Highly transferable pAQU-related plasmids encoding multidrug resistance are widespread in the human and fish pathogen *Photobacterium damselae* subsp. *damselae* in Aquaculture areas in the Black Sea

Ana Vences[a], Saqr Abushattal[a], Xosé M. Matanza[a], Javier Dubert[a], Ecren Uzun[b], Hamdi Ogut[c], Carlos R. Osorio[a]#

[a]*Departamento de Microbioloxía e Parasitoloxía, Instituto de Acuicultura, Universidade de Santiago de Compostela, Santiago de Compostela, Galicia, Spain;*

[b]*Karadeniz Technical University, Surmene Faculty of Marine Sciences, Department of Fisheries Technology Engineering, Trabzon, Turkey*

[c]*Bursa Technical University, Faculty of Natural Sciences, Architecture and Engineering, Department of Bioengineering, Bursa, Turkey*

Running title: pAQU-like MDR plasmids in *P. damselae* subsp. *damselae*

#For correspondence:

Carlos R. Osorio

Departamento de Microbioloxía e Parasitoloxía, Instituto de Acuicultura, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

Email: cr.osorio@usc.es

Tel. (+34) 881 816050

Fax. (+34) 881 816047

ABSTRACT

The marine bacterium *Photobacterium damselae* subsp. *damselae* is a pathogen that causes disease in diverse marine animals, and is also a serious opportunistic human pathogen that can cause fatal infections. Strains of this pathogen isolated from diseased European sea bass in aquaculture facilities in the Turkish coast of the Black Sea were found to exhibit reduced sensitivity to multiple antimicrobials. Selected representative strains were subjected to complete genome sequencing and plasmid characterization. It was found that multidrug resistant (MDR) isolates harboured large conjugative plasmids sharing part of their sequence backbone with pAQU-group plasmids, hitherto reported exclusively in China and Japan. Four new pAQU-group versions of plasmids were identified in the present study, containing distinct combinations of the resistance determinants *tetB*, *floR*, *sul2*, *qnrVC*, *dfrA* and *strAB*. Conjugative transfer of pPHDD2-OG2, a representative plasmid of 170,998 bp, occurred at high frequencies (2.2×10^{-2} transconjugants per donor cell), to *E. coli* and to pathogenic *P. damselae* subsp. *damselae* and subsp. *piscicida* strains. Upon transfer, pPHDD2-OG2 conferred reduced susceptibility to a number of antimicrobials to the recipient strains. Comparative genomics analysis of host strains suggested that these MDR plasmids of the pAQU-group were acquired by different genetic lineages of *Pdd*. This study provides evidence that *P. damselae* subsp. *damselae* isolated from diseased fish constitute a reservoir for conjugative MDR pAQU-group plasmids in the Mediterranean basin, and have the potential to spread to diverse bacterial species.

KEYWORDS: *Photobacterium damselae*, pAQU, MDR, plasmid, acquired resistance, aquaculture

INTRODUCTION

The marine bacterium *Photobacterium damselae* subsp. *damselae* (hereafter *Pdd*) is pathogenic for marine animals as well as for humans [1]. It is an emerging pathogen for fish species of financial importance in marine aquaculture, and outbreaks in different geographical locations of the globe have increased in the last years [2-5]. Human infections caused by *Pdd* originate from wounds exposed to seawater and by lesions inflicted during handling of fish and fishing tools, and may evolve into a severe necrotizing fasciitis with a fatal outcome, despite prompt antibiotic treatment [6-8]. In fact, some authors recommend to surgically debride and amputate at an early stage of the infection to save lives of patients [9]. Of note, *Pdd* is considered one of the main zoonotic pathogens acquired topically from fish [10,11].

The use of antibiotics in aquaculture contributes to the persistence of resistant strains, representing a major drawback for disease control of cultured fish as well as for human public health [12,13]. In fact, development of antimicrobial resistance in aquaculture environments is thought to contribute to the antimicrobial resistances of human bacterial pathogens [14]. In an earlier study, a conjugative multiple drug resistance (MDR) plasmid dubbed pAQU1 was identified in a *Pdd* strain isolated from an aquaculture site in Japan [15]. Subsequently, plasmids related to pAQU1 were characterized in species of *Vibrio*, *Photobacterium* and *Shewanella* from Japan and China [16,17]. The pAQU-group of plasmids contribute to dissemination of antibiotic resistance genes (*tetM*, *tetB*, *sul2*, *floR*, $bla_{CARDB\text{-}9}$*-like*, *mphA-like* and *mefA-like*, and others) among marine bacterial communities (15,16), and a study demonstrated that pAQU1 persists in the microbial

communities even under low antibiotic selection pressure [18]. Notably, despite the increasing number of reports on isolation of *Pdd* from diverse geographical locations, the identification of pAQU-group MDR plasmids is so far confined to Japan and China. In addition, studies aimed at the characterization of the genetic basis of antimicrobial resistance in *Pdd* isolates in other parts of the globe, from veterinary or from human clinical origin, are very scarce.

In the present study, it was found that *Pdd* isolates recovered in a previous study from diseased fish in the Turkish coast of the Black Sea [2] exhibited resistance to various antimicrobial classes. This information prompted us to examine the drug resistance markers carried by these strains. The results of the present study show that *Pdd* strains from the Black Sea harbour at least four new versions of MDR plasmids that share sequence blocks with pAQU1, indicating for the first time the presence of this family of plasmids in the Mediterranean basin. Notably, a representative plasmid identified in this study could be transferred to human enteric bacteria and to pathogenic *Photobacterium damselae* strains at a very high frequency. Whole genome sequencing and comparative genomics of six selected strains suggest that the multidrug resistance plasmids were acquired independently by different genetic lineages of *Pdd*, and not by a single virulent clone. Due to the high frequencies of conjugative transfer of these plasmids to other fish pathogens and to human enteric bacteria, staying vigilant about the spread of these MDR plasmids in bacteria in the Mediterranean and Atlantic basins is advised.

METHODS

Bacterial strains and antibiotic susceptibility disc diffusion tests

Thirteen *Pdd* strains isolated in a previous study from diseased European sea bass (*Dicentrarchus labrax*) in two different Aquaculture sites at the Turkish coast of the Black

Sea [2,3], were used in this study to investigate the genetic basis of reduced susceptibility to various antimicrobials (Table 1). Antimicrobial susceptibility patterns were determined by disc diffusion tests on tryptic soy agar plates supplemented with 1% NaCl (TSA-1), using bacterial suspensions adjusted to an optical density at 600 nm (OD_{600}) of 0.5 in saline solution (0.85 % NaCl wt/vol). The diameter (in mm) of the inhibition zones around the discs was measured after 24 h incubation at 25ºC. Three replicas for each strain and antimicrobial were measured. The following antimicrobial agents were tested (disc contents in parentheses): tetracycline (30 µg), chloramphenicol (2 µg), florfenicol (30 µg), trimethoprim-sulfamethoxazole (25 µg), trimethoprim (5 µg), streptomycin (10 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), flumequine (5 µg) nalidixic acid (30 µg) and oxolinic acid (2 µg). All antimicrobial discs were purchased from Oxoid, except for chloramphenicol and trimethoprim discs prepared in-house by soaking sterile paper discs with solutions of chloramphenicol (2 µg per disc), and with trimethoprim (5 µg per disc), respectively.

Genome sequencing, comparative genomics and molecular phylogeny analyses

Genome sequencing was performed using an Illumina platform as previously described [19]. In brief, high-purity genomic DNA of the six selected *Pdd* isolates listed in Table 2 was extracted using the G NOME DNA Kit (MPBio), following manufacturer's recommendations. For sequencing libraries preparation, genomic DNA was mechanically-sheared in a ultrasonicator (Covaris), ends were enzymatically repaired and adaptors (Illumina) ligated. Library was sequenced using Illumina MiSeq platform (2 × 150-bp paired-end reads). The reads were assembled with SPAdes 3.6 using default settings [20]. The assemblies consisted of the number of contigs detailed in Table 2. Draft genomes were submitted to the RAST tool for annotation [21], and gaps in a selected plasmid of strain OG2 (plasmid dubbed pPHDD-OG2) were closed by PCR and Sanger

sequencing. Draft genomes of the six selected strains were submitted to the NCBI database with accession numbers listed in Table 2. Easyfig v.2.2.3. [22], was used for analysis of plasmid synteny between pAQU1-like plasmids. The MLSA analysis was conducted using a concatenate of the sequences of nine genes (*ftsZ*, *gapA*, *gyrB*, *mreB*, *recA*, *rpoA*, *pyrH*, *topA* and *toxR*), selected for their demonstrated value for fine-tuned discrimination of taxa within species of the *Vibrionaceae* family [23,24]. Evolutionary analyses were conducted in MEGA6 [25]. The evolutionary history of the strains was inferred using the Neighbor-Joining method [26]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method [27], and are in the units of the number of base substitutions per site. The OrthoAni analysis [28] was used to quantify overall sequence similarity, based on comparison of orthologous fragments between pairs of genomes. Calculations of the core genome and of unique genes among strains were conducted using RAST [21].

PCR Screening of plasmid genes

Standard PCRs with suitable primers (Table 3) were performed to detect the *tetB*, *floR*, *sul2*, *qnrVC* and *dfrA* resistance genes and the plasmid backbone genes *repA* and *parAB* in a collection of *Pdd* strains from diverse geographical locations and host species.

Conjugation assays

Conjugation experiments were performed by a drop-mating assay essentially as previously described [29], on tryptic soy agar plates (TSA) prepared with seawater instead of with distilled water. Strain OG2, carrying MDR plasmid pPHDD-OG2 was used as donor. A collection of rifampicin resistant *Photobacterium damselae* strains, and the kanamycin resistant *E. coli* CAG18420 were used as recipient strains. Labelling of the

147 *Pdd* virulence plasmid pPHDD1 in strain RM-71 with a kanamycin-resistance cassette
148 was conducted by selecting for a single cross over of the suicide vector pNidKan into
149 damselysin toxin gene as reported in a previous study [30]. This labelled version was
150 conjugally transferred from donor RM-71 to a rifampicin resistant variant of the naturally
151 plasmidless *Pdd* strain LD-07 by drop-mating conjugation, selecting for kanamycin and
152 rifampicin resistance. Further verification that the transconjugants corresponded to LD-
153 07 derivatives and not to spontaneous rifampicin resistant mutants of RM-71 donor, was
154 achieved by PCR amplification of the collagenase gene *colP*, that is present in the LD-07
155 genome but absent from the RM-71 genome, as previously described [31]. The resulting
156 strain LD-07_pPHDD1 was subsequently used as recipient strain with OG2 as donor, to
157 assess the influence that pPHDD1 presence in the recipient strain might exert on
158 pPHDD-OG2 transfer. For all the mating pairs, transconjugants were recovered from
159 TSA-1 plates supplemented with 12 mg/L tetracycline, plus 25 mg/L rifampicin (when
160 rifampicin resistant recipients were used) or 50 mg/L kanamycin (when *E. coli*
161 CAG18420 was used as recipient).

162 **Data Availability**

163 The complete sequence of plasmid pPHDD-OG2 has been deposited in GenBank
164 database under Accession number VAUU02000098.1. The GenBank Accession numbers
165 for the draft genome sequences of *Pdd* strains obtained in this study are detailed in Table
166 2.

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169 **RESULTS AND DISCUSSION**

170 **Identification of multidrug resistance plasmids of the pAQU-type in *Pdd* strains**
171 **isolated from diseased European sea bass in the Black Sea**

Antimicrobial susceptibility tests revealed resistances to tetracycline, chloramphenicol, streptomycin, trimethoprim and SXT, as well as reduced susceptibility to florfenicol, in some of the *Pdd* strains isolated from the Black Sea (Table 1). As a control, RM-71, a *Pdd* highly virulent strain considered as non-multidrug resistant, exhibited sensitivity to all the antimicrobials tested. To gain an insight into the genetic basis of antimicrobial resistances in Black Sea strains, we selected strain OG2 as a donor in a conjugation experiment using kanamycin-resistant *E. coli* CAG18420 as recipient. The results showed that the tetracycline, florfenicol, trimethoprim and SXT resistance phenotypes were transferred to *E. coli* CAG18420 (Table 1). We therefore subjected strain OG2 to whole genome sequencing. Bioinformatics analysis identified the presence of several contigs containing antimicrobial resistance markers. Plasmid reconstruction and closing were accomplished through scaffolding, using the sequence of pAQU1 plasmid [15] as a reference, and gap closing using a PCR-Sanger sequencing strategy. This analysis unveiled the presence of a plasmid molecule in strain OG2 which was dubbed pPHDD-OG2. The complete nucleotide sequence of pPHDD-OG2 consisted of 170,998 bp, exhibited a G+C content of 43.7% and contained 183 predicted open reading frames (ORFs), including the resistance genes *tetB* (encoding a tetracycline efflux pump), *floR* (encoding a phenicol efflux transporter), *sul2* (encoding a dihydropteroate synthase for sulphonamide resistance), *qnrVC* (encoding a putative target-protection protein conferring quinolone resistance) and *dfrA* (encoding a dihydrofolate reductase for trimethoprim resistance) (Fig. 1). The similarity studies performed showed that the putative RepA and ParAB proteins of pPHDD-OG2 had 94% identity to RepA and ParAB counterparts of pAQU1, a plasmid previously characterized in a *Pdd* strain from Japan [15]. pPHDD-OG2 and pAQU1 plasmids also shared a 99.7 and a 100% identical TraI relaxase and Ter terminase, respectively. The 600-bp nucleotide region downstream

pPHDD-OG2 *repA* gene exhibited >98% identity to the putative replication origin of pAQU1. A comparative sequence analysis revealed a high degree of synteny between pPHDD-OG2 and pAQU1 (Fig. 1). All these lines of evidence suggest that pPHDD-OG2 is a member of the pAQU-group of MDR plasmids. However, pPHDD-OG2 harbours *qnrVC* and *dfrA* resistance genes, absent in pAQU1, and also a gene module that has not been reported in other pAQU-like plasmids so far, encoding an integrase, a restriction-modification system, and hypothetical proteins (module 4 in Fig. 1). Similarly, pAQU1 has three modules absent in pPHDD-OG2, one of these containing the *bla*$_{\text{CARB-9}}$-like gene (modules 1 to 3 in Fig. 1).

Different versions of pAQU-group plasmids are widespread in the Black Sea *Pdd* isolates

Using the pPHDD-OG2 sequence as a reference, primer pairs were designed to screen for presence of plasmid markers in 12 additional *Pdd* strains isolated from the Black Sea. All the strains with the exception of OG4A and OG7B tested positive for the plasmid backbone markers *repA* and *parAB* (data not shown). PCR tests for *tetB*, *floR*, *sul2*, *qnrVC* and *dfrA* markers revealed four distinct plasmid versions (Table 4). We thus selected five strains covering all the resistance marker profiles: OG12 (version 1), OG15A (version 2), OG16 and OG9 (version 3), and OG3 (version 4), and subjected them to whole genome sequencing. Contigs were assembled using pPHDD-OG2 as reference. Each sequenced genome contained a number of antimicrobial resistance genes whose loci tags are detailed in Table 2. The plasmid maps are shown in Fig. 1, and a comparative analysis of the plasmid modules containing the majority of the antimicrobial resistance markers is depicted in Fig. 2A. The comparative analysis of plasmid molecules confirmed the existence of four plasmid versions sharing a high degree of synteny and sequence identity,

but each plasmid version contained unique accessory modules (Fig. 1 and Fig. 2A). An *strAB* module providing streptomycin resistance was detected uniquely in plasmid version 3, represented by strains OG9 and OG16, confirming the results of the streptomycin susceptibility tests (Table 1). Further PCR tests demonstrated that *strAB* markers are unique to these two strains (Table 4). To the best of our knowledge, this constitutes the first report of a MDR plasmid of the pAQU-group that carries *strAB* resistance markers. Plasmid pPHDD-OG15A (representative of version 2), contained a large gene region unique to this plasmid (module 5 in Fig. 1), encoding hypothetical proteins, an integrase and other functions. With the exception of the putative quinolone resistance gene *qnrVC*, all the antimicrobial resistance genes were clustered within a module, and were often flanked by insertion sequence elements (*IS*) (Fig. 2A). This abundance of transposase genes suggests that the antimicrobial resistance genes were introduced by events of DNA transposition into an ancestral plasmid backbone, followed by selection aided by the use of antimicrobials in aquaculture environments. This might have contributed to a large extent to generate the diversity of plasmid versions found in this study.

The first plasmid-borne quinolone resistance gene was reported in 1998 [32], and subsequent studies described the presence of *qnr*-like genes harboured by plasmids [33]. Notably, we found that three of the four plasmid versions of *Pdd* contained a putative quinolone resistance gene which, based on its similarity to previously described genes, was dubbed *qnrVC* (Fig. 1). A closer look at the location of *qnrVC* in the different plasmid versions that contain this resistance marker (versions 1, 2 and 4) revealed that this gene disrupts an ORF encoding a hypothetical protein (locus tag FD719_00150 in plasmid pPHDD-OG-9) (Fig. 2B). The insertion was mapped in all the plasmid versions to codon 209 coding for an aspartate residue (data not shown).

None of the *Pdd* isolates tested exhibited full resistance to ciprofloxacin, enrofloxacin and flumequine, and resistances to oxolinic and nalidixic acid in some of the 13 strains studied did not correlate with presence of *qnrVC* gene (Table 1). These results suggest that resistance to quinolones might be due to base substitutions in chromosomal *gyrA* and *parC* genes [34], or that the plasmid-borne *qnrVC* gene does not provide detectable resistance levels to the tested quinolones under the conditions of the assay and within a *Pdd* genetic background. In support of this last hypothesis, we found that the *E. coli* transconjugant for pPHDD-OG2 exhibited reduced sensitivity to ciprofloxacin, enrofloxacin, flumequine, oxolinic acid and nalidixic acid (Table 1). These results clearly suggest that *qnrVC* might confer varying levels of quinolone resistance depending on the host strain. Of note, an earlier study reported that water-borne species of the *Vibrionaceae* family constitute reservoirs of *qnr*-like resistance genes [35].

pPHDD-OG2 transfers at a high frequency to a variety of recipient bacteria

pPHDD-OG2 was transferred with a high frequency, of 2.2×10^{-2} transconjugants per donor cell, to *E. coli* CAG18420 (Table 5), constituting the highest transfer frequency of a plasmid of this group reported so far (16). The transconjugant *E. coli* cells acquired resistance to tetracycline, florfenicol, trimethoprim and SXT, and reduced susceptibility to all the quinolones tested (Table 1). Notably, conjugative transfer of pPHDD-OG2 was also detected at very high frequencies to fish pathogenic *Pdd* LD-07 and to *P. damselae* subsp. *piscicida* DI21 (Table 5). We failed to detect transconjugants when the recipient strain was *Pdd* RM-71, a strain that harbours the virulence plasmid pPHDD1. However, pPHDD-OG2 was successfully transferred when a different pPHDD1-harbouring *Pdd* strain, 402O, was used as a recipient. To further investigate this potential incompatibility with pPHDD1, the native pPHDD1 plasmid of RM-71 was marked with a kanamycin

cassette and conjugally-transferred to the naturally plasmidless strain LD-07, rendering LD-07_pPHDD1. In this instance, we detected conjugal transfer of pPHDD-OG2 to LD-07_pPHDD1, indicating that pPHDD1 by itself does not prevent the acquisition of pPHDD-OG2, and suggests that other factors in RM-71 are involved.

For each mating pair experiment, one hundred individual transconjugant colonies were tested for co-transference of resistance to Cm^{20} (provided by *floR* to recipients upon conjugation, albeit donor strain OG2 itself did not resist Cm^{20}, likely due to unknown effects caused by the genetic background of donor cell), and colony-PCR was performed to test for presence of *tetB* and *floR* markers. As a whole, >97% of the transconjugants were also Cm^{20} resistant and tested positive for *tetB* and *floR*, and between 1-3% transconjugants were Cm^{20} sensitive and tested negative for *floR* while testing positive for *tetB*. PCR and sequencing analyses revealed that the Cm^{20} sensitive transconjugants had undergone the loss of the *floR* gene and flanking sequences (data not shown). The *floR* gene is flanked by *IS* elements (Fig. 2A) and is prone to deletion events, likely through *IS*-dependent rearrangements as described in other mobile elements [36]. Altogether, these results indicate that the *Pdd* MDR plasmids have the potential to spread among bacterial communities, and are prone to gene rearrangements that modify their resistance gene content, thus yielding new plasmid variants. Transmission of these plasmids to a variety of bacterial species might contribute to the resistome in diverse environments, both aquatic and terrestrial [13,37]. This link between aquatic and terrestrial resistomes is of special importance, because some antimicrobials authorised for use in farmed fish, as tetracyclines and phenicols, are of medical importance in human health, and the *Pdd* MDR plasmids reported here encode resistances to tetracyclines and phenicols, among others.

pPHDD-OG2 plasmid and relatives, are characteristic of isolates from the Black Sea and are absent from a large collection of *Pdd* strains from other geographical areas

To study the distribution of pPHDD-OG2-like plasmids among strains of *Pdd*, we PCR-screened the presence of *repA*, *parAB*, *tetB*, *floR*, *sul2*, *qnrVC* and *dfrA*, in a large collection of 70 *Pdd* strains of our laboratory (Table 6). Notably, 61 strains tested negative for all the markers, and none tested positive for *repA*. Only 9 strains yielded amplification of some of the assayed resistance markers, but the negative results for *repA* gene suggest that such isolates do not harbour a pAQU1-group plasmid. Of note, the Black Sea *Pdd* strains were isolated in 2011, whereas all the other *Pdd* strains of our collection were isolated previous to 2011, and many of them in the last two decades of the 20th century. Therefore, these results indicate that the pAQU-group plasmids, hitherto restricted to Japan and China [15-17], have undergone a recent expansion in the *Pdd* populations of the aquaculture environment in the Black Sea.

MLSA gene-based phylogenetic analysis and genome comparisons suggest a multiclonal origin of the Black Sea population of *Pdd* harbouring MDR plasmids

In order to assess whether the pAQU-like MDR plasmids were acquired independently by genetically diverse pre-existing bacteria, or whether the resistant *Pdd* are derived from a single virulent clone that thrived in this area following the acquisition of the resistance plasmid, we conducted a comparative and phylogenetic analysis of the six *Pdd* complete genomes obtained in this study. The average nucleotide identity analysis conducted with the core genome (OrthoANI), revealed a high degree of identity among the six genomes when common genes to all strains were compared, confirming their accurate taxonomic placement as *Pdd* and, at the same time, evidenced subtle differences in nucleotide sequences among strains (Fig. 3A). In addition, the MLSA analysis based on the

phylogenetic analysis of concatenated sequences of nine housekeeping genes demonstrated the existence of different genetic lineages of *Pdd* harbouring pAQU-group plasmids in the area (Fig. 3B). Most notably, the analysis of the pangenome and of the strain-specific genome demonstrated that each strain represents a distinct genetic lineage, with a large number of strain-specific protein coding genes (Table 2). These results altogether, suggest that the pAQU-like MDR plasmids were acquired independently by genetically diverse bacteria. Whether this plasmid acquisition occurred in the Black Sea environment by pre-existing plasmidless *Pdd* populations, or whether MDR strains were introduced into the Black Sea from other geographical sources, will need further investigation to be answered.

CONCLUSIONS

This study demonstrates that fish pathogenic *Pdd* constitute a reservoir for different variants of conjugative pAQU-group plasmids in the Mediterranean basin. The widespread use of oxytetracycline and florfenicol in Aquaculture areas may contribute to the maintenance and spread of MDR plasmids of the pAQU-group in the aquatic environment. To the best of our knowledge, this study has unveiled for the first time the presence of *strAB* resistance genes in a pAQU-like plasmid, demonstrating the plasticity of this family of MDR plasmids. Considering the recent expansion of MDR plasmid-containing *Pdd* strains, and the high frequency of conjugative transfer of these plasmids to other bacteria, it will be advisable to initiate a surveillance of this group of resistance plasmids in the Atlantic and Mediterranean basins. Horizontal transfer of these plasmids to other potentially pathogenic and environmental bacteria may pose additional implications for animal and human health.

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Author Contributions CRO and AVL conceived and designed the study. AVL and SA performed the experimental work. EU and HO isolated the strain collection from diseased fish and contributed to data interpretation. AVL, SA, XMM, JD and CRO analysed and interpreted the data. AVL, SA and XMM prepared tables and figures. CRO wrote the manuscript.

Conflict of Interest The authors declare no conflict of interest

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FIGURE LEGENDS

Fig. 1 Comparative analysis of pPHDD-OG2 and related plasmids from *Pdd* strains isolated from the Black Sea identified in the present study, and their genetic relationships with the previously characterized plasmid pAQU1 of a *Pdd* isolate from Japan. Sequence similarities (at the nucleotide sequence level) are depicted by shaded regions

493 **Fig. 2 (A)** Detailed comparative analysis of the modules harbouring antimicrobial
494 resistance genes in pPHDD-OG2 and related plasmids from *Pdd* strains isolated from the
495 Black Sea identified in the present study. The homologous region in the reference plasmid
496 pAQU1 is shown for comparative purposes. Sequence similarities (at the nucleotide
497 sequence level) are depicted by shaded regions. Colour code for gene categories is the
498 same as in Fig. 1. **(B)** Close view of the location of the putative quinolone resistance gene
499 *qnrVC* in the three plasmid versions 1, 2 and 4. Note that this gene is invariably inserted
500 disrupting the coding sequence of the gene of a hypothetical protein
501 **Fig. 3 (A)** Heatmap generated with OrthoANI values calculated from the OAT software,
502 and **(B)** Phylogenetic tree based on 9 concatenated genes (*ftsZ*, *gapA*, *gyrB*, *mreB*, *recA*,
503 *rpoA*, *pyrH*, *topA*, *toxR*), of six representative *Pdd* strains harbouring versions of pAQU-
504 like MDR plasmids

505 **Table 1** Antimicrobial resistance and sensitivity patterns for *Pdd* isolates from the Black
506 Sea, and for *Escherichia coli* CAG18420 and its respective transconjugant for plasmid
507 pPHDD-OG2

508

Strain		Diameter of inhibition zones around antimicrobial discs expressed in mm [a]									
	TET(30)[b]	CHL(2)	FFC (30)	TMP (5)	SXT (25)	STR (10)	CIP (5)	ENR (5)	UB (5)	OA (2)	NAL (30)
154dp-OG1	R	R	18	R	R	10	30	25	28	20	21
164dp-OG2	R	R	14	R	R	13	27	28	27	13	23
144bp-OG3	R	17	30	30	25	13	26	23	22	14	21
162bp-OG4A	20	16	21	31	22	12	34	26	29	18	24
158dp-OG5	R	R	16	R	R	11	22	20	20	11	19
189bp-OG7B	25	20	27	33	26	13	30	30	30	23	25
82dy-OG8	R	R	11	R	R	13	29	25	25	13	21
64bp-OG9	R	R	17	31	21	R	20	17	10	R	R
156dp-OG10A	R	R	17	R	R	13	30	26	27	17	22
70dps-OG12	R	R	14	R	R	11	9	8	9	R	R
164dpbuy-OG13B	R	R	17	R	R	10	30	26	25	30	22
111bp-OG15A	20	R	12	R	R	12	18	18	16	R	R
89dp-OG16	R	R	12	30	21	R	18	15	7	R	R
RM-71[c]	20	16	26	17	23	10	36	32	33	26	28
E. coli CAG18420	17	R	24	20	25	R	48	41	37	28	28
E. coli CAG18420 + pPHDD-OG2	R	R	R	R	R	R	30	30	27	17	20

509

510 [a] R denotes that the strain is resistant to the antimicrobial (complete absence of inhibition
511 zone)

512 [b] numbers between parenthesis denote the amount of antibiotic (in μg) in each test disc.
513 Abbreviations correspond to: tetracycline (TET); chloramphenicol (CHL); florfenicol
514 (FFC); trimethoprim (TMP); trimethoprim-sulfamethoxazole (SXT); streptomycin
515 (STR); ciprofloxacin (CIP); enrofloxacin (ENR); flumequine (UB); oxolinic acid (OA);
516 nalidixic acid (NAL).

517 [c] RM-71 is a non-multidrug resistant *Pdd* strain used as a control

518

519

520

521 **Table 2** A summary of the attributes of the six *Pdd* genomes determined in this study

522

	164dp-OG2	**144bp-OG3**	**64bp-OG9**	**70dps-OG12**	**111bp-OG15A**	**89dp-OG16**
Accession no.	VAUU00000000	VAND00000000	VANE00000000	VANF00000000	VANG00000000	VANH00000000
Genome size (bp)	4,596,602	4,760,721	4,474,417	4,778,025	4,585,436	4,504,167
Contigs	98	175	190	162	157	258
% GC	40.6%	40.5%	40.9%	40.5%	40.6%	40.9%
Genes (total)	4,071	4,283	4,092	4,277	4,105	4,129
CDSs	3,976	4,148	3,853	4,156	3,981	3,893
Unique CDSs	76	203	11	34	203	23
Resistance genes (locus_tag)						
tet(B)	FD717_020135	FD718_00810	FD719_00890	FD720_01180	-	FD722_00885
dfrA	FD717_020100	-	-	FD720_15195	FD721_00980	-
floR	FD717_020080	-	FD719_00840	FD720_00045	FD721_00010	FD722_00025
sul2	FD717_020050	-	FD719_00815	FD720_00010	FD721_00245	FD722_00050
strA	-	-	FD719_00820	-	-	FD722_00045
strB	-	-	FD719_00825	-	-	FD722_00040
qnrVC	FD717_020310	FD718_00155	-	FD720_14620	FD721_00175	-

523

524 **Table 3** Oligonucleotides used in this study

525

OLIGONUCLEOTIDES	SEQUENCE (5′-3′)	AMPLICON SIZE (bp)
Replicase *repA*		
Rep_int_F	ACATCAGCCGAACCAATAGC	333
Rep_int_R	GTTGGCTTTGCCAGAGTACC	
Plasmid partition *parAB*		
parB_F	CCCAGTTCCTCGATATTACG	358
parA_R	GGATCGTTCAGTGAACCACA	
Tetracycline efflux pump *tetB*		
3′_int_tetB	TGAAGTGGTTCGGTTGGTTA	319
5′_int_tetB	AATAGCACCCACACCGTTGC	
Florfenicol efflux pump *floR*		
floR_int_F	CCGTCTACTTCAAGCAGTGG	345
floR_int_R	GCGCTAAAGCCGACAGTGTA	
Sulfonamide-resistant dihydropteroate synthase sul2		
sul2_int_F	TCGCTCGACAGTTATCAACC	347
sul2_int_R	AATTCATCGAACCGCGCCAG	
Quinolone resistance *qnrVC*		
qnrVC_int_F	TATTGAGCAAGGCGAGTTGG	316
qnrVC_int_R	TGAAGCGCCTCTCAAGTTAG	
Trimethoprim-resistant dihydrofolate reductase *dfrA*		
dfrA2_int_F	ATCGGCTATCAAGCACCAGG	188
dfrA2_int_R	GTATCTGCGTCAAACACTCC	
Aminoglycoside phosphotransferases *strAB*		
strA_F	CTCTTCAATGCACGGGTCTG	380
strB_R	CGCGCAGTTCATCAGCAATG	

526

Table 4 Presence (1) and absence (0) of six antimicrobial resistance genes among the *Pdd* strains isolated from the Black Sea.

Strain	Plasmid version [a]	Presence of antimicrobial resistance genes					
		tetB	*floR*	*sul2*	*qnrVC*	*dfrA*	*strAB*
164dp-OG2	V1	1	1	1	1	1	0
154dp-OG1	V1	1	1	1	1	1	0
158dp-OG5	V1	1	1	1	1	1	0
82dy-OG8	V1	1	1	1	1	1	0
156dp-OG10A	V1	1	1	1	1	1	0
70dps-OG12	V1	1	1	1	1	1	0
164dpbuy-OG13B	V1	1	1	1	1	1	0
111bp-OG15A	V2	0	1	1	1	1	0
64bp-OG9	V3	1	1	1	0	0	1
89dp-OG16	V3	1	1	1	0	0	1
144bp-OG3	V4	1	0	0	1	0	0
162bp-OG4A	Ø	0	0	0	0	0	0
189bp-OG7B	Ø	0	0	0	0	0	0

[a] V1 to V4 denote the four distinct versions of MDR plasmids characterized in this study. The two strains with version Ø do not harbour pAQU-like MDR plasmids.

Table 5 Conjugative transfer of pPHDD-OG2 using *Pdd* OG2 as donor strain, and different *Escherichia coli* and *Photobacterium damselae* strains as recipients.

Recipient strain			**Transfer frequency** [b]
Strain name	**Relevant properties** [a]	**Reference**	
CAG18420	*E. coli*; Kan^R	[40]	2.2×10^{-2}
LD-07-Rif	*P. damselae* subsp. *damselae;* lacks pPHDD1 virulence plasmid; Rif^R	[31]	2.1×10^{-2}
DI21-Rif	*P. damselae* subsp. *piscicida*; Rif^R	[38]	3.5×10^{-3}
RM-71 -Rif	*P. damselae* subsp. *damselae*; harbours pPHDD1 virulence plasmid; Rif^R	[30]	$< 1.0 \times 10^{-8}$ [c]
402O-Rif	*P. damselae* subsp. *damselae*; harbours pPHDD1 virulence plasmid; Rif^R	[39]	1.6×10^{-2}
LD-07_pPHDD1	*P. damselae* subsp. *damselae*; transconjugant for pPHDD1 virulence plasmid; Rif^R	This study	1.1×10^{-2}

[a] Kan^R, kanamycin resistant; Rif^R, rifampin resistant,

[b] Transfer frequency was calculated as the number of transconjugants/donors. The results are the means of three independent assays.

[c] Assay detection limit

565 **Table 6** PCR-based screening for the distribution of 7 gene markers of plasmid pPHDD-
566 OG2 in 70 *Pdd* strains isolated from different geographical locations and hosts, indicating
567 the positive (1) and negative (0) amplification of the respective target gene

repA	*parAB*	*tet(B)*	*floR*	*sul2*	*qnrVC*	*dfrA*	Strain/Host	Country
0	0	0	0	0	0	0	9FT1M-3/ Shark	USA
0	0	0	0	0	0	0	RS80L1V1/ Red snapper	USA
0	0	0	0	0	0	0	ST-1/ Seatrout	USA
0	0	0	0	0	0	0	ATCC 33539/ Damselfish	USA
0	0	0	0	0	0	0	CDC-2227-81/ Human	USA
0	0	0	0	0	0	0	192/ Dolphin	USA
0	0	0	0	0	0	0	238/ Dolphin	USA
0	0	0	0	0	0	0	ATCC 35083/ Brown shark	USA
0	0	0	0	0	0	0	9FT2B-2/ Shark	USA
0	0	0	0	0	0	0	RS78SPL1/ Red snapper	USA
0	0	0	0	0	0	0	DCL 1.2/ Seabream	Spain (Canary Islands)
0	0	0	0	0	0	0	DCL 4.1/ Seabream	Spain (Canary Islands)
0	0	0	0	0	0	0	DCL 7.1/ Seabream	Spain (Canary Islands)
0	0	0	0	0	0	0	DCL 7.3/ Seabream	Spain (Canary Islands)
0	0	0	0	0	0	0	DCL 8.1/ Seabream	Spain (Canary Islands)
0	0	0	0	0	0	0	DCL 9.1/ Seabream	Spain (Canary Islands)
0	0	0	0	0	0	0	RG-91/ Turbot	Spain mainland
0	0	0	0	0	0	0	RM-71/ Turbot	Spain mainland
0	0	0	0	0	0	0	RG-153/ Turbot	Spain mainland
0	0	0	0	0	0	0	RI162/ Turbot	Spain mainland
0	0	0	0	0	0	0	RG-191/ Turbot	Spain mainland
0	0	0	0	0	0	0	RG-214/ Turbot	Spain mainland
0	0	0	0	0	0	0	309/ Mussel	Spain mainland
0	0	1	0	0	0	0	TW250.03/ Gilthead seabream	Spain mainland
0	0	0	0	0	0	0	TW462.02.1/ Gilthead seabream	Spain mainland
0	0	0	0	0	0	0	TW294L2/ Seabass	Spain mainland
0	0	0	0	0	0	0	AZ245.1/ Turbot	Spain mainland
0	0	0	0	0	0	0	AZ247.1/ Turbot	Spain mainland
0	0	0	0	0	0	0	USC-Viro-1/ Turbot	Spain mainland
0	0	0	0	0	0	0	LD-07/ Gilthead seabream	Spain mainland
0	0	0	0	0	0	0	ACR208.1/ Turbot	Spain mainland
0	0	0	0	0	0	0	ACRp72.1/ Turbot	Portugal
0	0	0	0	0	0	0	A-162/ Eel	Belgium
0	0	0	0	0	0	0	158/ Eel	Belgium
0	0	0	0	0	0	0	94-11-229 / Rainbow trout	Denmark
0	0	0	0	0	0	0	940804-1.1/ Rainbow trout	Denmark
0	0	0	0	0	0	0	940804-1.2/ Rainbow trout	Denmark
0	0	0	0	0	0	0	940804-2.1a/ Rainbow trout	Denmark
0	0	0	0	0	0	0	940804-2.3/ Rainbow trout	Denmark
0	0	0	0	0	0	0	940804-2.4/ Rainbow trout	Denmark
0	0	0	0	0	0	0	940804-2.5a/ Rainbow trout	Denmark
0	0	0	0	0	0	0	950810-3.2/ Rainbow trout	Denmark
0	0	0	0	0	0	0	950810-3.4/ Rainbow trout	Denmark
0	0	0	0	0	0	0	950810-3.5/ Rainbow trout	Denmark
0	0	0	1	1	0	0	950823-1.3b/ Rainbow trout	Denmark
0	0	0	0	0	0	0	950823-1.5/ Rainbow trout	Denmark
0	0	0	0	0	0	0	950825-2.4a/ Rainbow trout	Denmark
0	0	0	0	0	0	0	950828-1.3/ Rainbow trout	Denmark
0	1	0	1	1	0	1	950901-2.2b/ Rainbow trout	Denmark
0	1	0	1	1	0	1	950901-2.5b/ Rainbow trout	Denmark
0	1	0	1	1	0	1	96-2-25.1/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206308-4/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206306-2/ Rainbow trout	Denmark

568

569

570 **Table 6 (**Continued)

571

repA	*parAB*	*tet(B)*	*floR*	*sul2*	*qnrVC*	*dfrA*	Strain/Host	Country
0	0	0	0	0	0	0	206328-2/ Rainbow trout	Denmark
0	1	0	0	1	0	1	206306-6/ Raibow trout	Denmark
0	0	0	0	0	0	0	206328-5/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206303-14/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206302-7/ Rainbow trout	Denmark
0	0	0	1	1	0	0	206320-5/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206276-1/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206302-2/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206266-1/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206317-1/ Rainbow trout	Denmark
0	1	0	0	0	0	1	206303-1/ Rainbow trout	Denmark
0	1	0	0	0	0	1	206308-1/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206352-6/ Rainbow trout	Denmark
0	0	0	0	0	0	0	206351-4/ Rainbow trout	Denmark
0	0	0	0	0	0	0	PG801/ Shrimp	Taiwan
0	0	0	0	0	0	0	J3G801/ Shrimp	Taiwan
0	0	0	0	0	0	0	CDC1421-81/ Fish	Senegal

572

Figure 1

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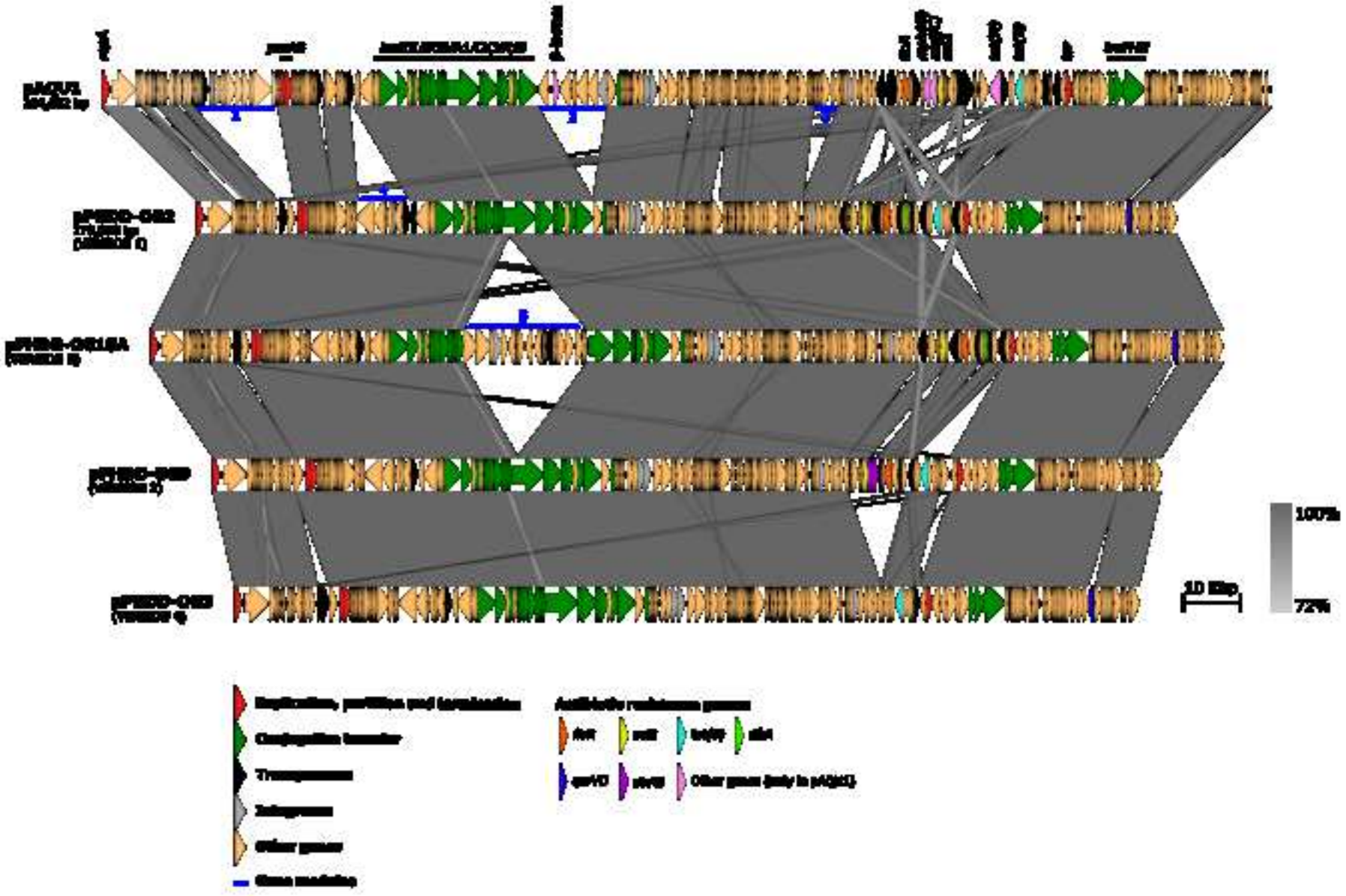

Figure 2

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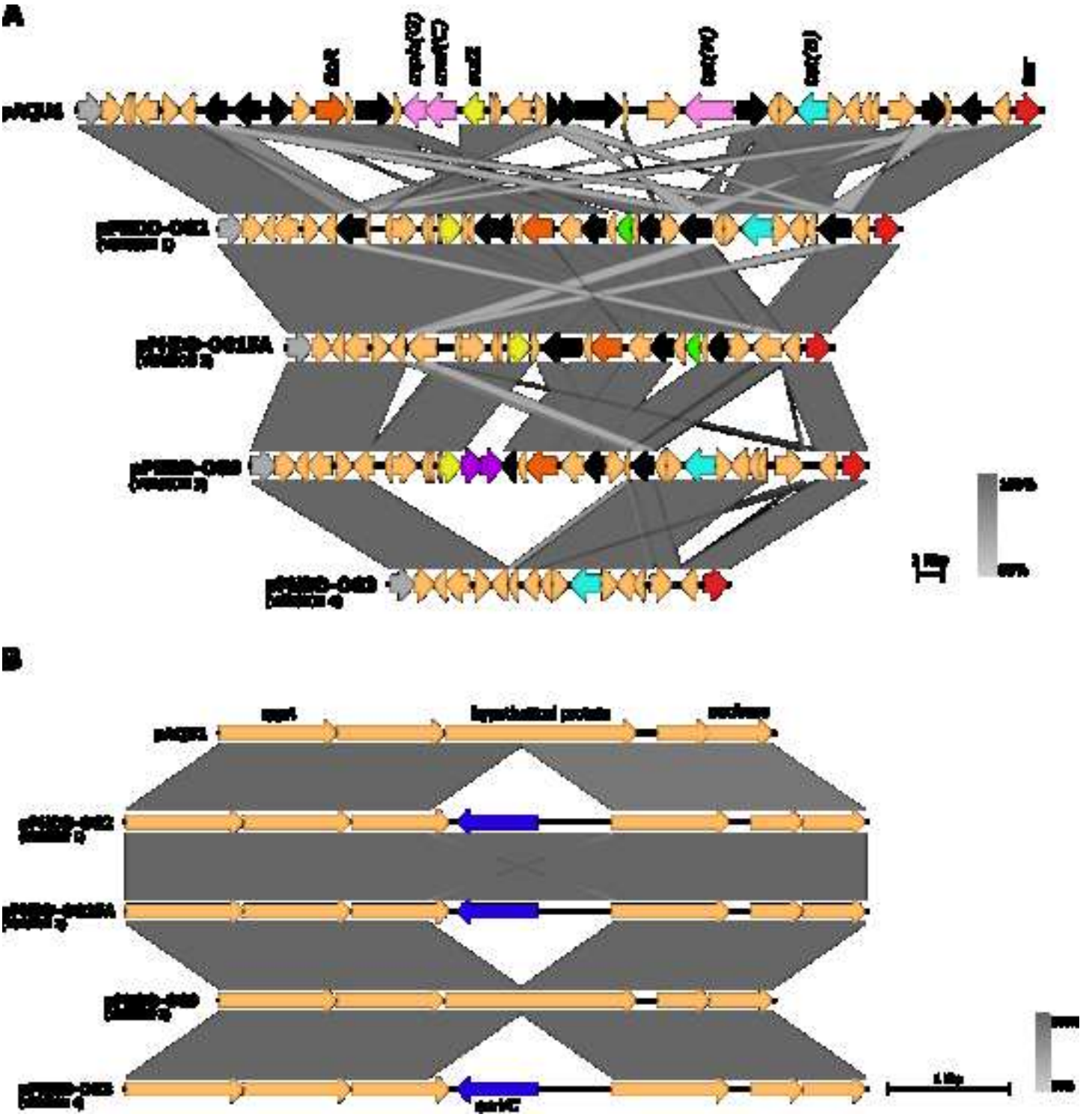

Figure 3