Chapter4. Cloning and nucleotide sequence analysis of a β-lactamase gene from *Photobacterium damsela* subsp. *piscicida*

INTRODUCTION

In Japan, various antimicrobial compounds have been used in fish culture for the prevention and treatment of fish diseases. However, the indiscriminate use of these antimicrobial agents has brought about infection with multiple drug-resistant strains of pathogens in cultured fish. The efficacy of drugs used for preventing the infection has been hindered by an increase of multiple drug resistance in *P. damsela* subsp. *piscicida*. Ampicillin (Ap) is one of the most important and extensively used antimicrobial compound in fish farms and other day to day life not only in Japan but also throughout the world. Due to the extensive use of Ap, fish pathogenic bacteria have developed resistance against it (Kim & Aoki, 1993a).

The Ap^r genes have been found in various bacterial chromosomes, in R-plasmids and also within transposable elements (Heffron et al., 1975). Various drug resistance genes could be located and sequenced, for example Kim &

Aoki (1993b) could locate and sequence the Cp^r gene from a transferable R plasmid. However, the occurrence of Ap^r in *P. damsela* ssp. *piscicida* though was confirmed, it`s location was unclear and was thought to be located on the chromosome, as it could not be transferred to another bacteria (Kim & Aoki, 1993a).

In the second and third chapter, it was observed that the Ap^r determinant from *P.damsela* ssp. *piscicida* could be conjugally transferred to *E.coli*. The aim of the study in this chapter was to clone the Ap^r gene from the 65 kb transferable R-plasmid from *P. damsela* ssp. *piscicida* strain PP8517, analyse it`s sequence and compare it with known sequences that are already reported.

MATERIALS AND METHODS

Bacteria and plasmid. *Photobacterium damsela* ssp. *piscicida* strain PP8517 studied was isolated from diseased cultured yellowtail *Seriola quinqeradiata* in 1985 in Kyushu, Japan. The PP8517 strain expressed Ap^r, Cp^r, Km^r, Na^r, Oa^r, Sa^r and Tc^r. *Escherichia coli* K-12 χ1037 Rp^r mutant strain (Iyobe et al., 1981, 1994) was used as the recipient for conjugal transfer of drug resistance.

Escherichia coli JM83 streptomycin-resistant (Sm^r) strain [F-, thi, ara, del(lac-pro), str A (fi80, lacZ, del M15)] was used as competent cell for transformation.

The R-plasmid pPDP8517 from PP8517 strain was used as the source of the Ap^r determinant and the vectors pHSG398 (Cp^r) and pUC19 (Ap^r, lacZ) plasmids were used for the cloning or nucleotide sequence analysis of the Ap^r determinant.

Media and growth conditions. *P. damsela* ssp. *piscicida* was incubated with BHI broth (Difco, Becton-Dickinson, Sparks, Maryland, USA) containing 2% NaCl at 28°C and *E.coli* with 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 drug susceptibility test. BTB-lactose nutrient agar [1% bactopeptone (Difco), 1% beef extract (Difco), 1% lactose, 0.0045% bromothymol blue, 0.5% NaCl, 1.5%agar, pH7.5] was used for mating assay.

Conjugal transfer assay. Mating procedures, the calculation of transfer frequency of drug resistance, and

drug susceptibility test of transconjugants obtained by mating were performed as described in the second chapter. The transconjugants obtained from mating with the donor *P. damsela* ssp. *piscicida* strains and the recipient *E. coli* K-12 χ1037 Rp^r strain were used for isolation of R-plasmids which were further used for cloning and nucleotide sequence analysis of the Ap^r determinant.

Plasmid manipulation and characterisation. R-plasmids of the transconjugants obtained by conjugal transfer assay were extracted by the method of Kado and Liu (1981) and electrophoresed on 0.7% agarose gel. A large amount of the purified DNA was prepared by rapid alkaline hydrolysis and cesium chloride/ethidium bromide density gradient purification method. The digested DNAs after restriction endonuclease treatment were electrophoresed on 1.0% low- melting-point agarose gel (Grosa et al., 1994) for separation of the DNA fragments. Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co. Ltd.(Shiga, Japan) and used according to the specifications of the manufacturer. All other DNA manipulations unless specified were performed

as described by Sambrook et al. (1989).

Cloning of Ap^r determinant. The DNA of pPDP8517 was completely digested with the restriction endonuclease *Hin* cII. The resulting fragments were cloned into the *Hin* cII site of the vector pHSG398. The competent cells of *E. coli* JM83, which were prepared according to the method of Hanahan (1983) with minor modifications were transformed with the recombinant plasmid DNAs by a slightly modified procedure of Lederberg and Cohen (1974). Clones carrying the Ap^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 Cp (25 μg ml^{-1}) and X-gal (5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside, Takara) and IPTG. The recombinant DNA insert was digested with *Hin* cII and was subcloned into the *Hin* cII site of vector pUC19. The recombinant plasmids containing the Ap^r determinant were used as the source of the determinant and it`s surrounding sequences.

Nucleotide sequencing and analysis. The subcloned fragment containing the Ap^r determinant was

sequenced by the dideoxy chain termination method using a Big Dye Terminator Cycle Sequencing kit and an ABI 377 DNA sequencer (Applied Biosystems, USA). DNA sequencing was performed by using M13 forward and reverse primers. Later, internal sequencing primers were constructed from the available DNA sequences to complete the sequence walk. The oligonucleotides were synthesised by Amersham Pharmacia (Tokyo, Japan). The nucleotide sequence of the Ap^r determinant was analysed by using the DNASIS Mac Ver 3.6 (Hitachi Software Engineering Co. Ltd., Japan). Databases similarity searches for both the nucleotide searches and deduced amino acid sequences were carried out at the National Center of Biotechnology Information website. Multiple sequence alignment of the deduced peptide sequence was carried out by using CLUSTAL W over the internet. The identification of signal peptides was carried out with the program SignalP V 1.1 at the center for Biological Sequence Analysis over the internet (http://www.cbs.dtu.dk/services/SignalP/) [Nielsen, et al, 1997].

Hydropathy plot. A hydropathy plot of *P. damsela*

ssp. *piscicida* Ap^r determinant ORF amino acid sequence was performed using the amino acid hydropathy values of Kyte and Doolittle (1982).

RESULTS

Drug resistance transfer

Transfer frequencies of drug resistances transferred from the donor *P. damsela* ssp. *piscicida* strains to the recipient *E. coli* K-12 χ1037 Rp^r strain are shown in Table 1. Ap^r, Cp^r, Km^r, Sa^r and Tc^r (except Na^r and Oa^r) carried by the donors were transferred to the recipient. The transfer frequencies were about 10^{-4} for Km^r, Sa^r or Tc^r and about 10^{-6} for Ap^r or Cp^r.

Drug resistance of transconjugants obtained by mating with the donor *P. damsela* ssp. *pisicicida* strains and the recipient *E. coli* K-12 χ1037 Rp^r strain is shown in Table 2. The transconjugants selected with Km, Sa or Tc (Km, Sa or Tc transconjugants) exhibited Km^r, Sa^r and Tc^r. The transconjugants selected with Ap or Cp (Ap or Cp transconjugants) expressed Ap^r and Cp^r or Ap^r, Cp^r, Km^r,

Table 1 Transfer frequency of drug resistances transferred from the donor *Photobacterium damsela* subsp.*piscicida* strain PP8517 to the recipient *Escherichia coli* K-12 χ 1037 Rp^r strain. The donor exhibited Ap^r, Cp^r, Km^r, Na^r, Sa^r, and Tc^r. See text for abbreviations

Donor strain	Drug resistance transferred	Transfer frequency
PP8517	Km^r	1.3×10^{-4}
	Sa^r	1.1×10^{-4}
	Tc^r	1.2×10^{-4}
	Ap^r	5.3×10^{-6}
	Cp^r	3.2×10^{-6}

Table 2 Drug resistance of transconjugants obtained in mating with the donor *Photobacterium damsela* subsp. *piscicida* strain PP8517 and the recipient *Escherichia coli* K-12 χ 1037 Rp^r strain. Symbols:+, resistant; -, susceptible; and /, not determined. See text for abbreviations

Donor strain	Transconjugant selected with individual drugs	Resistance to each drug: Km	Sa	Tc	Ap	Cp
PP8517	Km, Sa, Tc-transconjugant	+	+	+	-	-
	Ap, Cp-transconjugant	-	-	-	+	+
	Ap, Cp-transconjugant	+	+	+	+	+

Sa^r and Tc^r.

Agarose gel electrophoresis

Agarose gel electrophoresis of R-plasmids in transconjugants selected with respective drugs in mating with the donor *P. damsela* ssp. *piscicida* strain PP8517 and recipient *E. coli* K-12 χ1037 Rp^r mutant is shown in Fig1. Km, Sa or Tc transconjugants always harboured only an approximately 125-kb plasmid, which encoded Km^r, Sa^r and Tc^r and the plasmid was transferred at a transfer frequency of about 10^{-4}. Ap and Cp transconjugant possessed only an approximately 65 kb plasmid, which coded Ap^r and Cp^r and was transferred at a frequency of about 10^{-6}, or both 125 kb and 65 kb plasmids or a presumptive fused plasmid that might have presumably been created by the fusion of the two plasmids (125 kb and 65 kb plasmids) [chapter three].

Cloning of DNA fragment carrying the Ap^r gene (PDP-AP gene) from plasmid pPDP8517

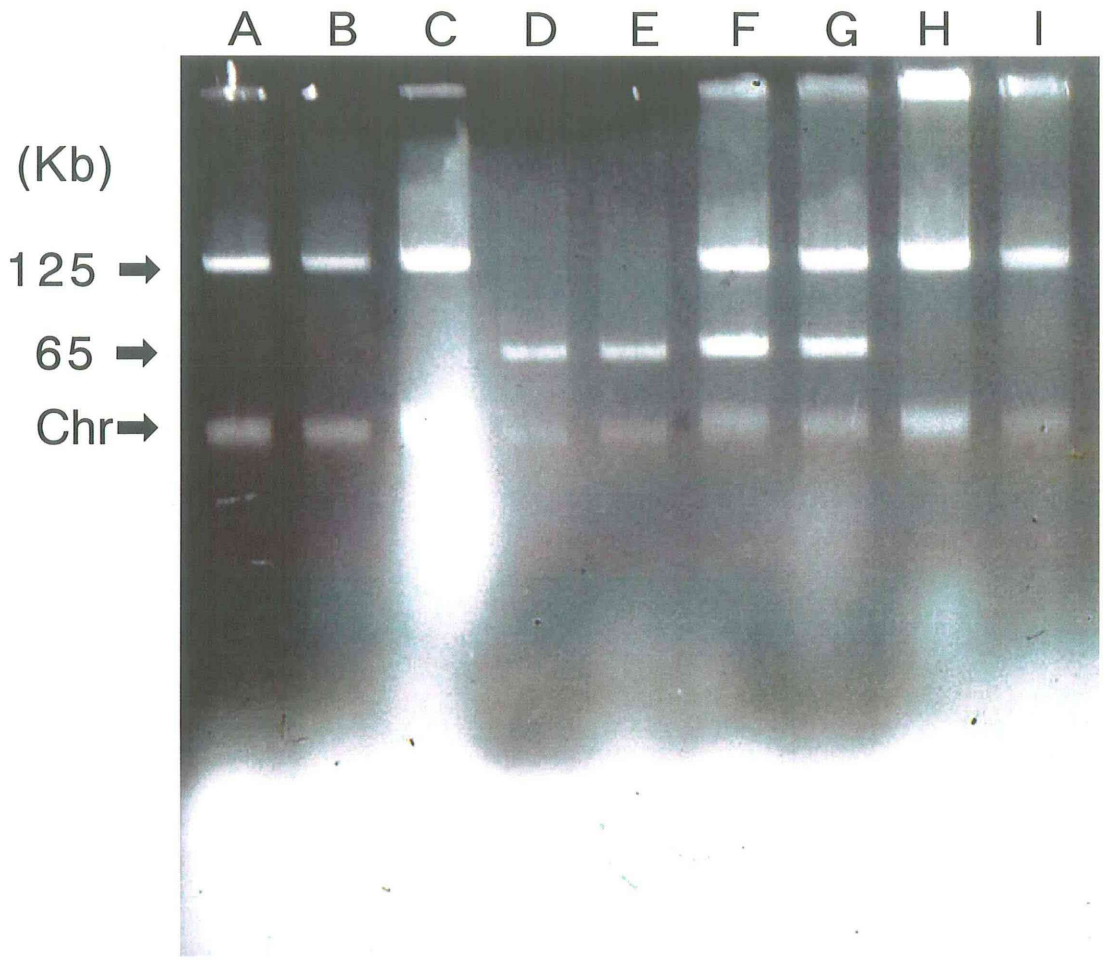

Fig . 1 Agarose gel eletrophoresis of plasmid DNA from transconjugants in mating with the donor *Photobacterium damsela* subsp. *piscicida* strain 8517 and the recipient *Escherichia coli* K-12 χ 1037 Rp^r strain. Lane A: transconjugants selected with Sa and showing resistances to Sa Km Tc; lane B, transconjugants selected with Km and showing resistances to Sa Km Tc; lane C, transconjugants selected with Tc and showing resistances to Sa Km Tc; lane D, transconjugants selected with Cp and showing resistances to Cp Ap; lane E, transconjugants selected with Ap and showing resistances to Cp Ap; lane F, transconjugants selected with Cp and showing resistances to Sa Km Tc Cp Ap; lane G, transconjugants selected with Ap and showing resistances to Sa Km Tc Cp Ap; lane H, transconjugants selected with Cp and showing resistances to Sa Km Tc Cp Ap; and lane I, transconjugants selected with Ap and showing resistances to Sa Km Tc Cp Ap.

The cloned *Hin*cII fragment carrying Ap^r determinant was approximately 1.8 kb.

Nucleotide sequence analysis and the deduced amino acid sequences

DNA sequence of *Hin*cII fragment of pPDP8517 plasmid carrying PDP-AP is shown in Fig. 2. Nucleotide sequence analysis of the 1.8 kb *Hin*cII fragment harbouring Ap^r determinant revealed the fragment to be 1,736 bp long. The analysis further revealed a 846 bp long open reading frame (ORF) of the Ap^r gene from nucleotide 183 to 1028. The Ap^r gene was predicted to contain 282 amino acid residues which had a calculated molecular mass of 31,292 Da, a size consistent with those of other known class A β-lactamases (Ambler, 1980). The first known base of the sequence for the prokaryotic ribosome association occurs from 12 nucleotide residues before the ATG initiation codon and is 5'-AGTCGAG-3'. The -10 upstream region (Pribnow box) was located from 51 to 46 nucleotide residues upstream of the ATG initiation codon, and was found to be 5'-TATTAT-3'. Also, the -35 region was found to be 5'-

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1 GA CTT ATG CGT ATG AGA TTC AGA TAG CGC TTC CTT CAT CCT AAG TCG AGC AGA AAA TAA CGC   62
                                                                  -35
63 TCC ATA AAC ACA CTC ATC TTT TCA TAC AAA CTT AAT ATG ACC CGC TGT TGA TTT ATA TCT   122
                  -10                                              SD
123 TTC GGG CTG TAT TAT TAC GCT CCG TAA ATT CAG TCT TAT TAC ATA AAT AGT CGA GTT ATC  182

183 ATG AAA AAA TCA ATC TTA CTT TCG AGC TGT TTG TTC ATT TCT TTT TTA TCG ACC GCT TCA  242
    Met Lys Lys Ser Ile Leu Leu Ser Ser Cys Leu Phe Ile Ser Phe Leu Ser Thr Ala Ser

243 ACA TTG AAC GAC TCG CTC TAT TCT ATA GAA CAA CGC ACC TTG GGA CGC ATA GGC GTA TCA  302
    Thr Leu Asn Asp Ser Leu Tyr Ser Ile Glu Gln Arg Thr Leu Gly Arg Ile Gly Val Ser

303 GTT TTA GAT TCA ACG GAT CAA CAA TGG CAC TAT AAA GGG AAT GAA AGG TTC CCT ATG ATG  362
    Val Leu Asp Ser Thr Asp Gln Gln Trp His Tyr Lys Gly Asn Glu Arg Phe Pro Met Met

363 AGT ACA TTC AAG ACA TTA GCA TGT GCA AAA ATG CTA CAG GAC TCT GAT AGA GAC ATT TTA  422
    Ser Thr Phe Lys Thr Leu Ala Cys Ala Lys Met Leu Gln Asp Ser Asp Arg Asp Ile Leu

423 GAT ATA AGT ACA ATG GCG CCA GTA AAA TCC GAT GAA CTA ATC GCT TGG TCA CCA ATA ACA  482
    Asp Ile Ser Thr Met Ala Pro Val Lys Ser Asp Glu Leu Ile Ala Trp Ser Pro Ile Thr

483 AAA AAC ATG GTT GGC AGT TCA ATT ACC ATT GAA AAT GCT TGT GAA GCT ACG ATG AAG ACT  542
    Lys Asn Met Val Gly Ser Ser Ile Thr Ile Glu Asn Ala Cys Glu Ala Thr Met Lys Thr

543 AGT GAT AAT ACT GCT GCA AAC ATA GTC TTA AAG CAC ATC GGA GGC CCA CAG GGT GTC ACT  602
    Ser Asp Asn Thr Ala Ala Asn Ile Val Leu Lys His Ile Gly Gly Pro Gln Gly Val Thr

603 GCT TTT CTA CGT TTG ATC GGA GAT AAA GTA ACT CAA TTA GAT CGT TTT GAA CCT GAA CTA  662
    Ala Phe Leu Arg Leu Ile Gly Asp Lys Val Thr Gln Leu Asp Arg Phe Glu Pro Glu Leu

663 AAC CAA GCC AAA GCT GAT GAC CTA CGT GAT ACA ACG ACG CCA TTT GCG ATG AAT AAG ACC  722
    Asn Gln Ala Lys Ala Asp Asp Leu Arg Asp Thr Thr Thr Pro Phe Ala Met Asn Lys Thr

723 CTA TAT CAT ATT TTA TTC GAA GAT GTA TTA GCT CAA AAT TCA AAA AAA CAA CTT AAG GAA  782
    Leu Tyr His Ile Leu Phe Glu Asp Val Leu Ala Gln Asn Ser Lys Lys Gln Leu Lys Glu

783 TGG ATG CAA GGG ACC ACT GTT TCC GAT TCT TTA CTC CGT TCT GTT TTA CCA AAA GGG TGG  842
    Trp Met Gln Gly Thr Thr Val Ser Asp Ser Leu Leu Arg Ser Val Leu Pro Lys Gly Trp

843 TCT ATT GCA GAT CGT TCT GGT GCA GGA GCT AAC GGT TCG CGC GGT ATT ACA GCA GCA ATT  902
    Ser Ile Ala Asp Arg Ser Gly Ala Gly Ala Asn Gly Ser Arg Gly Ile Thr Ala Ala Ile

903 TGG ACT GAC GAG CGC GAG CCA TTA ATC ATT AGC ATC TAC CTG ACA CAA ACC AAC CTT TCT  962
    Trp Thr Asp Glu Arg Glu Pro Leu Ile Ile Ser Ile Tyr Leu Thr Gln Thr Asn Leu Ser

963 ATG CCA GAA CGT AAT CAA GTT ATT AAT GAA ATT GGT AAG GCT ATT TTC GAA GAG TAT GCT 1022
    Met Pro Glu Arg Asn Gln Val Ile Asn Glu Ile Gly Lys Ala Ile Phe Glu Glu Tyr Ala

1023 GTA AAA TAA CTA GAT GAG ATT GTC ATA CTG TTA ACT GTC CCC TTC TTT TCT TGG CGT TCA 1082
     Val Lys ***

1083 GAA CGG TTA TAT TTG ACT TCT ATT TTT GCC GCT TCT TCC AAT ACT TTG CGC ATC TTG TTG  1142

1143 CTA TCA CTT GGG GTT TTA CCC CCC TGA GCT GTC ACA TCA ACA GTG GTA AAG TTT TCA TCC  1202

1203 AGC CAA TCA GCC CAC TTG ATC GCT TTT TTC ATC AAG TCA GCA CGA TAA ATT TGA TTG TAT  1262

1263 TTT GGC GTC CGT GAA TGG TAG TTA GCC GCT CTA GTC CAA AGG TCC CCT GAA TCT TTT AGG  1322

1323 ATA TGC CCG CGA AGT CGC CAT GCT GCG AGA TCG AAA GGG TAG CAG CCG GAC GCT GCT ACA  1382

1383 TGC TCT GCG GTA ATG CCA TAC TTT TTA AGA TCG CGT AAA TAA GCT GTA TTA AAC TGC ATA  1442
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1443 AAA CCA ACG TCA TGC GTA CCG TTT GAG TTT TTA ACC CAT TGC CCT GGT TTA CCT GAT TCT 1502

1503 TTT TCG GCC ACG GCC AAA ACA ATG TTG GCT GGT ACT TCA TAT TTG GCA GCT GCT GAA ATT 1 562

1563 GAG CAC ACA ACG CGC TCT TGT TCT AAA GGT GGC ATA TCT GCA ATA AAC GGT AAC ATG ACG 1622

1623 TAT TTC CTT ACC AGT AGT AAG TCG GCC CAG CCA ACT CAT TTT CTA TAT AAA CCT CAG CGA 1682

1683 ATC GTT CAG TTC TAG TAC AAC CTT TGG GGG TTC TAT AAC CAT CCC TAT CACG TC 1736

Fig. 2 Nucleotide sequence of the DNA fragment containing the β-lactamase gene from *Photobacterium damsela* subsp. *piscicida* plasmid pDP 8517 and predicted amino acid sequence of β-lactamase. A putative promoter region and possible ribosome binding site are underlined.

TTGATT-3' from 73 to 68 residues.

The PDP-AP ORF showed a maximum of 58% DNA homology with known β-lactamase ORF sequences of other bacteria from the data bank. Alignments of the amino acid sequences using CLUSTAL W of the *P. damsela* ssp. *piscicida* Ap^r gene (PDP-AP) and the sequences of β-lactamases from 9 other bacteria are shown in Fig.3. Alignment revealed that a total of 57 amino acid residues were conserved in all the aligned sequences. Also, all the aligned sequences were from Gram negative bacteria. The maximum homology was seen with the β-lactamase from *Vibrio harveyi* (56%). In addition to class A β-lactamases, three other molecular classes of β-lactamases (class B, C, and D) are recognised (Ambler, 1980). Alignment of the amino acid sequence of PDP-AP and various other classes of β-lactamases (class A, B, C and D) is shown in Fig.4. PDP-AP showed maximum homology (56%) with class A β-lactamases as compared to class B (11-12%), class C (10-11%) and class D (10-14%). Alignment between amino acid sequences of PDP-AP and 5 other β-lactamases belonging to classA is shown in Fig.5. A 19 amino acid signal peptide was deduced for PDP-AP (Fig. 5). Four important structural features found

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PDP-AP    ----------------------MKKSILLSSCLFISFLSTASTLNDSLYSIEQRTLGRIG
AF217649  ----------------------MKKLFLLLGLLACSSATYAANLMKTSPRLKKHISGRIG
AF030945  -------------------MKFLLAFSLLIPSVVFASSSKFQQVEQDVKAIEVSLSARIG
D13210    -------------------MKFLLVFSLLIPSVVFASSSKFRQVEQDVKAIEVSLSARIG
Z18955    -------------------MKFLLAFSLLIPSVVFASSSKFQQVEQDVKAIEVSLSARIG
AF071555  ---MLLYKMCDNQNYGVTYMKFLLAFSLLIPSVVFASSSKFQQVEQDVKAIEVSLSARIG
AJ277209  --MRNEEVISMWQRMKWGL-----CVLAALSGSAMAAPLTAQYVSTIATQEEQRLHARIG
U14748    MYVLSVEKPTLRNKFAAGIGVVLVCVVASFIPTPVFALDTTKLIQAVQS-EESALHARVG
AF135373  -------MNVRKHKASFFS-----VVITFLCLTLSLNANATDSVLEAVTNAETELGARIG
AF351241  ------------MSIQHFR-----VALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVG
                                                          :    .*:*

PDP-AP    VSVLDS-TDQQWHYKG-NERFPMMSTFKTLACAKMLQDSDRDILDISTMAPVKSDELIAW
AF217649  VSVWDTQTDEHWDYRG-DERFPMMSTFKTLACATMLSDMDNEKLDKNATAKVEERNMVVW
AF030945  VSVLDTQNGEYWDYNG-NQRFPLTSTFKTIACAKLLYDAEQGKVNPNSTVEIKKADLVTY
D13210    VSVLDTQNGEYWDYNG-NQRFPLTSTFKTIACAKLLYDAEQGKVNPNSTVEIKKADLVTY
Z18955    VSVLDTQNGEYWDYNG-NQRFPLTSTFKTIACAKLLYDAEQGKVNPNSTVEIKKADLVTY
AF071555  VSVLDTQNGEYWDYNG-NQRFPLTSTFKTIACAKLLYDAEQGKVNPNSTVEIKKADLVTY
AJ277209  IAVLDTATNSITHYRG-EERFPLNSTHKPLLCAALLREVDRKALALSASTQFESSQLVEY
U14748    MTVFDSNTGTTWNYRG-DERFPLNSTHKTFSCAALLAKVDGKSLSLGQSVSISKEMLVTY
AF135373  LAVHDLETGKRWEHKS-NERFPLSSTFKTLACANVLQRVDLGKERIDRVVRFSESNLVTY
AF351241  YIELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEY
              *  ..   .    ::***: **.* : *. :*   :      .     ..  :: :

PDP-AP    SPITKNMVGS-SITIENACEATMKTSDNTAANIVLKHIGGPQGVTAFLRLIGDKVTQLDR
AF217649  SPVMDRMASQ-TTRIEHACEAAMLMSDNTAANIVLRSIGGPRGVTTFLRSIGDKATRLDR
AF030945  SPVIEKQVGQ-AITLDDACFATMTTSDNTAANIILSAVGGPKGVTDFLRQIGDKETRLDR
D13210    SPVIEKQVGQ-AITLDDACFATMTTSDNTAANIILSAVGGSKGVTDFLRQIGDKETRLDR
Z18955    SPVIEKQVGQ-AITLDDACFATMTTSDNTAANIILSAVGGPKGVTDFLRQIGDKETRLDR
AF071555  SPVIEKQVGQ-AITLDDACFATMTTSDNTAANIILSAVGGPKGVTDFLRQIGDKETRLDR
AJ277209  SPITEKHVAPDAMSWAQLCSAAVSYSDNTAANLIARKLNGPQAVTQFLRDSGDTITRLDR
U14748    SPITEKSLSPETVTFGKICQAAVSYSDNTAANVVFDAIGGATGFNAYMRSIGDEETQLDR
AF135373  SPVTEKHVGKKGMSLAELCQATLSTSDNSAANFILQAIGGPKALTKFLRSIGDDTTRLDR
AF351241  SPVTEKHLTG-GMTVRELCSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDR
          **: ..          . * *::  ***:***.:   :.*.  .. :::  **  *:***

PDP-AP    FEPELNQAKADDLRDTTTPNAMNKTLYHILFEDVLAQNSKKQLKEWMQGTTVSDSLLRSV
AF217649  FEPRLNEANPGDKRDTTTPNAMVNTLHTLLEGDALSYESRIQLKIWMQDNKVSDSLMRSV
AF030945  IEPDLNEGKLGDLRDTTTPKAIASTLNQLLFGSTLSEASQKKLESWMVNNQVTGNLLRSV
D13210    IEPDLNEGKLGDLRDTTTPKAIASTLNKFLFGSALSEMNKKKLESWMVNNQVTGNLLRSV
Z18955    IEPDLNEGKLGDLRDTTTPKAIASTLNKFLFGSALSEMNQKKLESWMVNNQVTGNLLRSV
AF071555  IEPDLNEGKLGDLRDTTTPKAIASTLNKFLFGSALSEMNQKKLESWMVNNQVTGNLLRSV
AJ277209  YEPELNSAIPGDERDSTTPVAIAQTLNTLLLGNVLQPSSREQLMQWMRDDKVADGLLRSV
U14748    KEPELNEGTPGDVRDTTTPNAMVNSLRKILLGDALSASSRSQLTQWMLDDQVAGALLRAS
AF135373  WETELNEAVPGDKRDTTTPIAMVTTLEKLLIDETLSIKSRQQLESWLKGNEVGDALFRKG
AF351241  WEPELNEAIPNDERDTTMPAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSA
           *. **..  .* **:* * *:  :*  :*  . *   .: :*  *:    * . *:*

PDP-AP    LPKGWSIADRSGAGANGSRGITAAIWTDEREPLIISIYLTQTNLSMPERNQVINEIGKAI
AF217649  LPKGWSIADRSGAGGFGSRGITAMIWKENHKPVYISIYITETDLSLQARDQVIAQVSQLI
AF030945  LPVKWSIADRSGAGGFGARSITAIVWSEEKKTIIVSIYLAQTEASMAERNDAIVKIGRSI
D13210    LPAGWNIADRSGAGGFGARSITAVVWSEHQAPIIVSIYLAQTQASMAERNDAIVKIGRSI
Z18955    LPAGWNIADRSGAGGFGARSITAVVWSEHQAPIIVSIYLAQTQASMAERNDAIVKIGHSI
AF071555  LPAGWNIADRSGAGGFGARSITAVVWSEHQAPIIVSIYLAQTQASMAERNDAIVKIGHSI
AJ277209  LPDGWKIADKTGAGDNGSRSIVSVVWPTSQKPLLVVIYITQTPATMAQRDAAIVRIGESL
U14748    LPSDWKIADKTGAGGYGSRSIVAVIWPPSKQPLVVGIYITQTKASMQASNQAIARIGVVL
AF135373  VPSDWIVADRTGAGGYGSRAITAVMWPPNRKPIVAALYITETDASFEERNAVIAKIGEQI
AF351241  LPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASL
          :*  * :**::***  *:*.* : :    : .    :* : :  ::   :  * .:.  :
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PDP-AP     FEEYAVK---- 100%
AF217649   ----------  56%
AF030945   FEVYTSQSR—  49%
D13210     FDVYTSQSR--  48%
Z18955     FDVYTSQSR—  48%
AF071555   FDVYTSQSR—  48%
AJ277209   FSTLAVYD---  42%
U14748     KDTVAP-----  40%
AF135373   AKTVLMENSRN  40%
AF351241   IKHW-------  37%
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Fig. 3 Amino-acid sequence alignment of Ap^r gene of *Photobacterium damsela* subsp. *piscicida* with 9 other amino acid sequences from other bacteria. Asterisks indicate conserved amino acids in all Ap^r gene monomers. The sequences are indicated by their accession numbers. Abbreviations: PDP-AP, Ap^r gene encoded by the plasmid pPDP8517 from *P. damsela* subsp. *piscicida*; AF217649, Ap^r gene from *Vibrio harveyi*; AF030945, Ap^r gene from *Vibrio cholerae*; D13210, Ap^r gene from *Proteus mirabilis*; Z18955, Ap^r gene from *Salmonella typhimurium*; AF071555, Ap^r gene from *Pseudomonas aeruginosa*; AJ277209, Ap^r gene from *Citrobacter koseri*; U14748, Ap^r gene from *Aeromonas hydrophilia*; AF135373, Ap^r gene from *Acinetobacter calcoaceticus* var *anitratus*; AF351241, Ap^r gene from *Escherichia coli*.

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PDP-AP     ----------MKKSILLSSCLFISFLSTASTLNDSLYSIEQRTLGRIGVSVLDS-----
AF217649   ----------MKKLFLLLGLLACSSATYAANLMKTSPRLKKHISGRIGVSVWDTQ----
AF030945   --------MKFLLAFSLLIPSVVFASSSKFQQVEQDVKAIEVSLSARIGVSVLDTQ----
AAK13430   ------------MKKLFVLCVCFLCSITAAGAALPDLKIEKLEEGVYVHTSFEEVN---
M63556     ------------MKTVFILISMLFPVAVMAQKSVKISDDISITQLSDKVYTYVSLA----
A01007     --------MFKTTLCALLITASCSTFAAPQQINDIVHRTITPLIEQQKIPGMAVAVIYQG
BAB72158   MQQRQSILWGAVATLMWAGLAHAGEASPVDPLRPVVDASIQPLLKEHRIPGMAVAVLKDG
S38671     MKTFAAYVITACLSSTALASSITACLSSTALASSITENTFWTRVLCRSRHVFRACKSSSK
S22683     ----------MLKSTLLAFGLFIALSARAENQAIAKLFLRAGVDGTIVIESLTTGQ---
                                                    :

PDP-AP     --TDQQW---HYKGNERFPMMSTFKTLACAKMLQDSDRDILDISTMAPVKSDELIAWSPI
AF217649   --TDEHW---DYRGDERFPMMSTFKTLACATMLSDMDNEKLDKNATAKVEERNMVVWSPV
AF030945   --NGEYW---DYNGNQRFPLTSTFKTIACAKLLYDAEQGKVNPNSTVEIKKADLVTYSPV
AAK13430   ------------GWGVVSKHGLVVLVNTDAYLIDTP--------FTATDTEKLVNWF--
M63556     ----------EIEGWGMVPSNGMIVINNHQAALLDTP--------INDAQTEMLVNWVT-
A01007     KPYYFTWGYADIAKKQPVTQQTLFELGSVSKTFTGVLGGDAIARGEIKLSDPTTKYWPEL
BAB72158   KAHYFNYGVANRESGASVSEQTLFDIGSVSKTLTATLGAYAVVKGAMQLDDKASRHAPWL
S38671     LACATNN---LARASKEYLPASTFKIPNAIIGLETG----------VIKNEHQIFKWD--
S22683     --RLVHN---DPRAQQRYPAASTFKVLNTLIALEEG----------AISGENQIFHWN--
                                     .       :

PDP-AP     TKNMV-------GSSITIENACEATMKTSDNTAANIVLKHIGGPQGVTAFLRLIGDKVTQ
AF217649   MDRMA-------SQTTRIEHACEAAMLMSDNTAANIVLRSIGGPRGVTTFLRSIGDKATR
AF030945   IEKQV-------GQAITLDDACFATMTTSDNTAANIILSAVGGPKGVTDFLRQIGDKETR
AAK13430   ------------VERGYKIKGTISSHFHSDSTGGIEWLNSQSIPTYASELTNELLKKDGK
M63556     ------------DSLHAKVTTFIPNHWHGDCIGGLGYLQRKGVQSYANQMTIDLAKEKGL
A01007     TAKQWNGITLLHLATYTAGGLPLQVPDEVKSSSDLLRFYQNWQPAWAPGTQRLYANSSIG
BAB72158   KGSVFDSITMGELATYSAGGLPLQFPEEVDSSEKMRAYYRQWAPVYSPGSHRQYSNPSIG
S38671     ------------GKPRAMKQWERDLSLRGAIQVSAVPVFQQIAREVGEVRMQKYLKKFSY
S22683     ------------GTQYSIANWNQDQTLDSAFKVSCVWCYQQIALRVGALKYPAYIQQTNY
                                                                      .

PDP-AP     LDRFEPELN---------QAKADDLRDTTTPNAMNKTLYHILFEDVLAQNSK-KQLKEWM
AF217649   LDRFEPRLN---------EANPGDKRDTTTPNAMVNTLHTLLEGDALSYESR-IQLKIWM
AF030945   LDRIEPDLN---------EGKLGDLRDTTTPKAIASTLNQLLFGSTLSEASQ-KKLESWM
AAK13430   VQAKNSFSG---------VSYWLVKNKIEVFYPGPGHTQDNVVVWLPEKKIL-FGGCFVK
M63556     PVPEHGFTDS--------LTVSLDGMPLQCYYLGGGHATDNIVVWLPTENIL-FGGCMLK
A01007     LFGALAVKPSGLSFEQAMQTRVFQPLKLNHTWINVPPAEEKNYAWGYREGKA-VHVSPGA
BAB72158   LFGHLAASSLKQPFAQLMEQTLLPGLGMHHTYVNVPKQAMASYAYGYSKEDKPIRVNPGM
S38671     GNQNISGGI---------DKFWLEGQLRISAVNQVEFLESLFLNKLSASKE---NQLIVK
S22683     GHLLEPFNG---------TEFWLDGSLTISAEEQVAFLRQVVERKLPFKAS--SYDSLKK


PDP-AP     QGTTVSDSLLRSVLPKGWSIADRSGAGA-----------------NGSRGITAAIWTDER
AF217649   QDNKVSDSLMRSVLPKGWSIADRSGAGG-----------------FGSRGITAMIWKENH
AF030945   VNNQVTGNLLRSVLPVKWSIADRSGAGG-----------------FGARSITAIVWSEEK
AAK13430   PDGLGNLGDANLEAWPKSAKILMSKYG----------------------KAKLVVSSHS
M63556     DNQATSIGNISDADVTAWPKTLDKVKAK-----------------FP---SARYVVPGHG
A01007     LDAEAYGVKSTIEDMARWVQSNLKPLDINEKTLQQGIQLAQSRYWQTGDMYQGLGWEMLD
BAB72158   LADEAYGIKTSSADLLAFVKANIGGVDDKALQQAISLTHKG--HYSVGGMTQGLGWESYA
S38671     EALVTEAPEYLVHSKTGFSGVGTESNP------------------GVAWWVGWVEKGAE
S22683     VMFADENAQYRLYAKTGWATRMTPSVG----------------------WYVGYVEAKDD
```

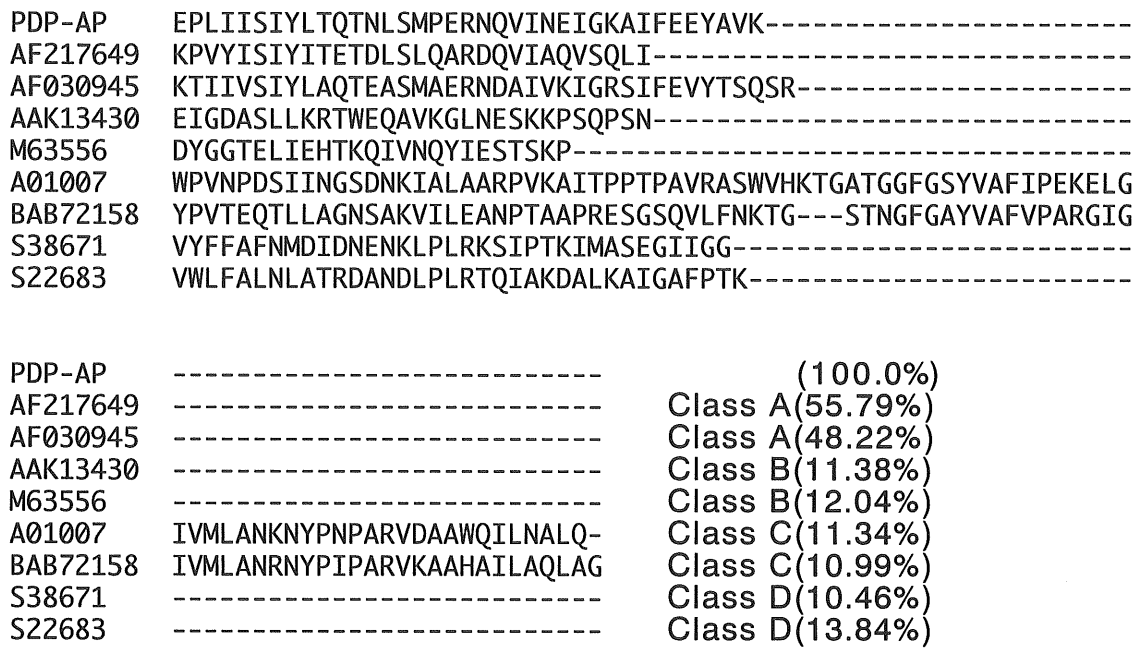

Fig. 4 Comparison of Ap^r gene of *Photobacterim damsela* subsp. *piscicida* with various classes of ß-lactamases. The sequences are indicated by their accession numbers. Abbreviations: PDP-AP, Ap^r gene encoded by the plasmid pPDP8517 from *P. damsela* subsp. *piscicida*; AF217649, Ap^r gene from *Vibrio harveyi*; AF030945, Ap^r gene from *Vibrio cholerae*; AAK13430, Ap^r gene from *Klebsiella pneumoniae*; M63556, Ap^r gene from *Bacteroides fragilis*; A01007, Ap^r gene from *Escherichia coli*; BAB72158, Ap^r gene from *Escherichia coli*; S38671, Ap^r gene from *Escherichia coli*; S22683, Ap^r gene from *Pseudomonas* sp.

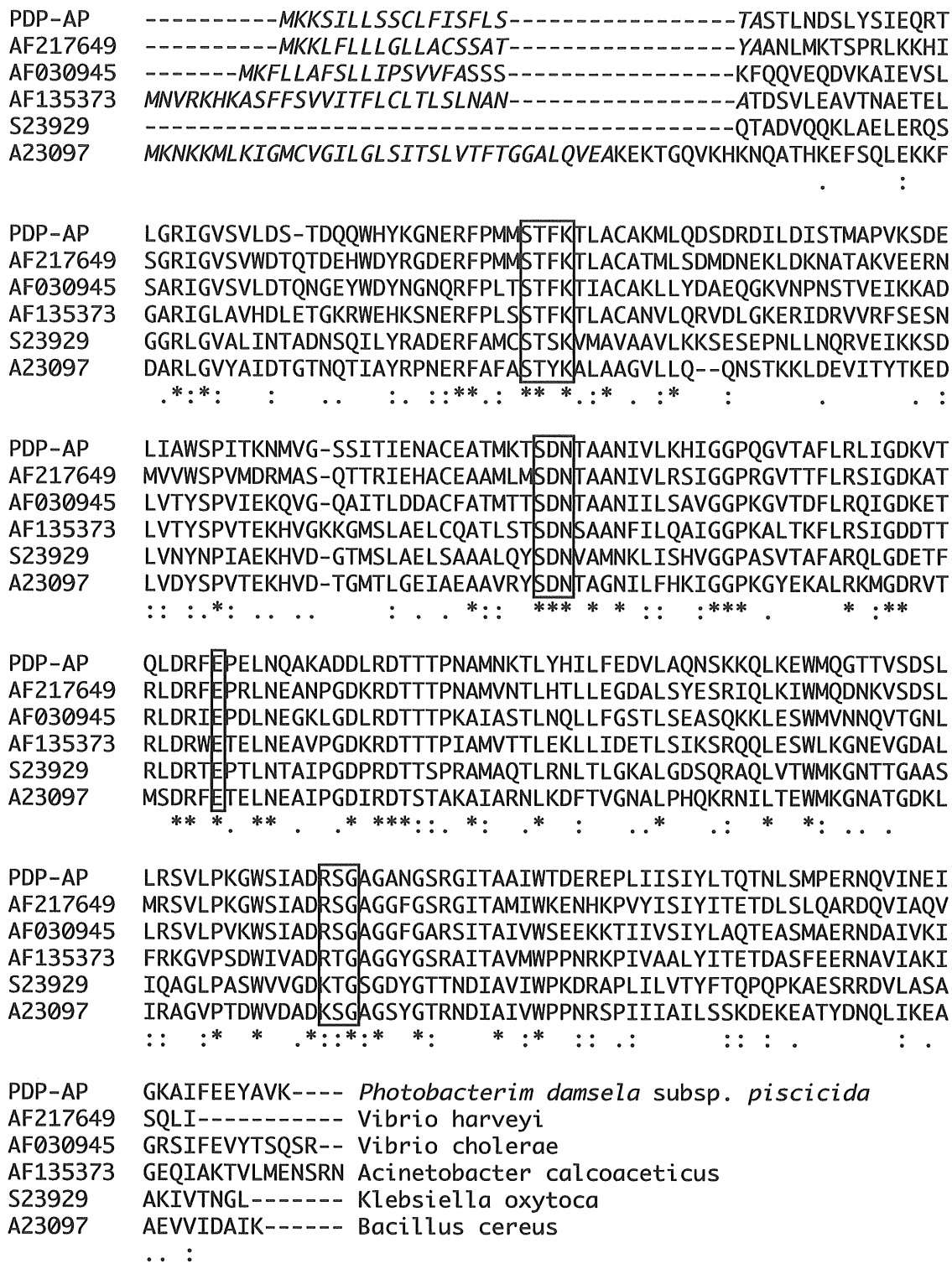

Fig. 5 Amino acid sequence alignment of Ap^r gene of *Photobacterim damsela* subsp. *piscicida* with various class A β-lactamases. The sequences are indicated by their accession numbers. Abbreviations: PDP-AP, Ap^r gene encoded by the plasmid pPDP8517 from *P. damsela* subsp. *piscicida* PP8517 strain; AF217649, Ap^r gene from *Vibrio harveyi;* AF030945, Ap^r gene from *Vibrio cholerae;* AF135373, Ap^r gene from *Acinetobacter calcoaceticus;* S23929, Ap^r gene from *Klebsiella oxytoca*; A23097, Ap^r gene from *Bacillus cereus*. Ambler's standard numbering of ß-lactamases was used. Conserved amino acid residues are shown with an asterisk below. Conserved amino acid regions important for catalytic function of ß-lactamases are boxed. The signal peptide sequence is indicated in italics.

conserved in class A β-lactamases were present in the deduced amino acid sequences of PDP-AP, which included an STKF active site tetrad at position 70 to 73 according to Ambler`s standard numbering of class A β-lactamases (Ambler, et al., 1991). This Ser-X-X-Lys tetrad is characteristic of serine β-lactamases. An SDN loop characteristic of class A β-lactamases was located at position 130 to 132 on PDP-AP, as well as the unique Glu residue at position 166. Lastly, an RSG triad was established at position 234 to 236 on PDP-AP. Normally, the triad at this position is KTG for the other serine β-lactamases (class C and D) and the RSG substitution if seen is only in class A β-lacatamases. The Gly at position 236 is always conserved (Matagne & Frere, 1995).

Sequence homology with other β-lactamases

Database searches of PDP-AP for β-lactamase genes generated a maximum homology of 58% with other β-lactamases in the database. The highest level of amino acid sequence identity (56%) was shown for the β-lactamase from *Vibrio harveyi* HB3 (Teo, et al., 2000).

Hydropathy plot

Hydropathy plot of *P. damsela* ssp. *piscicida* PDP-AP (Fig. 6) using the amino acid hydropathy values of Kyte and Doolittle(1982) indicated that PDP-AP is a globular soluble protein quiet usual of other known β-lactamases.

DISCUSSION

The resistances to Ap, Cp, Km, Sa and Tc carried by *P. damsela* ssp. *piscicida* strain PP8517 were transferred to *E. coli* K-12 χ1037 Rp^r strain. The transferability of the resistances differed with the pattern (different combinations of drug resistance markers) [Chapter two]. The resistances in each donor were seperately transmitted by the 65 kb plasmid bearing Ap^r, Cp^r and Tc^r at a frequency of about 10^{-6} and the 125 kb plasmid carrying Km^r and Sa^r at a frequency of about 10^{-4} because the resistances are separately transferred when Ap^r was transferred (as also discussed in chapter two). The resulting transconjugants harboured each or both of the two plasmids, or the presumptive fused plasmid from the two

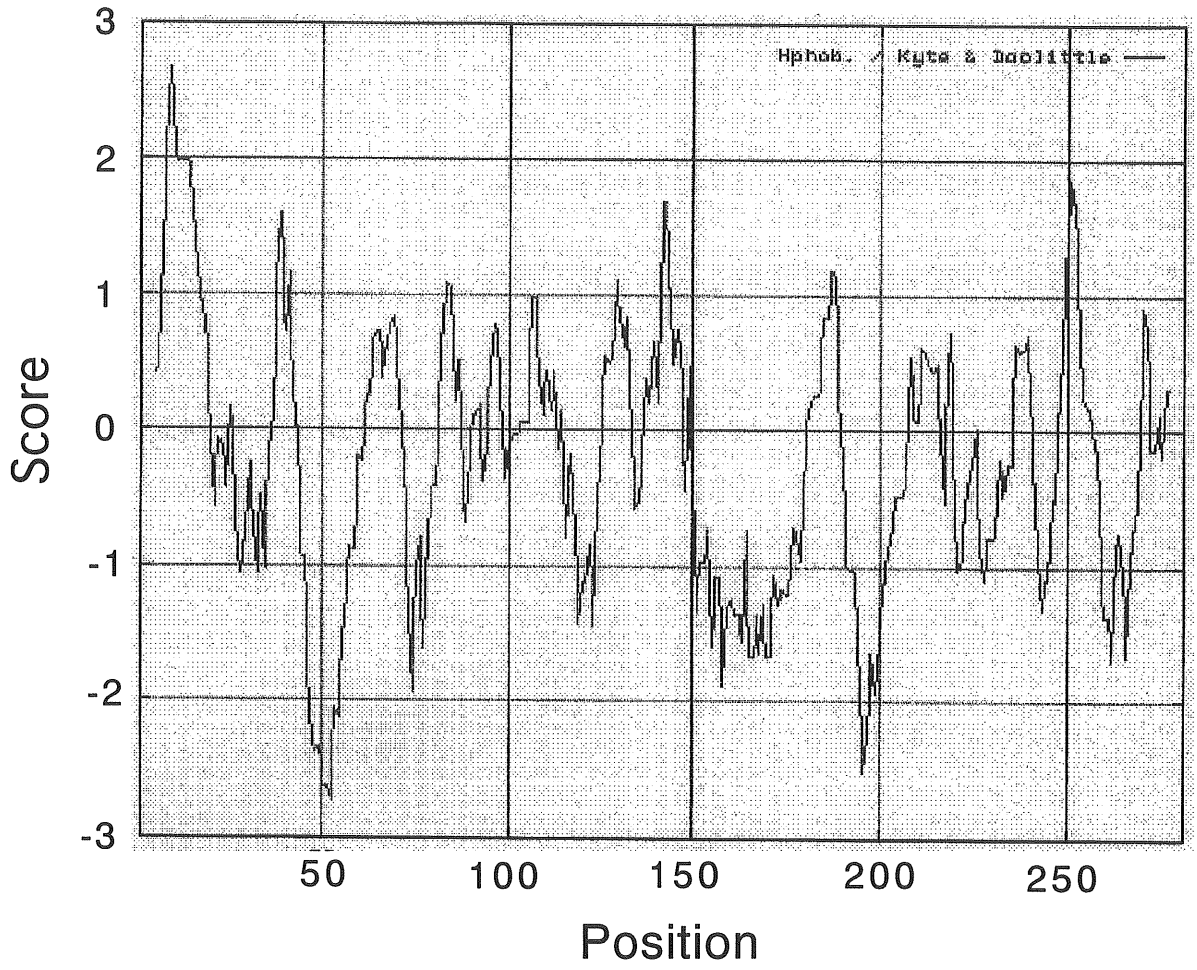

Fig. 6 Hydropathy plot for β-lactamase of *Photobacterium damsela* subsp. *piscicida* obtained according to the hydropathy values of Kyte & Doolittle (1982).

plasmids, which were dependent upon transfer frequencies of the plasmids. These results are the same as those discussed in chapter three. Therefore the present results lead credence to the previous results.

In this study,, we have cloned an Ap^r gene from a 65 kb plasmid of *P. damsela* ssp. *piscicida*. *E. coli* JM83 cells harbouring recombinant plasmid (pHSG398 having a fragment of pPDP8517) had a highly elevated level of resistance to Ap compared to that of the host strain alone, indicating that the cloned insert did contain the Ap^r determinant.

Sequence analysis of the Ap^r gene demonstrated that they did not have homology (maximum of 58% homology with *V. harveyi*) to any other β-lactamase genes. At the amino acid level, PDP-AP possessed low levels of homology ((56%) to other class A β-lactamases and regions which were strongly conserved are implicated in enzyme catalysis.

The results obtained indicate that the calculated molecular mass of 31,292 Da of the deduced amino acid sequences of PDP-AP was consistent with the masses of known class A β-lactamase amino acid sequences. The

highest level of identity (56%) was shown for the class A β-lactamase from *Vibrio harveyi* HB3 (Teo, et al., 2000). This clearly confirmed that the PDP-AP Ap^r determinant is none other than the β-lactamase gene. Therefore, PDP-AP is a globular soluble enzyme belonging to the class A β-lactamases and is a novel and distinctly different β-lactamase from the other known β-lactamases.

The genetic mechanism of Ap^r in *P. damsela* ssp. *piscicida* is very complex and the above results attest to it. Since, *P. damsela* ssp. *piscicida* is generally in contact with yellowtail and marine water, it may acquire foreign bacterial genes, the mechanism of which is still poorly known. Chemotherapeutic agents such as amoxicillin, Ap, florfenicol, flumequine, novobiocin, oxolinic acid, Sa and Tc have been used for treatment of pseudotuberculosis in fish farms (Aoki, 1992) and the recent rise in the occurrence of bacterial strains resistant to new chemotherapeutic agents has been extraordinarily quick among fish pathogens. So, it can be supposed that resistance is conferred by molecular mechanisms that enable easy aquisition of the resistance genes for recently introduced chemotherapeutic agents. The dynamics of infectious drug resistance dissemination

outside the laboratory is inherently complex and involves a host of variables. For example, Schaberg and coworkers (Schaberg et al., 1977) have reported the transfer of R-plasmids from *Serratia marcescens* to *E. coli* in urine! Also most organisms that acquire R-plasmids do not persist in the ecological niche in which they find themselves. For a newly transferred R plasmid to survive, it must find a host endowed with exceptional survival value when antibiotics are present as well as when they are not (Elwell, 1994).

We therefore suspect that plasmid pPDP8517 in *P. damsela* ssp. *piscicida* may have originally been a cryptic plasmid (Zhao & Aoki, 1992) into which the β-lactamase gene may have been added by transposition from a Tn3 like transposon. However, we do not rule out the possibility of the β-lactamase gene to be present on the chromosome too. This is because the gene was quite unstable and difficult to isolate. This assumption is also supported by the fact that the presence of multiple copies of a gene in a bacterium is often a source of genetic instability due to the recombination between the same genes as homologous recombination is mainly governed by the *rec*A gene product (Miller & Kokjohn, 1990). Therefore, it is more likely that the β-lactamase gene in *P.damsela* ssp. *piscicida*

came from a Tn3 like transposon a mechanism similar to that in *Haemophilus* species (Elwell, 1994).

To our knowledge this is the first ever report of a β-lactamase gene having been isolated from a fish pathogenic bacteria. This report is also significant as it differs from the previous reports about the status of Ap^r resistance in *P. damsela* ssp. *piscicida* (Kim & Aoki, 1993a) and proposes an altogether different concept of Ap^r in *P. damsela* ssp. *piscicida*. Also, it throws light on the mechanism of inheritance of resistance genes from foreign DNA into the plasmid DNA of *P. damsela* ssp. *piscicida* and the dangers of indiscriminate use of antibiotics in general and Ap in particular.

Further work has to be done to verify whether the non-transferable β-lactamase gene from *P. damsela* ssp. *piscicida* is encoded in plasmid or in chromosomal DNA and to get more insight about the mechanism of transfer of resistant genes. Our work is the beginning in that direction as it has clearly disproved the present assumption of the Ap^r to be confined to the chromosomal DNA and proposes an altogether new theory of Ap^r transfer in *P. damsela* ssp. *piscicida* in particular and fish pathogenic bacteria in

general.

Future work will involve studying the mechanism of transfer of the β-lactamase gene as well as the purification and characterization of the β-lactamase enzyme from pPDP8517.

SUMMARY

A novel β-lactamase gene has been found to be located on a 65 kb R-plasmid from a strain of *P.damsela* ssp. *piscicida* a fish pathogen isolated from cultured yellowtail *Seriola quinqueradiata*. The R-plasmid contained ampicillin (Ap) resistance and could be conjugally transferred to *E.coli* K-12 χ 1037 Rp^r. The Ap resistance gene encoding β-lactamase was localised to a 1.8 kb *Hin*cII fragment of a 65 kb transferable R plasmid pPDP8517 isolated from *P.damsela* ssp. *piscicida* strain PP8517 and was cloned into the plasmid vector pHSG398. The nucleotide sequence analysis revealed the 1.8 kb fragment to be 1,736 bp long. The analysis also identified an ORF encoding a protein of 282 amino acid residues with a calculated molecular mass of 31,292 daltons. Homology

between the predicted amino acid sequences for this β-lactamase gene(PDP-AP) and other β-lactamase proteins from other bacteria ranged from 38-56% . Amino acid regions important for the catalytic function and found conserved in class A β-lactamases were present in PDP-AP indicating that it belonged to class A. The possibility of the gene being located on a Tn3 like transposon is also discussed. This is the first ever report of cloning and sequencing of a β-lactamase gene from *P.damsela* ssp. *piscicida.*