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Resistance Determinants of *Pseudomonas* Species from Aquaculture in Australia

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Abstract

There is limited information on antibiotic resistance determinants present in bacteria of aquaculture origin in Australia. The presence of integron and other resistance determinants was investigated in 129 *Pseudomonas* isolates derived from nine freshwater trout farms in Victoria (Australia). Polymerase chain reaction (PCR) was carried out for the detection of integrase genes Int1, Int2 and Int3, gene cassette array, integron-associated *aad*A, beta-lactamase resistance genes blaTEM and blaSHV. Genes coding for efflux pump *mex*A, *mex*B and *opr*M were also investigated as well as *cad*A and czr which are known to mediate resistance to cadmium.

Class 1 integrons were detected in 30/129 (23 %) isolates while class 2 and class 3 was not detected in any of the isolates. *aad*A gene was detected in 28 of the 59 integrase positive isolates which are also resistant to streptomycin. The *str*A-*str*B, blaTEM or blaSHV genes were not detected in any of the strains. *mex*B was detected in 85/129 isolates and *cad*A gene in 59/92 isolates tested.

The sequence analysis *mex*B from this study demonstrated similarity to the RND multidrug efflux transporter *mex*B and its homologue *Ttg*B which in addition to multidrug efflux also transports toluene out of the cell. The sequence analysis of *cad*A confirms similarities to the cadmium translocating P-type ATPases, *cad*A of various *Pseudomonas* spp.

Pseudomonas spp. carrying integrons, efflux gene and cadmium resistance genes are present in farm-raised fish and sediments even though no antibiotics were licensed for use in Australian aquaculture at the time of the study.

Keywords: Antibiotic resistance; Heavy metal resistance; Aquaculture; *Pseudomonas* species; Australia

Introduction

Members of the genus *Pseudomonas* are a ubiquitous group of Gram-negative, motile, rod-shaped bacteria known for their metabolic versatility with genetic and physiologic capabilities that allow them to flourish in environments hostile to many other bacteria. It is one of the most diverse bacterial genera, containing over 60 validly described species [1]. Some species of Pseudomonad particularly *P. aeruginosa* are opportunistic pathogens that can cause serious and life-threatening infections in immunocompromised and cystic fibrosis patients [2,3]. In aquaculture, *P. aeruginosa* and *P. fluorescens* especially are the most frequently isolated opportunistic pathogenic species [4,5] although other species may also be serious opportunistic pathogens, including *P. anguilliseptica* in eels, *Anguilla japonica* [6] *P. chlororaphis* in amago trout, *Oncorhynchus rhodurus* [7], *P. plecoglossicida* in ayu, *P. altivelis* [8] and more recently *P. putida* in rainbow trout, *Oncorhynchus mykiss* [9].

Pseudomonas spp. express natural resistance to many antibiotics, heavy metals and biocides and this resistance is usually higher in *P. aeruginosa* than in other Pseudomonads [10,11]. *Pseudomonas* spp. have the capacity to acquire many additional mechanisms of resistance for antibiotics and heavy metals by integrons and (or) plasmids [12-14], as well as multidrug active efflux [15-20].

The objective of this study was to identify integrons and genes coding for multidrug efflux pumps such as *mex*AB-*opr*M which is known to contribute greatly to the natural resistance seen in *Pseudomonas* spp. These pumps have broad substrate specificity and may act synergistically with the permeability barrier to result in significant intrinsic resistance to many antimicrobials including β-lactams, β-lactamase inhibitors, quinolones, chloramphenicol, tetracycline, trimethoprim, sulphamethoxazole and novobiocin [21,22]. Although 12 potential efflux systems of this family have been identified in the *P. aeruginosa* genome [23], four of them are best characterised as antibiotic transporters [24]. This study also determined the mechanisms of intrinsic antibiotic resistance and resistance to cadmium, one of the heavy metals tested. Several mechanisms have been identified within bacteria that confer cadmium resistance. These include sequestration by metallothionein-like proteins and ATPase (cad operon) and non-ATPase (czc operon) efflux pumps. Metallothionein-like proteins that sequester cadmium have been identified in the Cyanobacterium *Synechococcus* and *P. putida*, whereas the czc resistance operon has been found in *Alcaligenes* spp [25,26] and *P. aeruginosa* [27].

Materials and Methods

Bacterial strains and plasmids

One hundred and twenty nine *Pseudomonas* spp. isolated from healthy fish and sediments from nine freshwater trout farms in Victoria (Australia) between August and December 2004 were used in this study. Isolation, identification, heavy metal and antibiotic sensitivity

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testing have been previously described [28]. The positive controls used were Escherichia coli strains containing integron plasmids; pR388, a class 1 integron with a two cassette array dfrB2 and orfA and pR1033 that contains Tn1496 which in turn contains a class 1 integron with four cassettes, aac, orfE, *aad*A2 and cmlA, (kindly provided by Hatch Stokes, Macquarie University, Sydney, Australia) and an *E. coli* strain with the Int2 gene. *Escherichia coli* strain JIR5431 (Burdett 1996) carrying a tetM gene (kindly provided by Julian Rood, Monash University, Melbourne, Australia) and Aeromonas strains WA13 (tetA), WA14 (tetD) and WA18 (tetE) were from the culture collection of our laboratory (University of south Australia).

Total and plasmid DNA extraction

DNA was extracted by inoculating isolates into Tryptone Soy Broth (Oxoid CM0129) and incubating overnight at 30°C. A 1.5-ml amount was dispensed into Eppendorf tubes and centrifuged at 12 662 g for 5 min. The supernatant was discarded and cell pellets re-suspended in 200 µl TE buffer, and they were then heated in the heating block at 95°C for 10 min. The samples were placed on ice to cool and then centrifuged at 12 662 g for 5 min, and the supernatants were transferred into fresh Eppendorf tubes and these served as DNA templates for polymerase chain reaction (PCR). For plasmid DNA extraction, cell pellets were extracted by alkaline lysis of cells [32].

PCR amplification of 16S rRNA

16S rRNA gene universal primers were used for the PCR amplification of the *Pseudomonas* spp. to confirm the identification obtained by the biochemical method. The primers used for PCR are listed in Table 1. The reaction mixture (final volume 25 µL) contained 10× PCR buffer (1.5 mm $MgCl_2$, 10 mm Tris–HCl (pH 8.8 at 25°C), 50 mm KCl and 0.1% Triton X-100), 200 µm of each deoxynucleotide triphosphate, 0.5 µm L^{-1} of each oligonucleotide primer and 0.25 µL (0.5 U μL^{-1}) of *Taq* polymerase (Promega) and 200ng μl^{-1} of Bovine serum albumin. A 5 µL volume of DNA template was added to each reaction tube. The cycling conditions were as follows: 95°C for 5 min, 30 cycles of denaturation 94°C for 30 s, annealing at 68°C for 60 s and extension at 72°C for 60 s and a final single cycle of extension at 72°C for 10 min.

The PCR products were analysed by electrophoresis on 1.5 % agarose gels, stained with ethidium bromide and visualised using the Biorad Gel Documentation system (GelDoc).

Integron PCR

Detection of integrons and the resistant gene cassette was performed using PCR amplification with the specific primers listed in Table 1. All PCR amplification was carried out in a BioRad MyCycler thermal cycler. Integrase genes *Int*1, *Int*2 and *Int*3 were targeted in a multiplex PCR as previously described [29] using primers Int1F–Int1R [30], Int2F–Int2R and Int3F–Int3R [31]. Assays were carried out in 25 µL volumes containing 2.5 µL of 10× PCR buffer [100 mM Tris–HCl (pH 8.8), 500 mM KCl, 15 mM $MgCl_2$ and 1% Triton X-100], 0.5 µL of 10 mM DNTP, 1 µL of each primer stock solution (25 pmol μL^{-1})] and 0.25 µL of Bio*Taq* polymerase (5 U μL^{-1}). Three microlitres of the prepared DNA extract was added to provide the DNA template. A volume of 16.75 µL sterile milliQ water was added to make up the volume to 25 µL. All PCRs were subjected to an initial denaturation step at 94°C for 5

Gene	Primer sequence	Product size (bp)	Accession number	Reference
Uni (16S rDNA) (F) Uni (16S rDNA) (R)	TGC CAG CAG CCG CGG TA GAC GGG CGG TGT GTA CAA	990		[60]
Int1- F* Int1-R	CAG TGG ACA TAA GCC TGT TC CCC GAG GCA TAG ACT GTA	160		[30]
Int2-F Int2-R	GTA GCA AAC GAG TGA CGA AAT G CAC GGA TAT GCG ACA AAA AGG T	788		[31]
Int3-F Int3-R	GCC TCC GGC AGC GAC TTT CAG ACG GAT CTG CCA AAC CTG ACT	979		[31]
bla_{TEM}-F bla_{TEM} -R	ATG AGT ATT CAA CAT TTC CG CTG ACA GTTACC AATGCT TA	867		[61]
bla_{SHV}-F bla_{SHV} -R	GGT TATGCG TTA TAT TCG CC TTAGCT TTGCCAGTGCTC	867		[61]
*mex*AB-*opr*M-F *mex*AB-*opr*M-R	CTC ATG AGG ACA ACG CTA TGC AAC GAA CG TGG GTC AGG TCG AAA CTC TTC TGG TAGGTG	4.9 kb	*a*	[62]
*mex*A-F *mex*A-R	ATC CTC AAG CGC CTG TTC AAG GAA ACT GCA GGC CTT CGG TAA TGA TCT	832	*a*	This study
*mex*B-F *mex*B-F	ACT TCT TCA GCT TCA AGG ACG CGA ACG GAA TCG ACC AGC TTT CGT ACA	732	*a*	This study
*Opr*M-F *Opr*M-R	ACC TAC CAG AAG AGT TTC GAC CTG TGA TGT CCT TCT GGA TCT TCG CGT	511	*a*	This study
*cad*A1-F *cad*A1-R	GCC TGC CCG TGT GCC CTG GTG A CGG CGG TCT CGA TGG CGG TGT C	823	AF333961	This study
*cad*A2-F *cad*A2-F	TTG GCG CCG AAG ACG ATA GCA CT GTC GCC CAC GGT CTT CTC CAC AGG	581	AF333961	This study
*czr*A-F *czr*A-R	CAG TGG CCG GAC CCG AAG AAG T GCC CGT GGG TCC AGC GAT AGA	1207	Y14018	This study
*czr*B-F *czr*B-R	GTG ATC AGC AGC CCG CAG TTG TC TGC CCA GTT CGG ATT TGA GGA TGA	694	Y14018	This study
*czr*C-F *czr*C-R	GGA AAC GCG CTG CTG GCT ACT G TTC CGA GGT GCG CAA CTG GTC	1020	Y14018	This study

[a]PCR primers for amplification of the *cat* and *mex*A-*mex*B region and its flanking regions were synthesized on the basis of the nucleotide sequences of the *Pseudomonas* genome sequencing project database (http://www.pseudomonas.com/).

Table 1: List of primers used in the study.

min and final extension step at 72°C for 5 min. The subsequent cycling conditions are as follows for *Int*1, *Int*2 and *Int*3: 94°C for 60s, 59°C for 60s, 72°C for 60s (30 cycles); *aad*A: 95°C for 60s, 48°C for 30s, 72°C for 60s (30 cycles). PCR amplicons were analysed by electrophoresis on 1.5 % agarose gels and a 100-bp ladder was used as the molecular size marker (New England Biolabs).

Detection of beta lactamase resistance genes

The PCR for the amplification of beta lactamase genes bla_{TEM} and bla_{SHV} was carried out using primers listed in Table 1. The reaction mixture was the same as those described for integron PCR but 1 µl of 10 mM DNTP was used. The cycling conditions used were denaturation at 96°C for 5 min; 35 cycles of 96°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final extension period of 72°C for 10 min. Electrophoresis was performed as described for integron PCR.

Detection of *mex*A, *mex*B, and *opr*M genes

PCR reaction mixture was as described above. PCR reactions were subjected to an initial denaturation for 5 min, then 30 cycles with 95°C for 30 s, followed by 54°C for 30 s and 72°C for 45 s with an additional elongation at 72°C for 5minutes. Electrophoresis was performed as described for integron PCR.

Detection of cadmium resistance genes

PCR reaction mixture was as described above. The cycling condition used for *cad*A was an initial denaturation step at 95°C for 4 min; then 35 cycles with 95°C for 30 s, followed by 61.5°C (*cad*A1) or 55°C (*cad*A2 and czrABC) for 30 s and 72°C for 80 s with an additional elongation at 72°C for 5minutes. Electrophoresis was performed as described for integrin PCR.

Sequencing

The PCR products of the gene cassette and other resistance genes were purified using an Ultraclean DNA purification kit (MO-BIO Laboratories, Inc.) and sequenced using the BigDye Terminator v3.1 1 cycle sequencing kit and analysed with an ABI 3100 Genetic Analyser (Flinders Medical Centre, South Australia). Primer sets used to sequence the genes are those listed in Table 1. Database similarity searches for the nucleotide sequences were carried out with BLAST at the National Centre of Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Southern hybridization

In order to check for the co-location of *cad*A and *aad*A gene, the PCR products were digoxigenin (DIG)-labelled as probes as described by the manufacturer (Boehringer, Mannheim, Germany) and used in subsequent southern hybridisation procedures on plasmids [32].

Results

Identification of isolates

A total of 129 *Pseudomonas* spp. were isolated, 85 of which were from sediments and 44 from fish. The strains identified based on biochemical tests included 58 (45%) *Pseudomonas* aeruginosa, 26 (20 %) *Pseudomonas* fluorescens, 37 (29 %) *Pseudomonas stutzeri* and eight (6%) *Pseudomonas* sp. A 990 bp PCR product was obtained for isolates using the 16S rRNA primers (Figure 1). There were differences in the identification obtained using 16S rRNA gene sequencing and the conventional biochemical tests [28]. With 16S rRNA methodology, 28 (22%) *Pseudomonas aeruginos*a, 41 (32%) *Pseudomonas fluorescens*, 9 (7%) *Pseudomonas putida*, 2 (2%) *Pseudomonas syringae* and 49 (38%) *Pseudomonas* spp., where GenBank database similarities match *Pseudomonas* sp. or where there was a match with more than one *Pseudomonas* species.

Detection of integrase, streptomycin and β-lactamase resistance genes

Class 1 integrons were detected in 30 of the 129 (23%) strains investigated whereas Class 2 and class 3 integrons were not detected.

β-lactamase resistance genes bla_{TEM} and bla_{SHV} were investigated however, the genes were not detected in any of the isolates tested. Suggesting a different mechanism for the ticarcillin resistance observed.

The presence of streptomycin resistance genes was investigated in representative isolates (with ≥ 16 µg/ml from previous study) using primers specific for *aad*A gene which is normally associated with integrons and codes for resistance to streptomycin and spectinomycin; *aad*A gene was detected in 28 of the isolates.

Detection of *mex*A, *mex*B, and *opr*M genes

*mex*B was detected in 85 of the 129 (66 %) isolates tested. Neither *mex*A nor *opr*M could be amplified with the primers used. No PCR product was obtained when the primers that amplifies the whole operon was used. Fifty-five of the *Pseudomonas* isolates positive for *mex*B, with a PCR product of 732 bp (Figure 2) were from sediments and thirty were from fish.

Some of the *mex*B genes obtained by PCR were sequenced to confirm the gene identity. The sequence analysis of our gene showed similarities to the Resistance-Nodulation-Division family (RND) multidrug efflux transporters including *mex*B and its different homolog *Ttg*B, so called because in addition to multidrug efflux it is also able to transport toluene; which have been described in *Pseudomonas* spp.

The sequences obtained have been deposited in the Genbank under the nucleotide sequence accession numbers listed in Table 2.

Detection of cadmium resistance genes

The *Pseudomonas* strains with cadmium Minimum Inhibitory Concentration (MIC) of ≥ 200 µg/ml were investigated for the presence of cadmium resistance gene. The *cad*A gene which was a PCR product

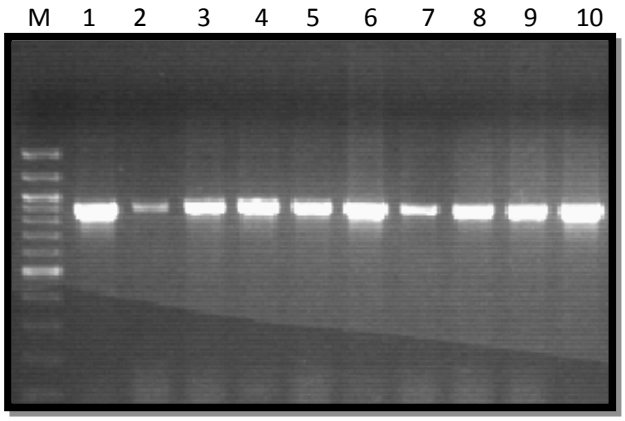

Figure 1: Example of an ethidium bromide stained gel of PCR products for the 16S rRNA identification of *Pseudomonas* spp.
M: 100 bp marker. Lane 1-10 shows different *Pseudomonas* isolates positive for the 990 bp PCR product of the universal 16S rRNA primers.

of 823 bp (Figure 3) was detected in 59 of the 92 (64%) isolates tested. The homolog of the czc genes, called czr, was not detected in any of the isolates. Thirty-five of the *Pseudomonas* strains carrying *cad*A were from sediments and twenty-four were from fish.

Plasmid DNA was extracted from 129 *Pseudomonas* isolates and 55 of them were found to possess plasmids. It was found that 29 of the 59 (49%) *cad*A positive isolates possessed plasmids.

The southern hybridization of plasmids with the *cad*A as probe did not reveal the presence of *cad*A on the plasmid, suggesting that *cad*A is likely to be chromosomally located. Some of the *cad*A genes obtained were sequenced to confirm the gene identity. The sequence analysis confirms similarities to the cadmium translocating P-type ATPases, *cad*A of various *Pseudomonas* spp. The sequences obtained have been deposited in the Genbank under the nucleotide sequence accession numbers and are listed in Table 2.

Discussion

The association of integrons with various resistance gene cassettes in aquatic environments has been well documented [33-37], however there are few reports of integron and associated resistance genes in *Pseudomonas* spp. There is limited information on the occurrence of integrons and other resistance genes in bacteria of aquaculture origin

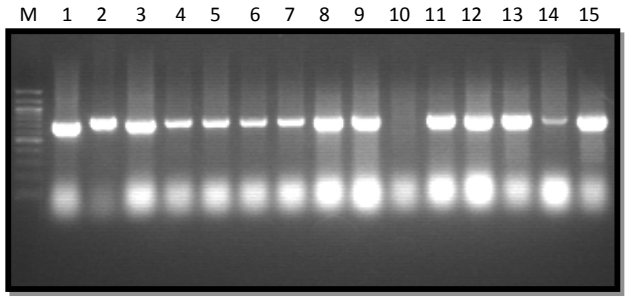

Figure 2: An example of ethidium bromide stained gel showing the 732 bp PCR product obtained for *mex*B in different *Pseudomonas* isolates obtained from sediment samples.
M: 100 bp marker; Lane: 1, 2, 6, 8-11, 13 (*P. aeruginosa*; lane 10 is negative for mexB); Lane: 3, 12, 15 (*P. fluorescens*); Lane: 4, 5, 7 14 (*P. stutzeri*).

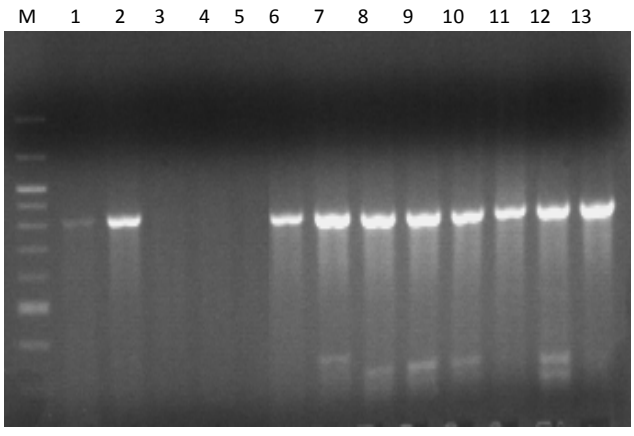

Figure 3: An example of ethidium bromide stained gel showing the 823 bp PCR product obtained for *cad*A in different *Pseudomonas* isolates obtained from fish samples.
M; 100 bp marker; lane: 1, 2, 6-13 shows strains that were positive for *cad*A and lane 3-5 shows strains that were negative for *cad*A.

in Australia [38,39]. Integrons are known to have the capacity to carry many antibiotic resistance genes and so the resistant *Pseudomonas* strains were investigated for the presence or absence of integrase genes for class 1, class 2 and class 3 integrons. In this study, Class 1 integron was detected in 30 of the 129 strains investigated. Streptomycin resistance gene *aad*A was detected in 28 of the 59 integrase positive strains; the primers used were specific for the detection of the *aad*A1a gene cassette and other closely related *aad*A gene cassettes (with the exception of *aad*A4 and *aad*A5). The presence of *aad*A and other resistance gene cassettes in different aquatic environments has been reported [33,35,37]; it is therefore not surprising to detect these genes in the isolates tested.

It is a well known fact that the members of the family *Pseudomonadaceae* show significant intrinsic resistance to a wide variety of structurally unrelated compounds [40,41]. The intrinsic resistance previously attributed only to non-specific impermeability of the outer membrane [42] is now known to result from a synergy with drug efflux pumps with a wide spectrum of activity [43].

One of the efflux system, *mex*AB-*opr*M that was investigated in this study encodes a tripartite pump is able to exports a wide range of antibiotics including chloramphenicol and β-lactams [21,44,45]. The detection of *mex*B, the major integral inner membrane efflux component of the *mex*AB-*opr*M pump in a large number of our isolates clearly suggests that some of the natural/intrinsic resistance of these isolates is partly as a result of this efflux system. A similar mechanism has been described in clinical *P. aeruginosa* strains which were meropenem resistant; where *mex*B was detected in all *bla*IMP, bla_{SPM} and bla_{VIM} negative isolates [46]. The sequence analysis of isolates from this study demonstrated similarity to the RND multidrug efflux transporter *mex*B and its homologue *Ttg*B which in addition to multidrug efflux also transports toluene out of the cell and have been described in *P. putida* [47,48], and in *P. fluorescens* Pf-5 genome sequence. No PCR product was obtained when the primers that amplifies the whole operon *mex*AB-*opr*M was used, this could be that it was too large to amplify.

β-lactamase resistance genes bla_{TEM} and bla_{SHV} were investigated however, the genes were not detected in any of the isolates tested suggesting a different mechanism for the ticarcillin resistance observed.

In addition to some antibiotic resistance mechanisms, we also report the incidence of the heavy metal resistance determinant, *cad*A gene in 59 of the 92 strains tested. A well characterised cadmium resistance system in Gram-negative bacteria is the cadmium, zinc and cobalt (*czc*) resistance determinant of *Alcaligenes eutrophus* where the *czc*C, *czc*B, and *czc*A proteins comprise an active efflux mechanism driven by antiporter, rather than a cation transporting ATPase [49]. A homolog of the *czc* gene, called *czr*, which confers cadmium and zinc resistance, have been identified in the chromosome of *P. aeruginosa* and appear to be highly conserved in environmental isolates of that species [27]; however this resistance determinant was not detected in our isolates. Instead we detected *cad*A which is mostly found in Gram-positive bacteria [50-53]. The genome sequences of several Gram-negative bacteria have revealed homologs of *cad*A [54,55] and there are some reports of *cad*A-encoded cadmium resistance being identified in some *Pseudomonas* species [56,57]. The *cad*A gene detected in this study is believed to be chromosomally located since southern hybridisation did not detect the presence of the gene on the plasmids in any of the isolates tested. Chromosomally located *cad*A have also been reported in *P. putida* [57].

There are reports of correlation between heavy metal contamination

ACCESSION NUMBER	GENE	CODE	ORGANISM	SOURCE
EF636685	mexB	SNFP3	*P. aeruginosa*	Fish
EF636686	mexB	SNSP18	*P. fluorescens*	Sediment
EF636687	mexB	SNSP28	*P. fluorescens*	Fish
EF636689	mexB	SNSP22	*P. stutzeri*	sediment
EF636688	mexB	SNFP21	*P. stutzeri*	Fish
EF636682	cadA	SNSP5	*P. aeruginosa*	Sediment
EF636683	cadA	SNFP24	*P. stutzeri*	Fish

Table 2: Nucleotide sequence accession numbers.

and antimicrobial resistance [58,59] and this is a potential public health concern. *Pseudomonas* spp. carrying integrons, efflux gene and cadmium resistance genes are present in farm-raised fish and sediments even though no antibiotics were licensed for use in Australian aquaculture at the time of the study.

Conclusion

*Int*1 of Class one integrons and *aad*A genes were detected in addition to *mex*B, the major integral inner membrane efflux component of the *mex*AB-*opr*M pump which would account for some of the intrinsic resistance. However, this pump can also mediate resistance to many antibiotics. Cadmium resistance gene *cad*A was also detected in the isolates. These results suggest that bacteria from farmed raised fish and environments in Australia are potential sources of antibiotic and heavy metal resistance genes in the environment. Established environmental reservoirs of resistance genes can lead to contamination of food and human water sources and this has great implications for public health.

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