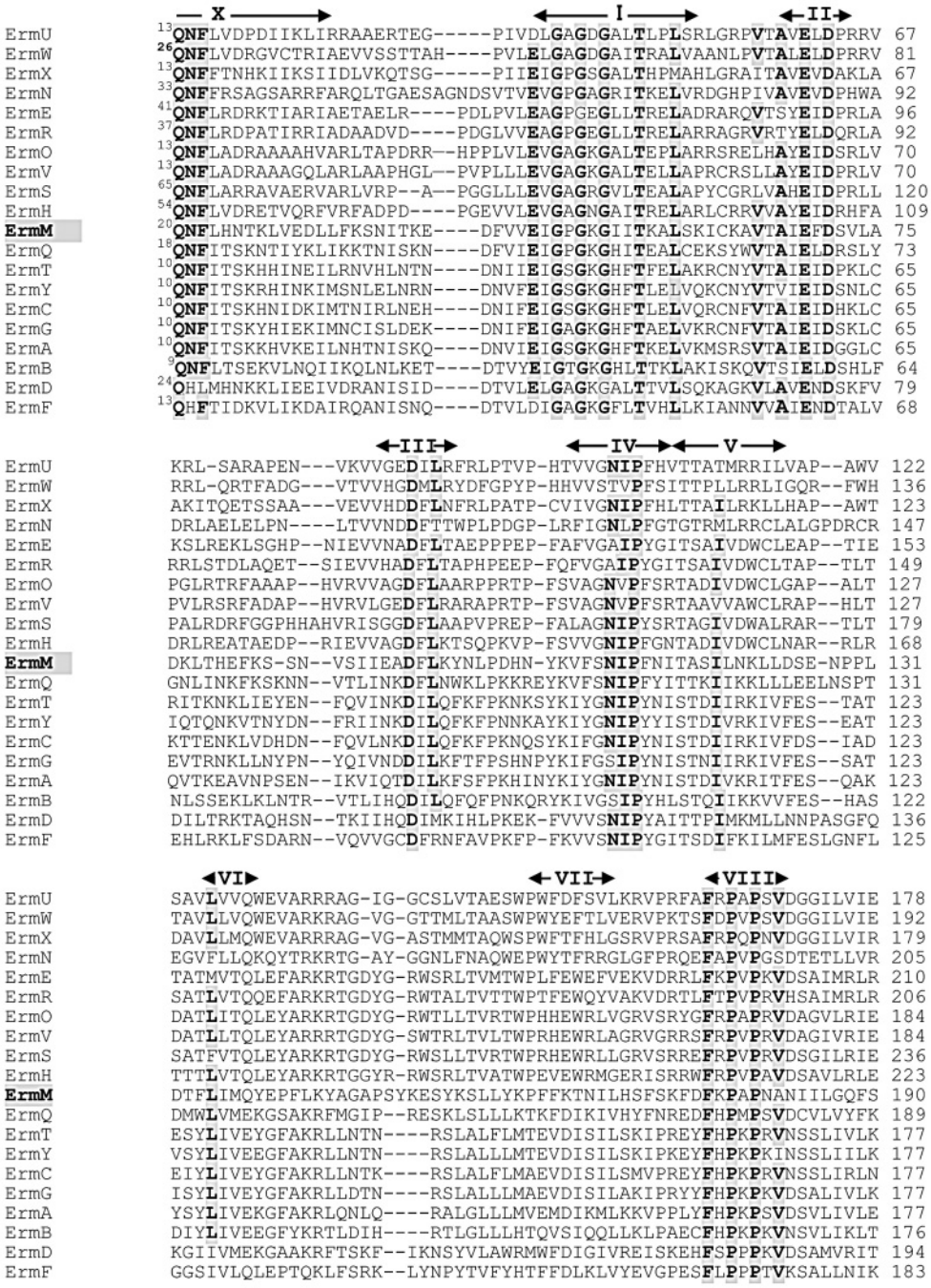

Fig.3 The partial amino acid sequence alignments of erythromycin resistance protein (ErmM) from *Photobacterium damselae* subsp. *piscicida* (DDBJ accession number AB601890) and 23S rRNA methyltransferases (ErmA to ErmY) from nineteen other bacteria. The position and extent of each of the conserved motifs, motifs I to VIII and X, are indicated by arrows above the sequence alignment. Regions of identity between the ErmM protein and the 23S rRNA methyltransferases are boxed in shadow. The superscript numbers to the left of the start of each amino acid sequence indicate the starting residue number. The sources of the respective 23S rRNA methyltransferases (Erm proteins) and the relevant accession numbers are as follows: ErmA, *Staphylococcus aureus*, AB435013; ErmB, *Clostridium perfringens*, U18931; ErmC, *S. aureus*, M17990; ErmD, *Bacillus licheniformis*, M29832; ErmE, *Saccharopolyspora erythraea*, X51891; ErmF, *Bacteroides fragilis*, M62487; ErmG, *Lysinibacillus sphaericus*, M15332; ErmH, *Streptomyces thermotolerans*, M16503; ErmN, *Streptomyces fradiae*, X97721; ErmO, *Streptomyces ambofaciens*, AJ223970; ErmQ, *C. perfringens*, L22689; ErmR, *Arthrobacter* sp., M11276; ErmS, *Streptomyces fradiae*, M19269; ErmT, *Streptococcus pasteurianus*, AY894138; ErmU, *Streptomyces lincolnensis*, X97146; ErmV, *Streptomyces viridochromogenes*, U59450; ErmW, *Micromonospora griseorubida*, D14532; ErmX, *Corynebacterium jeikeium*, AF338705; ErmY, *S. aureus*, AB014481. The figure was modified from reference 28.

of SAM[27, 28] and appeared to have mostly the consensus sequence of EXGXGXGXXTXXL, including the *P. damselae* Em resistance gene (ErmM: hereafter cut this word and phrase). Motif II contains a negatively charged amino acid that interacts with the ribose hydroxyls of SAM and a bulky hydrophobic side chain that makes contact with the adenine residue of SAM[28] and factually appeared to have generally the consensus sequence of VXAXEXD including glutamate and aspartate residues. Motif III contains a conserved residue that interacts directly with the exocyclic amino group of the target adenine residue[28] and appeared to have mostly the consensus sequence of DXL. Motif IV creates a structure known as the P loop that forms the active or catalytic site of the enzyme[27, 28] and appeared to have generally the consensus sequence of NIP. Motif V contains a conserved consensus sequence that contacts the SAM adenine residue and interacts with motif VII to form the SAM-binding site[28] and appeared to have generally a conserved isoleucine residue. Motif VI consists of a cluster of three hydrophobic residues that have been suggested to be involved in placement of the target adenine ring opposite motif IV[28] and appeared to have generally a conserved leucine residue. Motif VII was not strongly conserved but is beleaved to be involved in the folding of the catalytic region.[28, 33] Motif VIII contains a phenylalanine residue that is proposed to interact with the target adenine residue[28] and factually appeared to have mostly the consensus sequence of FXPXPXV including a phenylalanine residue. This region forms a loop that hangs over the active site and is reffered to as the adenine-binding loop.[26, 28] Motif X was appeared to have mostly the consensus sequence of QNF; incidentally, the loop formed by motif X, along with the G loop of motif I and the P loop of motif IV, forms the sides of the binding pocket for the methionine moiety of SAM.[27, 28] Consequently, the amino acid sequences of the ErmM protein from *P. damselae* and the 23S rRNA methyltransferases from nineteen other bacteria revealed the presence of the same nine sequence motifs, notwithstanding very low homology (15 to 35% identities) between the former and the latter (Table 2). These results suggest that the pPDP9106b Em resistance gene on the transferable R plasmid from *P. damselae* is a novel 23S rRNA methyltransferase. Moreover, our study is the first report of a Em resistance gene isolated from *P. damselae*.

Table 2 Relationship between Erm proteins*1. Relationship between the ErmM protein cloned in this study and the other Erm proteins is shaded.

Erm*2	ErmA	ErmB	ErmC	ErmD	ErmE	ErmF	ErmG	ErmH	**ErmM**	ErmN	ErmO	ErmQ	ErmR	ErmS	ErmT	ErmU	ErmV	ErmW	ErmX	ErmY
(Length)	(243)	(245)	(244)	(287)	(381)	(266)	(244)	(299)	**(303)**	(291)	(259)	(257)	(340)	(319)	(244)	(279)	(258)	(311)	(284)	(244)
ErmA	100	50	56	27	21	24	58	22	**27**	16	17	38	19	21	62	20	14	18	20	57
ErmB		100	48	25	22	24	49	15	**23**	14	13	38	20	14	51	17	12	15	17	51
ErmC			100	21	21	26	70	17	**27**	16	14	34	19	16	77	18	15	14	17	71
ErmD				100	22	32	22	24	**26**	17	22	30	21	20	21	19	19	19	22	21
ErmE					100	26	21	45	**16**	29	48	22	55	42	20	35	45	31	29	18
ErmF						100	23	24	**24**	14	22	29	24	21	22	23	21	20	17	20
ErmG							100	20	**28**	17	18	35	21	19	73	21	15	16	20	68
ErmH								100	**18**	30	54	21	45	46	18	32	49	31	26	16
ErmM									**100**	**16**	**17**	**38**	**15**	**16**	**29**	**17**	**15**	**15**	**23**	**25**
ErmN										100	27	14	28	27	12	29	24	29	29	12
ErmO											100	16	46	62	14	35	74	37	30	15
ErmQ												100	16	14	36	18	15	19	23	35
ErmR													100	41	22	35	44	30	30	20
ErmS														100	17	32	61	29	26	18
ErmT															100	20	14	15	20	77
ErmU																100	35	48	44	16
ErmV																	100	35	30	12
ErmW																		100	38	14
ErmX																			100	14
ErmY																				100

*1 Protein identities (%) were calculated by comparison of the amino acid sequence alignments and their parts are presented in Fig. 3.

*2 Erythromycine resistance methylase (the length of amino acid residues).

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魚病原細菌*Photobacterium damselae* subsp. *piscicida* の伝達性Rプラスミドのクロラムフェニコールおよびエリスロマイシン耐性因子のクローニングと塩基配列解析

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P. damselae subsp. *piscicida* PP9106株のクロラムフェニコール（Cm）およびエリスロマイシン（Em）耐性因子は40 kb（キロ塩基対）プラスミド（pPDP9106b）にコードされ伝達された。Cm耐性因子（アミノ酸残基数404）はフロルフェニコール耐性因子と交差耐性を示す*floR*であることが予想され，調節遺伝子（アミノ酸残基数101）およびトランスポザーゼ遺伝子*tnpA*（アミノ酸残基数497）とともに，環状のトランスポゾン（塩基数4279）を形成した。Em耐性因子（アミノ酸残基数303）はその保存配列領域と相同性から，新規な23S rRNAメチル基転移酵素（ErmMと命名）であることが示唆された。

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