TESIS DE DOCTORADO

STUDY OF THE VARIABLE GENOME OF *PHOTOBACTERIUM DAMSELAE*: ACQUISITION OF NEW METABOLIC TRAITS, ANTIMICROBIAL RESISTANCE AND A NOVEL VIRULENCE PLASMID

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Study of the variable genome of *Photobacterium damselae*: acquisition of new metabolic traits, antimicrobial resistance and a novel virulence plasmid

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My Lord says: ‘’ (Whoever chooses to be guided, it is only for their own good. And whoever chooses to stray, it is only to their own loss. No soul burdened with sin will bear the burden of another. And We would never punish ˹a people˺ until We have sent a messenger ˹to warn them) ‘’ (15)

Sura 17: AL-ISRA (ISRA') – Holy Quran

‘’ (Your Lord has ordered you to worship none except Him, and to be good to your parents. If either or both of them attain old age with you, do not say: "ufff on you", nor rebuke them, but speak to them with words of respect. (23) And lower to them the wing of humbleness out of mercy and say: 'My Lord, be merciful to them, as they raised me since I was little).’’ (24)

Sura 17: AL-ISRA (ISRA') - Holy Quran

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Our ability to overcome the difficulties that face us in this life is evidence of our beautiful life that we live with our loved ones, friends, and ***families****.*

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PUBLICATIONS

Publications derived from the present doctoral thesis:

Article 1.
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Article 2.
Abushattal S., Vences A., Barca A.V. and Osorio C.R. (2020). Diverse horizontally-acquired gene clusters confer sucrose utilization to different lineages of the marine pathogen *Photobacterium damselae* subsp. *damselae*. *Genes* **11**:1244.

Article 3.
Abushattal S., Vences A., dos Santos N.M., do Vale A. and Osorio C.R. (2019). Draft genome sequences of *Photobacterium damselae* subsp. *piscicida* SNW-8.1 and PP3, two fish-isolated strains containing a type III secretion system. *Microbiology Resource Announcements* **8**(21):e00426-19.

Article 4.
Abushattal S., Vences A. and Osorio C.R. (2020). A virulence gene typing scheme for *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish photobacteriosis, reveals a high prevalence of plasmid-encoded virulence factors and of type III secretion system genes. *Aquaculture* **521**:735057.

Paper that will be submitted for publication:

Article 5.
A highly unstable plasmid encoding the type III secretion system contributes to virulence of *Photobacterium damselae* subsp. *piscicida* for fish.

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ESPECIFICACIÓN DE LAS CONTRIBUCIONES A LOS ARTÍCULOS QUE COMPONEN LA TESIS

Article 1.
Vences A., Abushattal S., Matanza X.M., Dubert J., Uzun E., Ogut H. and Osorio C.R. (2020). 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. *Microbial Ecology* 80:507-518.

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Saqr Abushattal preparó las muestras de ADN de los seis genomas para su secuenciación completa. Realizó todos los tipados genéticos de los genes de resistencia en 70 cepas de *Pdd*, y llevó a cabo todos los experimentos de conjugación, así como parte de las pruebas de resistencia a antibióticos. Elaboró las Tablas 2, 3, 4, 5 y 6, y contribuyó al análisis e interpretación de los datos.

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Saqr Abushattal realizó todos los experimentos de este trabajo. Elaboró todas las Tablas, y las Figuras 3 y 4. Diseñó los experimentos, analizó los datos y escribió el artículo conjuntamente con CRO y AVL.

Article 3.
Abushattal S., Vences A., dos Santos N.M., do Vale A. and Osorio C.R. (2019). Draft genome sequences of *Photobacterium damselae* subsp. *piscicida* SNW-8.1 and PP3, two fish-isolated strains containing a type III secretion system. *Microbiology Resource Announcements* 8(21):e00426-19.

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Saqr Abushattal procesó las muestras de ADN de los 2 genomas de *Pdp* para su secuenciación y realizó toda la parte experimental de este trabajo, así como parte de los análisis de las secuencias de los genomas, conjuntamente con CRO y AVL.

Article 4.
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Saqr Abushattal realizó todos los experimentos de este estudio. Elaboró todas las Tablas y Figuras, analizó los datos e interpretó los resultados.

Article 5.

Abushattal S., Vences A. and Osorio C.R. A highly unstable plasmid encoding the type III secretion system contributes to virulence of *Photobacterium damselae* subsp. *piscicida* for fish. (Unpublished).

Saqr Abushattal realizó todos los experimentos de este estudio, con la única excepción de las inoculaciones en peces en el acuario experimental, que fueron realizadas por CRO. Diseñó la estrategia experimental, realizó todas las modificaciones genéticas, analizó e interpretó los datos. Elaboró las Figuras y Tablas, y escribió el artículo conjuntamente con CRO y AVL.

En Santiago de Compostela, 22 de Diciembre de 2020

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ABBREVIATIONS & ACRONYMS

bp: base pairs

°C: Celsius Degree

CDS: Predicted Coding Sequences

CM9: Minimal medium supplemented with casamino acids

Kb: Kilobase

LB: Luria Bertani medium

MDR: Multidrug Resistance

MLSA: Multilocus sequence analysis

nm: nanometer

OD: Optical Density

ORF: Open Reading Frame

PCR: Polymerase chain reaction

Pdd: *Photobacterium damselae* subsp. *damselae*

Pdp: *Photobacterium damselae* subsp. *piscicida*

T3SS: Type III Secretion System

TCBS: Thiosulfate Citrate Bile Sucrose medium

TSA-1: Tryptic Soy Agar supplemented with NaCl 1%

TSB-1: Tryptic Soy Broth supplemented with NaCl 1%

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RESUMO

A familia *Vibrionaceae* é unha das familias bacterianas máis comúns en medio acuático, especialmente en augas mariñas. Esta familia abrangue especies bacterianas patóxenas para diferentes animais, incluíndo peixes, crustáceos, moluscos e cetáceos entre outros. Ademais, algunhas especies da familia *Vibrionaceae* poden transmitirse ao ser humano a través da infección de feridas ou mediante a inxesta de marisco pouco cociñado, podendo causar a morte no caso de non tratarse rapidamente. *Photobacterium damselae* é un patóxeno mariño da familia *Vibrionaceae* que presenta dúas subespecies distintas, *damselae* e *piscicida*. A subespecie *damselae* é considerada un patóxeno non clonal e xeral para diversas especies de animais mariños e tamén o home, mentres que a subespecie *piscicida* e un patóxeno máis especializado, infectando unicamente peixes. As dúas subespecies constitúen importantes patóxenos na acuicultura mariña, causando gromos e perdas económicas en empresas acuícolas de todo o mundo. Previos estudos describiron amplamente a considerable diverxencia nos estilos de vida das dúas subespecies de *Photobacterium damselae*. Esta diverxencia pode ser entendida, no marco das dúas subespecies, pola adquisión de factores de virulencia por transferencia xénica horizontal e, nomeadamente na subsp. *piscicida*, pola proliferación masiva de secuencias de inserción, fenómeno que probablemente conduciu a un estilo de vida hóspede-dependente para esta última. A pesar de seren consideradas altamente relacionadas e semellantes no tocante ás secuencias de ADN, cada unha das duas subespecies difiren en moitos aspectos metabólicos e morfolóxicos, como poden ser a temperatura óptima de crecemento, taxa de crecemento, tipo de hóspede, viabilidade na auga do mar, lugares de illamento, factores de virulencia e contido de enzimas.

Esta tese estruturouse en dúas partes: a primeira trata do estudo da diversidade xenética dentro da subespecie *damselae*, centrándose na caracterización do xenoma accesorio e particularmente no estudo dos plásmidos de resistencia a antimicrobianos e no estudo de xenes metabólicos que exhiben presenza diferencial entre as cepas desta subespecie. A segunda parte da presente tese ocúpase do estudo xenético da subespecie *piscicida* e está maiormente centrada no estudo dun novo plásmido de virulencia, non caracterizado ata o momento, que codifica para un sistema de secreción de tipo III.

Estudo do xenoma accesorio en *P. damselae* subsp. *damselae* (*Pdd*)

O estudo de plásmidos e outros elementos móbiles portadores de xenes de resistencia a antimicrobianos en *Pdd* era moi escaso no momento de inicio desta tese. Concretamente, o noso coñecemento sobre a materia estaba reducido a poucos estudos en illados de *Pdd* de China e Xapón. Nesta tese abordamos o estudo dunha colección de illados de *Pdd* procedentes de gromos en granxas de robaliza no Mar Negro en Turquía, os cales amosaron resistencia a varios axentes antimicrobianos.

Realizamos un extenso estudo xenético que incluíu a secuenciación completa do xenoma dunha selección de 7 illados de *Pdd*, o estudo das resistencias antimicrobianas portadas nunha familia de plásmidos e a capacidade dos mesmos de seren transferidos por conxugación a outras bacterias. Este estudo levou á publicación dun artigo titulado "**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**" (*Microbial Ecology,* 2020. 80:507-518). Neste estudo, atopamos que illados multirresistentes de *Pdd* conteñen grandes plásmidos conxugativos que comparten

módulos base da secuencia con plásmidos da familia pAQU. Esta familia abarca plásmidos relacionados co pAQU1, o primeiro plásmido descrito deste grupo, e polo momento, soamente foron descritos en China e Xapón. Polo tanto, o noso estudo constitúe a primeira descrición dun plásmido deste grupo en illados de *Pdd* procedentes da cunca do Mediterráneo. Na presente tese, identificamos 4 novas variantes do grupo pAQU e asemade, cada variante contiña unha combinación única de xenes de resistencia, que inclúen *tetB* (que codifica para unha bomba de saída de tetraciclina), *floR* (que codifica para un transportador de saída de fenicol), *sul2* (que codifica para unha dihidropteroato sintase para resistencia a sulfonamida), *qnrVC* (que codifica para unha posible proteína de protección de diana que confire resistencia a quinolonas), *dfrA* (que codifica para unha dihidrofolato redutase para resistencia a trimetoprima) e os xenes *strAB* que confiren resistencia a estreptomicina. Notablemente, demostramos que estes plásmidos poden ser transferidos a *Escherichia coli* e a cepas de *P. damselae* subsp. *damselae* e subsp. *piscicida* a unha taxa elevada. Como exemplo, un plásmido de 170,998 pb caracterizado neste estudo e ao que nomeamos como pPHDD2-OG2, foi transferido a unha cepa bacteriana receptora a unha frecuencia de $2{,}2 \times 10^{-2}$ transconxugantes por célula doadora. Despois de ser adquirido por unha *E. coli* receptora, pPHDD2-OG2 conferiu reducida susceptibilidade a tódolos antimicrobianos para os cales se portaba un xene de resistencia no plásmido. Os resultados obtidos da análise comparativa dos xenomas destas cepas multirresistentes suxeriron que os plásmidos MDR (*multi drug resistance*) da familia pAQU foron adquiridos por diferentes liñaxes xenéticas de *Pdd*. Este estudo aportou polo tanto evidencias de que illados de *Pdd* procedentes de peixes enfermos constitúen un reservorio para plásmidos conxugativos MDR da familia pAQU na cunca do Mediterráneo e teñen o potencial de diseminarse a diversas especies bacterianas.

Continuando co estudo da diversidade xenética do xenoma accesorio de *Pdd*, decidimos abordar o estudo da base xenética da utilización da sacarosa nesta subespecie, un tema interesante que, ata o momento, non recibira atención. O estudo dos patróns de utilización das distintas fontes de carbono é un método tradicionalmente empregado para diferenciar especies dentro da familia das vibrionáceas. En particular, o agar tiosulfato citrato bile sacarosa (TCBS), é o medio de cultivo clásico e estándar para o illamento selectivo de especies da familia *Vibrionaceae*. O TCBS contén un 2% de sacarosa e indicadores de pH, o que permite distinguir aqueles microorganismos fermentadores de sacarosa, que forman colonias amarelas (fenotipo Scr^{+}), daqueles non fermentadores, que forman colonias verdes (fenotipo Scr^{-}). Durante os últimos anos, foron varios os estudos que indicaron que algúns illados pertencentes á subsp. *damselae* formaban colonias amarelas en TCBS, feito que indicaba a capacidade destes para fermentar sacarosa. Esta observación, que difire do observado na maioría de illados da subsp. *damselae* formadores de colonias verdes en TCBS, motivounos a estudar a base xenética da adquisición desta característica metabólica tan peculiar. Os resultados deste estudo deron lugar a un artigo científico titulado: "**Diverse horizontally-acquired gene clusters confer sucrose utilization to different lineages of the marine pathogen *Photobacterium damselae* subsp. *damselae*.**" (*Genes*, 2020. 11, 1244). Neste estudo, identificamos un total de oito illados de *Pdd* Scr^{+} na nosa colección do laboratorio e levamos a cabo a secuenciación completa do seus xenomas. A existencia dun gran número de xenomas de cepas de *Pdd* Scr^{-} nas bases de datos públicas (algúns deles secuenciados na presente tese e, como se describiu anteriormente, empregados no estudo dos plásmidos de multirresistencia a antibióticos), permitiunos levar a cabo un estudo exhaustivo de xenómica comparativa entre os xenomas daquelas cepas de *Pdd* Scr^{+} e Scr^{-}. As análises realizadas revelaron a existencia de dúas versións

diferentes dun clúster de catro xenes, exclusivo daqueles illados Scr$^+$, e que denominamos clúster *scr*. Este grupo de catro xenes codifica o compoñente IIBC do sistema PTS (*phosphotransferase system*) específico para sacarosa (xene *scrA*), unha fosfofrutoquinase (*scrK*), a sacarosa-6-fosfato-hidrolase (*scrB*) e un represor do operón da sacarosa (*scrR*). Das dúas versións identificadas, unha delas constitúese como a maioritaria (presente en 7 das 8 cepas Scr$^+$), mentres que a segunda versión foi atopada nun único illado. A diverxencia entre as dúas versións a nivel nucleotídico foi do 30%, o que suxire claramente que estamos ante dous clústers filoxenéticos distintos. O seguinte obxectivo consistiu en demostrar que este clúster era necesario para a utilización de sacarosa como fonte de carbono, e observouse que o mutante por deleción no xene *scrA* non fermentaba sacarosa e presentaba un crecemento defectivo utilizando este disacárido como fonte de carbono. Sorprendentemente, atopamos que o clúster de xenes para a sacarosa está localizado nunha rexión hipervariable do cromosoma I. Ademais, a análise desta rexión nos xenomas de *Pdd* Scr$^-$ revelou a presenza de combinacións xénicas específicas de cada cepa, o que suxire que representa un punto quente de recombinación de ADN adquirido por transferencia horizontal. Tamén tiñamos interese en descifrar se os clústers de sacarosa seguían a filoxenia do xénero *Photobacterium,* ou se, pola contra, foran adquiridos por un mecanismo de transferencia horizontal de xenes. Para o noso asombro, cando comparamos as árbores filoxenéticas construídas coas secuencias dos clústers *scr* e as árbores creadas coas secuencias de xenes conservados, atopamos que era probable que os clústers *scr* de *Pdd* foran adquiridos dunha especie do xénero *Vibrio* e non dunha especie doadora do xénero *Photobacterium*. Esta análise tamén revelou que as filoxenias dos clústers *scr* entre as especies de *Vibrio* e *Photobacterium* non seguían exactamente a filoxenia das especies, o que indicaba que a transferencia horizontal dos clústers de xenes da sacarosa foi ampla nestes dous xéneros.

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Estudo do xenoma accesorio en *P. damselae* subsp. *piscicida (Pdp)*

Na segunda parte da tese, estudamos a presenza de factores de virulencia que xorden de maneira diferencial ó longo dos xenomas e das coleccións de cepas de *Pdp* e realizamos un amplo estudo dos factores de virulencia. Adicionalmente, o noso estudo revelou, por primeira vez, a presenza dun plásmido que codifica o sistema de secreción de tipo III (T3SS), e que xoga un papel importante na patoxénese de *P. damselae* subsp. *piscicida* en peixes.

Á hora de iniciar o estudo do xenoma variable de *Pdp*, tomamos vantaxe dun estudo previo no que se indicaba a existencia dun plásmido que codificaba para un T3SS na cepa tipo da subespecie irmá, *Pdd*. Ata o de agora, este sistema de secreción nunca fora descrito, de por si, na subespecie *piscicida*, e preguntabámonos se algúns illados poderían codificar este potencial factor de virulencia. O T3SS é un artiluxio molecular, similar ó dunha xeringa, que inxecta toxinas bacterianas, denominadas efectores, no interior das células hóspedes.

Coa fin de realizar un cribado da presenza do T3SS nos illados de *Pdp*, comezamos co deseño de parellas de cebadores, nun estudo preliminar que serviu para detectar a existencia de xenes do T3SS en varias cepas desta subespecie. A continuación, decidimos enviar dúas cepas de *Pdp* para realizar a secuenciación completa do xenoma e esta análise desvelou a presenza de xenes do T3SS en dúas cepas, unha illada en España e a outra en Xapón. Este estudo publicouse no artigo titulado: **"Draft genome sequences of *Photobacterium damselae* subsp. *piscicida* SNW-8.1 and PP3, two fish-isolated strains containing a type III secretion system."** (*Microbiology Resource Announcements*, 2019. 8(21): e00426-19). Cómpre destacar que os xenes do T3SS non foran nunca anotados como factores de virulencia en *Pdp*. Estes resultados motiváronnos a estudar a presenza dos xenes

deste sistema nunha ampla colección de illados de *Pdp*. Este obxectivo foi alcanzado na segunda publicación que abarca o estudo do xenoma variable de *Pdp* nesta tese, que foi nomeado **"A virulence gene typing scheme for *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish photobacteriosis, reveals a high prevalence of plasmid-encoded virulence factors and of type III secretion system genes."** (*Aquaculture*, 2020. 521:735057).

Tendo en conta os estudos previos realizados en illados de *Pdp,* non había suficientes datos que permitisen trazar a prevalencia dos factores de virulencia deste patóxeno en coleccións amplas de procedencia distinta, tanto na orixe xeográfica coma de distintos hóspedes. Neste estudo, deseñamos cebadores específicos para a detección de xenes de virulencia en 103 illados de *Pdp* procedentes de distinta orixe xeográfica e obtidos ó longo dun longo periodo de tempo. Estas amplificacións enfocáronse no rastrexo de: (1) a presenza do xene *aip56* contido no plásmido pPHDP10, que codifica para a toxina inductora de apoptose AIP56; (2) a presenza do plásmido pPHDP70 apuntando ós xenes *irp2* e *frpA* que forman parte dun sistema de captación de ferro baseado no sideróforo piscibactina; e (3) a presenza de dous marcadores xenéticos do citado T3SS, cuxa existencia, como se mencionou anteriormente, foi descrita por primeira vez en dous illados de *Pdp,* SNW-8.1 e PP3. Os nosos resultados indicaron que o plásmido pPHDP10 estaba presente en 97 dos 103 illados, proporcionando probas contundentes da elevada estabilidade deste plásmido, considerado, ata o momento, como o principal factor de virulencia de *Pdp.* Cómpre sinalar que este cribado revelou a presenza do plásmido pPHDP70 nun total de 91 illados, dos cales 89 eran illados europeos e dous illados de procedencia xaponesa. En consecuencia, estes resultados demostraron por primeira vez que a presenza deste plásmido non se restrinxe exclusivamente a aqueles illados europeos, como fora suxerido en estudos anteriores.

O máis interesante é que este amplo estudo desvelou que os xenes pertencentes ó T3SS estaban presentes en 74 illados de *Pdp,* o máis antigo dos cales foi illado en Xapón no 1980. A pesar de que as infeccións causadas por *Pdp* son obxecto de estudo dende 1963 e da distribución xeneralizada dos xenes do T3SS entre os illados de *Pdp*, a súa presenza, ata onde sabemos, nunca fora recollida nos estudos dispoñibles, polo que podemos considerar este resultado de suma relevancia. Neste estudo, detectamos a perda de xenes do T3SS no que pareceu ser, inicialmente, un fenómeno inducido pola adición de rifampicina durante o proceso de construción de mutantes nos xenes do T3SS. Esta observación inicial, evidenciou a posibilidade de que os xenes deste sistema estiveran contidos nun plásmido inestable, xa que os marcadores xenéticos perdíanse espontáneamente e de xeito concomitante cos xenes plasmídicos *parAB* e *traC*.

Tendo en conta estes antecedentes, consideramos de especial interese continuar co estudo dos xenes do T3SS, en particular na cepa PP3, na que o fenómeno de perda espontánea de xenes fora xa descrito. O primeiro paso para desvelar a natureza plasmídica do T3SS consistiu na re-secuenciación do xenoma da cepa PP3 de *Pdp* usando a tecnoloxía PacBio, xa que o xenoma obtido previamente, mediante secuenciación Illumina, dera lugar a un xenoma moi fragmentado, impedindo a obtención da ensamblaxe do contexto xenético do T3SS. Unha vez completada a re-secuenciación do xenoma da PP3 mediante secuenciación PacBio, desvelouse que os xenes do T3SS estaban nun plásmido grande de 133 kilobases, e que denominamos pPDPT3. Inesperadamente, a secuencia do plásmido pPDPT3 incluía dúas copias parálogas, 100% idénticas a nivel de secuencia nucleotídica, dun conxunto de 24 xenes do T3SS, mentres que nunha soa copia existían 12 xenes adicionais relacionados co T3SS. Estas dúas secuencias parálogas estaban dispostas en imaxe especular. O resto da secuencia do plásmido, presentaba un alto

contido en xenes de transposases (elementos de inserción, *IS*), suxerindo que a rexión do T3SS eludiu a inactivación xénica mediada por elementos *IS* posto que é probable que este sistema xogue un papel na virulencia para o peixe hóspede.

A secuencia do pPDPT3 contíña os xenes *parAB* potencialmente implicados na partición do plásmido trala replicación. Sen embargo, e de xeito sorprendente, o plásmido non codificaba unha proteína *rep* manifesta, o que significa que os mecanismos de replicación empregados polo plásmido son, ata o momento, descoñecidos. Outra das características máis destacables do pPDPT3 consiste na presenza dun xene para unha posible adhesina do tipo YadA e para un regulador transcripcional hipotético ExsA. A análise da secuencia do pPDPT3 revelou a presenza dunha rexión de 15kb que contiña os xenes *traALEKBVC, trbI* e *traWDI.* Este módulo de xenes, responsable das funcións de transferencia conxugativa, divídese en tres submódulos por elementos de secuencias de inserción (*IS*). O elemento *IS*1 atopouse en dúas copias en dous sitios diferentes entre xenes *tra,* nomeados *traI,* corrente abaixo, e o xene *traD*, corrente arriba. O elemento *IS3* está localizado entre os xenes *traC* e *traV.* Entre os xenes *trbI* e *traW*, intercálanse cinco xenes que codifican para proteínas hipotéticas. Levamos a cabo unha análise comparativa das secuencias entre os xenes da rexión de transferencia do pPDPT3 e rexións homólogas secuenciadas previamente en 4 plásmidos noutras cepas de *Photobacterium damselae,* abarcando ambas subespecies. Esta análise comparativa revelou unha coincidencia case exacta entre o pPDPT3 e un posible plásmido na cepa OT-51443 de *Pdp*, que tamén contiña xenes para o T3SS. É de destacar que a rexión *tra* nestes dous plásmidos é moito máis curta que a rexión *tra* homóloga de tres plásmidos diferentes descritos en cepas de *Pdd,* feito que suxire claramente que os dous plásmidos de *Pdp* conteñen un conxunto incompleto de xenes de transferencia conxugativa. De feito,

a comparativa dos plásmidos proporcionou evidencias da existencia de pérdida de xenes de conxugación na rexión entre os xenes *traD* e *traW*, representada pola ausencia dos xenes *traGHFNU* e *trbBC* no pPDPT3 así coma no plásmido homólogo da cepa OT-51443 de *Pdp*. Os intentos por demostrar a mobilización das versións marcadas do pPDPT3 a unha cepa receptora en experimentos de conxugación, deron lugar a resultados negativos. Sen embargo, e como se detalla a continuación, obtívose evidencia de que todas as versións marcadas do pPDPT3 que contiñan clústers de xenes de resistencia a antibióticos integrábanse, de xeito sistemático, no cromosoma da bacteria hóspede. Polo tanto, a capacidade conxugativa do plásmido pPDPT3 precisaría dunha investigación máis profunda.

O estudo funcional dos xenes que porta este plásmido comezou a atopar numerosos problemas, debidos á grande inestabilidade do mesmo. Certamente, un dos achados máis inesperados do noso estudo foi a confirmación de que pPDPT3 pode curarse (perderse da célula hóspede) cun simple subcultivo nun medio de crecemento en condicións de laboratorio. De feito, na presente tese, demostramos que a perda do plásmido en cultivo non era unha característica única da cepa PP3 de *Pdp*, xa que a perda espontánea do T3SS rexistrouse noutras cepas de *Pdp* illadas de distintas localizacións xeográficas e cun lapso de tempo entre os illamentos de varios anos. Esta inestabilidade levounos a deseñar unha estratexia para construír mutantes dos xenes do T3SS, substituíndo o xene diana cun xene de resistencia a antibiótico. Deste xeito, dita mutación implicaría a integración dun xene de resistencia na estrutura do plásmido, de modo que a adición do respectivo antibiótico durante tódalas etapas de construción dos mutantes garantiría a estabilidade do pPDPT3 na célula. Como resultado, nesta tese construímos dous mutantes por inserción distintos no xene do T3SS *vcrD* (tamén denominado *escV* noutras especies). Un mutante simple (no que chamamos o alelo

$vcrD^{1}$) contiña un xene de cloranfenicol (*cat*) substituíndo a maior parte da secuencia do xene *vcrD*, mentres que o segundo tipo de mutante simple contiña o xene *cat* substituíndo a secuencia do outro alelo, denominado $vcrD^{2}$. Como engado, usando o mutante marcado co xene *cat* en $vcrD^{1}$ como base, mutamos o $vcrD^{2}$ inserindo nel un xene de resistencia a kanamicina (*kan*) para así xerar un dobre mutante $vcrD^{1}$-$vcrD^{2}$.

Os resultados insospeitados non tardaron en aparecer. Os experimentos de inoculación de peixes coa cepa parental PP3, xunto cos distintos mutantes e coa cepa curada do pPDPT3, aportaron resultados inesperados; en primeiro lugar e como sería de agardar, a cepa sen plásmido negativa para o pPDPT3 presentaba unha forte atenuación da virulencia en robaliza, en comparación coa cepa parental. Sen embargo, aumentando a dose bacteriana 100 veces (100×), a cepa negativa para o plásmido pPDPT3 foi quen de producir unha mortalidade do 100%. Este resultado axústase á situación esperada, considerando que esta cepa pPDPT3-negativa aínda produce outros factores de virulencia e, de feito, a mortalidade dos peixes en infeccións experimentais acadouse rutineiramente en estudos previos nos que, a día de hoxe, sabemos que se utilizaron cepas negativas para o T3SS. Pola contra, os resultados inesperados chegaron cando observamos que os mutantes simples e o mutante dobre para o *vcrD* non causaron mortalidade nos peixes á dose alta (100×). Os conseguintes estudos puxeron de manifesto a grande dificultade, a pesares de reiterados intentos, de seleccionar a partir destes mutantes un clon no que se curase espontaneamente o plásmido pPDPT3. De feito, ámbolos dous tipos de mutantes, tanto os simples como o dobre, semellaban conter o plásmido pPDPT3 integrado no cromosoma da célula hóspede e a nosa hipótese de traballo é que esta integración do plásmido ocorre a través da disrupción dun xene necesario para a virulencia e/ou a fitness en vivo.

En conxunto, os nosos resultados demostran que a perda do pPDPT3 causa unha disfunción na virulencia de *Pdp* en peixes, aínda que a doses infectivas maiores, as bacterias negativas para o pPDPT3 poden causar mortalidade. A sospeita de que os mutantes no T3SS marcados cos xenes de resistencia a antibióticos conteñen este plásmido integrado no xenoma bacteriano impídenos, polo momento, atribuír ao T3SS un rol innegable na virulencia. Estes mutantes no T3SS poderían volverse non virulentos ben pola mutación no T3SS, ou ben pola disrupción de xenes esenciais para a virulencia mediante a integración no cromosoma. Estudos futuros serán dirixidos a revelar a base xenética da atenuación na virulencia nestas cepas mutantes.

Consideramos que unha lección de maior importancia derivada dos nosos estudos da inestabilidade do pPDPT3 é o descubrimento de que, sorprendentemente, a existencia dos plásmidos que codifican para un T3SS pasou desapercibida durante as case cinco décadas de estudo deste importante patóxeno da acuicultura mariña. Todas estas observacións indican que a perda dos xenes do T3SS codificados no plásmido durante o illamento bacteriano e o seu subcultivo podería ter ocorrido de xeito sistemático nos laboratorios durante décadas e alertan de que a imaxe proxectada respecto ó contido xenético da virulencia de *Pdp* foi, historicamente, infraestimada e sesgada.

O nosos resultados, aínda non publicados, do estudo do plásmido pPDPT3 que codifica o T3SS en *Pdp*, da súa gran inestabilidade e do seu papel na virulencia, preséntanse neste traballo como un artigo de investigación preparado para o seu envío a publicar, que leva por título "**A highly unstable plasmid encoding the type III secretion system contributes to virulence of *Photobacterium damselae* subsp. *piscicida* for fish**".

SUMMARY

The family *Vibrionaceae* is one of the most common bacterial families spread in water bodies, especially marine ones. This family includes bacterial species that cause diseases for many different animals, including fish, crustaceans, molluscs and cetaceans among others. In addition, some species of the family *Vibrionaceae* can be transmitted to humans through wounds or through undercooked seafood, which causes death in some cases if not treated quickly. *Photobacterium damselae*, is a marine pathogenic species of the family *Vibrionaceae,* and includes two distinct subspecies, *damselae* and *piscicida*. The subsp. *damselae* is considered a non-clonal and generalist pathogen for diverse species of marine animals and even for humans, while subsp. *piscicida* is a more specialized pathogen and only infects fish. The two subspecies constitute important pathogens for marine aquaculture, causing disease outbreaks and financial losses in aquaculture facilities worldwide. A considerable divergence in the lifestyles of the two *Photobacterium damselae* subspecies has been largely established in previous studies. Such divergence can be understood in the framework of the acquisition of distinct virulence factors by horizontal gene transfer in each subspecies and of the massive proliferation of insertion sequences in *piscicida* subspecies in particular, that has likely led to a host-dependent lifestyle in the latter subspecies. Each of the two subspecies differ in many metabolic and morphological characteristics such as optimum growth temperature, growth rate, motility, host type, duration of stay in water, places of isolation, virulence factors, and enzymes content, even though they are considered to be highly related and similar in terms of overall DNA sequence relatedness.

This thesis has been structured in two parts. The first part deals with the study of genetic diversity within the subspecies *damselae*,

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focusing on the characterization of the accessory genome with attention to antimicrobial resistance plasmids and to metabolic genes that exhibit differential presence among strains of this subspecies. The second part of the present thesis deals with the genetic analysis of subspecies *piscicida*, and is majorly focused on the study of a novel, hitherto uncharacterized virulence plasmid that encodes a Type III secretion system.

Study of the accessory genome in *P. damselae* subsp. *damselae* (*Pdd*)

The study of plasmids and other mobile elements that carry antimicrobial resistance genes in *Pdd* was very scarce when this thesis was initiated. Indeed, our knowledge about this topic was reduced to few studies on *Pdd* isolates from China and Japan. In this thesis we tackled the study of a collection of *Pdd* isolates from disease outbreaks in sea bass farms in the Black Sea in Turkey, that exhibited resistance to several antimicrobial agents. We carried out an extensive genetic study that included the complete genome sequencing of a selection of six *Pdd* isolates, the study of the antimicrobial resistances carried on plasmids, and the ability of this family of plasmids to be transferred by conjugation to other bacteria. This study led to the publication of a research article entitled "**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**" (*Microbial Ecology,* 2020. 80:507-518). In this study, we found that multidrug resistant (MDR) isolates of *Pdd* contain large conjugative plasmids that share sequence backbone modules with plasmids of the pAQU-family. This family includes plasmids related to pAQU1, the first described plasmid of this group, and they have been hitherto reported exclusively in China and Japan. Therefore, our study has contributed

the first description of this plasmid group in *Pdd* isolates in the Mediterranean basin. We identified four novel pAQU-group variants of plasmids in the present thesis work, and each variant contained a unique combination of resistance genes, which include *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), *dfrA* (encoding a dihydrofolate reductase for trimethoprim resistance) and *strAB* genes that confer streptomycin resistance. Notably, we demonstrated that these plasmids could be transferred to *Escherichia coli* and to pathogenic *P. damselae* subsp. *damselae* and subsp. *piscicida* strains, at a very high rate. As an example, a plasmid of 170,998 bp characterized in our study and which we dubbed pPHDD2-OG2, was transferred to a recipient bacterial strain at 2.2×10^{-2} transconjugants per donor cell. After being acquired by a recipient *E. coli*, pPHDD2-OG2 conferred reduced susceptibility to all the antimicrobials for which a putative resistance gene was borne in the plasmid. A comparative genomics analysis of host strains genomes was carried out, and results suggested that the MDR plasmids of the pAQU-family have been acquired by different genetic lineages of *Pdd*. This study has thus provided evidence that *Pdd* isolated from diseased fish constitute a reservoir for conjugative MDR plasmids of the pAQU-family in the Mediterranean basin, and have the potential to spread to diverse bacterial species.

Continuing with the study of the genetic diversity of the accessory genome in *Pdd*, we next tackled the study of the genetic basis of sucrose utilization in this subspecies, an intriguing topic that so far had not received attention. Historically, carbon source utilization patterns have been extensively used to differentiate species within the family *Vibrionaceae*. Most notably, the thiosulfate citrate bile-salt

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sucrose (TCBS) agar constitutes a classical and standard culture medium used for the selective isolation of species of the family *Vibrionaceae*. TCBS contains 2% sucrose and pH indicators, and thus allows the differentiation between sucrose fermenters that produce yellow colonies (Scr$^+$ phenotype) and non-fermenters that produce green colonies (Scr$^-$ phenotype). During the past years, several studies reported that some subsp. *damselae* isolates formed yellow colonies on TCBS, thus indicative of sucrose fermentation, unlike most subsp. *damselae* isolates that are referred as green-colony formers in that medium. This observation prompted us to study the genetic basis of acquisition of this metabolic trait. The results of this study have produced a research article entitled: "**Diverse horizontally-acquired gene clusters confer sucrose utilization to different lineages of the marine pathogen *Photobacterium damselae* subsp. *damselae*.**" (*Genes*, 2020. 11, 1244). In this thesis, we have identified a total of eight Scr$^+$ *Pdd* isolates among our laboratory collection, and carried out their complete genome sequencing. The existence of a large number of genomes of Scr$^-$ *Pdd* strains in public databases (some of them sequenced in the present thesis, as part of the study of multidrug resistance plasmids as described above), allowed us to conduct an exhaustive study of comparative genomics among Scr$^+$ and Scr$^-$ *Pdd* genomes. This analyses unveiled the existence of two different versions of a four-gene cluster exclusive of Scr$^+$ isolates, which we dubbed *scr* cluster. This group of four genes encode a PTS system sucrose-specific IIBC component (*scrA* gene), a fructokinase (*scrK*), a sucrose-6-phosphate hydrolase (*scrB*), and a sucrose operon repressor (*scrR*). Of the two identified versions, one constituted the majority (occurred in 7 out of 8 Scr$^+$ strains), whereas the second version was found in a single isolate. The divergence between the two versions and the nucleotide sequence level was 30%, clearly suggesting that they constitute two phylogenetically distinct clusters. We next aimed at demonstrating that this cluster was necessary for sucrose utilization as

a carbon source, and it was found that a deletion mutant for *scrA* gene did not ferment sucrose and was impaired for growth with sucrose as carbon source. Interestingly, we found that the sucrose gene clusters (*scr* clusters) were located in a highly variable region of chromosome I. Indeed, the analysis of this region in the Scr$^-$ *Pdd* genomes revealed the presence of strain-specific gene combinations, suggesting that it represents a hot-spot for recombination of newly acquired DNA. We were also interested in deciphering whether the sucrose gene clusters followed the phylogeny of the *Photobacterium* genus, or whether they had been likely acquired by horizontal transfer. Surprisingly, when we compared the phylogenetic trees constructed with sequences of *scr* clusters, and the trees constructed with sequences of housekeeping genes, we found that the *Pdd scr* clusters were likely acquired from a *Vibrio*-like donor rather than from a *Photobacterium*-like donor species. This phylogenetic analysis also unveiled that the phylogenies of *scr* clusters among *Vibrio* and *Photobacterium* species did not exactly follow the phylogeny of the species, indicating that horizontal gene transfer of sucrose gene clusters has been extensive in these two genera.

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Study of the accessory genome in *P. damselae* subsp. *piscicida (Pdp)*

In the second part of this thesis, presence of differentially-occurring virulence factors was tracked through genomes and strain collections of *Pdp*, and a comprehensive survey study of virulence factors was made. In addition, our study unveiled for the first time the role of genes of the type three secretion system (T3SS) in *P. damselae* subsp. *piscicida* pathogenesis for fish.

Initially, our study of the variable genome in *Pdp* took advantage of a previous study that reported the existence of a large putative

plasmid encoding a T3SS in the type strain of the sibling subspecies, *Pdd*. So far, this secretion system had never been described as such in the subspecies piscicida, and we wondered whether some isolates might encode this potential virulence factor. The T3SS is a molecular device similar to a syringe that injects bacterial toxins, named effectors, into host cells. We started by designing some primer pairs for PCR screening of presence of genes of the T3SS in *Pdp* isolates, and this preliminary study already unveiled the existence of T3SS genes in a number of *Pdp* strains. Then we decided to submit two selected *Pdp* strains for complete genome sequencing, and such analysis disclosed the presence of T3SS genes in two strains, one isolated in Spain and the other in Japan. This study was published in the article entitled **"Draft genome sequences of *Photobacterium damselae* subsp. *piscicida* SNW-8.1 and PP3, two fish-isolated strains containing a type III secretion system."** (*Microbiology Resource Announcements*, 2019. 8(21): e00426-19). T3SS genes had not previously been listed as a virulence factor in *Pdp*. These results prompted us to analyse the presence of these genes in a large and comprehensive collection of *Pdp* isolates. This aim was accomplished in the second published study about the variable genome of *Pdp* in this thesis, that was entitled **"A virulence gene typing scheme for *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish photobacteriosis, reveals a high prevalence of plasmid-encoded virulence factors and of type III secretion system genes."** (*Aquaculture*, 2020. 521:735057).

In view of the many previous studies related to *Pdp* isolates, there were not sufficient surveys that tracked the prevalence of virulence factors in large collections of this pathogen, from different geographical locations and different hosts. In this study, we designed specific PCR amplifications to detect selected virulence genes in 103 *Pdp* isolates from different geographical regions isolated within a long

period of time. These amplifications were targeted to tracking: (1) the presence of *aip56* gene borne in pPHD10 plasmid, that encodes the apoptosis-inducing toxin AIP56; (2) presence of pPHD70 plasmid by targeting *irp2* and *frpA* genes which are part of the siderophore piscibactin iron-acquisition system; and (3) presence of two gene markers of the aforementioned T3SS that was reported for the first time in the two *Pdp* isolates SNW-8.1 and PP3, as mentioned above. Our results indicated that pPHDP10 plasmid was present in 97 isolates out of 103, providing strong evidence of the high stability of this plasmid, which was considered the main virulence factor of *Pdp* described to date. Of note, this screening revealed a total of 91 isolates harbouring pPHDP70 plasmid, of which 89 were European isolates and two were isolated in Japan. Consequently, this results demonstrated for the first time that this plasmid is not restricted to European isolates, as previous studies had suggested. Most interestingly, this extensive gene survey unveiled that the genes belonging to the T3SS were present in 74 *Pdp* isolates, the oldest of which was isolated in Japan in 1980. This result can be considered as of utmost relevance, because *Pdp* infections in fish have been studied since as early as 1963 and, despite the widespread nature of T3SS genes among *Pdp* isolates, they were not previously recorded or tracked to the best of our knowledge. Interestingly, in this study we detected the loss of T3SS genes in what appeared, at first, to be a phenomenon induced by the addition of rifampicin during the process of constructing mutants for T3SS genes. Such initial observations clearly indicated the possibility that T3SS genes are carried on an unstable plasmid, as T3SS gene markers were spontaneously lost concomitantly with plasmid genes *parAB* and *traC*.

Therefore, we considered of outmost interest to continue the study of the T3SS genes, in particular in PP3 strain, in which the phenomenon of spontaneous gene loss was recorded. The first step to

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unveil the plasmid-like nature of the T3SS included the re-sequencing of the genome of *Pdp* strain PP3 using a PacBio approach, since this genome had been previously sequenced using an Illumina approach and it yielded a high number of contigs that prevented from stablishing the genetic context of the T3SS genes. The completion of PP3 genome sequencing by PacBio, unveiled that the T3SS genes are carried within a large plasmid of 133 kilobases, that we dubbed pPDPT3. Unexpectedly, the sequence of pPDPT3 included two paralogous copies, 100% identical at the nucleotide sequence level, of a set of 24 T3SS genes, whereas 12 additional T3SS-related genes existed in single copy. These two paralogous copies were arranged in a mirror-like structure. The rest of the plasmid sequence exhibited a high content of transposase genes (insertion elements, *IS*), suggesting that the T3SS gene region eluded *IS*-mediated gene inactivation because this system likely plays a role in virulence for the fish host.

pPDPT3 sequence contained the *parAB* genes putatively involved in plasmid partition after replication. However, much surprisingly, the plasmid did not encode an evident *rep* protein, which means that the replication mechanisms used by this plasmid is so far unknown. Other interesting features of pPDPT3 include the presence of a gene for a putative YadA-like adhesin and for a putative transcriptional regulator ExsA. Sequence analysis of pPDPT3 revealed the presence of a 15 kb region containing 11 predicted genes *traALEKBVC, trbI and traWDI*. This module of genes for conjugative transfer functions is divided into three submodules by insertion sequence (*IS*) elements. The *IS1* element was found in two copies in two different sites between *tra* genes, namely downstream *traI* and upstream *traD* gene. The *IS3* element is located between *traC* and *traV* genes. Five genes encoding hypothetical proteins are interspersed among *trbI* and *traW* genes. We conducted a comparative sequence analysis between pPDPT3 transfer region genes and homologous regions previously sequenced in 4

plasmids in other *Photobacterium damselae* strains, covering the two subspecies. This comparative analysis showed an almost exact match between pPDPT3 and a putative plasmid in *Pdp* strain OT-51443, that also contains genes for a T3SS. Notably, the *tra* region in these two *Pdp* plasmids is much shorter than the homologous *tra* region of three different plasmids of *Pdd* strains, clearly suggesting that the two *Pdp* plasmids contain an incomplete set of conjugative transfer genes. Indeed, the plasmid comparison provides evidence that there is a loss of conjugation genes in the region between *traD* and *traW* genes, which is represented by the absence of *traGHFNU* and *trbBC* genes in pPDPT3 and also in the homologous plasmid of *Pdp* OT-51443. Our attempts to demonstrate mobilization of labelled versions of pPDPT3 to a recipient strain in conjugative matings yielded negative results. However, as explained below, we further gained evidence that all the labelled versions of pPDPT3 containing antibiotic resistance gene cassettes, systematically underwent integration into the host bacterial chromosome. Therefore, the conjugative nature of pPDPT3 will need further investigation.

The functional study of the genes carried on this plasmid started to be full of difficulties, due to the high instability of the plasmid. Indeed, one of the most unexpected findings of our study was the confirmation that pPDPT3 can be curated (lost from the host cell) as fast as upon a single subculture on growth medium in laboratory conditions. Indeed, we demonstrate in this thesis work that plasmid loss in culture was not a characteristic unique to *Pdp* strain PP3, since spontaneous loss of T3SS was recorded for other *Pdp* strains isolated from different geographical locations and with many years of isolation lapse in between. This instability prompted us to design a strategy to construct T3SS gene mutants by replacing the target gene with an antibiotic resistance gene. Hence, such mutation would involve the integration of a resistance cassette into the plasmid structure, so that

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addition of the respective antibiotic during all stages of mutant construction would guarantee the stability of pPDPT3 into the cell. As a result, in the present thesis work we generated two distinct insertional mutants in the T3SS gene *vcrD* (also dubbed *escV* in other species). One single mutant (in what we dubbed *vcrD*^{1} allele) contained a chloramphenicol (*cat*) gene substituting most of the *vcrD* gene sequence, whereas the second type of single mutant contained the *cat* gene substituting the sequence of the other allele, dubbed *vcrD*^{2}. In addition, using the *cat*-labelled mutant in *vcrD*^{1} as a basis, we mutated *vcrD*^{2} by inserting within it a kanamycin resistance gene (*kan*) to generate a double mutant *vcrD*^{1}-*vcrD*^{2}.

The unsuspected results did not take long to appear. Fish experimental inoculations with the PP3 parental strain, with all the different mutants, and with the spontaneously pPDPT3-curated strain, yielded unexpected results. First, and as it might be expected, the plasmidless, pPDPT3-negative strain was strongly impaired in virulence for sea bass fish compared to the parental strain. However, raising the inoculated bacterial dose 100×, the pPDPT3-negative strain was able to cause 100% mortality. This result fits within the expected situation considering that the pPDPT3-negative strain still produced other virulence factors, and indeed, fish mortality in experimental infections was routinely achieved in previous studies that now we know they used T3SS-negative strains. However, the unexpected results came when we observed that the single and double *vcrD* mutants did not cause any fish mortality at the higher dose (100×). Subsequent studies demonstrated our inability, despite reiterated attempts, to cure pPDPT3 plasmid from any of these mutants. Indeed, both the single and double mutants seemed to contain pPDPT3 plasmid integrated within the host cell chromosome, and our working hypothesis is that this plasmid integration occurs through disrupting a gene necessary for virulence and/or fitness in vivo.

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Altogether, our results demonstrate that loss of pPDPT3 causes an impairment of virulence of *Pdp* for fish, although at higher infection doses the pPDPT3-negative bacteria can still cause mortality. The suspect that T3SS mutants labelled with antibiotic resistance cassettes contain this plasmid integrated into the bacterial genome, prevents us for the moment to attribute the T3SS an undeniable role in virulence. These T3SS mutants might become nonvirulent either due to T3SS mutation, or due to the disruption of essential genes for virulence upon chromosomal integration. Further studies are prompted to disclose the genetic basis of virulence attenuation in these mutant strains.

We consider that a major lesson derived from our results on the instability of pPDPT3, is the realization that for almost five decades of study of this important pathogen of marine aquaculture, the existence of plasmids encoding a T3SS has remained astonishingly elusive. All these observations indicate that the loss of plasmid-encoded T3SS genes during bacterial isolation and subculture may have occurred systematically in laboratories for decades, and warn that the picture of *Pdp* virulence gene content has been historically biased and underestimated.

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The yet-unpublished results of our study on pPDPT3 plasmid encoding the T3SS in *Pdp*, its high instability and its role in virulence, are here presented in the form of a ready-to-submit research article entitled "**A highly unstable plasmid encoding the type III secretion system contributes to virulence of *Photobacterium damselae* subsp. *piscicida* for fish**".

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INTRODUCTION

1. INTRODUCTION

1.1 THE ROLE OF AQUACULTURE IN RELATED LIFESTYLES AND ECOSYSTEMS

Aquaculture systems, as reservoirs for many different organisms, constitute miniature environmental ecosystems providing opportunities for humans to increase the growth of marine organisms independently and to study the components of these systems, and have a great impact on large aquatic environments such as oceans, seas, and lakes. Food supply is one of the most worrisome challenges in the future of humanity, especially with a large increase in the world's population (Goddard and Al-Abri, 2019). The steady increase in population growth numbers has created pressure on vital resources by consuming them quickly and making the process of controlling these resources very difficult (Chen et al., 2016). Hence, the pursuit of sustaining vital resources and trying to keep pace with the depletion of vital resources by preparing plans and working to increase food production in a way that does not harm nature, such as the aquaculture and fish ponds.

Attention to many aspects that include food supply in places of aquaculture are represented in several things namely, enhancing the production of desired species with high quality, contributing to increasing production through attention to designs and construction, attracting the most qualified administrators, seeking to combat pollution, diseases and parasites, as well as contributing to improving

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forage and food in the aquaculture (Fisheries, 2010). The hunger rate increased from 873 million to slightly more than one billion people between 2006 and 2009, and then it decreased again in 2010 to 925 million, along with the global economic recovery (Fan, 2010; Senker, 2011). Depending on this economic imbalance, several mechanisms have been conducted to produce and distribute enough food to an expected global population of 9 billion in 2050, a major concern of development policy (Allison, 2011).

Aquaculture plays an important role in food security supply and economic balance (income), especially in many developing countries, by interfering with many other economic sectors, such as crop cultivation and livestock rearing (Ahmed and Lorica, 2002). This is in addition to being an economic multiplier in marginal rural areas. Countries with natural fisheries or possessing conditions conducive to the development of aquaculture can also make important contributions to the national economy through trade, increased exports, tax revenues, and customs duties (Allison, 2011; Harper et al., 2013).

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Many types of aquaculture can be classified in different ways. One way is to describe the location of the aquaculture facilities beside a freshwater or marine water body, either by the type cultivation of kinds of seafood, fishes, algae, or the method that is used to get complete mixed nutrients of different fish rest. Based on the above methods, aquaculture can be divided into mariculture, fish farming, algaculture, and integrated multitrophic aquaculture. Aquaculture also plays a crucial role to protect biodiversity by reducing the fishing activities on wild stocks in ecosystems and at the same time, allows farmers to increase wild species stocks in fish farms and allow the wild populations to revitalize (Beardmore et al., 1997). On the other hand, many studies have shown the importance of aquaculture seafood such as fish, shellfish, and seaweed in immunity responses, reducing obesity, improvement of mental abilities, decreasing the nervous

system diseases, and diabetes as well (Hosomi et al., 2012; Nkondjock and Receveur, 2003). This demonstrates the growing demand, according to the annual reports of the Food and Agriculture Organization (FAO), for fish and seafood and the huge consumption of these resources. Therefore, aquaculture appears to fulfil the quantities required by consumers. The intersection of the economic factor with the objectives of establishing aquaculture, a food security valve, has changed management protocols in the aquaculture so that it helps to reduce the cost of production and speed up the production process and also includes maximizing food conversion, and reducing water, energy, processing and storage costs.

Management of aquaculture has faced numerous challenges to obtain large quantities at the lowest costs such as food, water quality, and population growth. The fish growth rate makes food management difficult, especially in extensive fish growth when the fish hatch and there is a lack of ensuring access to food to all fish at the same time. Water is also the main factor that carries all the elements of aquaculture, which must have a constant temperature, a suitable pH, and high oxygen content. This is reflected in the fish growth, which in turn makes it more difficult in a situation of extensive growth (Allen and Steeby, 2012).

The difficulty in managing the extensive growth of fish in aquaculture in terms of food access, water quality, and the lack of control over the number of fish produced permanently contributed to the unintended emergence of diseases. The spread of diseases in aquaculture is easier than in any other place because of the presence of water as an environment for living and as a carrier of all components of aquaculture. In aquaculture, diseases are transmitted vertically through the parents to progeny or horizontally, through direct contact with the affected fish or surrounding water, which makes disease

control more difficult (Manin and Ransangan, 2011; Pradeep et al., 2016).

The emergence of marine diseases caused by parasites or pathogens has led to a significant change in the composition of the aquatic ecosystem in the recent years. This results in the transmission of the diseases to other living organisms, whether terrestrial or aquatic, especially those considered wild type stock that are used in aquaculture (Ward and Lafferty, 2004). As it happened when the crayfish plague was transferred to Europe from the United States, which resulted in the eradication of the original crayfish from most of Europe (Alderman, 1996).

The emergence of diseases in aquaculture has caused huge financial losses which negatively affected the expansion of production and the preservation of high-quality species (Murray and Peeler, 2005). Spread of some diseases in aquaculture proved to be difficult to control, such as sea lice in Scottish Atlantic-salmon farms (Pike and Wadsworth, 1999), amoebic gill disease (Nowak et al., 2002), furunculosis (McIntosh and Austin, 1993), infectious pancreatic necrosis (Hill, 1982), and infectious salmon anemia (Anon, 2000), among others. The prevalence of diseases in aquaculture depends on several factors, including the population of fish (extensive populations), fish immunity, water flow rate, fish stress (stressful fish more susceptible to disease), and the virulence of the pathogen (Reno, 1998). Control of diseases in aquaculture has brought us back to the review of the aquaculture management methods through the above-mentioned factors and also work to increase fish protection against pathogens using antibiotics, disinfectants, and anthelmintic agents (Romero et al., 2012). The use of antibiotics in aquaculture came after taking into account measures used to reduce disease such as reducing stress, managing water flow, and preventing transmission of infection to wild species. Antibiotics are given to fish in several ways, including

oral (in feed) treatment, which is the preferred method, immersion therapy, and injection (Inglis, 2000). Also, some measures must be taken into consideration to reduce the chances of disease transmission in aquaculture systems such as rapid removal of sick fish or dead stock, rotation of treatment plans to prevent the development of resistance in the pathogen, low stocking density, and regulation of the fish movement between fish farms (Murray and Peeler, 2005).

1.2 ANTIBIOTIC RESISTANCE GENES EMERGENCE, SPREAD, AND CONCERNS

The intersection between marine organisms and pathogens in aquaculture has led to the emergence of diseases, especially in intensive aquaculture. The outbreaks of diseases in aquaculture and fish farms have caused severe financial losses as well as a breach of one of the most important sources of food supply. This has forced those in charge of aquaculture facilities to improve vaccines and use antibiotics to control these diseases and reduce their severity (Defoirdt et al., 2011). Also, in some instances these diseases can be transmitted to humans, causing death in some cases, which makes it all the more important.

Several attempts were made to use chemotherapy in aquaculture diseases for a long time before it entered in force during the 1970s (Inglis, 2000). The use of antibiotics in aquaculture was accompanied by many public health concerns, which led to the establishment of strict regulations to control the use of antibiotics (Defoirdt et al., 2011). As previously mentioned, adding antibiotics with food or in water directly in aquaculture is considered the ideal method among other methods. This method promotes the delivery of antibiotics to all targeted fish and other aquatic living beings equally. Besides, this method involves a wide environmental application that affects a wide

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range of bacteria. Intestinal tracts of healthy fish include microbiota that plays an important role in controlling diseases, innate immune responses, and the promotion of nutrient metabolism in fish (Irianto and Austin, 2002; Llewellyn et al., 2014). As said above, the use of antibiotics in aquaculture does not distinguish between pathogenic and beneficial bacteria, and this constitutes a major impediment to the use of antibiotics (Marshall and Levy, 2011).

The use of antibiotics in aquaculture has been accompanied by many fears on the emergence of resistance in the target bacteria, according to some reports indicating that this has occurred outside of aquaculture (Hamilton-Miller, 1990; Prescott and Baggot, 1988). The efficacy of antibiotics such as quinoline, oxolinic acid, in controlling furunculosis in Europe was praised in 1983 (Austin et al., 1983), and as well as it was used in Scotland effectively against the furunculosis, but later the antibacterial resistance appeared and failed to respond to this therapy in 1987 (Hastings and McKay, 1987). Furthermore, 40-50% of *Aeromonas salmonicida* isolates in Scotland during the 1990s showed the resistance of bacteria for antibiotic therapy (Inglis et al., 1991). In Japan, amoxycillin has been widely used in the treatment of pasteurellosis in yellowtail, but in 1982 bacterial resistance to the antibiotic appeared (Inglis, 2000). The isolates of *A. salmonicida* in the United Kingdom also indicated sensitivity to amoxycillin during the period 1988-1990 (Barnes et al., 1994; Inglis et al., 1991), but three years later, Inglis and colleagues indicated that the drug was ineffective in furunculosis treatment and bacterial resistance appeared (Inglis et al., 1993). The emergence of bacterial resistance to antibiotics varies depending on the time required for resistance, from one species to another, the geographical area, and how antibiotics are used in aquaculture (Inglis, 2000).

The ability of bacteria to live in harsh conditions and under pressure reflects an improvement in their fitness to deal with these

conditions, especially antibiotics. On the one hand, bacteria can resist the effect of antibiotics either because the antibiotic is unable to enter the bacterial cell or the target of the antibiotic does not exist within the bacterial cell (Romero et al., 2012). On the other hand, bacteria can obtain mobile genetic elements, such as plasmids and transmissible elements, between bacteria in their surroundings. Hence, bacteria can obtain resistance genes by acquiring them in a number of ways, such as transformation, transduction, and conjugation (Romero et al., 2012).

Acquired genes by bacteria to resist antibiotics can be in the form of efflux pumps that work to excrete the antibiotic out of the bacterial cell before it reaches the target (Van Bambeke et al., 2000). It is also able to acquire antibiotic-breaking enzymes such as beta-lactamases (break of penicillin) or enzymes that inhibit antibiotic activity such as phosphorylation (antibiotic-inactivating enzymatic reactions) (Kumarasamy et al., 2010; Romero et al., 2012). Moreover, bacterial populations may acquire resistance to antimicrobial agents by mutation or by obtaining genetic information that encodes resistance from other bacteria.

Horizontal gene transfer plays a crucial role to acquire resistance to multiple classes of antibiotics in bacteria, which led to acquiring more than three resistance genes (multidrug resistance) in some species of bacteria (Von Wintersdorff et al., 2016). Bacteria containing multidrug resistance genes (MDR) will become difficult to control. In aquaculture, the use of various antibiotics has led to the emergence of MDR in pathogenic bacteria. Romero et al (Romero et al., 2012), indicated the possibility of generating MDR in bacteria by successive accumulation of multiple genes in a single cell, which ultimately leads to the creation of plasmids that contain many resistance genes, and by the increase in the number of multidrug efflux pumps or by increasing the expression of the genes that code

the multidrug efflux pumps, thus leading to drug resistance. The antibiotic-resistant genes described in aquaculture have been observed to be regularly located on mobile genetic elements. Resistance genes have also been identified in transferable plasmids in some pathogenic bacteria such as *Aeromonas spp., Edwardsiella spp.,* and *Vibrio spp.* (Defoirdt et al., 2011). *Vibrio harveyi* strains bearing multiple resistance genes for cotrimoxazole, chloramphenicol, erythromycin, and streptomycin caused mass mortality in *Penaeus monodon* larvae (Karunasagar et al., 1994).

The plasmid pAQU1, which contains numerous resistance genes (MDR), was discovered for the first time in a tetracycline-resistant *Photobacterium damselae* subsp. *damselae* (*Pdd*) strain isolated from an aquaculture site in Japan. This plasmid harbored many resistance genes such as *bla*CARB-9-like, *floR*, *mphA*-like, *mefA*-like, *sul2*, *tetM* and *tetB*. It also showed the ability to transfer to other species of bacteria such as *Escherichia coli* by conjugation (Nonaka et al., 2012).

Studies of resistance genes present in pAQU plasmids have prompted researchers to assume that the overuse of antibiotics in aquaculture will increase the diversity of pAQU plasmids, increase the introduction of resistance genes using mobile elements, and the potential for widespread of pAQU plasmids to human pathogens (Li et al., 2017; Nonaka et al., 2014). Nowadays, numerous studies related to the use of antibiotics in aquaculture advocate the need to diminish the level of stress on fish and reduce the possibility of infection that requires treatment with antibiotics. Moreover, some alternative solutions to antibiotics, such as probiotics, have been introduced to control bacterial infections in aquaculture, phage therapy and essential oils (Irianto and Austin, 2002).

1.3 *PHOTOBACTERIUN DAMSELAE* INCLUDES TWO SUBSPECIES THAT ARE RELATED TO FISH DISEASE AND HAVE DIFFERENT LIFESTYLES

The sudden outbreak of diseases in aquaculture, especially caused by *Vibrio* spp, is one of the problems affecting the economic sector with huge financial losses annually in aquaculture worldwide (Dadar et al., 2017; Toranzo et al., 2005). Two of the most frequently isolated species of *Vibrio* spp are *V. harveyi* and *V. anguillarum*, which have been associated with many diseases in aquaculture such as losses of larval and juvenile and several opportunistic diseases to fishes (Chatterjee and Haldar, 2012). An important species belonging also to the *Vibrionaceae* family is *Photobacterium damselae*, which causes severe diseases in aquaculture. *Photobacterium damselae* is a pathogenic marine bacterium that includes two distinct subspecies, *piscicida* and *damselae*. The subspecies *damselae* is a pathogen for a wide variety of homeotherm and poikilotherm animals, while the subsp. *piscicida* is a pathogen exclusively of fish (Rivas et al., 2013b; Osorio, 2019). *Photobacterium damselae* subsp. *damselae* (hereafter *Pdd*) and *Photobacterium damselae* subsp. *piscicida* (hereafter *Pdp*) are the most widespread species of the *Photobacterium* genus causing disease in aquaculture systems. The acquisition of different virulence factors by horizontal gene transfer in the two *P. damselae* subspecies plays a significant role in shaping their lifestyles. Besides, the massive presence of insertion sequences (ISs) in the *Pdp* genome and in consequence, the high number of gene deletions and pseudogenes, has contributed to a host-adapted lifestyle (Osorio, 2019). Next, we will describe each of the two *P. damselae* subspecies separately to illustrate some of their different virulence strategies.

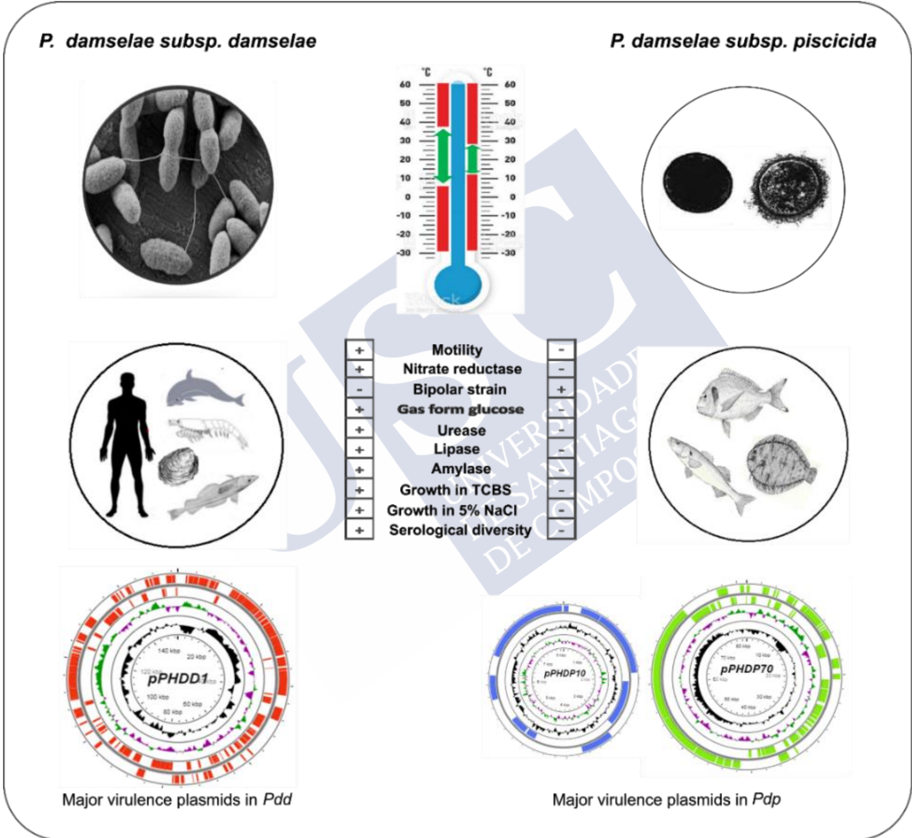

Figure 1.1 Schematic comparison between *damselae* and *piscicida* subspecies.

1.3.1 *Photobacterium damselae* subsp. *damselae* (*Pdd*)

1.3.1.1 Taxonomical position of *Pdd*

The marine bacterium *Photobacterium damselae* currently includes two subspecies, namely subsp. *damselae* and subsp. *piscicida*. However, these two subspecies have undergone numerous taxonomical changes in the past, since they were originally named *Vibrio damsela* and *Pasteurella piscicida*, respectively. In order to understand the story behind their current taxonomical placement, it is necessary to go through the changes in nomenclature undergone by these microorganisms.

Photobacterium damselae subsp. *damselae* (*Pdd*) (formerly *Vibrio damsela*) was described during the time period from 1981 to 1997 by several changed names until it reached its current name. It was isolated for the first time in 1971 causing infection in humans, and was described as an "unnamed marine *Vibrio*" (Morris et al., 1982). A few years later, a microorganism with very similar characteristics was isolated from skin ulcers in natural populations of damselfish (*Chromis punctipinnis*) in California, being dubbed *Vibrio damsela* based on the sharing of some general characteristics with other *Vibrio* species, and it was deposited in American Type Culture Collection after it was identified by the Centers for Disease Control (Atlanta, USA) at that time (Love et al., 1981). Building on the evolutionary trees created by comparing the sequences of rRNA genes, MacDonell & Colwell proposed a restructuring of the family *Vibrionaceae* by creating two new genera (*Listonella* and *Shewanella*) and the change of *V. damsela* to *Listonella* genus to become *Listonella damsela* (MacDonell and Colwell, 1985). In 1991, *L. damsela* taxonomical position was reassessed by *Smith et al* on the basis of a comparison of phenotypic characteristics in addition to some

distinctive features of *Photobacterium* species such as the absence of a flagellar sheath, accumulation of poly-P-hydroxybutyrate during growth on glucose, and inability to utilize DL-P-hydroxybutyrate as a sole carbon source and as a result, *L. damsela* strains were added to the genus *Photobacterium* to be *Photobacterium damsela* (Smith et al., 1991). Thereafter, the reclassification and description of the formerly known *Pasteurella piscicida* in the genus *Photobacterium*, and the evidence that these two bacterial entities (*Photobacterium damsela* and *Pasteurella piscicida*) shared a high percentage of DNA-DNA relatedness, led to the necessity of creating two new subspecies within the *P. damsela* species, so that the name of the previously known as *P. damsela* would change to *P. damsela* subsp. *damsela* (Gauthier et al., 1995), which differed in some attributes with *P. damsela* subsp. *piscicida* such as the presence of a flagellum and urease and nitrate reductase activities. After a revision of *Photobacterium* species names in 1997, based on Rule 12c of the International Code of Classification of Bacteria, *P. damsela* subsp. *damsela* name was corrected to *P. damselae* subsp. *damselae* to conform with rules of the International Code of Nomenclature of Bacteria (Trůper and De'clari, 1997).

1.3.1.2 The emergence of *Pdd*-related disease

Pdd is a marine bacterium that thrives in coastal marine ecosystems, and it has been isolated from sea water, oysters, mussels, seafood, and from apparently healthy marine animals (Grimes et al., 1985; Ortigosa et al., 1989; Buck et al., 2006; Richards et al., 2008; Serracca et al., 2011; Chiu et al., 2013). However, this bacterium is best known for its potential to cause infections and fatal diseases in a wide range of marine animals including fish, crustaceans, and molluscs, and is also capable of causing fatal disease in humans (Rivas et al., 2013b). In 1971, the emergence of symptoms associated

with a bacterial infection in a 41-year-old man after being injured in his foot while in the Gulf of Mexico 18 hours before, necessitated treatment with antibiotics appropriate for these symptoms. Thereafter, a bacterial swab was taken from the wound and cultured to be described later as belonging to the *Vibrio* family without giving it a specific name at that time (Morris JR et al., 1982). Based on a personal communication, *Love et al* determined the beginning of the observation of the appearance of skin ulcers in damselfish (*Chromis punctipinnis*) in King Harbor, Redondo Beach, California from August through October, and from June through August off Ship Rock, Catalina Island (Love et al., 1981). In 1981, *Love* and colleagues described the *skin ulcers disease* caused by *V. damsela* as having several symptoms and how it appeared on the fish skin (Love et al., 1981). Not only did the disease appear in fish, but *Love et al* also reported that two human wounds were infected by *V. damsela* and might be a cause of human disease (Love et al., 1981). *V. damsela* was again isolated from six wound infections during the period between 1970 to 1981 from people between the ages of 26 and 55 (Morris JR et al., 1982). In addition, *V. damsela* was isolated associated with a fatal wound infection in humans in 1985 (Clarridge and Zighelboim-Daum, 1985). Later, it was reported a severe and rapid progression of infection caused by *V. damsela*, which required amputation of the right arm within 36 hours after the patient was injured by a puncture from a fish fin (Coffey et al., 1986). In 1993, a pathological condition caused by *V. damsela* after injury by a rabbitfish forced doctors to a radical surgical intervention because the patient did not respond to antibiotics (Yuen et al., 1993), and *V. damsela* was isolated to the first time from both blood and wound of a 70-year-old patient infected by a hunting knife who died later (Perez-Tirse et al., 1993). Thereafter, there were several cases of human infection by *V. damsela* (already dubbed as *Pdd* in most studies) during the years 1996, 1997, and 2000, two of them resulting in death (Fraser et al., 1997; Shin et al.,

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1996), while the other resulted in septicemia and the appearance of skin ulcers, which was subsequently treated (Barber and Swygert, 2000). The reports on human infections caused by *Pdd* continued until present, and indeed three recent cases of human infection with their primary origin in the European continent have been recently reported (Chochlakis et al., 2019; Guimaraes et al., 2020; Schröttner et al., 2020).

Although the first isolations of *Pdd* corresponded to human clinical cases, year after year the diversity of animal species infected by this pathogen was increasing. Thus, *Pdd* has been subsequently isolated from brown shark (*Carcharhinus plumbeus*) (Colwell and Grimes, 1984; Grimes et al., 1984a; Grimes et al., 1984b), octopuses (Hanlon et al., 1984), leatherback turtles (*Dermochelys coriacea*) (Obendorf et al., 1987; Oliver-Guimerá et al., 2019), Bryde's whale (*Balaenoptera edeni*) (Buck et al., 1991), bottlenose dolphins (*Tursiops truncatus*) (Fujioka et al., 1988), stingrays (*Dasyatis pastinaca*), and nurse shark (*Orectolobus ornatus*) (Pedersen et al., 1997) among others.

Of note, *Pdd* is pathogenic for a large diversity of fish and crustacean species of financial importance in marine aquaculture, causing infections and hemorrhagic septicaemia. The list of fish species reported to be affected by *Pdd* is extensive. Some examples include yellowtail (*Seriola quinqueradiata*) (Sakata, 1989), turbot (*Scophthalmus maximus*) (Fouz et al., 1992; Fouz et al., 1991), sea bream (*Sparus aurata*) (Vera, 1991), eel (*Anguila reinhardtii*) (Ketterer and Eaves, 1992), barramundi (*Lates calcarifer)* (Renault et al., 1994), rainbow trout (*Oncorhynchus mykiss*) (Pedersen et al., 1997; Pedersen et al., 2009), common dentex (*Dentex dentex*) (Company et al., 1999), ovate pompano (*Trachinotus ovatus*) (Zhao *et al.*, 2009), redbanded seabream (*Pagrus auriga*), white seabream

(*Diplodus sargus*) and meagre (*Argyrosomus regius*) (Labella *et al*., 2006; Labella et al., 2010a; Labella *et al*., 2010b), sea bass (*Dicentrarchus labrax*) (Uzun and Ogut, 2015; Essam et al., 2016), and Nile tilapia (*Oreochromis niloticus*) (El-Son et al., 2020). The number of reports of *Pdd* infections in cultivated species of crustaceans is also increasing, and some examples include black tiger shrimp (*Penaeus monodon*) (Song et al., 1993; Vaseeharan et al., 2007), Pacific white shrimp (*Litopenaeus vannamei*) (Bachand et al., 2019; Singaravel et al., 2020; Wang et al., 2020), and mud crab (*Scylla paramamosain*) (Xie et al., 2020).

The notable increase in the diversity of species susceptible to infection by *Pdd*, and the expansion in the geographical area from which it has been reported in recent years, clearly suggests that *Pdd* can be considered as an emerging pathogen in marine aquaculture.

1.3.1.3 Phenotypic characteristics of *Pdd*

Numerous studies describing the phenotypic characteristics of *Pdd* during the last 4 decades have contributed to enrich the characterization reported in the original publication that described *Pdd* for the first time as *V. damsela* (Love et al., 1981). Important phenotypical features originally considered for identification of *Pdd* included, as general characteristics: Gram negative rod-shaped cells, motile, oxidase-positive, halophilic, facultative anaerobic, and grows well on thiosulfate citrate bile salts agar as 2- to 3-mm green colonies. Other features of utility to differentiate *Pdd* from other *Vibrio* species included the following: indole negative, urease positive, nitrate reduction to nitrite, methyl red- and Voges-Proskauer-positive, arginine dihydrolase (Moeller's)-positive, lysine and ornithine decarboxylase-negative, gas produced during fermentation of sugars, and only D-glucose, D-mannose and maltose are fermented (Love et

al., 1981). Subsequent studies reported variable results for phenotypical characteristics that were initially judged either fully positive or negative, demonstrating the existence of intrasubspecies diversity (Farmer et al., 1985; Fouz et al., 1992; Botella et al., 2002; Terceti et al., 2016). This diversity not only pertained to classical biochemical tests, but also to enzymatic activities with potential roles in virulence. Hence, it was demonstrated that a fraction of *Pdd* strains are able to hydrolyse gelatin but casein is not hydrolysed, and phospholipase phenotypes are variable depending on the presence of one (*plpV*) or two (*plpV* and *dly*) phospholipase-encoding genes (Vences et al., 2017). Similarly, the observation that *dly*-negative isolates still produced hemolysis of sheep erythrocytes (Osorio et al., 2000) lead to the further identification of additional hemolysins in this pathogen (Rivas et al., 2013). According to (Labella et al., 2010), the extracellular products (ECPs) in *Pdd* contain several enzymatic activities such as amylase, lipase, phospholipase, alkaline phosphatase, esterase lipase, acid phosphatase, and β-glucosaminidase that are associated with virulence, and for some of these activities the genetic basis still awaits investigation.

Of note, several phenotypical features that exhibit intra-subspecies heterogeneity remain unstudied at the genetic level. Among these, the initially described ability of *Pdd* to grow on TCBS forming green colonies was soon contradicted by reports of *Pdd* isolates that formed yellow colonies on TCBS indicative of fermentation of sucrose (Farmer et al., 1985). This tendency was increased in subsequent studies, that reported existence of sucrose-fermenting *Pdd* isolates (Thyssen et al., 1998; Botella et al., 2002; Zhao et al, 2009; Terceti et al., 2016; Terceti et al., 2018; Tao et al., 2018). However, the genetic basis of sucrose utilization in *Pdd* has never been studied and, indeed, the scarcity of information about the genetics of sucrose utilization is a generalized weak point in the whole

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family *Vibrionaceae*. In this regard, only some classical studies are available about sucrose utilization genes in *V. alginolyticus* (Scholle et al., 1987; Scholle et al., 1989; Blatch et al., 1990; Blatch and Woods, 1991), and some scattered studies have included some data on sucrose utilization genes in *V. cholerae* but in this last case sucrose genes were not the primary research topic of the publications (Houot et al., 2010; Wang et al., 2011; Hayes et al., 2017). Last, no studies are available about sucrose utilization genes in the genus *Photobacterium*. Hence, investigation of what is the genetic basis of sucrose utilization in the *Pdd* isolates that grow as yellow colonies on TCBS is prompted, and will constitute one of the objectives of the present thesis.

1.3.1.4 Pathological characteristics and virulence factors of *Pdd*

The occurrence of factors such as fish overpopulation, low dissolved oxygen and low water flow quality, negatively affect the immune system of aquatic organisms, and if these variables are accompanied by increases in the density of the pathogen in the environment, all together would enhance the opportunity of a specific pathogen to cause disease (Post, 1987). Reports on the isolation of *Pdd* from healthy hosts indicate the ability of *Pdd* to be present in the marine environment before the appearance of disease outbreaks (Buck et al., 2006; Lozano-León et al., 2003; Serracca et al., 2011). Moreover, increases in water temperature during warmer summer months contribute to providing the opportunity for *Pdd* to cause disease (Matanza and Osorio, 2018). Raised temperatures, coupled with increases of *Pdd* numbers, likely enhance the opportunity of the pathogen to penetrate the host skin and cause ulcers (Love et al., 1981). In addition to transmitting the disease through fish contact, the ability of *Pdd* to be present in seawater for a long time increases the chance of disease taking advantage of stressed host (Fouz et al.,1998;

Fouz et al., 2000). The production of different enzymatic activities by *Pdd* strains, especially sphingomyelinase enzymes and phospholipases, are expected to contribute to its pathogenic ability by adhesion and cell penetration, thus avoiding the host's immune system and increasing the chances to cause disease (Flores-Díaz et al., 2016; Rivas et al., 2015).

Typical signs of *Pdd* infection in fish include hemorrhaged areas and ulcerative lesions on the body surface (Love et al., 1981; Fouz et al., 1995). In humans, infections have their primary origin in wounds, which are followed by bullae formation and marked edema. Infection may progress into a necrotizing fasciitis with multiple organ failure, leading to a fatal outcome (Fraser et al., 1997; Yamane et al., 2004).

The first studies aimed at the identification of the molecular basis of virulence in *Pdd* (in those times still known as *V. damsela*) detected the production of high amounts of a cytolytic toxin by some *Pdd* strains, and such strains were more pathogenic for mice than the strains that did not produce such toxin (Kreger, 1984). Subsequent studies reported that this toxin is a phospholipase D with the capacity to cleave sphingomyelin, removing choline phosphate head groups in the plasma membrane of host cells. The toxin was named damselysin (Dly), it was further characterized and its gene was cloned for the first time (Kothary and Kreger, 1985; Kreger et al., 1987; Cutter and Kreger, 1990).

More than 20 years after the cloning of *dly* gene, it was discovered that damselysin toxin is encoded in a virulence plasmid, that was named pPHDD1 (Rivas et al., 2011). The size of this plasmid in strain RM-71 was reported to be of 153 kb and encodes 172 proteins (Rivas et al., 2011). This plasmid was found to encode other virulence factors in addition to damselysin toxin. The ORF located immediately besides *dly* gene was found to encode a pore-forming

toxin with haemolytic activity which initially was dubbed $HlyA_{pl}$ and in a later study it was renamed as phobalysin P (PhlyP) (Rivas et al., 2011; Rivas et al., 2013b; Rivas et al., 2015).

It was soon afterwards demonstrated that almost all the *Pdd* strains, regardless of whether they contain pPHDD1 plasmid or not, encode an additional hemolysin in the chromosome I. This toxin, encoded by $hlyA_{ch}$ gene, was named initially $HlyA_{ch}$ (Rivas et al., 2013b). Its amino acid sequence is 92% identical to the plasmid-encoded homologue $HlyA_{pl}$, and is responsible for the haemolytic activity detected in strains that do not contain pPHDD1 plasmid. Currently, this chromosome-encoded toxin is known as PhlyC (Phobalysin C), due to its similarity to PhlyP (Osorio et al., 2018).

The presence of pPHDD1 confers to the bacterial host cell a strong hemolytic phenotype. Therefore, two main categories of strains can be distinguished in *Pdd*: strains with strong haemolytic activity and high virulence on the one hand (strains possessing pPHDD1), and strains with weak haemolytic activity and lower virulence degree on the other hand (strains lacking pPHDD1 plasmid). Dly toxin cooperates with PhlyP and PhlyC cytotoxins in a synergistic manner, whereas the two phobalysins PhlyC and PhlyC cooperate among them in an additive manner (Rivas et al., 2013b; Rivas et al., 2015). PhlyC alone, also contributes to virulence for fish and to toxicity for fish and human cell lines in strains without pPHDD1 plasmid (Vences et al., 2017). Plasmidless strains do not produce damselysin phospholipase, but they exhibit phospholipase activity due to the production of a ubiquitous, chromosome-encoded, phospholipase that was recently identified in all the *Pdd* isolates and was dubbed PlpV (Vences et al., 2017). It was demonstrated the role of phospholipase PlpV in cell toxicity and virulence for fish, and its hemolytic activity against fish erythrocytes but not against sheep erythrocytes. The contribution of

PlpV to virulence was found to be minor in comparison to that of Dly (Vences et al., 2017).

Presence of collagen in many parts of the body in vertebrate hosts such as skin, cartilage, ligaments, tendons, teeth, cornea, and connective tissue, has made it an additional target for the pathogens that able to degrade the collagen to penetrate their bodies (Awad et al., 2000). The ability of pathogenic bacteria to produce collagenases contributes to the breakdown of collagen and may be considered in many cases an additional virulence factor (Beecher et al., 2000). The collagenase *colP* gene was identified and mutated in a model *Pdd* strain (LD-07) that does not contain pPHDD1 plasmid. The *colP* gene was mapped to the *Pdd* chromosome, and its role in gelatin degradation was proved. A minor role was demonstrated for *colP* gene in virulence for sea bass (Vences et al., 2017).

The production of the three major toxins Dly, PhlyP, and PhlyC is regulated by the two-component regulatory system RstAB that is homologous to the *V. cholerae* CarSR system (Terceti et al., 2017). In addition, the RstAB system was found to regulate additional biological activities and production of other putative virulence factors in *Pdd*. Hence, it was demonstrated the role of RstAB system in regulating virulence factors, motility, cell morphology, antimicrobial resistance, and production of type II secretion system-dependent factors in this subspecies (Terceti et al., 2019). Indeed, previous recent studies demonstrated that The T2SS (Type II secretion system) plays a major role in the secretion of Dly, PhlyP, PhlyC and PlpV toxins and therefore in virulence (Rivas et al., 2015; Vences et al., 2017). Proteins secreted by the type II secretion system in Gram negative bacteria include proteases, cellulases, pectinases, phospholipases, lipases, and toxins that contribute to tissue damage and cause disease (Sandkvist, 2001).

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Iron Uptake in Pdd

Most organisms depend on iron as an essential contributor in many biological cellular processes, the most important of which are redox enzymes. Although iron is ranked fourth on the level of elements in terms of availability on the globe, the presence of iron in marine environments is less than in terrestrial environments (Tortell et al., 1999). Hence, it is imperative for microorganisms to develop and create mechanisms that help them to obtain iron from the surrounding environment. One of the most common mechanisms in pathogenic bacteria to obtain iron is the synthesis of siderophores, which are iron chelators that form a Fe-siderophore complex to transfer it into the bacterial cell (Ratledge and Dover, 2000; Miethke and Marahiel, 2007; Amin et al., 2009). Siderophores play crucial roles in microbial growth rate, regulation of quorum sensing and regulation of virulence factors as signal molecules (Chen et al., 2019; McRose et al., 2018). According to (Thode et al., 2018), at least nine different siderophore biosynthesis systems and thirteen siderophore receptors were identified in the family *Vibrionaceae*. Vibrioferrin is an acidic and very hydrophilic siderophore that was originally observed in *Vibrio parahaemolyticus* strains in iron-deficient media and is produced under low-iron conditions. It also had a different composition than the types of siderophores discovered at that time because it contains four constituents, alanine, citric acid, ethanolamine, and two ketoglutaric acids (Yamamoto et al., 1994). Braun and colleagues indicated that all siderophore systems for obtaining iron in gram-negative bacteria need specific outer membrane receptors as well as ATP-binding cassette (ABC) transport systems (Braun et al., 1998). The receptor PvuA was identified in *V. parahaemolyticus* as involved in utilization of the siderophore vibrioferrin (Funahashi et al., 2002). Therefore, a genetic analysis of the regions surrounding *pvuA* gene was performed, which showed that there are two operons involved in the synthesis and

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utilization of vibrioferrin. These operons contained nine genes, five genes in the *pvs* operon (*pvsABCDE*) related to vibrioferrin synthesis, and four genes in the *pvu* operon (*pvuBCDE*) related to vibrioferrin utilization (Tanabe et al., 2003).

In two separate studies, Fouz and colleagues indicated the ability of some *Pdd* isolates to use hemoglobin and ferric ammonium citrate as iron source, and at the same time their ability to produce siderophores in low-iron concentration media. Virulence of *Pdd* for fish and mice was found to increase when an iron excess was provided, and the production of siderophores of the hydroxamate type was observed (Fouz et al., 1994; Fouz et al., 1997). Later studies demonstrated that some strains of *Pdd* produce the siderophore vibrioferrin (Balado et al., 2017b), and a proteomic study demonstrated the production of iron-regulated proteins similar to proteins involved in vibrioferrin synthesis and transport in other *Vibrio* species (Puentes et al., 2017). Interestingly, only a fraction of *Pdd* strains produced vibrioferrin, and such strains tested positive for PCR detection of the vibrioferrin biosynthetic gene *pvsD* (Balado et al., 2017b). According to (Terceti et al., 2018), the genes responsible for vibrioferrin synthesis and transport might be located within a large segment of DNA in the genome of some *Pdd* strains while being absent from other *Pdd* strains. These results suggested that this piece of DNA might constitute a pathogenicity island that was acquired by some *Pdd* strains through horizontal gene transfer and inserted within a tRNA-Ser gene in chromosome II (Osorio, 2019).

1.3.1.5 Current knowledge on genetics of antibiotic resistance in *Pdd*

The use of antibiotics in aquaculture facilities promotes the maintenance and the spread of mobile genetic elements that carry

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antimicrobial resistance determinants (Cabello et al., 2013). Previous studies have analysed the antimicrobial resistance profiles of *Pdd* strains causing disease in fish farms. The detection of some resistances to antibiotics suggested that strains of this subspecies might harbour antimicrobial resistance genes (Fouz et al., 1992; Pedersen et al., 2009; Chiu et al., 2013). However, genetic studies aimed at characterizing the genes responsible for these resistances are very scarce. Of note, recent studies reported for the first time the isolation and characterization of a *Pdd* strain from seawater in an aquaculture site in Japan, that harboured a large conjugative plasmid which was dubbed pAQU1 (Nonaka et al., 2012). pAQU1 is a large plasmid of 204,052 bp and encodes seven antimicrobial resistance genes: *bla*$_{\mathrm{CARB\text{-}9}}$-like, *floR*, *mph*(A)-like, *mef*(A)-like, *sul2*, *tet*(M) and *tet*(B). Thus, it is considered a multidrug-resistance (MDR) plasmid. This plasmid is stably maintained in *Pdd* cells even with low antibiotic selection pressure (Bien et al., 2015). Later studies reported the presence of pAQU1-like plasmids in *Pdd* strains and in species of the *Vibrio* genus isolated in different areas of China and Japan (Nonaka et al., 2014; Nonaka et al., 2015; Li et al., 2017).

This presence of MDR plasmids in Asian countries contrasts with the absence of reports of MDR plasmids from European and American *Pdd* isolates so far. In this regard, the analysis of genome sequences of *Pdd* strains from different European locations deposited in Genbank database and characterized in recent studies, did not reveal presence of MDR plasmids or other mobile elements (Vences et al., 2017; Terceti et al., 2018). Therefore, the presence of pAQU1-like plasmids in *Pdd* strains from geographical areas other than China and Japan still awaits investigation, and will constitute one of the Objectives of this thesis.

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1.3.2 *Photobacterium damsela subsp. piscicida (Pdp)*

1.3.2.1 Taxonomical position of *Pdp*

Much controversy accompanied the taxonomic position of the pathogen causing pasteurellosis/pseudotuberculosis from its discovery until the adoption of its current taxonomical placement as *Photobacterium damselae* subsp. *piscicida*. Initially, this disease was reported in wild populations of fish and placed within the genus *Pasteurella* (Snieszko et al., 1964). A relationship was suggested linking the pathogen of pasteurellosis disease and the coryneform group of bacteria, based on its fastidious nutritional properties, carbohydrate metabolism and penicillin sensitivity (Ajmal and Hobbs, 1967). In addition, a relationship was suggested between the genus *Arthrobacter* and the pathogen of pasteurellosis as publishers thought it was appropriate for the pathogen of pasteurellosis (Simidu, 1972). Thereafter, based on many of its main characteristics, one of the studies suggested a strong relationship between the pathogen of pasteurellosis and the genus *Pasteurella*, and the name *Pasteurella piscicida* was proposed (Janssen and Surgalla, 1968). Although some researchers had ruled out the idea of *Pasteurella* joining the *Vibrio* group, other researchers suggested adding the *P. piscicida* to the *Vibrio* group (De Ley et al., 1990). Based on the 16S rRNA gene sequence and DNA–DNA hybridization data, the researchers demonstrated the relationship between *P. piscicida* and *Photobacterium damsela* with the necessity to add *P. piscicida* to the species *Photobacterium damsela* and to include it as a subspecies. Hence, it resulted in its renaming as *Photobacterium damsela* subsp. *piscicida* (Gauthier et al., 1995). In 1998, a study that included a comparison of 113 *P. damselae* subsp. *piscicida* strains and 20 *P. damselae* subsp. *damselae* strains based on the analysis of 129 morphological and biochemical tests showed that there was no

definitive evidence for the inclusion of *Photobacterium damselae* subsp. *piscicida* in *Photobacterium damselae* as subspecies, despite the great convergence using DNA–DNA hybridization data (Thyssen et al., 1998).

1.3.2.2 The emergence of pasteurellosis disease

Pdp is the etiological agent of fish pasteurellosis or photobacteriosis, a bacterial septicaemic disease affecting wild and farmed fish. It is also referred to as pseudotuberculosis because of the presence of whitish nodules with bacterial aggregations in the internal organs of moribund fish (Romalde, 2002). The fluctuation in its taxonomic position resulted in the renaming of pasteurellosis also as photobacteriosis.

The emergence of pasteurellosis disease was initially in USA in wild fish during the period between 1963 to 1967, while the first cases of this disease were recorded in farmed fish in 1970 (Toranzo et al., 1991). Snieszko and collaborators described a massive epidemic that spread to various places during the summer due to a pathogen that infected white perch (*Roccus americanus*) and striped bass (*Morone saxatilis*) in Chesapeake Bay, Maryland, USA (Snieszko et al., 1964). This disease also caused financial losses in wild fish such as wild menhaden (*Brevoortia tyrantis*) and striped mullet (*Mugil cephalus*) in Galveston Bay - Texas, and it was initially described that the bacterium that caused the disease was a *Pasteurella*-like bacterium (i.e., a *Photobacterium damselae* subsp. *piscicida*-like) (Lewis et al., 1970). Nearly 13 years later, pasteurellosis disease notation returned to wild fish since it was described in Chesapeake Bay (Paperna and Zwerner, 1976) and in West Long Island Sound, USA in wild populations of striped bass (Robohm, 1979). The huge financial losses caused by pasteurellosis in farmed fish, especially in cultured juvenile

yellowtail (*Seriola quinqueradiata*) (Egusa, 1983; Kimura and Kitao, 1971; Kubota et al., 1970; Kusuda, 1972) in Japan gave the utmost importance to pasteurellosis disease that was described at that time as pseudotuberculosis (Kubota et al., 1970). Thereafter, pasteurellosis disease outbreaks were widely noted in farmed fish and in different fish species such as ayu (*Plecoglossus altivelis*) (Kusuda and Miura, 1972), Black Sea Denis (*Mylio macrocephalus*) (Muroga et al., 1977; Ohnishi et al., 1982) red Sea Denis (*Acanthopagrus schlegeli*) (Yasunaga et al., 1983), oval file fish (*Navodan modestus*) (Yasunaga et al., 1984) and red grouper (*Epinephelus okaara*) (Ueki et al., 1990) in Japan; striped bass in the USA (Hawke et al., 1987); and snake-head fish (*Channa maculata*) in Taiwan (Tung et al., 1985). The emergence of *Pasteurella*-like infection in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) in Norway (Hårstein and Bullock, 1976), rudd (*Scardinius erythrophthaimus*) and chub (*Coregonus zenithicus*) in England (Ajmal and Hobbs, 1967), and sheat fish (*Silurus glanis*)) in Hungary (Farkas and Oláh, 1984) has shown the extent in the geographic and host ranges of pasteurellosis disease, and indicates that the pathogen has a worldwide distribution. Toranzo and colleagues (Toranzo et al., 1991), indicated the first report of pasteurellosis disease in Spain. In the same year (1991), pasteurellosis disease appearance was recorded in Korea and European countries such as Italy and France (Bakopoulos et al., 1995; Baudin-Laurencin et al., 1991), while it appeared in Greece and Israel in 1995 (Bakopoulos et al., 1997). In addition, the first documented report of pasteurellosis in Malta was in 1997 (Bakopoulos et al., 1997). Reports of *Pdp* causing disease in a variety of species of cultivated fish are published very frequently. In recent years the disease has been reported in Taiwan, in cultured cobia (*Rachycentron canadum*) (Liu et al., 2003), in paradise fish (*Macropodus opercularis*) (Liu et al., 2011), and in semicircle angelfish (*Pomacanthus semicirculatus*) (Leu et al., 2019); in meagre

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(*Argyrosomus regius*) in Canary Islands, Spain (Costa et al., 2017); and in cobia (*Rachycentron canadum*) in Brazil (Shimada et al., 2020), among others.

1.3.2.3 General characteristics of *Pdp*

P. damselae subspecies differ in many features such as host type, phenotypic characteristics, motility, growth rate, growth at different temperatures and salinities, and different responses in many culture media. *Pdp* is a Gram-negative, rod shaped cell, halophilic, nonmotile, oxidase-positive, catalase-positive, facultative anaerobic, sensitive to the vibriostatic agent 0/129, incapable of reducing nitrate, fermentative on glucose without production of gas and with ability to ferment maltose and mannose as sole carbon source, ornithine decarboxylase positive and lysine decarboxylase negative, and indole negative. It fails to grow at NaCl concentrations lower than 0.5% or higher than 4.0%, or at temperatures below 15 or above 30 °C. Brain heart infusion (BHI) agar supplemented with 2% NaCl or blood agar (2% sheep blood) are recommended to isolate this pathogen. Single colonies on blood agar are grayish-white in color, non-hemolytic, and clearly visible after 48 h incubation. It does not grow on thiosulphate-citrate bile-salt sucrose (TCBS) agar, is negative for methyl red and Voges-Proskauer, and does not produce lipase, amylase, and urease enzymes.

1.3.2.4 Pathological characteristics of pasteurellosis

Observing the characteristics associated with the disease during the early infection period helps to control the disease and the ability to treat it if possible. Several studies have described some external symptoms associated with pasteurellosis, which show a slight

difference in symptoms between the different types of fish (Hawke et al. 1987; Kubota et al. 1970; Kusuda and Miura 1972; Robohm 1979; Toranzo et al. 1991). One of the constant signs of pasteurellosis among fish is paleness of the gills and the area adjacent to the gill region. In addition, the pathological signs of pasteurellosis included lethargy with an inability to swim normally, failure to regulate pigmentation, and an increased respiratory rate. Reddening of the operculumand darkening of the skin were also observed in some others.

Acute pasteurellosis appears as necrosis of the liver, spleen, and kidney in conjunction with bacterial accumulation outside and inside phagocytic cells and in capillaries. In chronic infection, inflammation appears as white granulomas and is noticeable at the histological level (pseudotuberculosis) (Varello et al., 2014). However, the presence of granulomas may vary depending on several factors such as the type of affected fish, whether the mechanism of infection is experimental or natural, and the use of antibiotics and medications during the period of infection and diagnosis (Kubota et al., 1970; Thune et al., 1993). Some studies indicated that the emergence of pasteurellosis during the summer period has more chances to occur than during the rest of the year (Magariños et al., 2001; Magariños et al., 1996). These studies attributed the reason for this to the rise in temperature, which was associated with changing water quality, oxygen level, and population density of fish, which provided the ability for the pathogen to overcome the immune system of fish.

Internally, the histopathological description of fish infected with pasteurellosis showed bacterial clusters in the spleen and liver tissues associated with hemorrhage, an apparent decrease in inflammatory cell response, and necrotic changes in the kidneys (Hawke et al., 1987; Toranzo et al., 1991; Wolke, 1975). Kubota (1970) indicated the

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ability of *Pdp* to penetrate phagocytes and to be able to accumulate in them, causing swelling of macrophages in large cells in the spleen and kidneys, assuming that this leads to blocking capillary blood flow in the organs, which leads to ischemia (Kubota et al., 1970). Later, do Vale et al. demonstrated that *Pdp* causes the apoptosis of macrophages and neutrophils, forming bacterial clusters to cause a degradation of the macrophage cells to bypass the immune system (do Vale et al., 2003).

Many challenges made it difficult to control pasteurellosis, such as the ability of *Pdp* to remain within the macrophage, the emergence of many antibiotic-resistant *Pdp* strains, and the ineffectiveness of antibiotic treatment due to bacterial resistance. Also, bacterial vaccines showed limited efficacy, which indicated the importance of giving dietary probiotics to enhance the immune system (Smith, 2019).

1.3.2.5 Virulence factors of *Pdp*

The level of virulence differs from one pathogen to another because it depends on several factors such as the pathogen's ability to attach to host cells, how the pathogen enters into the host cells, the mechanism to overcome the host's immune system, the pathogen's production of virulence factors and its ability to destroy host cells. The pathogen interaction with host cells is the first step in entering the host. In 1994, Bonet et al described for the first time the ability of *Pdp* strains to produce a capsule and suggested a major role for capsule in *Pdp* pathogenicity as a virulence factor (Bonet et al., 1994). Several, but not all, *Pdp* strains produce a capsule with a polysaccharide structure that is hypothesized to enhance adhesion to host cells. Based on the amount of capsular material, a significant difference was observed between *Pdp* strains grown under iron-limited conditions and those grown under iron supplemented conditions (do Vale et al.,

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2001). Bacteria that produce capsules have been shown to be resistant to bactericidal effects of fish serum, as well as leading to lower lethal dose values (Kusuda et al., 1988). According to (Arijo et al., 1998), experiments on non-capsulated *Pdp* strains showed higher activity and abundance of macrophage in non-immune serum, unlike capsulated *Pdp* strains. These results indicate that the capsule is considered a virulence factor and suggest the ability of capsulated *Pdp* strains to cause more severe disease.

In 2003, do Vale and colleagues reported the observation of apoptosis of sea bass (*Dicentrarchus labrax L.*) macrophages and neutrophil cells after infection with *Pdp* strains, which led to the conclusion that there should be a strong virulence factor produced by *Pdp* causing such apoptotic effects (do Vale et al., 2003). Therefore, do Vale et al demonstrated that the cause of programmed cell death in macrophages and neutrophils is a toxic protein (56kDa) secreted outside the *Pdp* cells called Aip56 (apoptosis-inducing protein of 56 kDa) and encoded within the pPHDP10 plasmid (do Vale et al., 2005). These results also considered the Aip56 protein is the main virulence factor in *Pdp* strains. Later, it was demonstrated that secretion of AIP56 to the extracellular milieu is mediated by the type II secretion system that involves a 12-gene operon covering a 11,233 bp region of the *Pdp* genome (do Vale et al., 2017). In recent studies, these authors described the biochemical state of the main virulence factor AIP56, such as the role of the AIP56 disulphide bond in toxicity, and also indicated the role played by the TrxR-Trx redox system in reducing that bond in the cytosol in addition to the common features with other AB toxins (Pereira et al., 2020).

Iron Uptake in Pdp

The pioneering studies aimed at analysing the importance of iron uptake in the pathogenicity of *Pdp* reported that it can obtain iron from human transferrin by a mechanism that involves the production

of a siderophore (Magariños et al., 1994b). Later studies reported the presence in some *Pdp* strains of a gene cluster (dubbed *irp* cluster) required for the biosynthesis of a siderophore of the phenolate type (Osorio et al., 2006). The chemical structure of this siderophore, which was named piscibactin, was then determined (Souto et al., 2012). Its structure is similar but not identical to yersiniabactin produced by species of the genus *Yersinia*. Indeed, the genes of the *irp* cluster conform pathogenicity island-like structure very similar to the *Yersinia* HPI, a chromosome-borne High Pathogenicity Island that encodes the biosynthesis and transport of yersiniabactin siderophore in pathogenic *Yersinia* (Osorio et al., 2006). A subsequent study demonstrated that the pathogenicity island containing the piscibactin *irp* genes is part of a mobilizable plasmid that was named pPHDP70 (Osorio et al., 2015). pPHDP70 structure and sequence has been characterized exclusively in *Pdp* strain DI21, it consists of 68,686 bp and encodes 53 open reading frames. The *repA* and *parAB* genes of pPHDP70 are highly similar (99% identical) to homologous genes reported in the multidrug resistance plasmid pAQU1 of *Pdd* (Nonaka et al., 2012). The sequence of pPHDP70 includes three distinct insertion sequence elements, namely IS*1*, IS*3* and IS*91*. The largest element is IS*91* and has six copies in the plasmid, which accounts for 10% of the plasmid sequence. IS*1* consists of two genes *tnpA* and *tnpB*, and together with IS*3* they occur in double and single copy, respectively. IS*1* and IS*91* elements are present also in the *Pdp* plasmid pPHDP10 that encodes AIP56 toxin. Of note, the IS elements of pPHDP70 map to plasmid zones distant to the *irp* cluster, so that they do not inactivate any siderophore-related synthesis or transport function. This observation suggests that selective pressure has kept piscibactin-related genes functional.

As said above, pPHDP70 is defined as a mobilizable plasmid, not conjugative. It lacks genes for a conjugation machinery, but it was

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demonstrated that it can be transferred at very low frequencies to recipient strains, likely facilitated by coresident plasmids encoding a conjugative machinery. *Pdp* strains cured of pPHDP70 plasmid, and strains with disrupted gene functions related to piscibatin synthesis show attenuated virulence for fish (Osorio et al., 2006; Osorio et al., 2015). It is interesting to note that not all *Pdp* strains contain pPHDP70 plasmid. The most recent studies that evaluated the distribution of pPHDP70 plasmid in *Pdp* collections, reported that this plasmid was so far detected exclusively in European strains, and was absent from Asian and American strains (Osorio et al., 2015). Therefore, there is a need to further our understanding on the geographical distribution of pPHDP70-related plasmids in *Pdp*. Due to the demonstrated role of piscibactin in virulence for fish, the instability and loss of pPHDP70 might have consequences in the virulence of *Pdp* strains. One of the objectives of the present thesis will include an exhaustive analysis of pPHDP70 prevalence in *Pdp* strains, as well as a preliminary study on pPHDP70 plasmid stability.

Type Three Secretion System (T3SS)

In bacteria, different types of proteins are synthesized and secreted for use in many functions such as signal molecules, metabolic activities, cell communication, environmental responses, and essential roles in the pathogenicity of bacteria (Dautin and Bernstein, 2007; Green and Mecsas, 2016). Variation in bacterial secretion systems award the bacteria more flexibility in the transport of different protein types within the bacterial and host cells (Maffei et al., 2017). Bacterial secretion systems can be compared and differentiated according to the location of secretion, the secretion signal, the number of secretion steps, the number of skipped membranes, and their presence in Gram-negative or Gram-positive bacteria (Green and Mecsas, 2016).

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In Gram-negative bacteria, eight secretion systems (Sec, Tat, T1SS, T2SS T3SS, T4SS, T5SS, and T6SS) differ in shape, function, and the phospholipids membranes they cross (Saier, 2006). Three out of eight secretion systems (T3SS, T4SS, and T6SS) can be able to transport protein across three phospholipids membranes (inner membrane, outer membrane, and host membrane) (Bleves et al., 2019). These three secretion systems contribute to additional functions such as flagellar machinery (T3SS), spread of antibiotic resistance genes within bacterial populations (T4SS), and antibacterial injection machinery (T6SS), in addition to their role in disease (Bleves et al., 2019; Coulthurst, 2019; Juhas et al., 2008).

T3SS is one of the most common bacterial secretion systems in Gram-negative bacteria and one of the most important virulence factors in Gram-negative bacteria that attack vertebrates, plants, and insects (Hu et al., 2017; Hueck, 1998). According to previous studies, T3SS and the bacterial flagellum share many structural and functional proteins in their synthesis (Abrusci et al., 2014; Bhattacharya et al., 2019). The phylogenetic and comparative analysis suggested a high evolutionary relationship between T3SS and the flagellum (Bhattacharya et al., 2019). Additionally, one of these studies assumes different mechanisms for the evolution of T3SS from the flagellum (Abby and Rocha, 2012). T3SS is a macromolecular protein nanomachine that delivers the bacterial toxins (effectors protein) into the target eukaryotic cell (Abrusci et al., 2014; Spreter et al., 2009). The T3SS can be found in the surface of gram-negative bacteria (Thanassi et al., 2012). It is based on the inner membrane and passes to the periplasm to the outer membrane of bacteria, and then the needle extends out of the bacterial surface (Kubori et al., 1998). A three-dimensional structure of T3SS has been created by high-resolution microscopic images and demonstrates that the base (basal body) of the T3SS involves the inner ring that depends on the inner

membrane, a neck in the periplasm, and an outer ring that depends on the outer membrane, and then the needle extends outwards of the bacterial cell (Hu et al., 2015; Marlovits et al., 2004).

The construction of the T3SS system requires the gradual and serial steps to recruit the T3SS components into the bacterial membrane to create a functional T3SS capable of transmitting bacterial effectors (Burkinshaw and Strynadka, 2014; Marteyn et al., 2012). The export apparatus assembly (inner membrane) includes two proteins that are combined with two inner rings (inner membrane) connected to an outer ring (outer membrane) through its neck. Thereafter, the complex (inner rings, outer rings, and export apparatus) is linked with the cytoplasmic sorting platform that involves three scaffolding proteins and an ATPase (Büttner, 2012; Dey et al., 2019). Once the complex and the sorting platform are linked, functional T3SS will be able to secrete the inner channel and needle filament proteins (Blocker et al., 2008). The helical needle filament, consisting of a single self-polymerizing protein, appears 6 nm wide and surrounds a 2.5 nm inner channel through which the unfolded proteins are directed to their final destination within the eukaryotic target cells (Blocker et al., 2001).

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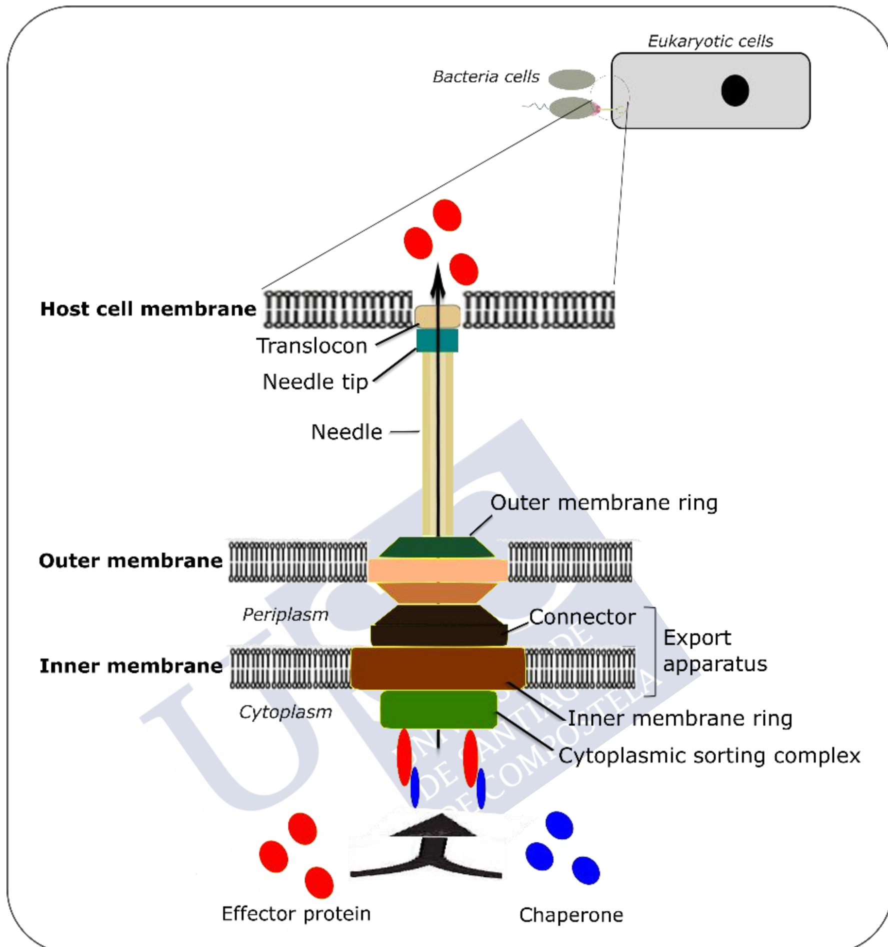

Figure 1.2 Schematic diagram illustrating type III secretion system (T3SS) architecture of Gram negative bacteria.

The Gram-negative pathogenic bacteria that use the T3SS have to ensure appropriate proximity to the target host cells. Mostly, the T3SS is not in an active mode in all Gram-negative bacteria, but in the event of contact between the T3SS needle and the eukaryotic cell, it becomes active and can be able to initiate secretion (Lara-Tejero and

Galán, 2019). Presumably, this occurs via a signal that these pathogens sense upon direct contact with the host cell. It is still not known precisely how Gram-negative bacteria sense contact with the host cell surface and which is the triggering signal to initiate the secretion process after transmitting the signals to start producing effectors. Needle contact with a eukaryotic cell will induce the T3SS to secrete the translocase proteins, thus acting as an activation signal to initiate the effector transport process (Kim et al., 2013). Upon activation of the secretion process and initiation of effector production, the chaperon will transfer the effector to prevent it from converting into a folded protein during transport within the bacterial cytoplasm to the ATPase region of the export platform (Roblin et al., 2015). The effectors travel to the inner membrane and then to the outer ring's neck in the outer membrane of the bacterial cell to reach the needle (Burkinshaw and Strynadka, 2014). The needle's inner channel transports the effectors as unfolded proteins to the host cell's cytoplasm, which is penetrated by the translocase proteins (Holland, 2004).

Several studies have been conducted on T3SS for animal pathogens (*Yersinia, Shigella, Salmonella, Escherichia, Pseudomonas*, *Vibrio* and *Chlamydia*) and plant pathogens (*Pseudomonas, Erwinia, Ralstonia, Xanthomonas, and Rhizobium*), which demonstrate the role of T3SS in bacterial pathogenicity and high distribution of T3SS within different species of bacteria (Hueck, 1998; Miller et al., 2019; Wu et al., 2020).

The discovery of T3SS in a *Vibrio* species took place for the first time in *V. parahaemolyticus* (Makino et al., 2003) and it has led to a succession of studies related to T3SS within other species of *Vibrio* related to human diseases. In fact, in *V. parahaemolyticus*, two sets of T3SS gene clusters have been described, and are dubbed T3SS1 and

T3SS2 (Makino et al., 2003). The T3SS1 is found in all *V. parahaemolyticus* strains, while T3SS2 is present only in KP-positive strains. T3SS gene clusters were also subsequently reported in non-O1, non-O139 strains and in non-toxigenic O1 *V. cholerae* (Tam et al., 2007; Mahmud et al., 2014) (Dziejman et al., 2005). Similar T3SS gene clusters were identified in other *Vibrio* species, including *V. alginolyticus* (Zhao et al., 2010), *V. mimicus* (Okada et al., 2010) and *V. harveyi* (Henke & Bassler, 2004), among others.

Thereafter, studies related to T3SS continued to reach as much information as possible about the structure, function, architecture of genes, and presence of this system among genomes of *Vibrio* species (Miller et al., 2019). Nowadays, researchers are seeking to figure out the complete secretion mechanism, T3SS activation, and nature of the effectors and the mechanism of its transport from the bacterial cell to the host cell (Miller et al., 2019; Osorio, 2018; Zhao et al., 2018).

According to (Vences et al., 2017), the *Photobacterium damselae* subsp. *damselae* type strain CIP 102761 genome, includes a large plasmid dubbed pPHDD203 that encodes a putative T3SS. Interestingly, a close look at two *Pdp* genomes deposited in Genbank database and published as draft genome sequences (Aoki et al., 2017; Teru et al., 2017) reveals the presence of genes related to the T3SS with high identity to the *Pdd* CIP 102761 genes. However, at the beginning of this thesis work, no scientific publication was available that reported the presence of a T3SS in *Pdp*.

Thus, a major objective of the present thesis work will be the analysis of a large collection of *Pdp* isolates to assess the presence of genes related to the T3SS. In the event of finding strains with such a secretion system, we will attempt to carry out a functional analysis by mutagenesis in order to ascertain its putative role in virulence for fish.

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JUSTIFICATION AND OBJECTIVES

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2. JUSTIFICATION AND OBJECTIVES

Current state of the art on the knowledge of the genetic diversity of Pdd and Pdp: immediate perspectives for research and Objectives of this study

A very recent review has highlighted the great contribution of mobile genetic elements to the diversification of *Pdd* and *Pdp* populations, as well as their contribution to shaping the two distinct pathogenic lifestyles exhibited by *Pdd* on the one side, and *Pdp* on the other side (Osorio, 2019). The recent publication of several *Pdp* genomes has unveiled that the subsp. *piscicida* has undergone a generalized loss of biochemical and cellular features with respect to subsp. *damselae*. The *Pdp* genomes have undergone a massive proliferation of insertion sequence (*IS*) elements, and even though natural selection is expected to have preserved essential gene functions the spread of *IS* elements has caused an extensive number of pseudogenes as well as large deletions in the *Pdp* chromosomes (Balado et al., 2017a). Due to this process of gene decay it is hypothesized that *Pdp* is adapted to a host-dependent lifestyle. In contrast, the subsp. *damselae*, in addition to be an opportunistic and also a primary pathogen for many animal species, is also considered a free-living bacterium that is usually isolated from seawater and healthy animals. Further on, the acquisition of distinct virulence factors by horizontal gene transfer in each subspecies has strongly

contributed to these two different lifestyles of the two *P. damselae* subspecies.

Recent studies have unveiled a significant amount of genetic data about *Pdp* and *Pdd* pathobiology. However, many questions arise, though, about the genetic diversity within each of the two *P. damselae* subspecies. Below we summarize some intriguing points that would deserve investigation, and that have not been tackled so far. These points will constitute the framework and the Objectives of the present thesis work:

1.- *P. damselae* subsp. *damselae* (*Pdd*) genetic diversity:

a) Very recent studies have tackled the study of the prevalence of the virulence plasmid pPHDD1 and of other virulence determinants in large collections of *Pdd* strains (Rivas et al., 2014; Vences et al., 2017; Terceti et al., 2018; Terceti et al., 2019). However, the study of plasmids and other mobile elements that carry antimicrobial resistance determinants in *Pdd* is very scarce and is reduced to only a few studies on isolates from China and Japan. There is thus a necessity to tackle the study of genetic basis of antimicrobial resistance in *Pdd* collections.

b) The identification of *Pdd* by phenotypical and biochemical traits is highly extended in laboratories worldwide, and the preliminary isolation of this subspecies from veterinary and environmental samples usually relies on the cultivation on the selective and differential medium TCBS (thiosulfate-citrate-bile salt-sucrose). This subspecies is generally defined as non-sucrose fermenter, and therefore the search of *Pdd* colonies growing on TCBS is biased to look for the green ones, whereas yellow colonies would be preliminarily considered as non-*Pdd*

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colonies. Curiously enough, some studies have reported putative *Pdd* isolates that grow as yellow colonies, but the genetic basis of this differential phenotype remains unstudied to date. Here we will attempt to discover the genetic basis underneath these two phenotypes.

2.- *P. damselae* subsp. *piscicida* (*Pdp*) genetic diversity:

a) Two currently known virulence factors of *Pdp* are encoded within plasmids, namely pPHDP10 (Aip56 toxin) and pPHDP70 (siderophore piscibactin). However, very little is known on the prevalence and distribution of these plasmids among *Pdp* populations. Presence of these plasmids has been assayed in a very reduced number of isolates to date. In addition, very little is known about the stability of these plasmids and about their proneness to be lost from the populations. Therefore, one of the major objectives of this thesis will include the extensive analysis of plasmid prevalence in a large collection of *Pdp* strains from different hosts and diverse geographical locations.

c) The so far uncharacterized (putative) existence of a type III secretion system in this subspecies: There is increasing evidence that a fraction of *Pdp* strains might encode a type III secretion system. However, at the beginning of the present thesis study, there were no published evidence on the existence of this secretion system in *Pdp*. A major objective of the present thesis is the elucidation of the existence of a T3SS in *Pdp* and its functional analysis, to ascertain its prevalence and its putative role in virulence for fish.

SPECIFIC OBJECTIVES (with mention to the articles in which they are addressed)

1. **Study of the genetic basis of antimicrobial resistance in *Pdd*:**
   Vences A., Abushattal S., Matanza X.M., Dubert J., Uzun E., Ogut H. and Osorio C.R. (2020). 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. *Microbial Ecology* 80:507-518.

2. **Genetic analysis of sucrose utilization ability in *Pdd*:**
   Abushattal S., Vences A., Barca A.V. and Osorio C.R. (2020). Diverse horizontally-acquired gene clusters confer sucrose utilization to different lineages of the marine pathogen *Photobacterium damselae* subsp. *damselae*. *Genes* 11:1244.

3. **Analysis of plasmid and virulence markers prevalence in *Pdp*:**
   Abushattal S., Vences A. and Osorio C.R. (2020). A virulence gene typing scheme for *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish photobacteriosis, reveals a high prevalence of plasmid-encoded virulence factors and of type III secretion system genes. *Aquaculture* 521:735057.

4. **Elucidation of the existence of a T3SS in *Pdp:* functional analysis, prevalence and role in virulence for fish:**
   Abushattal S., Vences A., dos Santos N.M., do Vale A. and Osorio C.R. (2019). Draft genome sequences of *Photobacterium damselae* subsp. *piscicida* SNW-8.1 and PP3, two fish-isolated strains containing a type III secretion system. *Microbiology Resource Announcements* 8(21):e00426-19.

   Abushattal S., Vences A. and Osorio C.R. (unpublished). A highly unstable plasmid encoding the type III secretion system contributes to virulence of *Photobacterium damselae* subsp. *piscicida* for fish.

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MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

3.1.1 *Photobacterium damselae subsp. damselae (Pdd)* culture

Pdd cells were routinely grown at 25°C on tryptic soy agar and broth supplemented with 1% NaCl (TSA-1 and TSB-1, respectively), supplemented with antibiotics when appropriate. For sucrose fermentation phenotypical tests, *Pdd* strains were grown on TSA-1 plates overnight at 25°C, and isolated colonies were seeded on Thiosulfate-citrate-bile salts-sucrose agar plates (TCBS) and incubated at 25°C for 18-24 h. Alternatively, API-20E galleries (Biomerieux) were used for sucrose fermentation assays, following the manufacturer's recommendations.

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3.1.2 *Photobacterium damselae subsp. piscicida (Pdp)* culture

Pdp cells were routinely grown on tryptic soy agar (TSA-1) or in tryptic soy broth (TSB-1) supplemented with 1% NaCl at 25°C. The bacterial stocks, kept at -80ºC, were thawed and cultured onto Trypticase Soy Agar (Difco) supplemented with 1% NaCl (TSA-1) at 25ºC for 48 h.

3.1.3 *Escherichia coli* culture

Escherichia coli strains were grown on Luria Bertani agar (LB) plates or Luria Bertani Broth at 37°C. Antibiotics were used at the following concentrations: kanamycin (Kan) at 50 µg ml^{-1}, ampicillin

(Amp) at 50 µg ml^{-1}, chloramphenicol (Cm) at 20 µg ml^{-1}, rifampin (Rif) at 50 µg ml^{-1}, streptomycin (Strep) 50 µg ml^{-1} and nalidixic acid (Nal) at 40 µg ml^{-1}.

3.2 GENOME SEQUENCING, COMPARATIVE GENOMICS AND MOLECULAR PHYLOGENY ANALYSES

3.2.1 DNA sequencing and functions annotation

High-purity genomic DNA of *Pdp* and *Pdd* strains was extracted using the G NOME DNA kit (MP Biomedicals) according to the manufacturer's instructions. Genome sequencing in this work was implemented using two techniques, namely the Illumina MiSeq platform or the PacBio procedure (SNPsaurus, OR, USA).

Annotation of the specific function of ORFs was performed using rapid annotations of subsystems technology (RAST) and the BLASTP database (Altschul et al., 1997; Aziz et al., 2008). The gaps in a selected plasmid of strain PP3 (pPDPT3 plasmid) were closed by PCR and Sanger sequencing.

3.2.2 Genome and plasmid comparison

Comparative analysis of plasmid and genome sequences were performed using BLASTN and BLASTP databases (Altschul et al., 1997), and MAUVE and EasyFig programs (Sullivan et al., 2011; Darling et al., 2010). The CGView server database was used to obtain a circular graphical map representation of the pPDPT3 plasmid genome (Grant and Stothard, 2008).

3.2.3 Phylogenetic tree of genomes

The phylogenetic tree of *Pdd* complete genomes was constructed using the guide tree obtained by the MAUVE genome

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alignment program (progressive Mauve option) (Darling et al., 2004; Darling et al., 2010).

3.2.4 OrthoAni analysis

The OrthoAni analysis (Lee et al., 2015) was used to quantify overall sequence similarity, based on a comparison of orthologous fragments between pairs of genomes. Calculations of the core genome and of unique genes among strains were conducted using RAST (Aziz et al., 2008).

3.2.5 MLSA gene-based phylogenetic analysis

The MLSA (Multilocus sequence analysis) analysis was conducted using a concatenate of the sequences of nine housekeeping 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 (Pérez-Cataluña et al., 2016; Labella et al., 2018).

On the other hand, the sucrose genes tree was constructed using the concatenated amino acid sequences of the proteins encoded by the 4 sucrose cluster genes *scrRAKB*. The Mesquite 3.61 program was used to concatenate the protein sequences (Maddison and Maddison, 2019).

Evolutionary analyses were conducted in MEGA6 (Takuma et al., 2013). The evolutionary history of the strains was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). 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 (Tamura et al., 2004), and are in the units of the number of base substitutions per site.

3.3 DATABASE SUBMISSION

The draft genome sequences and the plasmids sequences of the *Pdp* and *Pdd* strains determined in this study are available from GenBank. Table 3.1 shows the names of the sequenced strains, their corresponding access numbers and the sequencing technique employed.

Table 3.1: Genomes and plasmids deposited in GenBank

strain/plasmid	sequencing technique	Genbank Acc. No.
Ph. damselae* subsp *damselae		
164dp-OG2	Illumina MiSeq	VAUU00000000
144bp-OG3	Illumina MiSeq	VAND00000000
162bp-OG4A	Illumina MiSeq	JABWTP000000000
189bp-OG7B	Illumina MiSeq	JABXOP000000000
82dy-OG8	Illumina MiSeq	JABXOQ000000000
64bp-OG9	Illumina MiSeq	VANE00000000
125dy-OG11	Illumina MiSeq	JACFTX000000000
70dps-OG12	Illumina MiSeq	VANF00000000
111bp-OG15A	Illumina MiSeq	VANG00000000
89dp-OG16	Illumina MiSeq	VANH00000000
TW250/03	Illumina MiSeq	JABXOR000000000
DK32	Illumina MiSeq	JABWTO000000000
CDC-1421-81	Illumina MiSeq	JABXYE000000000
pPHDD2-OG2 plasmid	Illumina MiSeq	VAUU02000098.1
Ph. damselae* subsp *piscicida		
PP3	PacBio	SAMN11269591
SNW-8.1	Illumina MiSeq	SRHT00000000
pPDPT3 plasmid	PacBio	NZ_SRHT02000010

3.4 PCR CONDITIONS

For PCR template preparation, isolated single colonies of each strain were picked with sterile tips, resuspended in 20 µL sterile water, and 1 µL suspension was added as a template to the PCR tube containing the NZYTaq II 2_ green master mix (NZYTech, Lisbon, Portugal), followed by cell lysis and denaturation step of 5 min at 95 ºC. When possible, PCR conditions were standardized for more than

one primer combination to allow the use of a single PCR protocol for the simultaneous analysis of multiple target genes: 94ºC denaturation for 30 s, primer annealing at (*) ºC for 30 s, elongation at 72ºC for (30s/Kb). After 30 cycles of amplification, a final elongation step at 72ºC for (1min/Kb) was added. (*) Annealing temperature optimized for each primer set based on the primer T_m.

For the amplification of the fragments used to generate allelic-exchange mutants, NZYProof DNA polymerase (NZYTech, Lisbon, Portugal) with 3′→5′ exonuclease activity has been used. This enzyme has a proofreading capacity, which enables it to amplify DNA with increased accuracy.

3.5 CONSTRUCTION OF MUTANT STRAINS

3.5.1 Spontaneous rifampicin-resistant mutants

For obtention of spontaneous rifampicin-resistant mutants, *Pdp* strain PP3 was cultured in Trypticase Soy Broth (Difco) supplemented with 1% NaCl (TSB-1) until stationary phase, and samples were plated onto TSA-1 agar plates supplemented with rifampicin at 50 mg L^{-1}.

3.5.2 Mutagenesis construction of a *scrA* mutant in sucrose operon of *Pdd* DK32

A nonpolar deletion of the *scrA* gene was constructed in the Scr+ *Pdd* parental strain DK32 using PCR amplification of the ca. 2,000 bp sequences upstream and downstream the *scrA* coding sequence, which, when fused together, would result in an in-frame deletion of more than 90% of the coding sequence. Amplification was carried out with primer pair *scrA* 1-2 to get the 5’ fragment, and with primer pair *scrA* 3-4 to get the 3’ fragment. The PCR products were cut with

suitable restriction enzymes and sequentially ligated into the multiple cloning site of pKWS30 plasmid. This cloned fragment involving the deleted region was excised with NotI and ApaI and then ligated into the Kan^R suicide vector pNidKan containing the *sacB* gene, which confers sucrose sensitivity, and R6K ori, which requires the *pir* gene product for replication. The pNidKan plasmid construct containing the deleted allele was transferred from E. coli S17-1-λ*pir* into parental *Pdd* strain DK32. After conjugation for 48 h on TSA plates prepared with seawater, cells were scrapped off the plate and suspended in TSB-1. Next, 100-µl aliquots of serial decimal dilutions were spread on TCBS agar supplemented with kanamycin to select for *Pdd* clones that have undergone a first recombination event, as the growth of *E. coli* donors is inhibited on TCBS agar. Insertion of the suicide vector into the *Pdd* genome by homologous recombination results in kanamycin resistance, and in sucrose sensitivity due to *sacB* gene. Kanamycin resistant colonies were subsequently cultured in TSB-1 without antibiotic selection, and serial decimal dilutions were spread on TSA-1 plates supplemented with sucrose [15% (wt/vol)] to select for a second recombination event. Re-isolated colonies were tested by PCR using primer pair *scrA*-mutant-test to identify recombinants in which the mutant allele of the *scrA* gene replaced the parental allele. This led to mutant strain DK32 Δ*scrA* (SSS165).

3.5.3 Mutagenesis construction of *vcrD* gene in *Pdp* PP3

The coding sequence of each of the two *vcrD* alleles was replaced with antibiotic resistance genes following an allelic exchange procedure using a suicide vector of the pCVD442 family. Two fragments of ~2kb, upstream (fragment 1-2) and downstream (fragment 3-4) of *vcrD* coding sequence were PCR-amplified. Fragment 1-2 was amplified using primers *vcrD*_XhoI_1 and *vcrD* _BamHI_2 while fragment 3-4 was amplified using primers *vcrD*_BamHI_3 and

*vcrD*_NotI_4. After being digested with restriction enzymes, the two fragments were ligated so that the resulting DNA sequence generated an in-frame, 1697-bp deletion, of the *vcrD* coding sequence. In order to fix the unstable pPDPT3 plasmid, the construction containing the deletion was cut with *Bam*HI and a 882 bp chloramphenicol resistance gene (*cat*) amplified from pKD3 plasmid (Datsenko and Wanner, 2000) was inserted. In addition, another version for mutant construction was generated by cutting the aforementioned deletion construction with *Bam*HI and inserting there a 1190 bp PCR-amplified kanamycin resistance gene (*kan*) from pKD4 plasmid (Datsenko and Wanner, 2000). The *cat*-labelled construction for insertional mutation of *vcrD* alleles was used to obtain single *vcrD*^{1} and *vcrD*^{2} mutants independently (SSS256 and SSS325 mutant strains, respectively). Besides, in order to obtain a double mutant, the *cat*-labelled *vcrD*^{1} mutant was used as the basis to construct a double mutant (SSS299 mutant strain) by using the *kan*-labelled construction that was used to insertionally inactivate *vcrD*^{2} gene. The mutant construction containing the *cat* gene was introduced into the suicide vector pNidKan and propagated into *E. coli* β3914. The mutant construction containing the *kan* gene was inserted into the suicide vector pNidKan and propagated into *E. coli* S17-1-λ*pir*. *E. coli* donor strains were conjugated with *Pdp* recipient strains by mixing equal volumes of log-phase cultures of donor and recipient strains on TSA plates prepared with seawater. First recombinants for single *vcrD*^{1} mutant construction were selected on TSA-1 plates supplemented with chloramphenicol at 5μg ml^{-1} (Cm^{5}). For double mutant construction, the colonies resulting from the conjugation between *E. coli* S17-1-λ*pir* and *Pdp* PP3 SSS256 Δ*vcrD*^{1}-*cat* (single mutant) were selected on TSA-1 plates supplemented with Cm^{5} and kanamycin at 50 μg ml^{-1} (Kan^{50}). To select for the second recombination of the single mutant construction containing the *cat* gene, TSA-1 plates supplemented with Cm^{5} and sucrose (15% [wt/vol]) were used. TSA-1 plates supplemented with Cm^{5} + Kan^{50} and sucrose

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(15% [wt/vol]) were used to select double mutant colonies containing the *kan* gene plus the *cat* gene.

3.6 CONSTRUCTION OF A TRANSCRIPTIONAL FUSION AND Β-GALACTOSIDASE ASSAYS

The putative *scrA* gene promoter was PCR amplified with primer pair *scrA*-promoter and fused to a promotorless *lacZ* gene in the low-copy-number reporter plasmid pHRP309. The plasmid with the transcriptional fusion construct p*scrA*::*lacZ* (pSSS250) was mobilized from *E. coli* S17-1-λ*pir* into *Pdd* parental strain DK32 by conjugation. After conjugation for 24 h on TSA plates prepared with seawater, cells were scrapped off the plate, suspended in TSB-1, and 100 µL aliquots of serial decimal dilutions were spread on TCBS agar supplemented with gentamicin (resistance provided by pHRP309). DK32 transformants harboring pSSS250 plasmid (DK32 p*scrA*::*lacZ*) were grown in CM9 medium supplemented either with 1% glucose or with 1% sucrose, and β-galactosidase activities were measured as previously described and expressed in Miller units (Miller, 1992). Three independent experiments with 3 replicates each were conducted. Mean values are reported and error bars represent the standard deviations. The statistical analysis of the gene expression data was carried out with Mann–Whitney test.

3.7 CONJUGATION ASSAYS AND PLASMID TRANSMISSIBILITY ASSAY

3.7.1 Conjugation assays

Conjugation experiments were performed by a drop-mating assay essentially, as previously described (Osorio et al., 2008), on tryptic soy agar plates (TSA) prepared with seawater instead of with distilled water.

3.7.2 Plasmid transmissibility assay

Conjugation experiments to assess pPDPT3 conjugative transfer were performed by mixing equal volumes of log-phase cultures of donor and recipient strains on TSA plates prepared with seawater. In order to test pPDPT3 transmissibility, *E. coli* CAG18420-Kan^{R} and AVL49 (LD-07-Rif^{R}) strains, separately, were used as a recipient, while strain SSS299 (PP3 Δ*vcrD*^{1}*-cat* +Δ*vcrD*^{2}*-kan*) was used as a donor. In another conjugation, HW220 (*E. coli* CAG18439 strain which carries the Integrative and Conjugative Element SXT^{MO10}) that is resistant to streptomycin, SXT, and chloramphenicol, was used as a donor. The SSS299 mutant strain, which is resistant to kanamycin and chloramphenicol, was used as the recipient strain. pPDPT3 plasmid of PP3 strain was labeled using a kanamycin and chloramphenicol resistant cassette. The PP3 transconjugants acquiring SXT^{MO10} were selected on TSA-1 plates supplemented with streptomycin and kanamycin and confirmed by PCR amplification of the *floR* and *sul2* genes carried on ICE SXT^{MO10} element. The resulting strain SSS312 was subsequently used as a donor strain to BI533 (*E. coli* MG1655-Nal^{R}) as the recipient to assess the potential role of ICE SXT^{MO10} elements in pPDPT3 transfer. Conjugation was performed for 24 h at 25°C on TSA plates prepared with seawater, and then serial decimal dilutions were spread on selective LB plates supplemented with Nal and Kan to determine whether the pPDPT3 plasmid had been acquired by *E. coli* MG1655-Nal^{R}.

3.8 GROWTH ASSAYS WITH SUCROSE/GLUCOSE AS A CARBON SOURCE

Pdd parental DK32, and DK32 Δ*scrA* strain were streaked on a TSA-1 plate and incubated overnight at 25°C. A loopful of cells was resuspended in phosphate buffered saline (PBS) to achieve an optical density of 0.3 at 600 nm (OD_{600}: 0.3). For the assay, 1 µl of the bacterial cell suspensions was aliquoted into the wells of a 96-well plate

containing 100 µl of M9 minimal medium (Miller, 1972) supplemented, when necessary, with 0.2% (wt/vol) Casamino Acids (Difco) (CM9), and with a sugar (0.5% glucose or 2% sucrose (wt/vol)) depending on the aim of the experiment. Final concentration of NaCl was adjusted to 1% in all the assays. For each assay, OD_{600} values were recorded every 10 min for 20 h. This experiment was automated using the spectrophotometer Epoch2 microplate reader (Biotek). The 96-well plates were continuously incubated at 25°C in the plate reader with shaking. Three replicates were performed per assayed condition and strain. Mean values are reported and error bars represent the standard deviations.

3.9 ANTIBIOTIC SUSCEPTIBILITY DISC DIFFUSION TESTS

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 25°C. 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 inhouse by soaking sterile paper discs with solutions of chloramphenicol (2 µg per disc), and with trimethoprim (5 µg per disc), respectively.

3.10 EVALUATION OF PPDPT3 PLASMID STABILITY UNDER DIFFERENT TEMPERATURE CONDITIONS

In order to evaluate the pPDPT3 thermostability, *Pdp* PP3 cells were grown from frozen glycerol stock on tryptic soy agar (TSA-1) plates. Then, the plates were incubated for three days at 25°C to obtain single colonies, and the colony PCRs were performed to determine positive colonies for the T3SS genes. Ten positive colonies for T3SS genes were suspended in 50 ml TSB-1 and incubated at 18°C for 3 h, then divided into two equal parts into two 25 ml flasks. One of these flasks was incubated at 25°C and the other incubated at 18°C. Every 2 h during the first 6 h of incubation, and after 24 h incubation, appropriate volumes of the two bacterial cultures at 18°C and 25°C were diluted and spread onto TSA-1 plates. Colony PCRs for six different gene markers were conducted on the colonies that grew from each bacterial culture (at either 18°C or 25°C incubation). The selected genes to test the stability of pPDPT3 plasmid were *parAB, vscJ,* and *yopD*. Concurrently, PCR amplifications were conducted to test for presence of *aip56* gene (pPHDP10) and for *irp2* and *frpA* (pPHDP70).

3.11 VIRULENCE ASSAYS

European sea bass fish (*Dicentrarchus labrax*) with an average weight of 5 g were selected to perform virulence tests using the parental *Pdp* PP3, the pPDPT3-cured strain (SSS260) and three *vcrD* mutant strains (SSS256, SSS299 and SSS325). Fish were acclimated in 100 l aquaria at 24ºC for three days before injection. For dose preparation, bacterial biomass at the confluent growth zone of the agar plate from 48 h cultures was suspended in saline solution (0.85% NaCl) and adjusted at either a sharp OD_{600} = 0.3 or at sharp OD_{600} = 1. Serial decimal dilutions were prepared in saline solution and bacterial counts were carefully calculated. Fish were inoculated intraperitoneally with

0.1 ml of bacterial suspensions in saline solution (0.85% NaCl) using either a low dose (4.61×10^4 CFU/fish) or a high dose (4.61×10^6 CFU/fish). A group of 10 fish were inoculated with saline solution as control. For each strain and dose assayed, ten fish were inoculated, and mortality was recorded over a period of 7 d after injection. Dead fish were aseptically dissected, bacteria were recovered from kidney by culturing on TSA-1 agar plates, and colonies screened by PCR using gene markers for pPDPT3, pPHDP10 and pPHDP70 plasmids.

RESULTS

Photobacterium damselae subsp. *damselae*

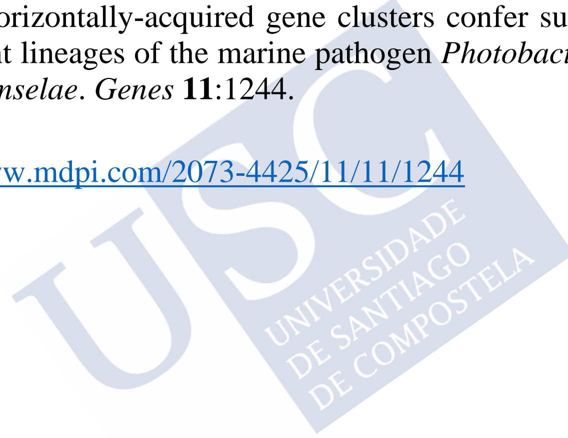
4.1. Article 1

Vences A., Abushattal S., Matanza X.M., Dubert J., Uzun E., Ogut H. and Osorio C.R. (2020). 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. *Microbial Ecology* **80**:507-518.

https://link.springer.com/article/10.1007/s00248-020-01519-4

4.2. Article 2

Abushattal S., Vences A., Barca A.V. and Osorio C.R. (2020). Diverse horizontally-acquired gene clusters confer sucrose utilization to different lineages of the marine pathogen *Photobacterium damselae* subsp. *damselae*. *Genes* **11**:1244.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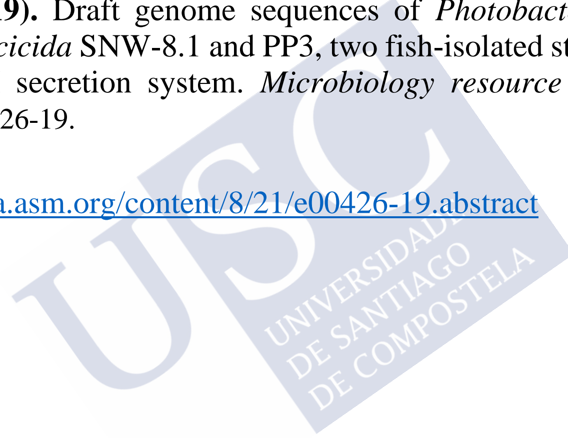
https://www.mdpi.com/2073-4425/11/11/1244

Photobacterium damselae subsp. *piscicida*

4.3. Article 3

Abushattal S., Vences A., dos Santos N.M., do Vale A. and Osorio C.R. (2019). Draft genome sequences of *Photobacterium damselae* subsp. *piscicida* SNW-8.1 and PP3, two fish-isolated strains containing a type III secretion system. *Microbiology resource announcements* **8**(21):e00426-19.

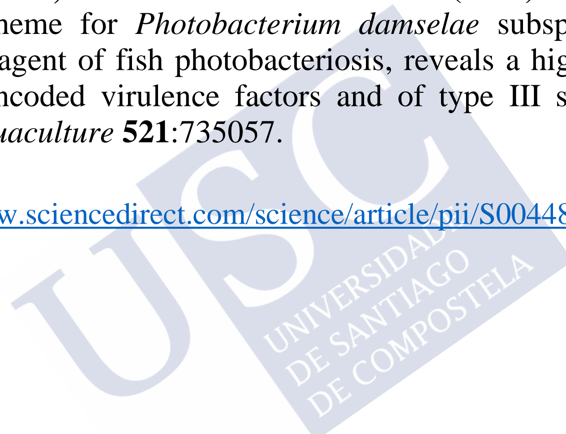
https://mra.asm.org/content/8/21/e00426-19.abstract



4.4. Article 4

Abushattal S., Vences A. and Osorio C.R. (2020). A virulence gene typing scheme for *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish photobacteriosis, reveals a high prevalence of plasmid-encoded virulence factors and of type III secretion system genes. *Aquaculture* **521**:735057.

https://www.sciencedirect.com/science/article/pii/S0044848619331126



4.5. Article 5 (*Results not yet published*)

A highly unstable plasmid encoding the type III secretion system contributes to virulence of *Photobacterium damselae* subsp. *piscicida* for fish.

In this section we describe the partial results obtained in this doctoral thesis that have not yet been published because they require additional experimental studies in order to elucidate the various unknowns generated from these results.

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A highly unstable plasmid encoding the type III secretion system contributes to virulence of *Photobacterium damselae* subsp. *piscicida* for fish

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Abstract

The marine pathogenic bacterium *Photobacterium damselae* subsp. *piscicida* (*Pdp*), the causative agent of photobacteriosis in fish, causes important financial loses in marine aquaculture. Recently, it was shown that genes of a type III secretion system (T3SS) are highly prevalent in *Pdp* strains. Here, we demonstrate that the T3SS is encoded within a novel plasmid of 133 Kb (pPDPT3) in *Pdp* strain PP3. The most prominent feature of pPDPT3 is the presence of two paralogous copies of twenty-four T3SS-related genes (*vscNOPQRSTU*, *vscXY*, *yopN*, *tyeA*, *scyN*, *vcrDRGVH, vopBD, yopH, tir, exsA* and *vscW*), and twelve genes (*vscABCDEFGHIJKL*) in single copy. The plasmid also carries two paralogous genes encoding a YopH-like tyrosine phosphatase effector and a single copy gene of a YadA-like adhesin. pPDPT3 showed to be highly unstable, its loss being detected upon a single subculture step on agar plates. Plasmidless strains resulting from spontaneous loss of pPDPT3 under laboratory conditions, exhibited reduced virulence levels compared to the parental strain, suggesting for the first time a role of T3SS in *Pdp* virulence. Construction of independent mutants by insertion of antibiotic resistance cassettes into each of the two paralogous *vcrD* genes seemed to select for the integration of pPDPT3 in the *Pdp* genome, as these mutants maintained the plasmid stably for generations. These disrupted T3SS mutants were non virulent for fish even at very high doses, a finding that might be explained by plasmid integration in the chromosome disrupting gene functions necessary for virulence. We also demonstrate that T3SS genes in other *Pdp* strains have a high tendency to be lost in cultivation, suggesting the existence of unknown mechanisms for plasmid maintenance *in vivo* but not *in vitro*. All these observations warn that the picture of *Pdp* virulence gene content has been historically biased and underestimated, since the loss of plasmid-encoded T3SS genes during bacterial isolation and

subculture may have occurred systematically in laboratories for decades. Collectively, our results provide evidence that the T3SS constitutes a novel, hitherto unreported virulence factor of *Pdp* that is encoded within a highly unstable plasmid.

INTRODUCTION

Photobacterium damselae subsp. *piscicida* (hereafter *Pdp*), a bacterium of the family *Vibrionaceae*, is one of the most devastating pathogens in marine aquaculture, causing a septicaemic disease in fish termed pasteurellosis or photobacteriosis (Romalde, 2002; Andreoni et al., 2014). Infections by *Pdp* are characterized by a bacteraemia with disseminated tissue necrosis and a pronounced cytopathology (do Vale et al., 2003). The best characterized virulence factor of *Pdp* is AIP56 toxin, that is systemically disseminated and induces apoptosis of macrophages and neutrophils (do Vale et al., 2005). AIP56 is an AB-type toxin with a metalloprotease A domain that cleaves the p65 subunit of the transcription factor NF-kB (Silva et al., 2013; Pereira et al., 2014) and is secreted through the type II secretion system (do Vale et al., 2017). AIP56 is encoded within the small virulence plasmid pPHDP10 (do Vale et al., 2005) that has a high prevalence and stability in *Pdp* populations (Abushattal et al., 2020). In addition, many *Pdp* isolates harbour the 70 kb plasmid pPHDP70 that encodes the functions for synthesis and utilization of siderophore piscibactin (Osorio et al., 2015). Curing of pPHDP70 caused reduction of virulence in a turbot fish model (Osorio et al., 2015). A recent study reported that *frpA* and *irp2* genes of pPHDP70 were present in 91 of 103 *Pdp* isolates tested, demonstrating a high prevalence, but not ubiquity, of this putative virulence factor in the subspecies (Abushattal et al., 2020).

The type III secretion system (T3SS) is a needle-like macromolecular apparatus used by several Gram negative pathogenic bacteria to inject proteins, termed effectors, into the target eukaryotic host cells by crossing three membranes: the inner and outer membrane of the bacterial cell and the plasma membrane of the host cell (Abrusci et al., 2014; Spreter et al., 2009). The effectors manipulate host cell physiology and cause diverse cellular responses and cell damage (Galan & Wolf-Watz, 2006; Abrusci et al., 2014). T3SS genes have been reported in a few species of the genus *Vibrio* as *V. parahaemolyticus* (Makino *et a*l., 2003), *V. alginolyticus* (Zhao et al., 2010), *V. harveyi* (Henke & Bassler, 2004) and non-O1, non-O139 *V. cholerae* strains (Dziejman et al., 2005; Tam et al., 2007), among others. As recently as 2019, it was reported for the first time the presence of genes of the type III secretion system (T3SS) in two *Pdp* strains, PP3 and SNW-8.1 (Abushattal et al., 2019). Unexpectedly, a subsequent study demonstrated that the T3SS genes were highly prevalent in *Pdp* strains from different hosts and geographical locations since as early as 1980 (Abushattal et al., 2020). Notably, the same study warned about the spontaneous loss of T3SS genes in *Pdp* PP3 during the attempts to genetically modify this strain, and it was demonstrated that the loss of T3SS gene markers *vscJ*, *vopD* and *vopB* occurred simultaneously with the loss of putative plasmid-borne genes *traC* and *parAB*, suggesting that the T3SS might be encoded within an unstable plasmid (Abushattal et al., 2020). However, the draft genome sequence available for *Pdp* PP3 was highly fragmented into numerous contigs due to the presence of transposase genes in a multicopy fashion, which prevented from deciphering the genome context of the T3SS genes.

In the present study, the genome sequencing of *Pdp* PP3 genome using a PacBio approach unveiled the location of the T3SS within a large plasmid of 133 kb that we dubbed pPDPT3. This plasmid

showed to be highly unstable in culture conditions, its loss occurring as fast as after a single subculture step on agar plates. Cured strains were still virulent for fish, but its virulence degree was notably reduced in comparison with the parental, plasmid-containing strains. The two individual *vcrD* insertional mutants plus the double *vcrD* mutant were strongly impaired in virulence for sea bass. Much surprisingly, plasmid loss was not detected in these *vcrD* mutant strains. These observations suggest that the selection for the recombination of the resistance cassette into pPDPT3 structure likely forced the integration of pPDPT3 in the bacterial chromosome in a way that it disrupted essential functions for virulence and/or fitness inside fish. Collectively, our results demonstrate that the T3SS constitutes a novel, hitherto unreported virulence factor of *Pdp* encoded within a highly unstable plasmid.

RESULTS

Identification of a 133 Kb plasmid that encodes a T3SS in *Pdp* PP3

In a recent study, we found the presence of T3SS-related genes in the draft genome sequence of *Pdp* PP3 (Abushattal et al., 2019). This genome sequence was fragmented into 520 contigs due to the presence of a high number of insertion sequences in multicopy, a characteristic of all the *Pdp* genomes analysed to date (Balado et al., 2017; Aoki et al., 2017; Abushattal et al., 2019). Subsequently, we found that T3SS genes are highly prevalent in *Pdp* isolates from different fish hosts and geographical sources and it was proposed that they might be encoded within an unstable plasmid (Abushattal et al., 2020). In order to investigate the genetic context of the T3SS genes in the genome of *Pdp*, we carried out the genome sequencing of *Pdp* PP3 using a PacBio approach. This sequencing strategy yielded a total of 10 contigs, one of them corresponding to a plasmid of 133,065 bp that

contained the T3SS genes and was dubbed pPDPT3. The remaining contigs corresponded to chromosomes I and II, a pPHDP10-like plasmid, a pPHDP70-like plasmid, a putative 286-Kb plasmid, and 4 unassembled contigs with sizes ranging from 25 to 2.9 Kb (Table 1). None of the unassembled contigs was part of pPDPT3, as demonstrated by PCR amplifications (data not shown).

Table 1: General features of *Pdp* PP3 genome sequenced with the PacBio approach.

PP3 genome (PacBio)		
Accession no.	SRHT02000000	
Genome size (bp)	4,918,480 bp	
% GC	40.90 %	
Genes (total)	4,868	
CDSs (total)	4,687	
Contigs	**Accession no.**	**size (bp)**
Chromosome I	SRHT02000009.1	3,184,080
Chromosome II	SRHT02000005.1	1,164,185
pPHDP10-like	SRHT02000004.1	8,115
pPHDP70-like	SRHT02000008.1	81,347
pPDPT3	SRHT02000010.1	133,065
putative 286-Kb plasmid	SRHT02000006.1	286,884
Unassembled contig	SRHT02000001.1	2,958
Unassembled contig	SRHT02000002.1	9,318
Unassembled contig	SRHT02000007.1	23,195
Unassembled contig	SRHT02000003.1	25,333

Sequence analysis and general features of pPDPT3

The structure and gene organization of pPDPT3 plasmid is summarized in Figure 1. It has an average G+C content of 43%, similar to that of the host genome (40.9%). A total of 146 coding sequences (CDS) were predicted by the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016), of which 28 were annotated as hypothetical proteins (Table 2). Notably, 33 CDS correspond to insertion sequences (IS) that sum up a total of 29.6 Kb, thus account for 22% of the total pPDPT3 sequence length. As many as six different families of transposases are present in pPDPT3, and most of them occur in a multicopy fashion (Table 2 and Figure 1). The IS elements are not uniformly distributed along the plasmid sequence, and concentrate in specific regions that separate putative functional modules in pPDPT3 sequence. A plasmid region (coordinates 69,703-90,775), contains four identical copies in tandem of a multigene sequence of 4,609 bp per copy (Figure 1), composed of IS elements and hypothetical proteins. Also, some of these CDSs occur, individually or in groups, in other regions of the plasmid, such as at the borders of the T3SS gene clusters and at the borders of and interspersed within the conjugation genes. Of note, genes related to plasmid replication and stability/maintenance were not predicted in pPDPT3. The pPDPT3 partitioning system is represented by *parA* and *parB* genes.

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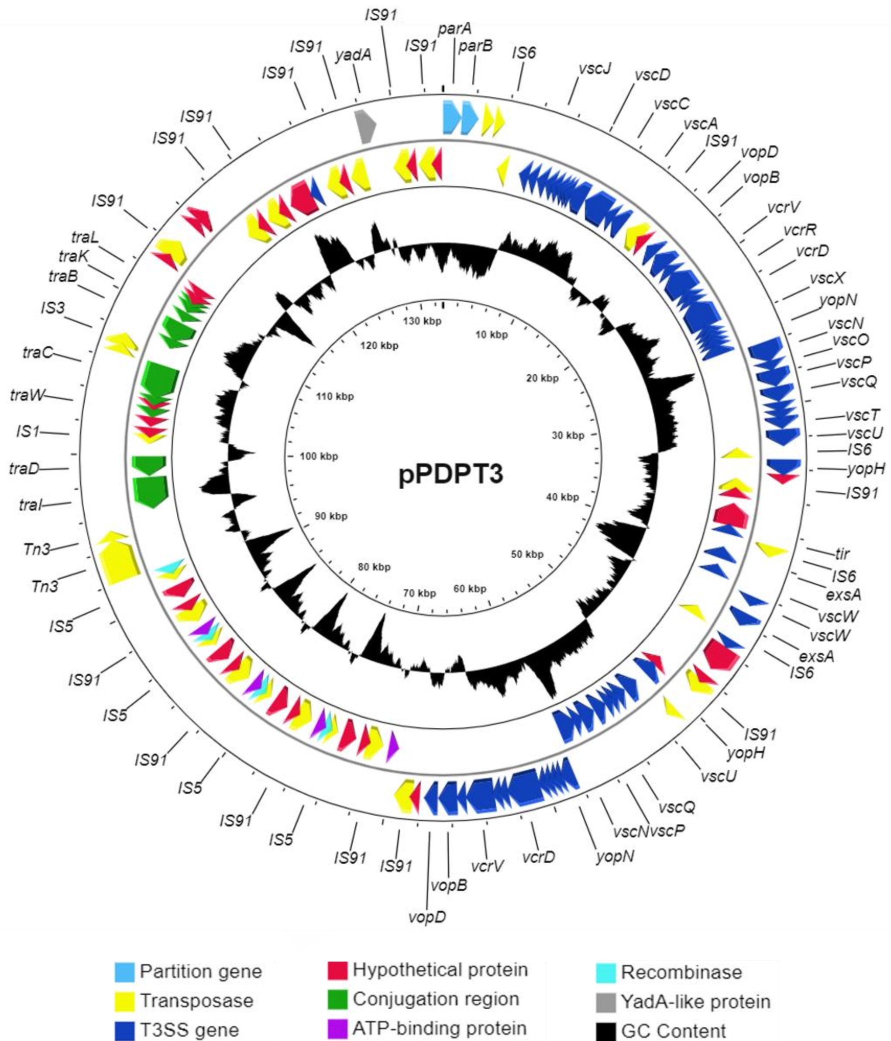

Figure 1: Circular genetic map of pPDPT3 plasmid described in *P. damselae* subsp. *piscicida* PP3 (GenBank accession no. SRHT02000010.1). Arrows represent predicted open reading frames of pPDPT3 plasmid. The orientation of the arrows indicate the direction of transcription for each gene. Map was generated using GCView server (Grant and Stothard, 2008).

Table 2: List of coding sequences (CDS) in pPDPT3 plasmid. The prediction and annotation of the functions was carried out using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016) (*asterisk represents pseudogenes).

CDS	Gene	Functional category	T3SS part and position		Putative function	Locus tag
1	*parA*	Partition	-	-	ParA family protein	E4T26_023615
2	*parB*	Partition	-	-	chromosome partitioning protein ParB	E4T26_023620
3	*IS**	Mobile element	-	-	IS1 family transposase	E4T26_023625
4	*IS**	Mobile element	-	-	IS6 family transposase	E4T26_023630
5	*IS**	Mobile element	-	-	IS6 family transposase	E4T26_023635
6	*IS*	Mobile element	-	-	IS1-like element ISPda1 family transposase	E4T26_023640
7	*vscL*	T3SS	Base	Cytoplasmic	HrpE/YscL family type III secretion apparatus protein	E4T26_023645
8	*vscK*	T3SS	Base	Cytoplasmic	type III secretion system sorting platform protein	E4T26_023650
9	*vscJ*	T3SS	Base	IM ring int	type III secretion inner membrane ring lipoprotein	E4T26_023655
10	*vscI*	T3SS	Base	inner rod	type III secretion system inner rod subunit	E4T26_023660
11	*vscH*	T3SS	Needle	Polimeryzation control	type III export protein	E4T26_023665
12	*vscG*	T3SS	Needle	Chaperone	YscG family type III secretion system chaperone	E4T26_023670
13	*vscF*	T3SS	Needle	Body	type III secretion system needle filament subunit	E4T26_023675
14	*vscE*	T3SS	Needle	Chaperone	EscE/YscE/SsaE family type III secretion system needle protein co-chaperone	E4T26_023680
15	*vscD*	T3SS	Base	IM ring ext	type III secretion system inner membrane ring subunit	E4T26_023685
16	*vscC*	T3SS	Base	OM ring	type III secretion system outer membrane ring subunit	E4T26_023690
17	*vscB*	T3SS	Regulators	Secretion	YscB family type III secretion system chaperone	E4T26_023695
18	*vscA*	T3SS	Regulators	T3SS transcription	T3SS regulon anti-activator ExsD family protein	E4T26_023700
19	*IS*	Mobile element	-	-	IS91 family transposase	E4T26_023705
20	hp	-	-	-	hypothetical protein	E4T26_023710
21	*vopD*	T3SS	Translocon	Pore	type III secretion system translocon subunit	E4T26_023715
22	*vopB*	T3SS	Translocon	Pore	type III secretion system translocon subunit	E4T26_023720
23	*vcrH*	T3SS	Translocon	Chaperone	SycD/LcrH family type III secretion system chaperone	E4T26_023725
24	*vcrV*	T3SS	Needle	Tip	Type III secretion cytoplasmic inhibitor	E4T26_023730
25	*vcrG*	T3SS	Needle	Chaperone	LcrG family type III secretion system chaperone	E4T26_023735
26	*vcrR*	T3SS	Needle	Chaperone	LcrR family type III secretion system chaperone	E4T26_023740
27	*vcrD*	T3SS	Base	C-ring, socket/Cup	type III secretion system export apparatus subunit	E4T26_023745
28	*vscY*	T3SS	Base	C-ring, secretion specificity	type III secretion system chaperone	E4T26_023750
29	*vscX*	T3SS	Base	C-ring, secretion specificity	type III secretion system protein	E4T26_023755
30	*sycN*	T3SS	Regulators	Secretion	type III secretion chaperone	E4T26_023760
31	*tyeA*	T3SS	Regulators	Secretion	TyeA family type III secretion system gatekeeper subunit	E4T26_023765
32	*yopN*	T3SS	Regulators	Secretion	type III secretion system gatekeeper subunit	E4T26_023770
33	*vscN*	T3SS	Base	ATPasa	SctN family type III secretion system ATPase	E4T26_023775
34	*vscO*	T3SS	Regulators	substrate recycling?	YscO family type III secretion system apparatus protein	E4T26_023780
35	*vscP*	T3SS	Needle	length control	type III secretion system needle length determinant	E4T26_023785
36	*vscQ*	T3SS	Base	Cytoplasmic	YscQ/HrcQ family type III secretion apparatus protein	E4T26_023790
37	*vscR*	T3SS	Base	C-ring, socket/Cup	SctR family type III secretion system export apparatus subunit	E4T26_023795
38	*vscS*	T3SS	Base	C-ring, socket/Cup	type III secretion system export apparatus subunit	E4T26_023800
39	*vscT*	T3SS	Base	C-ring, socket/Cup	type III secretion system export apparatus subunit	E4T26_023805
40	*vscU*	T3SS	Base	C-ring, socket/Cup	type III secretion system export apparatus subunit	E4T26_023810
41	*IS**	Mobile element	-	-	IS6 family transposase	E4T26_023815
42	*yopH*	T3SS	Effector	Phosphatase	dual specificity protein phosphatase family protein	E4T26_023820
43	hp	-	-	-	hypothetical protein	E4T26_023825
44	*IS**	Mobile element	-	-	IS91 family transposase	E4T26_023830
45	hp	-	-	-	hypothetical protein	E4T26_023835
46	hp	-	-	-	hypothetical protein	E4T26_023840
47	*tir*	T3SS	Effector	Chaperone	type III secretion system chaperone	E4T26_023845
48	*IS*	Mobile element	-	-	IS6 family transposase	E4T26_023850
49	*excA*	T3SS	Regulators	T3SS transcription	helix-turn-helix transcriptional regulator	E4T26_023855
50	*vscW*	T3SS	Base	Chaperone	YscW family type III secretion system pilotin	E4T26_023860
51	*vscW*	T3SS	Base	Chaperone	YscW family type III secretion system pilotin	E4T26_023865
52	*excA*	T3SS	Regulators	T3SS transcription	helix-turn-helix transcriptional regulator	E4T26_023870
53	*IS*	Mobile element	-	-	IS6 family transposase	E4T26_023875
54	*tir*	T3SS	Effector	Chaperone	type III secretion system chaperone	E4T26_023880
55	hp	-	-	-	hypothetical protein	E4T26_023885
56	hp	-	-	-	hypothetical protein	E4T26_023890
57	*IS**	Mobile element	-	-	IS91 family transposase	E4T26_023895
58	hp	-	-	-	hypothetical protein	E4T26_023900
59	*yopH*	T3SS	Effector	Phosphatase	dual specificity protein phosphatase family protein	E4T26_023905
60	*IS**	Mobile element	-	-	IS6 family transposase	E4T26_023910
61	*vscU*	T3SS	Base	C-ring, socket/Cup	type III secretion system export apparatus subunit	E4T26_023915
62	*vscT*	T3SS	Base	C-ring, socket/Cup	type III secretion system export apparatus subunit	E4T26_023920
63	*vscS*	T3SS	Base	C-ring, socket/Cup	type III secretion system export apparatus subunit	E4T26_023925
64	*vscR*	T3SS	Base	C-ring, socket/Cup	SctR family type III secretion system export apparatus subunit	E4T26_023930
65	*vscQ*	T3SS	Base	Cytoplasmic	YscQ/HrcQ family type III secretion apparatus protein	E4T26_023935
66	*vscP*	T3SS	Needle	length control	type III secretion system needle length determinant	E4T26_023940
67	*vscO*	T3SS	Regulators	substrate recycling?	YscO family type III secretion system apparatus protein	E4T26_023945
68	*vscN*	T3SS	Base	ATPasa	SctN family type III secretion system ATPase	E4T26_023950
69	*yopN*	T3SS	Regulators	Secretion	type III secretion system gatekeeper subunit	E4T26_023955
70	*tyeA*	T3SS	Regulators	Secretion	TyeA family type III secretion system gatekeeper subunit	E4T26_023960
71	*sycN*	T3SS	Regulators	Secretion	type III secretion chaperone	E4T26_023965
72	*vscX*	T3SS	Base	C-ring, secretion specificity	type III secretion system protein	E4T26_023970

Table 2 (continued))

CDS	Gene	Functional category	T3SS part and position		Putative function	Locus tag
73	*vscY*	T3SS	Base	C-ring, secretion specificity	type III secretion system chaperone	E4T26_023975
74	*vcrD*	T3SS	Base	C-ring, socket/Cup	type III secretion system export apparatus subunit	E4T26_023980
75	*vcrR*	T3SS	Needle	Chaperone	LcrR family type III secretion system chaperone	E4T26_023985
76	*vcrG*	T3SS	Needle	Chaperone	LcrG family type III secretion system chaperone	E4T26_023990
77	*vcrV*	T3SS	Needle	Tip	Type III secretion cytoplasmic inhibitor	E4T26_023995
78	*vcrH*	T3SS	Translocon	Chaperone	SycD/LcrH family type III secretion system chaperone	E4T26_024000
79	*vopB*	T3SS	Translocon	Pore	type III secretion system translocon subunit	E4T26_024005
80	*vopD*	T3SS	Translocon	Pore	type III secretion system translocon subunit	E4T26_024010
81	hp	-	-	-	hypothetical protein	E4T26_024015
82	*IS*	Mobile element	-	-	IS91 family transposase	E4T26_024020
83	ATP-binding	ATP-binding protein	-	-	ATP-binding protein	E4T26_024025
84	*IS*	Mobile element	-	-	IS91 family transposase	E4T26_024030
85	hp	-	-	-	hypothetical protein	E4T26_024035
86	hp	-	-	-	hypothetical protein	E4T26_024040
87	*IS**	Mobile element	-	-	IS5/IS1182 family transposase	E4T26_024045
88	recombinase	Recombinase family protein	-	-	recombinase family protein	E4T26_024050
89	ATP-binding	ATP-binding protein	-	-	ATP-binding protein	E4T26_024055
90	*IS*	Mobile element	-	-	IS91 family transposase	E4T26_024060
91	hp	-	-	-	hypothetical protein	E4T26_024065
92	hp	-	-	-	hypothetical protein	E4T26_024070
93	*IS**	Mobile element	-	-	IS5/IS1182 family transposase	E4T26_024075
94	recombinase	Recombinase family protein	-	-	recombinase family protein	E4T26_024080
95	ATP-binding	ATP-binding protein	-	-	ATP-binding protein	E4T26_024085
96	*IS*	Mobile element	-	-	IS91 family transposase	E4T26_024090
97	hp	-	-	-	hypothetical protein	E4T26_024095
98	hp	-	-	-	hypothetical protein	E4T26_024100
99	*IS**	Mobile element	-	-	IS5/IS1182 family transposase	E4T26_024105
100	recombinase	Recombinase family protein	-	-	recombinase family protein	E4T26_024110
101	ATP-binding	ATP-binding protein	-	-	ATP-binding protein	E4T26_024115
102	*IS*	Mobile element	-	-	IS91 family transposase	E4T26_024120
103	hp	-	-	-	hypothetical protein	E4T26_024125
104	hp	-	-	-	hypothetical protein	E4T26_024130
105	*IS**	Mobile element	-	-	IS5/IS1182 family transposase	E4T26_024135
106	recombinase	Recombinase family protein	-	-	recombinase family protein	E4T26_024140
107	*IS*	Mobile element	-	-	Tn3 family transposase	E4T26_024145
108	*IS*	Mobile element	-	-	Tn3 family transposase	E4T26_024150
109	*IS*	Mobile element	-	-	IS1-like element ISPda1 family transposase	E4T26_024155
110	*traI**	Conjugation region	-	-	AAA family ATPase	E4T26_024160
111	*traD**	Conjugation region	-	-	type IV conjugative transfer system coupling protein	E4T26_024165
112	*IS*	Mobile element	-	-	IS1-like element ISPda1 family transposase	E4T26_024170
113	hp	-	-	-	hypothetical protein	E4T26_024175
114	hp	-	-	-	NERD domain-containing protein	E4T26_024180
115	hp	-	-	-	DUF3465 domain-containing protein	E4T26_024185
116	*traW*	Conjugation region	-	-	type-F conjugative transfer system protein	E4T26_024190
117	hp	-	-	-	hypothetical protein	E4T26_024195
118	*trbI*	Conjugation region	-	-	TrbI F-type domain-containing protein	E4T26_024200
119	*traC**	Conjugation region	-	-	type IV secretion system protein	E4T26_024205
120	*IS*	Mobile element	-	-	transposase	E4T26_024210
121	*IS*	Mobile element	-	-	IS3 family transposase	E4T26_024215
122	*traV*	Conjugation region	-	-	type IV conjugative transfer system lipoprotein	E4T26_024220
123	*traB*	Conjugation region	-	-	conjugal transfer protein	E4T26_024225
124	*traK*	Conjugation region	-	-	type-F conjugative transfer system secretin	E4T26_024230
125	*traE*	Conjugation region	-	-	type IV conjugative transfer system protein	E4T26_024235
126	*traL*	Conjugation region	-	-	type IV conjugative transfer system protein	E4T26_024240
127	*traA*	Conjugation region	-	-	type IV conjugative transfer system pilin	E4T26_024245
128	hp	-	-	-	hypothetical protein	E4T26_024250
129	hp	-	-	-	hypothetical protein	E4T26_024255
130	*IS*	Mobile element	-	-	IS91 family transposase	E4T26_024260
131	hp	-	-	-	hypothetical protein	E4T26_024265
132	hp*	-	-	-	hypothetical protein	E4T26_024270
133	*IS*	Mobile element	-	-	IS91 family transposase	E4T26_024275
134	hp	-	-	-	hypothetical protein	E4T26_024280
135	*IS**	Mobile element	-	-	IS91 family transposase	E4T26_024285
136	hp	-	-	-	hypothetical protein	E4T26_024290
137	hp	-	-	-	hypothetical protein	E4T26_024295
138	chaperone	T3SS	-	Chaperone	type III secretion system chaperone	E4T26_024300
139	*IS*	Mobile element	-	-	IS91-like element ISPda2 family transposase	E4T26_024305
140	hp	-	-	-	hypothetical protein	E4T26_024310
141	*IS*	Mobile element	-	-	IS91 family transposase	E4T26_024315
142	*yadA*		-	-	YadA-like family protein	E4T26_024320
143	*IS**	Mobile element	-	-	IS91 family transposase	E4T26_024325
144	hp	-	-	-	hypothetical protein	E4T26_024330
145	*IS**	Mobile element	-	-	IS91 family transposase	E4T26_024335
146	hp	-	-	-	hypothetical protein	E4T26_024340

The most prominent organizational feature of pPDPT3 is the 62,153 bp region that accounts for half of the plasmid sequence (coordinates 5,573 to 67,725) and contains the genes of the T3SS. This region consists of two 100% identical, paralogous copies of a set of T3SS genes organized in opposite orientations (Figure 2). This repeated region is flanked, at both sides, by the genes of a hypothetical protein and a transposase respectively, which are located downstream *vopBD* genes. These two divergently-oriented gene sets differ by the presence, in one of the duplicated sets, of a module that contains the genes *vscABCDEFGHIJKL* in single copy. Thus, it may be considered that pPDPT3 contains a "*complete*" T3SS gene cluster comprised of 36 genes, and an "*incomplete*" T3SS gene cluster comprised of 24 genes (Figure 2). This unusual and intricate structure of T3SS genes occurring in double copy in the plasmid, was further demonstrated in this study by inserting a distinct antibiotic resistance gene into each of the two *vcrD* gene alleles (see below).

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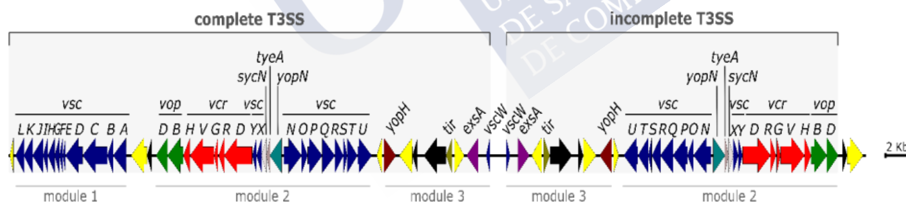

Figure 2: Schematic representation of the complete (E4T26_023645-E4T26_023860) and the incomplete (E4T26_023865- E4T26_024010) T3SS gene clusters. T3SS genes are arranged in three modules separated by transposases. The yellow arrows correspond to transposases and the black arrows to hypothetical proteins.

T3SS complete structure includes a basal body that crosses the inner and outer membranes of the bacterial cell, a cytoplasmic sorting platform that plays a role in effector selection and needle creation, and a needle that extends to the extracellular space and capable to interact with the host cell membrane (Burkinshaw and Strynadka, 2014). The T3SS gene cluster of pPDPT3 includes the structural genes of the secretion machinery (Table 2) and is divided into three modules separated by transposase genes that do not disrupt any gene function, clearly suggesting the existence of a selective pressure to maintain the T3SS functional in this pathogen. The first module includes *vscABCDEFGHIJKL* genes which are all transcribed from the same strand and are flanked by two different transposase genes. A central, second module includes *vscNOPQRSTUYX*, *yopN*, *tyeA, sycN, vcrDRGVH,* and *vopBD* genes. A third module contains genes encoding a predicted effector protein YopH-like, a Tir type III secretion system chaperone, a putative transcriptional regulator ExsA, VscW type III secretion system pilotin, and a number of transposases and hypothetical proteins (Figure 2). This third module constitutes the symmetry axis point that separates the two paralogous copies of the T3SS genes. The incomplete T3SS gene cluster is a 100% identical (at the nucleotide sequence level) paralogous copy of the complete T3SS cluster, oriented in opposite direction, but lacks genes *vscABCDEFGHIJKL.*

The T3SS gene cluster encoded within pPDPT3 is homologous to T3SS gene clusters encoded in chromosomes and in plasmids of *Vibrio* and *Photobacterium* species

The type III secretion system cluster of pPDPT3 shows a high degree of similarity and gene synteny with the chromosome-encoded T3SS1 of *Vibrio parahaemolyticus* RIMD 2210633 (Park et al., 2004), but pPDPT3 lacks a specific region otherwise present in *V.*

parahaemolyticus RIMD 2210633 which encodes effector proteins VopQ, VopR, and VopS (Figure 3). pPDPT3 also shows conserved synteny with T3SS gene clusters found in a putative plasmid (pPHDD203) of *Photobacterium damselae* subsp. *damselae* CIP 102761 (Vences et al., 2017), a plasmid that has not been functionally characterized so far. pPDPT3 structure also exhibits conserved synteny with a sequence scaffold reported in the genome of the *Pdp* strain OT-51443 and that corresponds to a putative plasmid not characterized so far (Aoki et al., 2017) (Figure 3). Several specific features of pPDPT3 which are absent from the homologous clusters include, first, the presence of two paralogous copies of a set of T3SS genes as mentioned above. And, second, pPDPT3 contains a specific region close to the symmetry axis between the two paralogous copies, that include the aforementioned genes encoding the predicted effector protein YopH-like, a Tir type III secretion system chaperone, a putative transcriptional regulator ExsA and VscW type III secretion system pilotin.

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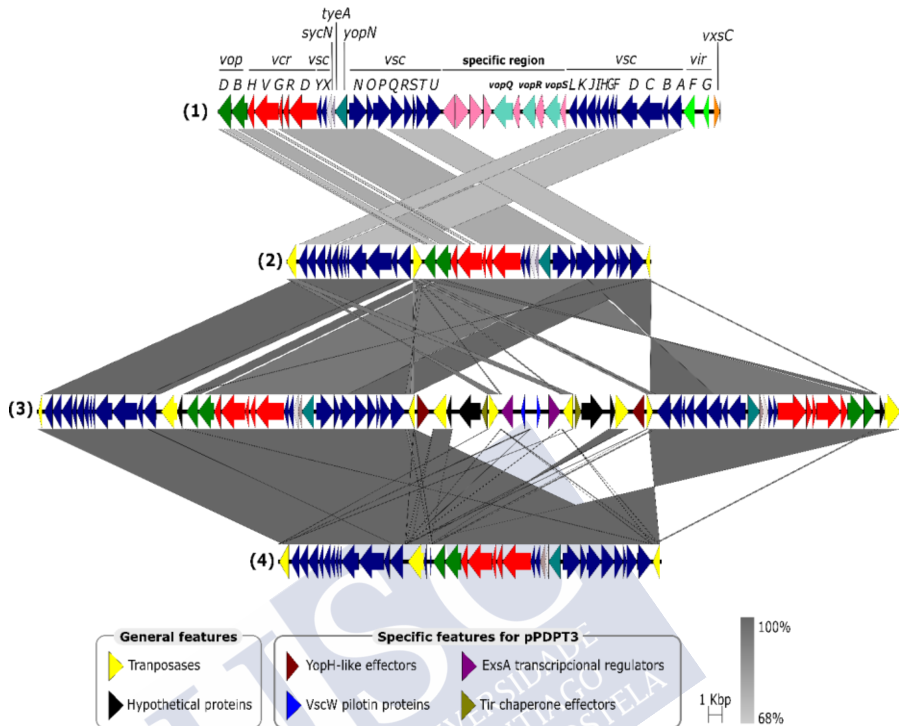

Figure 3: Linear genetic representation of T3SS gene clusters region in four strains: (1) *Vibrio parahaemolyticus* RIMD 2210633, (2) *Pdd* CIP102761, (3) *Pdp* PP3, and (4) *Pdp* OT-51443. The arrows depict the open reading frame organization within each cluster, and the orientation of the arrows indicate the direction of transcription for each gene. Grey-shaded vertical blocks between sequences indicate the homologous regions, the colour intensity denoting the identity levels (from 68% to 100%). GenBank accession numbers are: (1) BA000031, (2) ADBS01000003, (3) SRHT02000000 and (4) BDMQ01000003.

A comparison of the whole sequences of these three plasmids, namely pPDPT3, pPHDD203 and the putative plasmid of *Pdp* OT-51443, reveals conserved genetic architecture among them, as well as specific regions (Figure 4).

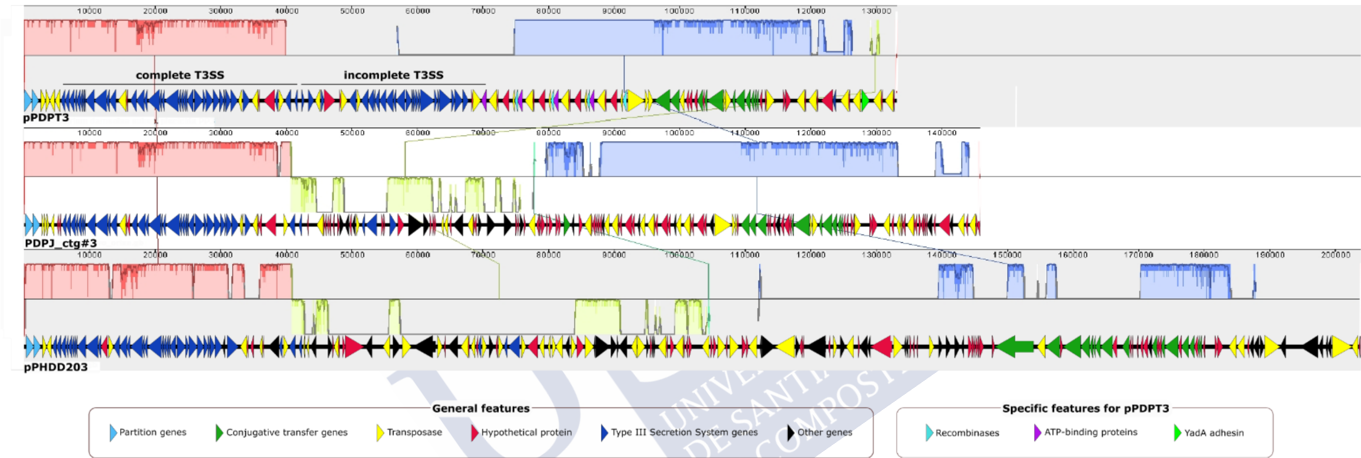

Figure 4: Mauve progressive alignment (Darling *et al.*, 2010) of the sequences of pPDPT3 (*Pdp*), pPHDD203 (*Pdd*) and PDPJ_ctg#3 (*Pdp*) plasmids. Genes are color-coded according to function. Lines are drawn to connect the similar blocks based on the sequence identity. GenBank accession numbers are: pPDPT3 (SRHT02000000), pPHDD203 (ADBS01000003) and PDPJ_ctg#3 (BDMQ01000003).

pPDPT3 is highly unstable and undergoes curing upon cultivation in laboratory

In a recent study, we observed that the T3SS genes were spontaneously lost concomitantly with some plasmid-related genes in *Pdp* Rif^{R} (rifampicin resistant) mutants, a phenomenon that was initially suggested to be enhanced by presence of rifampicin (Abushattal et al., 2020). In order to gain an insight into the role of the T3SS in virulence of *Pdp* PP3 for fish, we here attempted to construct an in-frame deletion mutant in *vcrD* gene by a classical allelic exchange procedure, in the wild type PP3 strain. Surprisingly, even though it was feasible to select a first recombination that integrated the suicide plasmid into the target gene in pPDPT3 (this was possible by selecting for Kan^{R}), we were unable, despite reiterated attempts, to obtain a second recombinant that would eliminate the suicide plasmid leaving the deleted allele in the genome. Instead, the *Pdp* PP3 colonies grown under the conditions that would select the second recombination (growth in presence of 15% sucrose without pressure for kanamycin resistance) all tested negative for *vcrD* and additional pPDPT3 genes (data not shown), demonstrating that plasmid loss occurred spontaneously in absence of rifampicin and other antibiotics. We further observed that *Pdp* PP3 streaked on a TSA-1 agar plate directly from a glycerol stock that had been maintained at -80ºC, tested positive for T3SS markers on condition that PCR was conducted using the bacterial biomass of the confluent growth on the first streaks as source of DNA template for PCR. However, a variable number of isolated colonies from the same plate tested negative, indicating pPDPT3 plasmid loss in the isolated colonies (Figure 5). These colonies tested positive for pPHDP10 and pPHDP70 gene markers, two co-resident virulence plasmids in *Pdp* PP3. Furthermore, we demonstrated that plasmid loss is not a feature unique to *Pdp* PP3 but a generalized feature of *Pdp* strains, since the same phenomenon of pPDPT3 curing was observed in 3 *Pdp* strains isolated in our laboratory directly from diseased fish, and that had undergone only one subculture pass between fish sampling and frozen stock preparation (Figure 5).

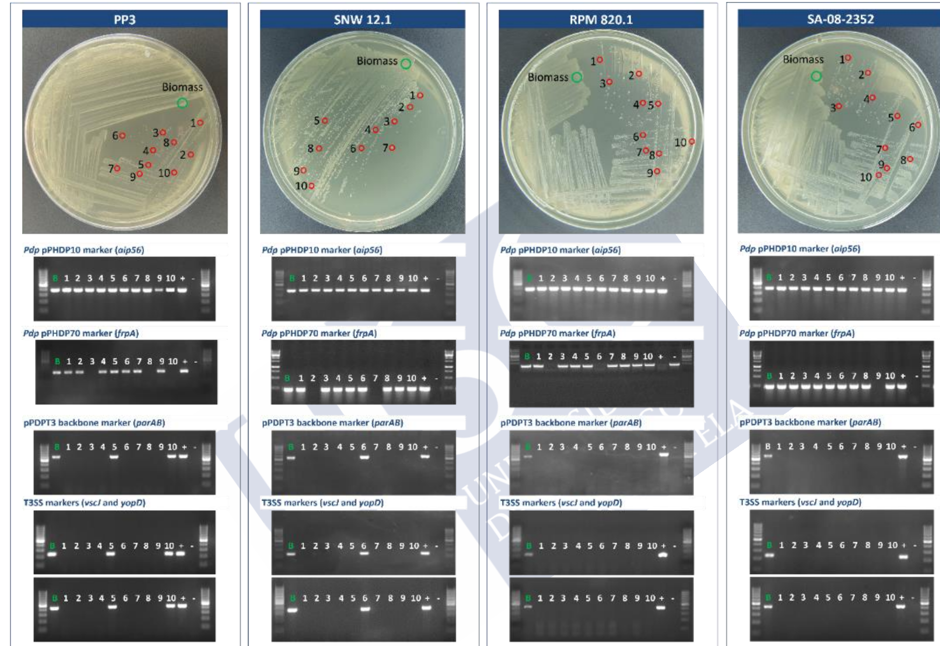

Figure 5: Detection of pPDPT3 loss in *Pdp* isolated colonies and in biomass by direct colony-PCR. A colony screening was conducted by PCR amplifications targeted to gene markers of plasmids pPHPP10 (*aip56*), pPHDPP70 (*frpA*), and pPDPT3 (*parAB*, *vscJ*, and *yopD*). Four different *Pdp* strains, PP3, SNW12.1, RPM 820.1 and SA-08-2352 were used. Purified DNA of *Pdp* PP3 was used as positive control. Ten single colonies were randomly selected for each strain to test the stability of pPDPT3 plasmid plus the biomass of at the confluent growth for each strain. Lane numbers from one to ten denote single colonies of each strain and the biomass is labeled as B-letter. All the chosen colonies yielded a positive result for the *aip56* gene. The absence of amplification of *parAB*, *vscJ*, and *yopD* markers was indicative of pPDPT3 curing, and demonstrates the high instability of pPDPT3 plasmid. Molecular mass ruler of 100 bp (Fermentas) is shown at the left and right ends.

Additional evidence of spontaneous pPDPT3 loss upon subcultivation of *Pdp* strains arose in our laboratory during the process of DNA purification for genome sequencing. When the present study was conceived, the available draft genomes for *Pdp* strains PP3 and SNW-8.1 were fragmented into numerous contigs, a fact that prevented from deciphering the genetic context of the T3SS genes. We thus subjected the genomes of PP3 and SNW-8.1 to a PacBio sequencing. For DNA purification, the two strains were streaked on TSA-1 plates from frozen stocks. Genomic DNA of strain PP3 was purified using the whole biomass grown in the agar plate, including the thick streak of growth with no isolated colonies. However, SNW-8.1 plate was subjected to a subsequent step of colony isolation so that a new plate subculture was grown and used for genomic DNA sequencing. As a result, we found that the PacBio sequence of PP3 contained the T3SS genes within the novel plasmid that is described in the present study. Unexpectedly, the PacBio sequence of strain SNW-8.1 did not contain neither the T3SS genes nor other plasmid genes that are otherwise present in pPDPT3 (data not shown). The same unexpected result of loss of T3SS genes was obtained when we subjected to complete DNA sequencing 2 additional *Pdp* isolates whose genomic DNA was purified from subculturing plates originated from isolated colonies from the plate originally derived from the frozen stock (data not shown).

Virulence for fish is reduced in pPDPT3-cured strains

In order to gain an insight into the role of pPDPT3 in virulence, we selected an isolated colony of *Pdp* PP3 that underwent pPDPT3 loss and that tested positive for the virulence plasmids pPHDP10 and pPHDP70 (SSS260). Bacterial suspensions derived from the pPDPT3-negative colony and from the original parental strain (testing positive for pPDPT3) were inoculated into European sea bass fish and

mortalities were quantified for 7 d. Remarkably, the parental strain killed 100% fish within the first 4 d post-inoculation, whereas the pPDPT3-cured strain only killed 19% fish, at a dose of 4.61 × 10^4 CFU/fish, thus demonstrating a strong impairment of virulence in absence of pPDPT3 plasmid (Figure 6A).

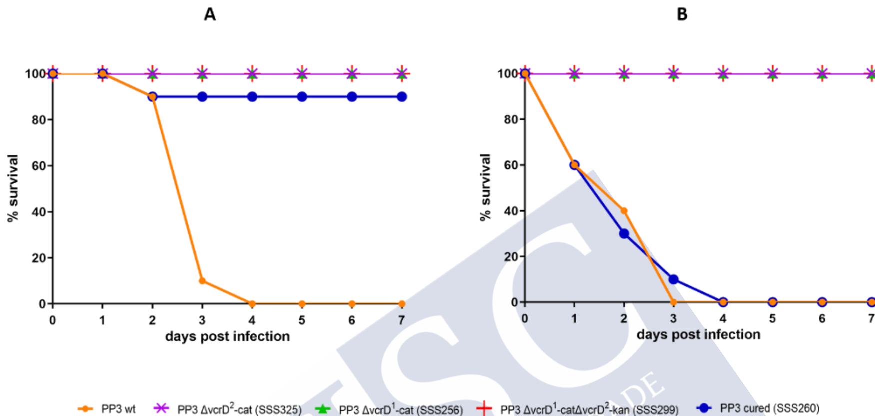

Figure 6: Survival (%) of sea bass fish after intraperitoneal injection of the *P. damselae* subsp. *piscicida* wild type strain (PP3), pPDPT3-cured strain (SSS260), single *vcrD¹* (SSS256) and *vcrD²* (SSS325) mutants, and double *vcrD* mutant (SSS299). A total of 10 fish were inoculated per strain at two different doses of 4.61 X 10^4 CFU/fish (A), and 4.61 X 10^6 CFU/fish (B). The respective control fish group (10 fish inoculated with 0.1 ml of sterile 0.85% NaCl solution) did not register any mortalities (data not shown).

Insertional inactivation of *vcrD* causes a strong impairment in virulence for fish: a phenotype likely explained by forced integration of pPDPT3 in the chromosome disrupting essential genes for virulence

Next, we aimed at specifically inactivating the T3SS secretory machinery to assess the role of this secretion system in virulence. We thus designed an allelic exchange strategy for inserting a chloramphenicol cassette (*cat* gene) in substitution of the coding

sequence of *vcrD* gene. Hence, by adding chloramphenicol in all the steps during mutant construction process, it was feasible to maintain the plasmid stable avoiding its spontaneous loss and, eventually, to insertionally-inactivate one of the two *vcrD* paralogous copies (hereafter dubbed $vcrD^{1}$ and $vcrD^{2}$) (Figure 7). Further on, the single $vcrD^{1}$ mutant was used as parental strain for construction of a double *vcrD* insertional mutant, by substituting the sequence of the other *vcrD* gene copy by a kanamycin resistance cassette. In order to obtain the two different versions of single mutants, we repeated the same procedure used for the first, *cat*-labelled single $vcrD^{1}$ mutant, to obtain a mutant in the other, paralogous and 100% identical, $vcrD^{2}$ copy. The distinction between each of the two mutants was achieved by using unique primer combinations that, by Long-PCR, would yield specific amplification products depending of which *vcrD* allele was being mutated. This mutagenesis approach, in addition to fulfil the aim of generating T3SS-defective mutants, also allowed us to corroborate the results of the PacBio sequencing and clearly demonstrated that the plasmid indeed contains two copies of *vcrD* gene (Figure 7).

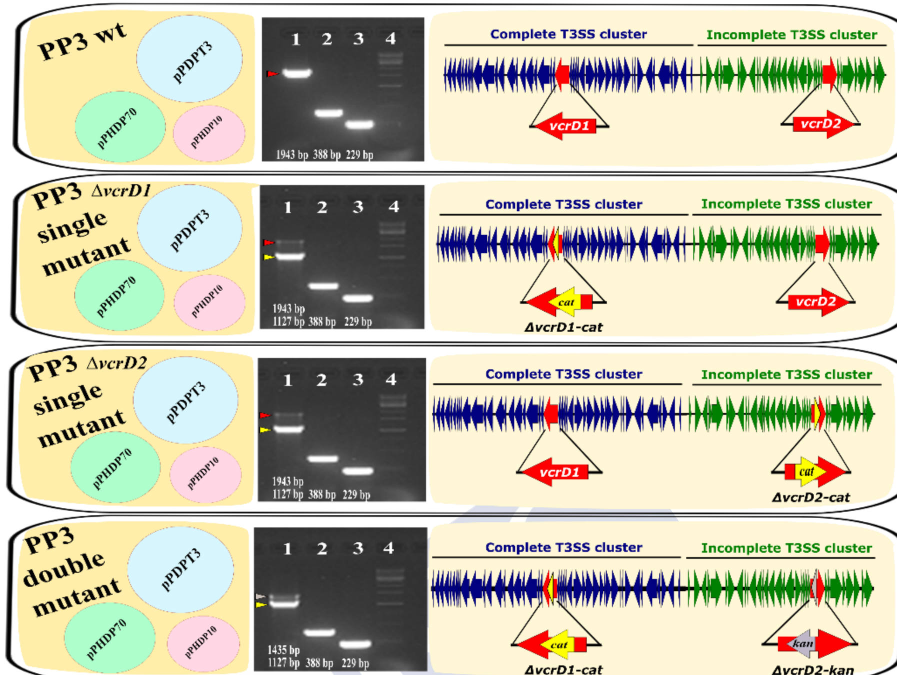

Figure 7: Schematic illustration to show the *vcrD*[1] and *vcrD*[2] gene location within the T3SS genes clusters and the strategy to generate single and double *vcrD* gene mutants. Demonstration of *vcrD* alleles disruption was conducted with PCR amplifications using specific primer combinations as described in table 4. Gene markers for other plasmid genes were amplified as controls. Agarose gel lanes are labeled as follows: (1) pPDPT3_*vcrD* gene: 1943 bp, (2) pPHDP10_*aip56* gene: 388 bp, (3) pPHDP70_*frpA* gene: 229 bp, and (4) ladder: 1Kb (Fermentas). The arrows at the left side of the agarose gels indicate the PCR amplicons for either the wild type or deletion-mutant versions of each *vcrD* allele. The yellow arrows indicate the chloramphenicol cassette location and orientation and the gray arrows indicate the kanamycin cassette location and orientation.

Notably, virulence assays in sea bass conducted with the single $vcrD^1$ and $vcrD^2$ mutants (SSS256 and SSS325, respectively) and with the double $vcrD^1$-$vcrD^2$ mutant (SSS299) demonstrated abolition of virulence at the same dose reported above, of 4.61×10^4 CFU/fish since no fish deaths were monitored after 7-day post-inoculation

(Figure 6A). These observations might be considered to constitute a strong evidence that the T3SS encoded within pPDPT3 is a major contributor of *Pdp* virulence for fish. Insertional disruption of $vcrD^{1}$ would be predicted to cause polar effects in the expression of the single copy genes *vscABCDEFGHIJKL*, and thus explain the abolition of virulence in this single mutant. However, it was at first surprising that the single $vcrD^{2}$ mutant appeared to be equally impaired in virulence, because this single mutant has an intact copy of the complete T3SS cluster. The fact that the two T3SS clusters (complete and incomplete) are virtually free of inactivating *IS* insertions, might suggests that each of the two clusters play an important function in virulence.

In order to gain a further insight into the degree of virulence attenuation in cured strains and *vcrD* mutants, we conducted a fish virulence assay using a dose of 4.61×10^{6} CFU/fish, i.e, 100× higher than the previously used dose. At this dose, the pPDPT3-cured strain killed 100% fish and, much unexpectedly, the single and double *vcrD* mutants did not cause any fish mortality ((Figure 6B). Also of note, we were unable to obtain plasmid-cured strains derived from the single and double *vcrD* mutants despite reiterated attempts. This impossibility to obtain pPDPT3-cured derivatives was in high contrast with the easiness with which cured strains were isolated from the parental strain after single passages on agar plates (see above). Such striking observations led us to hypothesize that the combination between the high instability of pPDPT3 and the antibiotic selective pressure (Cm and/or Kan depending on the marker used) invariably forced the integration of pPDPT3 into the *Pdp* chromosomes. This would explain the failure in selecting a spontaneously cured strain when any of the single and double *vcrD* mutants is used. In addition, the observed strong impairment in virulence for fish in these mutants (an impairment that proved to be more acute than in the spontaneously

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cured strains), might be explained by the integration of pPDPT3 in a chromosomal locus that causes an impairment in a fitness and/or a virulence-related function.

pPDPT3 plasmid is unstable under different temperature conditions

We wanted to gain an insight into the stability of pPDPT3 when *Pdp* PP3 strain is grown at 25ºC vs. 18ºC (see methods). Plasmid presence was monitored by specific PCRs targeting genes related to the T3SS and also genes related to the backbone structure of pPDPT3. Simultaneously, specific PCR amplification targeting the *aip56* gene carried on pPHDP10 plasmid and targeting *frpA* gene of pPHDP70 plasmid were conducted in parallel, to ensure the stability of these two co-resident plasmids. The results demonstrated that pPDPT3 is lost in a variable fraction of colonies while it is preserved in others, and the percentage of colonies that have lost the plasmid is similar at 25 and at 18ºC (Figure 8). Also, it was corroborated that pPHDP10 plasmid in highly stable, with 100% colonies testing positive for it. Of note, spontaneous loss of pPHDP70 was also detected in a fraction of colonies.

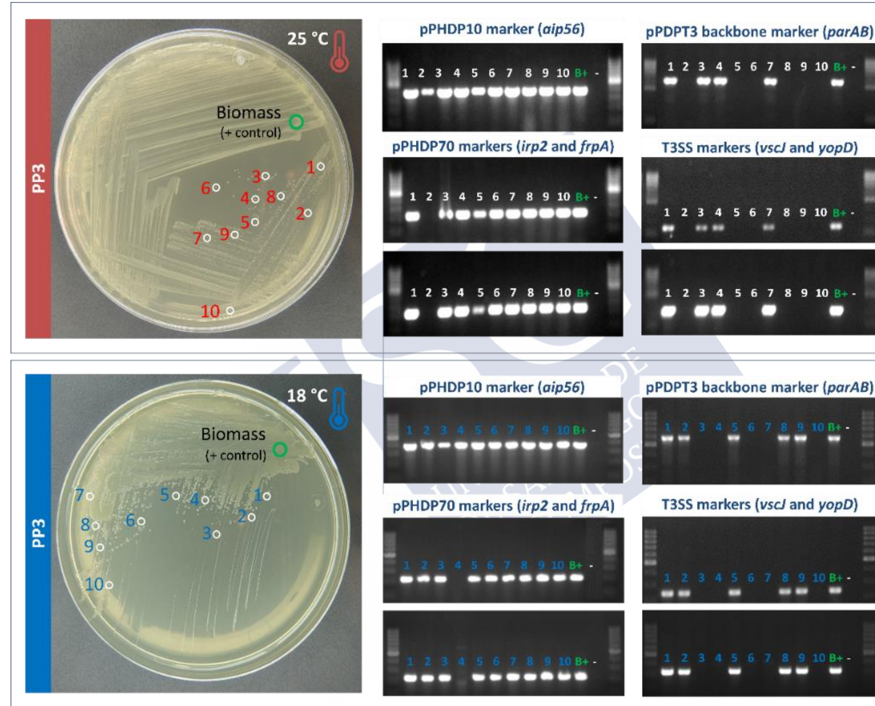

Figure 8: PCR screening of gene markers of pPHPP10 (*aip56*), pPHDPP70 (*frpA*), and pPDPT3 (*parAB*, *vscJ*, and *yopD*) plasmids, in *Pdp* PP3 cultivated at 25ºC and at 18ºC. Ten randomly selected single colonies (lane numbers from one to ten) plus the biomass of the confluent growth (denoted by a "B" lane) were PCR-tested for plasmid stability. All the colonies yielded a positive result for the *aip56* gene. The absence of amplification of *parAB*, *vscJ*, and *yopD* markers showed instability of the pPDPT3 plasmid in a fraction of colonies. Notably, curation of pPHDP70 was also detected in some colonies. Molecular mass ruler of 100 bp (Fermentas) is shown at the left and right ends.

pPDPT3 contains an incomplete set of conjugative transfer genes, and the resistance-marker labelled versions are not transmissible under laboratory conditions

Sequence analysis of pPDPT3 reveals the presence of a 15 kb region containing 11 predicted genes *traALEKBVC, trbI and traWDI* of the conjugative transfer apparatus (Figure 1). This module of genes for conjugative transfer functions is divided into three submodules by insertion sequence (*IS*) elements. The *IS1* element was found in two copies in two different sites between *tra* genes, namely downstream *traI* and upstream *traD* gene. The *IS3* element is located between *traC* and *traV* genes. Five genes encoding hypothetical proteins are interspersed among *trbI* and *traW* genes. We conducted a comparative sequence analysis between pPDPT3 transfer region genes and homologous regions previously sequenced in 4 plasmids in other *Photobacterium damselae* strains, covering the two subspecies (Figure 9). This comparative analysis shows an almost exact match between pPDPT3 and a putative plasmid in *Pdp* strain OT-51443, that also contains genes for a T3SS (Figure 3). Notably, the *tra* region in these two *Pdp* plasmids is much shorter than the homologous *tra* region of three different plasmids of *P. damselae* subsp. *damselae* strains, clearly suggesting that the two *Pdp* plasmids contain an incomplete set of conjugative transfer genes. Indeed, the plasmid comparison provides evidence that there is a loss of conjugation genes in the region between *traD* and *traW* genes, which is represented by the absence of *traGHFNU* and *trbBC* genes in pPDPT3 and also in the homologous putative plasmid of *Pdp* OT-51443.

Due to the plasmid-borne nature of T3SS genes in *Pdp*, we judged interesting to investigate the ability of pPDPT3 to be transferred by conjugation from *Pdp* PP3 to a recipient bacterium, by selecting for presence in the recipient cells of the chloramphenicol and kanamycin cassettes that were inserted into each of the two *vcrD* paralogous

copies. After reiterated attempts of conjugation, we systematically failed to detect transconjugants when double *vrcD*^{1}–*vcrD*^{2} mutant of *Pdp* PP3 was mated with *E. coli* CAG18420 strain. Negative results were also obtained when *Pdd* LD-07-Rif^{R} was used as recipient. These observations initially suggested that pPDPT3 was not a conjugative plasmid, likely motivated by the loss of some essential *tra* genes as said above.

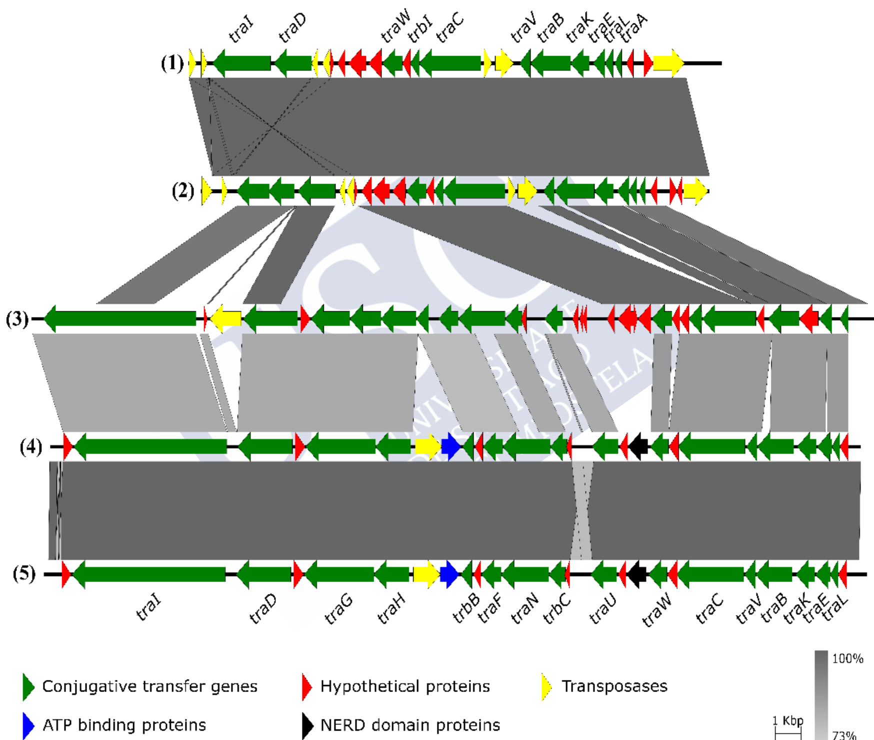

Figure 9: Linear genetic representation of the conjugation genes region in five plasmids: (1) pPDPT3 of *Pdp* PP3 (SRHT02000010.1); (2) contig 3 of *Pdd* OT-51443 (BDMQ01000003); (3) pPHDP203 of *Pdd* CIP 102761 (NZ_ADBS01000003); (4) pPDD-Na-1-3 of *Pdd* KC-Na-NB1 (NZ_CP021155) and (5) pFPPDNB1-3 of *Pdd* KC-Na-NB1 (CPO35461). The arrows depict the open reading frame organization within each cluster and the orientation of the arrows indicates direction of transcription. Grey-shaded vertical blocks connect homologous sequences, the color intensity indicating the identity levels (from 73% to 100%).

We next aimed at investigating whether pPDPT3 still retained the ability to be mobilized with the help of a co-resident mobile element that encodes a complete set of *tra* genes. We thus hypothesized that an integrative conjugative element (ICE) of the SXT/R391 family might provide the necessary functions *in trans* as to allow the mobilization of pPDPT3. Indeed, the integrative and conjugative element ICE*Pda*Spa1 belonging to the SXT/R391 family was described in a virulent *Pdp* strain (Osorio et al., 2008). However, we failed to select for conjugation of ICE*Pda*Spa1 into *Pdp* PP3 strain, because ICE*Pda*Spa1 carries a Tc^R marker (that would serve to select for ICE*Pda*Spa1 acquisition by PP3), and we discovered that *Pdp* PP3 also harbours a tetracycline resistance loci (E4T26_RS07625 - E4T26_RS07635). Thus, we selected for this experiment the SXT^{MO10} ICE element, originally described in *V. cholera*e (Beaber et al.,2002). The SXT^{MO10} element, that confers resistance to streptomycin and chloramphenicol among other resistances, was conjugated from *E. coli* HW220 (CAG18439 *prfC*::SXT^{MO10}) to *Pdp* SSS299 strain, which containing pPDPT3 labelled with a kanamycin and a chloramphenicol marker into each of the paralogous copies of *vcrD* gene. A PP3 transconjugant (PP3 Δ$vcrD^1$-*cat* +Δ$vcrD^2$-*kan* + SXT^{MO10}) containing the SXT^{MO10} element (corroborated by positive amplification of the SXT_MO10-borne *floR* and *sul2* genes) was used as donor with *E. coli* BI533 (Nal^R) as a recipient to assess transfer of pPDPT3. However, despite numerous attempts, no transfer of pPDPT3 could be detected. This inability of the labelled versions of pPDPT3 to be conjugated, might be explained either by pPDPT3 being *ab initio* a non-transferable plasmid, or by the suspected integration of the antibiotic resistant *vcrD*-mutant versions of pPDPT3 in the chromosome (see above).

DISCUSSION

After more than 55 years since it first report as a fish pathogen (Snieszko et al., 1964), *Pdp* nowadays continues to be a major threat to the cultures of many species of fish of importance in marine aquaculture worldwide (Liu et al., 2003; Liu et al., 2011; Essam et al., 2016; Costa et al., 2017; Leu et al., 2019; Pham et al., 2020; Shimada et al., 2020). In the past two decades, research has focused on the identification and the study of virulence factors in *Pdp*, the most important so far being the apoptosis-inducing toxin AIP56 encoded within plasmid pPHDP10 (do Vale et al., 2005) and the siderophore piscibactin system encoded within plasmid pPHDP70 (Osorio et al., 2015). In the current study, we investigated for the first time the role of a plasmid-borne T3SS system in *Pdp* ability to cause disease in fish. Notably, although *Pdp* has been causing important financial losses in marine aquaculture for longer than 50 years, it was not until 2019 that the presence of genes of the type III secretion system was reported in this pathogen (Abushattal et al., 2019). Soon afterwards, it became evident that genes of the T3SS were more the norm than the exception in *Pdp* isolates, and it was suggested that the T3SS might be encoded within an unstable plasmid (Abushattal et al., 2020).

In the current study, we demonstrate that the T3SS genes are carried on a large 133 Kb plasmid in *Pdp* PP3, and this plasmid is highly unstable during the growth of *Pdp* strains under laboratory conditions. The most characteristic feature of pPDPT3 are the two T3SS gene sets that account for 55% of the total plasmid length. These genes occur in two copies, one of which is so-called *incomplete* and the other *complete*, and the two sets are flanked by *IS* elements. A total of 33 *IS* of six different families account for 22% of the total plasmid sequence and many of them occur in multicopy. The pPDPT3 IS1 have 99% sequence similarity with IS1 of the co-resident plasmids pPHDP10 and pPHDP70, demonstrating the high capacity of expansion of this

IS, that is present in very high copies in the two *Pdp* chromosomes (Balado et al., 2017). These elements confer a high degree of genetic plasticity to pPDPT3, with the potential to undergo structural rearrangements, fast sequence variations and acquisition of new genes. The location of the T3SS genes is not restricted to either chromosomes or plasmids through bacterial species. In *Yersinia*, T3SS genes are carried on a 70 Kb plasmid (pYV) (Trosky et al., 2008), while T3SS genes in *Aeromonas salmonicida* are located on the chromosome and on plasmids (Burr et al., 2002; Stuber et al., 2003). The presence of T3SS genes on plasmids enhances the chance of their spread among bacterial species through horizontal gene transfer. The presence of T3SS in numerous species of Gram-negative bacteria provide them additional power to attack the host's cells and cause severe disease, in animal pathogens (*Yersinia, Shigella, Salmonella, Escherichia, Pseudomonas, and Chlamydia*) and also in plant pathogens (*Pseudomonas, Erwinia, Ralstoniav, Xanthomonas, and Rhizobium*) (Hueck, 1998). In *Vibrio* species, one of the most prevalent groups in aquaculture, T3SS genes were first discovered in *Vibrio parahaemolyticus* carrying the T3SS1 and T3SS2 gene clusters (Makino et al., 2003). Nowadays, the number of *Vibrio* and *Photobacterium* species in which T3SS genes are being discovered, is increasing (Osorio, 2018). This high dissemination of T3SS among bacterial species is a clear indicator of the role of T3SS in bacterial pathogenicity.

A major contribution of the present study is the demonstration that pPDPT3 is highly unstable in laboratory culture conditions, and it becomes lost in the majority of isolated colonies after a single passage on an agar plate. The reasons for this high instability are so far unknown. According to previous studies in *Aeromonas salmonicida*, a major pathogen of fish in aquaculture, a plasmid encoding the T3SS in this species is highly prone to undergo genetic rearrangements and

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T3SS gene losses when *A. salmonicida* is grown at 25ºC, but not when it is grown at 18ºC (Stuber et al., 2003; Tanaka et al., 2017). However, in this study we noticed that pPDPT3 was equally unstable during cultivation under laboratory conditions at either 25ºC or at 18ºC. This high instability of pPDPT3 prompted us to label each of the two paralogous copies of *vcrD* gene with distinct antibiotic cassettes in order to be able to study this plasmid as well as to assess its role in virulence. To date, there are no studies related to T3SS genes and their role in virulence in *Pdp* strains. Challenge assays with sea bass fish, yielded unexpected and intriguing results. Plasmidless strains exhibited reduced virulence than parental strains for sea bass. However, *Pdp* PP3 insertional mutants in either of the two paralogous copies of *vcrD* gene did not cause any fish mortality, even when the inoculated dose was doubled. As expected, a double mutant with the two *vcrD* gene copies insertionally inactivated, was equally non-virulent. Thus, our results indicated that a mutation in one of the two copies of the *vcrD* gene was sufficient to abolish the pathogenicity of *PP3* strain. We thus hypothesized that the obtention of a plasmid-cured version of any of these T3SS mutants, would render the strain virulent again. However, much surprisingly, after reiterated attempts, we were unable to isolate a pPDPT3-cured strain starting from a single or a double *vcrD* mutant. This new and unexpected scenario, suggests that, somehow, the presence of pPDPT3 in the cell with an inactivated T3SS system, would maintain the plasmid stable in vitro, likely by integration of pPDPT3 into the *Pdp* chromosomes. Such integration would be expected to inactivate genes essential for virulence and/or for in vivo fitness. Previous studies pointed at the existence of intricate mechanisms of cross-talk between plasmids and the rest of the bacterial genome. In some instances, plasmid-encoded regulators (either regulatory proteins or regulatory small RNAs) have been demonstrated to repress gene functions encoded in other replicons, namely in chromosomes. This is the case for the pAB5

plasmid that represses chromosomal virulence genes in *Acinetobacter baumannii* (Di Venanzio et al., 2019). Notably, a virulence plasmid of *Shigella* spp. is known to exhibit a high degree of unstability, and under laboratory conditions this plasmid can integrate into the bacterial chromosome and disrupt single copy genes necessary for the bacterial fitness (McVicker and Tang, 2016; Pilla et al., 2017).

The putative integration of pPDPT3 during T3SS-genes mutant construction, and the differences in virulence between cured strains and *vcrD* mutants, prevents so far from attributing a definite role in virulence to the T3SS itself. The plasmid encodes a YopH-like tyrosine phosphatase effector, whose homologues in other bacterial species play a major role in virulence, as reported in *Aeromonas salmonicida* (Dacanay et al., 2006) and in *Yersinia spp*. (Guan and Dixon, 1990; Bliska et al., 1991; Shaban et al., 2020). However, at present, it cannot be ruled out the possibility that other functions encoded within pPDPT3, different to the T3SS, play roles in virulence. pPDPT3 encodes a YadA-like putative adhesin, whose homologue constitutes a virulence factor in *Yersinia enterocolitica* (El Tahir and Skurnik, 2001; Mühlenkamp et al., 2015). Further studies will be necessary to unravel the roles of these proteins encoded by pPDPT3 in the pathogenesis of *Pdp*.

The high instability of pPDPT3 detected in the present study warns about the possibility that similar, unstable plasmids might be ubiquitous in isolates of this pathogen, and that its proneness to be lost in cultivation would prevent researchers from detecting and studying them in *Pdp* isolates worldwide. Laboratory handling and culturing may cause the loss of these unstable genetic elements, with special risk when isolated colonies are picked with the intention of stablish new bacterial stocks and to start cultures for research. This would undoubtedly cause an underestimation of the actual virulence gene content of the *Pdp* cells infecting fish in the field.

MATERIALS AND METHODS

Bacteria, plasmid, and media

Bacteria and plasmids used in this study are listed in Table 3. *Pdp* strains were routinely grown on tryptic soy agar (TSA-1) or in tryptic soy broth (TSB-1) supplemented with 1% NaCl at 25°C. *Escherichia coli* strains were grown on Luria Bertani agar (LB) plates or Luria Bertani Broth at 37°C. Antibiotics were used at the following concentrations: kanamycin (Kan) at 50 µg ml^{-1}, ampicillin (Amp) at 50 µg ml^{-1}, chloramphenicol (Cm) at 5 µg ml^{-1} or 20 µg ml^{-1}, rifampin (Rif) at 50 µg ml^{-1}, streptomycin (Strep)–50 µg ml^{-1} and nalidixic acid (Nal) at 40 µg ml^{-1}.

Table 3: Bacteria strains and plasmids used in this study.

Strain or plasmid	Description	References / Source
P. damselae* subsp. *piscicida		
PP3	Diseased yellowtail (*Seriola quinqueradiata*) in Japan	(Abushattal et al., 2019)
SNW 12.1	Diseased *Salmo salar* in Spain	(Abushattal et al., 2020)
RPM 820.1	Diseased *Solea senegalensis* in Spain	(Abushattal et al., 2020)
SA-08-2352	Diseased *Mugil cephalus* in France	(Abushattal et al., 2020)
SSS256	PP3 Δ *vcrD^{1}-cat* (carrier of pPHDP10 and pPHDP70)	This study
SSS325	PP3 Δ*vcrD^{2}-cat* (carrier of pPHDP10 and pPHDP70)	This study
SSS299	PP3 Δ*vcrD^{1}-cat* + Δ*vcrD^{2}-kan* (carrier of pPHDP10 and pPHDP70)	This study
SSS260	PP3 cured (carrier of pPHDP10 and pPHDP70)	This study
SSS312	PP3 *prfC*::SXT^{MO10} ($Strep^{R}$ Cm^{R})	This study
P. damselae* subsp. *damselae		
AVL49	LD-07, spontaneous rifampin-resistant mutant (Rif^{R})	(Vences et al., 2017)
Escherichia coli		
DH5α	Cloning strain	Laboratory stock
S17-1-*λpir*	RP4-2 (Kan::Tn7,Tc::Mu-1) *pro-82 λpir recA1 endA1 thiE1 hsdR17 creC510*	(Herrero et al., 1990)
β3914	RP4-2-Tc::Mu Δ*dapA*::(*erm-pir*) *gyrA462 zei-298*::Tn*10* (Kan^{R} Em^{R} Tc^{R})	(Le Roux et al., 2007)
BI533	MG1655 (Nal^{R})	(Prüss and Matsumura, 1996)
CAG18420	MG1655 *lacZU118 lacI42*::Tn*10kan* (KanR)	(Singer et al., 1989)
HW220	CAG18439 *prfC*::SXT^{MO10} (Cm^{R} $Strep^{R}$ SXT^{R} Tc^{R})	(Hochhut et al., 1999)
Plasmids		
pKD3	Template for *cat* gene amplification (Cm^{R})	(Datsenko and Wanner, 2000)
pKD4	Template for *kan* gene amplification (Kan^{R})	(Datsenko and Wanner, 2000)
pKWS30	Low-copy-number cloning vector (Amp^{R})	(Wang and Kushner, 1991)
pNidkan	Suicide vector derived from pCVD442 (Kan^{R})	(Mouriño et al., 2004)
pUC118	High-copy-number cloning vector (Amp^{R})	(Vieira and Messing, 1989)

DNA sequencing, annotation, and comparative genomics analyses

High-purity genomic DNA of *Pdp* PP3 strain was extracted using the G NOME DNA kit (MP Biomedicals) according to the manufacturer's instructions, and the DNA was subjected to sequencing following the PacBio procedure (SNPsaurus, OR, USA). Annotation of the specific function of ORFs was performed using rapid annotations of subsystems technology (RAST) and the BLASTP database (Altschul et al., 1997; Aziz et al., 2008). The gaps in a selected plasmid of strain PP3 (pPDPT3 plasmid) were closed by PCR and Sanger sequencing, and the CGView server database was used to obtain a circular graphical map representation of the pPDPT3 plasmid genome (Grant and Stothard, 2008). Comparative analysis of plasmid sequences were performed using BLASTN and BLASTP databases (Altschul et al., 1997), and MAUVE and EasyFig programs (Sullivan et al., 2011; Darling et al., 2010).

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PCR Conditions

Genomic DNA of *Pdp PP3* strain was extracted in this study using the G NOME DNA kit (MP Biomedicals). PCR analysis was used to identify clones that harbored the pPDPT3 plasmid using nine genes; all primers used are listed in Table 4. These genes involved four markers related to the backbone of pPDPT3 plasmid, *parAB, traB, traI,* and *traC*, while four genes were used to test T3SS genes, namely *vcrD, vscJ, vopD,* and *yopN*. We also used additional PCRs to test *aip56* gene and *frpA* and *irp2* genes located in pPHDP10 and pPHDP70 plasmids, respectively, to trace the virulence plasmids in parallel. PCR amplification was performed using the NZYTaq II 2x Green Master kit following the manufacturer's instructions; annealing temperatures were adjusted according to the corresponding primer pairs.

Table 4: Oligonucleotides used in this study.

Oligonucleotides	Sequence (5´-3´)[a]	Size (bp)
vcrD_XhoI_1	GCCTCGAGGAGAAGAGCCATCAGTACCT	2041
vcrD_BamHI_2	GCGGATCCTTCTAGCGGTGTGGTGATGT	
vcrD_BamHI_3	GCGGATCCTTAGCGCTTGACCCTTCTGT	2027
vcrD_NotI_4	GCGCGGCCGCACTTGAACATCCGCTAAGCC	
vcrD_int_F	ATGCTTGCCGTGATGCTACT	1943
vcrD_int_R	GACACCACCAGTACAGGTTT	
kanR_pKD4_BamHI_5	GCGGATCC TAGAAAGCCAGTCCGCAGAA	1190
kanR_pKD4_BamHI_3	GCGGATCCGAAGCCCAACCTTTCATAGA	
cat_pKD3_BamHI_5	GCGGATCCTACCTGTGACGGAAGATCAC	882
cat_pKD3_BamHI_3	GCGGATCCGGAACTTCATTTAAATGGCG	
aip56_F (pPHDP10)	TCACGTTACAGGCTCTAGTG	388
aip56_R (pPHDP10)	GCATTCAACTGAACTGTCGG	
irp2_5 (pPHDP70)	TTTGATCGCCCTGATGTGCA	217
irp2_3 (pPHDP70)	CTGGCATACTTGCACTAGAC	
frpA_5 (pPHDP70)	GTGGTGTCACTTACAGCGAT	229
frpA_3 (pPHDP70)	GAGACAGAAAACGTCACAGC	
parA_PP3_F	TCGTTCGTTTGGAGAATGGC	582
parB_PP3_R	GCCAATCGCAGGGTAGAACT	
traB_PP3_F	CTAACGGTCGAGTCAATCCC	402
traB_PP3_R	CGATACCGCGATTGGAGGAA	
traC_PP3_F	GCGTTCTTACGTGAAGAGTG	645
traC_PP3_R	GTGAGCATCGTCACCGTCAT	
traI_PP3_F	TGCCAACACCCTGAATAACG	551
traI_PP3_R	TTGTCCAGCGAGTGCCATCA	
vscJ_PP3_F	GCGGAGACGAAATCAGATCG	239
vscJ_PP3_R	TCAGGCCGAACTTTACACCG	
vopD_PP3_F	GTAATACCTGCAAGCACACC	322
vopD_PP3_R	CAATCGGCGATCAAGCTAGA	
yopN_PP3_F	GCGCTCAACCACATCCTTGT	431
yopN_PP3_R	AAGCGCATGAGCTGGTTTCC	
vscQ_PP3_F	GGCGTTGCTCAGTAGCCAAA	397
vscQ_PP3_R	ATTGTCGAGTTGGCATCGCC	
floR_int_F	CCGTCTACTTCAAGCAGTGG	345
floR_int_R	GCGCTAAAGCCGACAGTGTA	
sul2_int_F	TCGCTCGACAGTTATCAACC	347
sul2_int_R	AATTCATCGAACCGCGCCAG	

[a]underlined letters indicate the target of restriction enzymes

Mutagenesis construction of vcrD gene alleles in the Type 3 Secretion System (T3SS) gene clusters

The coding sequence of each of the two *vcrD* alleles was replaced with antibiotic resistance genes following an allelic exchange procedure using a suicide vector of the pCVD442 family. Two fragments of ~2kb, upstream (fragment 1-2) and downstream (fragment 3-4) of *vcrD* coding sequence were PCR-amplified. Fragment 1-2 was amplified using primers *vcrD*_XhoI_1 and *vcrD* _BamHI_2 while fragment 3-4 was amplified using primers *vcrD*_BamHI_3 and *vcrD*_NotI_4. After being digested with restriction enzymes, the two fragments were ligated so that the resulting DNA sequence generated an in-frame, 1697-bp deletion, of the *vcrD* coding sequence. In order to fix the unstable pPDPT3 plasmid, the construction containing the deletion was cut with *Bam*HI and a 882 bp chloramphenicol resistance gene (*cat*) amplified from pKD3 plasmid (Datsenko and Wanner, 2000) was inserted. In addition, another version for mutant construction was generated by cutting the aforementioned deletion construction with *Bam*HI and inserting there a 1190 bp PCR-amplified kanamycin resistance gene (*kan*) from pKD4 plasmid (Datsenko and Wanner, 2000). The *cat*-labelled construction for insertional mutation of *vcrD* alleles was used to obtain single $vcrD^1$ and $vcrD^2$ mutants independently (SSS256 and SSS325 mutant strains, respectively). Besides, in order to obtain a double mutant, the *cat*-labelled $vcrD^1$ mutant was used as the basis to construct a double mutant (SSS299 mutant strain) by using the *kan*-labelled construction that was used to insertionally inactivate $vcrD^2$ gene. The mutant construction containing the *cat* gene was introduced into the suicide vector pNidKan and propagated into *E. coli* β3914. The mutant construction containing the *kan* gene was inserted into the suicide vector pNidKan and propagated into *E. coli* S17-1-λ*pir*. *E. coli* donor strains were conjugated with *Pdp* recipient strains by mixing equal volumes of log-phase cultures of donor and recipient strains on

TSA plates prepared with seawater. First recombinants for single *vcrD*[1] mutant construction were selected on TSA-1 plates supplemented with chloramphenicol at 5µg ml^{-1} (Cm^5). For double mutant construction, the colonies resulting from the conjugation between *E. coli* S17-1-λ*pir* and *Pdp* PP3 SSS256 Δ*vcrD*[1]*-cat* (single mutant) were selected on TSA-1 plates supplemented with Cm^5 and kanamycin at 50 µg ml^{-1} (Kan^{50}). To select for the second recombination of the single mutant construction containing the *cat* gene, TSA-1 plates supplemented with Cm^5 and sucrose (15% [wt/vol]) were used. TSA-1 plates supplemented with Cm^5 + Kan^{50} and sucrose (15% [wt/vol]) were used to select double mutant colonies containing the *kan* gene plus the *cat* gene.

Virulence assays

European sea bass fish (*Dicentrarchus labrax*) with an average weight of 5 g were selected to perform virulence tests using the parental *Pdp* PP3, the pPDPT3-cured strain (SSS260) and three *vcrD* mutant strains (SSS256, SSS299 and SSS325). Fish were acclimated in 100 l aquaria at 24ºC for three days before injection. For dose preparation, bacterial biomass at the confluent growth zone of the agar plate from 48 h cultures was suspended in saline solution (0.85% NaCl) and adjusted at either a sharp OD_{600} = 0.3 or at sharp OD_{600} = 1. Serial decimal dilutions were prepared in saline solution and bacterial counts were carefully calculated. Fish were inoculated intraperitoneally with 0.1 ml of bacterial suspensions in saline solution (0.85% NaCl) using either a low dose (4.61×10^4 CFU/fish) or a high dose (4.61×10^6 CFU/fish). A group of 10 fish were inoculated with saline solution as control. For each strain and dose assayed, ten fish were inoculated, and mortality was recorded over a period of 7 d after injection. Dead fish were aseptically dissected, bacteria were recovered from kidney by culturing on TSA-1 agar plates, and colonies screened by PCR using gene markers for pPDPT3, pPHDP10 and pPHDP70 plasmids.

Plasmid transmissibility Assay

Conjugation experiments to assess pPDPT3 conjugative transfer were performed by mixing equal volumes of log-phase cultures of donor and recipient strains on TSA plates prepared with seawater. In order to test pPDPT3 transmissibility, *E. coli* CAG18420-Kan^R and AVL49 (LD-07-Rif^R) strains, separately, were used as a recipient, while strain SSS299 (PP3 Δ*vcrD*^{1}-*cat* +Δ*vcrD*^{2}-*kan*) was used as a donor. In another conjugation, HW220 (*E. coli* CAG18439 strain which carries the Integrative and Conjugative Element SXT^{MO10}) that is resistant to streptomycin, SXT, and chloramphenicol, was used as a donor. The SSS299 mutant strain, which is resistant to kanamycin and chloramphenicol, was used as the recipient strain. pPDPT3 plasmid of PP3 strain was labeled using a kanamycin and chloramphenicol resistant cassette. The PP3 transconjugants acquiring SXT^{MO10} were selected on TSA-1 plates supplemented with streptomycin and kanamycin and confirmed by PCR amplification of the *floR* and *sul2* genes carried on ICE SXT^{MO10} element. The resulting strain SSS312 was subsequently used as a donor strain to BI533 (*E. coli* MG1655-Nal^R) as the recipient to assess the potential role of ICE SXT^{MO10} elements in pPDPT3 transfer. Conjugation was performed for 24 h at 25°C on TSA plates prepared with seawater, and then serial decimal dilutions were spread on selective LB plates supplemented with Nal and Kan to determine whether the pPDPT3 plasmid had been acquired by *E. coli* MG1655-Nal^R.

Evaluation of plasmid stability under different temperature conditions

In order to evaluate the pPDPT3 thermostability, PP3 cells were grown from frozen glycerol stock on tryptic soy agar (TSA-1) plates. Then, the plates were incubated for three days at 25°C to obtain single colonies, and the colony PCRs were performed to determine positive

colonies for the T3SS genes. Ten positive colonies for T3SS genes were suspended in 50 ml TSB-1 and incubated at 18°C for 3 h, then divided into two equal parts into two 25 ml flasks. One of these flasks was incubated at 25°C and the other incubated at 18°C. Every 2 h during the first 6 h of incubation, and after 24 h incubation, appropriate volumes of the two bacterial cultures at 18°C and 25°C were diluted and spread onto TSA-1 plates. Colony PCRs for six different gene markers were conducted on the colonies that grew from each bacterial culture (at either 18°C or 25°C incubation). The selected genes to test the stability of pPDPT3 plasmid were *parAB, vscJ,* and *yopD*. Concurrently, PCR amplifications were conducted to test for presence of *aip56* gene (pPHDP10) and for *irp2* and *frpA* (pPHDP70) (see Table 4).

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DISCUSSION

5. DISCUSSION

One of the most common bacterial families distributed in marine water bodies, is the family *Vibrionaceae* (Thompson et al., 2004). This family includes several bacterial species that cause diseases to many different marine organisms (Baker-Austin et al., 2018). Via wounds or through undercooked fish, some of these bacterial species can be transmitted to humans, causing death in some instances if not treated quickly (Hundenborn et al., 2013; Love et al., 1981). *Photobacterium damselae*, is a marine pathogenic species of the family *Vibrionaceae,* and includes two distinct subspecies, *piscicida* and *damselae*. The subsp. *damselae* (hereafter *Pdd*) is considered a non-clonal and generalist pathogen for diverse species of marine animals and even for humans, while subsp. *piscicida* (hereafter *Pdp*) is a more specialized pathogen and only infects fish (Osorio, 2019). The acquisition of different virulence factors by horizontal gene transfer in each subspecies and the massive proliferation of insertion sequences in *piscicida* subspecies in particular, is considered to be a major driving force of the speciation process and the divergence in the lifestyles of the two *Photobacterium damselae* subspecies (Osorio, 2019).

In the past recent years, several studies have tackled the analysis of the genetic heterogeneity among isolates of the two subspecies of *Photobacterium damselae*. Initially, *Pdd* and *Pdp* were considered to be two closely related subspecies in terms of whole DNA-DNA

relatedness and of their consensus 16S rRNA gene sequences (Gauthier et al., 1995; Osorio et al., 1999). Further on, it was observed that even the intergenic transcribed spacer regions between the rRNA genes, widely considered to be highly variable among related bacterial taxa, exhibited a high degree of sequence conservation between *Pdd* and *Pdp* strains (Osorio et al., 2004; Osorio et al., 2005). However, it soon became evident that each subspecies encoded distinct virulence factors. On the one side, some strains of *Pdd* harbour the virulence plasmid pPHDD1 gene encoding damselysin and phobalysin P toxins, and its presence is variable among strains, without any correlation with geographical origin or host source (Osorio et al., 2000; Rivas et al., 2011; Rivas et al., 2014). The repertoire of virulence plasmids in *Pdp* is also noticeable, with pPHDP10 encoding the apoptotic toxin AIP56 (do Vale et al., 2005) and pPHDP70 encoding the siderophore piscibactin iron acquisition system (Osorio et al., 2015). Despite these discoveries, the genetic diversity of the two subspecies remained largely unstudied. As described in the Objectives of the present thesis, we aimed at unravelling several aspects about the variable (accessory) genome of *Pdp* and *Pdd* using next-generation sequencing technologies and functional studies on bacterial genetics and physiology.

5.1 STUDY OF THE ACCESSORY GENOME IN *P. DAMSELAE* SUBSP. *DAMSELAE* (*PDD*)

5.1.1 Characterization of multidrug resistance plasmids in *Pdd*

In the first part presented in this thesis and related to *Pdd*, the recent emergence of some multidrug resistant isolates among the subspecies *damselae* indicated the necessity of studying the transmission mechanisms of resistance genes, especially those that are

carried by plasmids. To track these genes and how they are transmitted and spread through subspecies *damselae* isolates, we have here characterized the plasmid pPHDD2-OG2, which is very similar to the previously reported pAQU plasmid that contains several resistance genes.

The spread of antibiotic-resistant bacteria, and of antibiotic resistance genes among bacterial species is an ever-increasing problem, threatening different life forms, especially humans (Llor and Bjerrum, 2014). The increasing use of antibiotics to control pathogenic bacteria has created direct pressure on bacteria to develop resistance mechanisms, including the acquisition of antibiotic resistance genes (Cabello, 2006). Antibiotics were used to treat human diseases and treat fish diseases in aquaculture, facilitating the spread of resistance among bacterial species (Cabello et al., 2013). Environmental changes and unfavourable conditions often force bacteria to improve their fitness and ability to live in new conditions. Moreover, the bacterial ability to take advantage of mobile genetic elements such as plasmids that contain resistance genes provides them with the ability to resist better the effects of antibiotics (Romero et al., 2012). One of the most important sources of transferring resistance genes between bacteria is plasmids, particularly those that harbour a group of resistance genes on the same plasmid. Among these plasmids, pAQU1 and pAQU-like plasmids transmit resistance genes between bacteria at an accelerated rate, especially in an aquatic ecosystem. The pAQU plasmid was first reported in a *Pdd* strain isolated in Japan and was shown to harbour several resistance genes (Nonaka et al., 2012). Some pAQU-like plasmids have been described in bacterial strains of the genera *Vibrio*, *Photobacterium* and *Shewanella* in Japan (Li et al., 2017; Nonaka et al., 2014). In the present thesis, by the study of *Pdd* strain OG2, which is an isolate from the Black Sea region, we observed that the OG2 strain had the ability to be resistant to tetracycline. Tetracycline is one

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of the most widely used antibiotics in aquaculture (Chopra and Roberts, 2001; Suzuki, 2010). The prevalence of tetracycline-resistant bacteria has been indicated in several previous studies (Kim et al., 2004; Miranda et al., 2003; Nonaka et al., 2000; Sandaa et al., 1992). Using bioinformatics analysis of OG2 genome, we unveiled a plasmid, dubbed pPHDD2-OG2, that shows high similarity to pAQU1 plasmid carrying multidrug-resistant genes (MDR). This similarity was clearly demonstrated by having RepA, ParAB, TraI relaxase, and Ter terminase proteins highly identical to those encoded in pAQU1 (Nonaka et al., 2012), in addition that it carries several multidrug-resistant genes. Five of the resistance genes, namely *tetB*, *floR, sul2, qnrVC,* and *dfrA* were detected in pPHDD2-OG2 plasmid. These genes were traced in *Pdd* isolates to find similarity or identity in these genes among the 13 *Pdd* strains that were isolated from the Black Sea region. The results indicated four versions of the pPHDD2-OG2 plasmid depending on the number of resistance genes carried on each version. These versions were as follows, the first version included (*tetB, floR, sul2, qnrVC,* and *dfrA*), the second version (*floR, sul2, qnrVC,* and *dfrA*), the third version (*tetB, floR, sul2,* and *strAB*), while the fourth version involved (*tetB* and *qnrVC*). However, two isolates did not show any tested genes (Vences et al., 2020). This difference in the number of bacterial resistance genes indicated a mechanism for these gene acquisitions, which may be horizontal gene transfer. Several studies have described horizontal gene transfer's role in the acquisition and transfer of genes through different types of bacteria (Abe et al., 2020; Deng et al., 2019; Osorio, 2019). Besides, the presence of these genes interspersed among insertion sequence elements, as demonstrated by our study, leads to the conclusion of these elements' role, which is evident in the second version of the plasmid (Agersø et al., 2002; Vences et al., 2020).

The study of bacterial resistance genes in different bacterial species contributes to tracking these genes and their transmission mechanisms (Shah et al., 2014; Su et al., 2020). In order to study the distribution of the pPHDD2-OG2 plasmid through other *Pdd* isolates, PCR amplifications were designed targeting the *repA* and *parAB* genes in addition to those previously used to track these genes in the *Pdd* collection. Testing the *Pdd* isolates collection of our laboratory, which contains 70 isolates from different geographic regions, only nine isolates showed positive results for amplification, but not for all target genes. The main problem we face with multidrug-resistant bacteria is their ability to spread the resistance genes widely and rapidly among the different types of bacteria (Christaki et al., 2020; Levy, 1998; Levy, 2002). Hence, it was necessary to measure the ability of the pPHDD2-OG2 plasmid to conjugate with varying types of bacteria and within the isolates of *Pdd* and *Pdp*. The conjugation results indicated that the pPHDD2-OG2 plasmid is transmissible between different types of bacteria and at high frequency in some, especially in *E. coli* and *Pdp* DI21. In our study, the highest transfer frequency of pPHDD-OG2 plasmid was recorded during conjugation to *E. coli* CAG18420 compared with the transfer frequency of pAQU plasmids group (Nonaka et al., 2014). In one of the previous studies, the possibility of transferring the tetracycline resistance gene from marine bacteria to *E. coli* and fish pathogen *Lactococcus* to *Enterococcus* that causes disease in humans was indicated (Neela et al., 2009).

Moreover, determining the mechanism of spread of multidrug-resistant genes in bacteria requires research into the resistant bacteria's genetic origins and how these genes accessed them. In order to obtain phylogenetic tree and genomic comparisons, using the housekeeping genes for the comparative studies showed a clear and significant convergence between the genomes of *P. damselae* subsp. *damselae* isolates that contained the different versions of the pPHDD2-OG2

plasmid. The different types of resistance genes distributed among pAQU plasmids that have demonstrated abuse or overuse of antimicrobials in aquaculture may increase the organisms harboring pAQU plasmids. New resistance genes might have been introduced into these plasmids via mobile elements (transposons for example), and horizontal gene transfer is a common event (Cabello et al., 2016; Santos and Ramos, 2018). Thus, it can be considered that horizontal gene transfer plays a vital role in gene exchange and transfer between different bacterial species, allowing for the emergence of a greater number of plasmids that involve multidrug-resistance in aquaculture environments.

5.1.2 The study of the accessory genome unveils a gene cluster that confers sucrose utilization ability in some *Pdd* isolates

Continuing with the study of the accessory genome in *P. damselae* subsp. *damselae* in the present thesis, another part of our study has focused on the differential ability of some subsp. *damselae* isolates to ferment sucrose. This is a metabolic trait that has long been used as a method to selectively isolate and differentiate among members of the *family Vibrionaceae*. During the past years, several studies reported the growth of some subsp. *damselae* isolates forming yellow color colonies on TCBS medium, which indicates the fermentation of sucrose, unlike most subsp. *damselae* isolates that grow green. This observation suggested the necessity of studying the genetic change that led to this new metabolic pattern and the mechanism of acquiring this metabolic trait among isolates of subsp. *damselae*. This study produced a research article entitled: **Diverse horizontally-acquired gene clusters confer sucrose utilization to different lineages of the marine pathogen *Photobacterium damselae* subsp. *damselae*.** The continuous change in bacterial

genomes, whether by gaining or losing genes, makes bacteria prone to acquiring genes that may change their metabolic behaviour.

The members of the *Vibrionaceae* family are considered natural inhabitants of the marine environment. Temperature fluctuations and other environmental factors often lead to the uncontrolled spread of these bacteria, especially in seafood, and it represents the most common way of transmitting pathogens to humans (Shikongo-Nambabi and Schneider, 2012). This requires that seafood is routinely examined for presence of different pathogenic *Vibrio* species. In line with this, Thiosulphate-Citrate-Bile-Sucrose (TCBS) agar is the standard culture medium used for the selective isolation of *Vibrio* species and performs better than Thiosulphate-Chloride-Iodide agar (Pfeffer and Oliver, 2003).
One of the most common carbohydrates is sucrose, which is made up of fructose and glucose, which are linked by a glycosidic bond. The transport of carbohydrates within bacterial cells is through membrane proteins, which differ in their number and distribution across bacterial membranes from one type to another (Saier Jr, 1998). In a genomic analysis of *Bacillus subtilis* indicated the presence of 50 carbohydrate transporters (Deutscher et al., 2001), while another genomic analysis of *Bifidobacterium longum* indicated that 8.5% of the genes were involved in carbohydrate transport (Schell et al., 2002). In the *Vibrio* family, as far as we know, few studies have been concerned with studying the proteins that transport sucrose, which did not adequately address the gene regulation and the phylogenetic analysis of genes related to these proteins (Blatch et al., 1990; Blatch and Woods, 1991; Hayes et al., 2017; Scholle, 1989). One of these studies on *V. cholerae* indicated the role that *scrA* gene plays in sucrose transport proteins (Hayes et al., 2017), while the other studies stated the organization of sucrose operon and genes architecture in *V. alginolyticus* (Blatch and Woods, 1991). Also, the survey conducted on the bacterial isolates

related to *Vibrio*, indicated that 5% of the isolates belonging to *damselae* subspecies could ferment sucrose (Farmer et al., 1985). After that, some studies on *Pdd* began to indicate the ability or not of isolates to ferment sucrose as an additional trait for these isolates. Among these studies, one was conducted on 71 isolates of *Pdd* from Spain indicating that 4% of isolates had the ability to ferment sucrose (Botella et al., 2002). Then came another study indicating that 3% of the *Pdd* strains isolated from marine rainbow trout in Denmark were able to ferment sucrose (Terceti et al., 2018). Although isolation of *Pdd* strains positive for sucrose fermentation and negative ones from the same fish has been reported, the frequency is still low for isolates capable of fermenting sucrose (Zhao et al., 2009). This low frequency of sucrose-fermenting isolates did not last long, and this is evident in one of the recent studies, which indicated that 40% of *Pdd* isolates in infected seabass of the Turkish coast of the Black Sea were able to ferment sucrose in TCBS medium (Terceti et al., 2016). Based on the best of our knowledge and on previous studies of the sucrose fermentation ability in *Pdd*, no strain has so far been subjected to a detailed study of this trait from a genetic or structural point of view. Based on the above, we selected DK32, an isolate capable of fermenting sucrose described in a previous study (Terceti et al., 2018), in order to initiate an study to describe and characterize the genes responsible for fermentation of sucrose (Abushattal et al., 2020a).

Our study has demonstrated the role of *scrA* gene in uptake and utilization of sucrose because mutants were unable to metabolize sucrose whereas they were still found to be capable of metabolizing glucose as a carbon source in the same degree as the parental strain. A deletion mutant of *Pdd* DK32 strain in the *scrA* gene of the sucrose utilization gene cluster resulted in the inability to utilize sucrose (Abushattal et al., 2020a).

As inferred from the nucleotide sequence of the *scrR* gene encoding a putative transcriptional repressor, the mechanism for controlling the

expression of the sucrose operon in *Pdd* is similar to what is found in some species of *Enterobacteriaceae* where these genes are negatively regulated by the repressor encoded by *scrR* (Bogs and Geider, 2000). The transcriptional activity of *scrA* gene promoter in DK32 strain showed that the transcriptional activity was doubled by 2.5 in medium containing sucrose as the sole carbon source compared with the glucose medium (Abushattal et al., 2020a).
The change in the bacterial phenotype depending on the growth media in which they are grown, as occurs to the biofilm of bacterial strains when grown in a media that includes sucrose as a source of carbon, raises questions about the genetic content role (Lambert et al., 2016; Nácher-Vázquez et al., 2017). During our study of the *Pdd* sucrose operon, the mutation in the *scrA* gene led to a change in the colony phenotype when grown on TCBS and TSA-1 supplemented by 15% sucrose compared with the parental strain (Abushattal et al., 2020a). Our study on the sucrose operon showed that horizontal gene transfer made the difference in the sucrose genes cluster transfer and its acquisition by the studied bacterial strains. We proposed some hypothesis about the putative mechanisms that might have facilitated the process of sucrose genes acquisition, based on the existence of recombination hot spots in bacterial genomes and these genes' presence or not on the chromosomes or plasmids. Also, the presence of the sucrose operon was tracked within 36 *Pdd* strains isolated from different geographical areas. Only eight isolates showed the ability to ferment sucrose and all of them invariably possessed the sucrose operon carried on the chromosome except for one isolate in which the sucrose operon might be potentially harboured within a plasmid because the genetic context of the sucrose genes differs with respect to the other seven isolates (Abushattal et al., 2020a). One previous study described the role of the abundance of hot spots in *Pdd* chromosomal regions (Terceti et al., 2018), and another study indicated the sucrose operon's presence within chromosomal regions rich in repetitive

sequences (Treviño-Quintanilla et al., 2007). Furthermore, the phylogenetic analysis conducted with homologous sucrose genes present in *Vibrio* and *Photobacterium* species, clearly suggested that the sucrose clusters have undergone extensive horizontal transfer within species of the family *Vibrionaceae*. The widespread acquisition of genes plays a major role in bacterial diversification, and the contribution of horizontal gene transfer can convert an environmental organism into a successful pathogen or even a symbiont (Hacker and Kaper, 2000). Gene acquisition through horizontal gene transfer is one of the most important mechanisms that have an important role in the evolution of the genome of an organism that is reflected in the new metabolic capacity, such as the acquisition of antibiotic resistance genes, metabolic activity genes, and virulence factors (Juhas et al., 2009). Many evolutionary biologists point to the role of acquired genes in bacteria, which leads to adaptation, an increased ability to coexist, and an increase in genetic diversity between the different types of bacteria. This acquisition of genes contributed to the development of pathogens and changed the relationship's nature with the host, and gave a remarkable development in the bacterial genome (Ochman and Moran, 2001). Sucrose is one of the most abundant carbohydrates on the earth's surface due to its production in many sources such as trees and terrestrial plants (Sabri et al., 2013). On the other hand, the abundance of sucrose in the aquatic environment components such as microalgae and cyanobacteria was indicated in many studies (del Pilar Bremauntz et al., 2011; Hagemann, 2011; Kirsch et al., 2019; Kolman and Salerno, 2016). In addition, it has been reported that sucrose in the marine environment acts as an attraction factor to a pathogenic *Vibrio* carrying an intact PTS system for sucrose, which means that there exist a strong chemotaxis for sucrose (Yu et al., 1993). All these lines of evidence suggest that the presence of a gene cluster for sucrose utilization might provide a

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selective advantage in the marine environment for the use of sucrose as a carbon source.

5.2 STUDY OF THE ACCESSORY GENOME IN *P. DAMSELAE* SUBSP. *PISCICIDA* (*PDP*)

The second part of this thesis focused on the subspecies *piscicida* and, most notably, on the identification and characterization for the first time in this subspecies, of a type III secretion system (T3SS), that is proposed to be encoded within a highly unstable plasmid. The first approach of this study tackled the identification of the T3SS genes in the genomes of two subsp. *piscicida* isolates, which resulted in the research article entitled **Draft genome sequences of *Photobacterium damselae* subsp. *piscicida* SNW-8.1 and PP3, two fish-isolated strains containing a type III secretion system.** This article led to the necessity to trace the genes of the T3SS in conjunction with other virulence factors that were previously recorded as major virulence factors in isolates of *piscicida* subspecies, which resulted in the research article entitled: **A virulence gene typing scheme for *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish photobacteriosis, reveals a high prevalence of plasmid-encoded virulence factors and type III secretion system genes**. This paper indicated the widespread nature of T3SS genes among subspecies *piscicida* isolates and prompted the necessity to study the role that T3SS genes play in virulence of subsp. *piscicida* for fish, a study that resulted in the yet unpublished research paper entitled: **A highly unstable plasmid encoding the type III secretion system contributes to virulence of *Photobacterium damselae* subsp. *piscicida* for fish.**

5.2.1 First evidences of presence of a Type III secretion system in *Pdp*

The study of bacterial genomes, in general, provides a wide knowledge in the sequences and architecture of genes and the stability of the bacterial genomes. Genome analysis of pathogenic bacteria plays a major role in identifying virulence genes, giving us an in-depth look into bacterial pathogens' pathogenesis. Virulence genes are located in specific regions of the chromosomes or plasmid of bacteria (Donkor, 2013). Genome analysis studies contribute to finding appropriate solutions to overcome the diseases caused by the pathogenic bacteria and anticipating and following up the change in bacterial behaviour. In the present study, two strains of *Pdp* were selected, one of which is PP3, which was isolated in Japan from diseased yellowtail (*Seriola quinqueradiata*), while SNW-8.1 was isolated in Spain from salmon (*Salmo salar*). The study of the genome of isolates of the same subspecies that have been isolated from different geographical regions helps to get a general idea of those isolates associated with them in the same geographical region. *Bakopoulos* et al. (Bakopoulos et al., 1995), pointed out some differences between the *Pdp* isolated from European countries (Italy, France, and Greece) and those isolated in Japan. Genomic changes in *Pdp* isolates are considered to be pervasive and increasing, due to the high plasticity related to the presence of a large number of insertion sequences and mobile elements (Osorio, 2019).

The genome sequences of two *Pdp* strains, SNW-8.1 and PP3 were analyzed to show the genome architecture and discover the virulence genes associated with these isolates, causing photobacteriosis. The genome sequencing showed that their genomes contain the type three secretion system (T3SS), one of the most important virulence factors in Gram-negative bacteria. The two strains also possessed pPHDP10 (encoding the AIP56 toxin) and pPHDP70 (encoding the siderophore

piscibactin iron sequestering system). The coexistence of these three distinct virulence factors is suggested to contribute to a highly pathogenic profile in *Pdp* SNW-8.1 and PP3. According to (Balado et al., 2017a), two draft genomes of *Pdp* strains isolated in Spain showed absence in their genomes of genes for the type III secretion system. This observation suggested the existence of an unsuspected variation in virulence gene content among strains of the same subspecies. This preliminary study thus unveiled the existence of genes for the T3SS export apparatus (EscV) as well as for T3SS effector proteins (YopR). Comparison of the *EscV* and *YopR* genes of SNW-8.1 and PP3 strains with those in the genome of *Pdp* OT-51443 and in the pPHDD203 plasmid of the type strain CIP102761 of *Pdd* showed a 99% to 100% identity among homologues (Aoki et al., 2017; Vences et al., 2017).

5.2.2 Study of the prevalence of virulence plasmids and of T3SS genes in *Pdp* isolates

Pdp causes high mortality rates in marine aquaculture leading to significant financial losses and the considerable disruption of the economic factors associated with aquaculture (Snieszko et al., 1964; Toranzo et al., 1991). Moreover, the spread of *Pdp* in many geographical regions in different countries during a long time indicates its role in causing disease and mortality of different types of fish (Hawke et al., 1987; Robohm, 1979; Snieszko et al., 1964; Toranzo et al., 1991). Many studies have focused on studying the pathogenic factors in *Pdp* to decipher the main factors causing the disease and the degree of virulence in them (do Vale et al., 2005; Hawke, 1996; Magariños et al., 1994b; Osorio et al., 2015). Among the most important virulence factors that have been studied, particularly in *Pdp* is the *aip56* gene (do Vale et al., 2017; do Vale et al., 2005) and the *irp2* and *frpA* genes (Osorio et al., 2006; Osorio et al., 2015). Before this thesis study was started, our knowledge on the

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prevalence of virulence genes and plasmids in *Pdp* populations was very scarce. It had been previously observed that the *aip56* gene was present in pathogenic isolates of *Pdp* and absent in two non-pathogenic isolates (do Vale et al., 2005). It had also been observed that *Pdp* DI21 cured of pPHDP70 plasmid (containing *irp2* and *frpA* genes) exhibited reduced virulence compared to the parental strain (Osorio et al., 2015). Unexpectedly, there were no comprehensive studies to track the virulence factors of *Pdp* isolates over a long period of time and from different geographical origins. In line with the above, a collection of 103 *Pdp* strains isolated from different geographical locations and various hosts during the time 1963 to 2015, were analysed in the present thesis study. These isolates possessed different plasmids that harboured virulence genes, such as *aip56* gene located in pPHDP10 plasmid, *irp2* and *frpA* genes in pPHDP70 plasmid, and T3SS genes were recorded for the first time in *Pdp* isolates (Abushattal et al., 2019). Specific PCRs were designed to test all the genes mentioned above to monitor their distribution in *Pdp* isolates. Our results indicated that pPHDP10 was present in 97 isolates out of 103, providing evidence of the high stability of this plasmid (Abushattal et al., 2020b), which is the main virulence factor in *Pdp* (do Vale et al., 2005). The genetic study of pPHDP10 plasmid indicated that the origin of this plasmid is unknown and that it is present only in *Pdp* strains. However, a recent study has indicated the presence of an homologue of *aip56* gene in a *Vibrio campbelli* strain and some strains of insect pathogenic bacteria (Silva et al., 2010). Hence, the *aip56* gene can be used for rapid and appropriate diagnosis of *Pdp* isolates.

pPHDP70 plasmid carries the genes for synthesis and utilization of siderophore piscibactin, presumably used by *Pdd* to obtain iron from the surrounding environment (likely from host tissues and iron-containing proteins) (Osorio et al., 2006; Souto et al., 2012).

Concerning the *irp2* and *frpA* genes traceability, in two separate studies, Osorio and colleagues indicated the presence of pPHDP70 in European and Israeli isolates while it was not recorded in Asian and American isolates (Osorio et al., 2006; Osorio et al., 2015). Moreover, the pPHDP70 plasmid was observed in some isolates and was not detected in the rest of the isolates from the same region. During the tracking of these genes in *Pdp* isolates, we here found that there were a total of 91 isolates harbouring pPHDP70 plasmid, of which 89 were European isolates and two were isolated in Japan. Consequently, this result demonstrated for the first time that this plasmid is not restricted to European isolates (Abushattal et al., 2020b).

As mentioned above, through the genome analysis of two *Pdp* isolates (PP3 and SNW-8.1), the type three secretion systems (T3SS) genes were reported for the first time in *Pdp* (Abushattal et al., 2019). This analysis gave us a deep insight into the T3SS genes, which indicated the presence of 34 putative T3SS-related genes with structural and secretory functions within one cluster. A BLAST search showed a high similarity between the T3SS genes of *Pdp* and those in *Vibrio paraheamolyticus* RIMD 2210633 (Ono et al., 2006; Park et al., 2004), but *Pdp* genomes were found to lack a specific region encoding effector proteins in *V. parahaemolyticus*. Concurrently, a high similarity was also observed with a putative T3SS recently reported in the sibling subspecies *Pdd* (Vences et al., 2017), with few differences as presence of transposase and hypothetical protein genes interspersed within the T3SS gene cluster of *Pdp*. Our study showed the widespread nature of the genes of the T3SS among *Pdp* strains that were isolated during a period of time spanning between 1963 and 2015 (Abushattal et al., 2020b). Interestingly, genes belonging to the T3SS were observed in 74 *Pdp* isolates, the oldest of which was isolated in Japan in 1980. Despite this widespread presence of T3SS genes among *Pdp* isolates, they were not previously recorded or

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tracked to the best of our knowledge. Strangely, the PCR analysis of rifampicin-resistant *Pdp* PP3 isolates demonstrated the loss of T3SS genes compared to the parental PP3 strain (Abushattal et al., 2020b). This loss of T3SS genes led us to initially suspect that its cause was the treatment of the bacterial strain with rifampicin when selecting for a spontaneous resistant mutant, as happened to other plasmids in *E. coli* (Bazzicalupo and Tocchini-Valentini, 1972). On the other hand, some studies had indicated instability of a plasmid harboring the T3SS Island in *Aeromonas salmonicida* subsp. *salmonicida* (Stuber et al., 2003; Tanaka et al., 2017). Taken together all these observations, we initially hypothesized that T3SS genes in *Pdp* PP3 are located in an unstable plasmid. T3SS is one of the most common virulence factors in Gram-negative bacteria, and it has a significant role in causing disease. While many studies have indicated the presence of T3SS genes within chromosomal regions of the bacterial genome (Bijlsma and Groisman, 2005; Merda et al., 2017; Okada et al., 2009), other studies have indicated the presence of T3SS genes on plasmids within islets (Hueck, 1998; Pilla et al., 2017; Stuber et al., 2003; Winstanley and Hart, 2001). Moreover, T3SS genes carried on plasmids played an essential role in increasing virulence or reducing the level of virulence in the event that these plasmids were lost (Daher et al., 2011; Tanaka et al., 2012).

5.2.3 Evidence that the *Pdp* T3SS is encoded within a highly unstable virulence plasmid

T3SS genes play a significant role in the level of bacterial virulence-associated with animal and plant diseases (Hueck, 1998). Also, T3SS genes play an important role among the different types of bacteria in many different lifestyles (Cornelis, 2006; Dale et al., 2001; Nazir et al., 2017; Nelson and Sadowsky, 2015; Tseng et al., 2009). As mentioned above, we reported for the first time the presence of

T3SS genes in two *Pdp* isolates (Abushattal et al., 2019). Although we studied the genome sequences of these two *Pdp* isolates using the Illumina approach (Abushattal et al., 2019), this sequence resulted in a relatively large number of contigs, one of which contained T3SS genes. In the context of studying the prevalence of T3SS genes, during our previous study, we referred to the instability of T3SS genes, which led to the conclusion that these genes are located on an unstable plasmid (Abushattal et al., 2020b). Hence, it was necessary to re-sequence the *Pdp* PP3 stain genome, using the PacBio approach, which indicated that the T3SS genes were carried on a plasmid of the size of a 133 Kilobases, dubbed pPDPT3. By using the new pPDPT3 plasmid sequence, T3SS genes were observed duplicated as two identical paralogous copies, and additional 12 genes in single copy. These two copies, which include T3SS genes, occupied more than half the size of the pPDPT3 plasmid while the second half of the plasmid largely consisted of insertion sequences (ISs). Although the genetic organization of the T3SS gene clusters of pPDPT3 was quite similar to that of a putative plasmid in *Pdp* strain OT-51443 (Aoki et al., 2017), the T3SS gene clusters of pPDPT3 were represented by two copies, which likely constitutes a unique feature of strain PP3. Also, pPDPT3 shows conserved synteny with T3SS gene clusters found in a putative plasmid (pPHDD203) of *Photobacterium damselae* subsp. *damselae* CIP 102761 strain (Vences et al., 2017). Moreover, despite the similarity of the T3SS gene clusters in pPDPT3 with those of *Vibrio parahaemolyticus* RIMD 2210633 (Park et al., 2004), pPDPT3 missed a specific region containing the effector's genes in the T3SS-linked gene clusters of *V. parahaemolyticus RIMD 2210633*. In contrast, pPDPT3 contains a particular region that includes the predicted effector protein YopH-like, a Tir type III secretion system chaperone, a putative transcriptional regulator ExsA and VscW type III secretion system pilotin.

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In light of the preceding, it is necessary to study the pPDPT3 plasmid in several aspects, including the stability of the plasmid in the host strain at different temperatures, the ability of pPDPT3 plasmid to transfer during bacterial conjugation to other bacterial species, and the role of the T3SS genes in the virulence of *Pdp* for fish. From here, we began to investigate the reasons for the instability of pPDPT3 plasmid under laboratory culture conditions, as was indicated in our previous study (Abushattal et al., 2020b). In order to assess the stability status of the pPDPT3 plasmid, we incubated the host strain of the pPDPT3 plasmid at different temperatures, which are 18°C and 25°C. This test led to the conclusion that the host strain's incubation temperature had no direct relationship to the loss of the pPDPT3 plasmid in contrast to what happened to plasmids carrying the T3SS genes harbored in *Aeromonas salmonicida* (Stuber et al., 2003; Tanaka et al., 2017). Based on the above, the instability of the pPDPT3 plasmid prompted us to generate a deletion-insertion mutation using the chloramphenicol cassette in one of the T3SS genes, *vcrD* gene, in order to fix the plasmid under laboratory selection conditions. This insertional mutation contributed to preserving the pPDPT3 plasmid in *Pdp* PP3 strain when cultured on TSA-1 plates supplemented by chloramphenicol.

In order to evaluate the ability of pPDPT3 plasmid to transfer during bacterial conjugation to other bacterial species, a number of conjugations were performed with *E. coli* and *Pdd* LD-07 as recipient strains. These conjugations resulted in the inability of pPDPT3 to transfer to different bacterial strains by conjugation. Furthermore, using the SXT^{MO10} ICE element (Beaber et al., 2002) to improve the chance of pPDPT3 plasmid transfer, also failed. In light of this result, the analysis of the conjugation genes region present in pPDPT3 plasmid was reviewed in particular, taking into account some studies that indicated the role of conjugation genes region in bacterial

plasmids during the conjugation (Fernandez-Lopez et al., 2016; Maneewannakul et al., 1991). BlastN search revealed the high match between the conjugation region in the pPDPT3 plasmid with a putative plasmid in the *Pdp* OT-51443 strain (Aoki et al., 2017) as well as the matching of the conjugation genes located at both ends of the conjugation region in the pPDPT3 plasmid with those in the three plasmids of *Pdd* strains. The above shows that the pPDPT3 plasmid has lost several conjugation genes, with incomplete genetic sequences of some conjugation genes compared with those present in *Pdd* plasmids. In line with this, other studies indicated the absolute importance of the presence of some other conjugation genes, such as *traN* and *traG*, that were lost in the pPDPT3 plasmid (Klimke and Frost, 1998; Low et al., 2020). Besides, the presence of the insertion elements between the conjugation genes of pPDPT3 plasmid could lead to disruption of the transcriptional activity of the conjugation genes (Vandecraen et al., 2017).

According to the above, in order to study the role of T3SS genes in virulence, we wanted to use a strain cured of plasmid pPDPT3 and challenge the fish in conjunction with the parental PP3 strain to evaluate the virulence, especially since some studies indicated a decrease in bacterial virulence in the event of losing or curing virulence plasmids (Osorio et al., 2015; Xiao et al., 2013). In this challenge, we used two doses, the low dose and the high dose, for both the cured pPDPT3 strain and the parental PP3 strain. The high dose of both strains caused mortalities in all of the inoculated fish, while the low dose resulted in the death of fish that were challenged with the parental PP3 strain only. According to (Frey and Origgi, 2016), *Frey and Origgi* pointed to studies using a strain of *A. salmonicida*, which has been shown to be non-pathogenic due to loss of virulence plasmids *pASA5* / *pASvirA* containing the T3SS. This study emphasized the necessity to link and report the concept of

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pathogenicity in *A. salmonicida* strain with clear data related to the presence or absence of T3SS.

In light of these results, we had to generate a deletion mutation - insertion in each of the two copies of the *vcrD* gene of pPDPT3 in *Pdp* PP3, in addition to creating a deletion – insertion mutation in the *vcrD* gene in the complete cluster and another in the incomplete cluster for *T3SS* genes of two different strains. So, we generated the two distinct types of single *vcrD* mutants, plus a double *vcrD* mutant. While some studies have described the adequacy of T3SS genes within a single cluster in inducing virulence (Ideses et al., 2005; Warawa and Woods, 2005), other studies have demonstrated the complementary role of two different systems of T3SS genes clusters in bacterial virulence (Finn et al., 2017; Kim et al., 2018; Procyk et al., 1999). We have used the parental PP3 strain in addition to each of the mutated strains in which one copy or both copies of the *vcrD* gene was mutated to assess the role of T3SS genes in virulence. As expected, mortality was recorded for all fish that were injected with the parental strain PP3, and no mortalities were recorded for fish that were challenged with the strain in which both copies of the *vcrD* gene were mutated. In contrast, unexpectedly, no mortalities were reported in fish that were inoculated with strains in which one copy of the *vcrD* gene was mutated, either in the complete cluster or incomplete cluster. Based on these results, we wanted to re-obtain a cured pPDPT3 strain from the mutant strains of the *vcrD* gene. Unexpectedly and strangely, we have not yet obtained a cured pPDPT3 strain from the mutant strains. This result led us to believe that the pPDPT3 plasmid was integrated into the bacterial genome, in a similar way as it has been reported to happen to the pINV plasmid of *Shigella flexneri* in previous studies (McVicker and Tang, 2016; Pilla et al., 2017).

Through our study of pPDPT3 and preparing this thesis, we would like to draw attention to the fact that we have recorded for the first

time the widespread presence of the T3SS, an important virulence factor, in one of the most devastating pathogens in aquaculture. And what is striking about this matter, is that despite *Pdp* strains having been isolated for over more than fifty years, the existence of the T3SS passed unadvertised. All these observations warn that the picture of *Pdp* virulence gene content has been historically biased and underestimated, since the loss of plasmid-encoded T3SS genes during bacterial isolation and subculture may have occurred systematically in laboratories for decades.

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CONCLUSIONS

6. CONCLUSIONS

From the results obtained along this work it can be concluded that:

1. *Pdd* strains isolated from diseased fish in the Mediterranean basin constitute a reservoir for multidrug-resistance plasmids of the pAQU-family that have the ability to spread to various bacterial species.

2. The difference in the resistance genes content between the different versions of pAQU-like plasmids of *Pdd* and the presence of *strAB* resistance genes for the first time in pAQU-like plasmids, demonstrate the high flexibility of MDR plasmids to acquire resistance genes.

3. Comparative genomics analyses of *Pdd* isolates indicate that the genes for sucrose metabolism were obtained by horizontal transfer by different *Pdd* lineages and inserted into a hotspot for DNA recombination in the *Pdd* genome.

4. Phylogenetic analysis reveals that the sucrose gene clusters do not follow the phylogeny of the species and have likely undergone extensive horizontal transfer among *Vibrio* and *Photobacterium* species.

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5. Identification for the first time of a type III secretion system (T3SS) in two *Pdp* genomes has enhanced our understanding of the pathogenesis of this fish pathogen and recommends that virulence factors be tracked through complete genomic sequencing to give a deep and clear picture.

6. A large-scale survey of *Pdp* isolates has revealed an unexpectedly high prevalence of T3SS genes encoded within a novel plasmid dubbed pPDPT3.

7. pPDPT3 contributes to *Pdp* virulence for fish, it is highly unstable in laboratory culture conditions and becomes lost from the bacterial population upon a single subculture step.

8. The loss of T3SS genes encoded within pPDPT3 during bacterial isolation and subculture may have occurred systematically in laboratories for the last 5 decades, a phenomenon that has largely distorted the picture of the virulence gene content of *Pdp*.

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ANEXO

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ANEXO 1

ASPECTOS ÉTICOS

- En esta tesis se incluyen estudios que utilizan peces como animales de experimentación, en cumplimiento con el RD 53/2013.

- El proyecto de investigación se titula “Inoculaciones experimentales de peces con bacterias patógenas y con proteínas bacterianas”, del que es investigador responsable el Prof. Dr. Carlos Rodríguez Osorio, tutor y director de la presente tesis doctoral.

- El número de autorización de este proyecto de experimentación animal es: **15004/14/003** (se adjunta copia de la autorización).

- El centro de usuario en el que se realizó el trabajo, es el Animalario Experimental de la Facultad de Biología de la Universidade de Santiago de Compostela, con código de centro: **15004AE: ES150780263301**.

- Los experimentos con peces fueron realizados por el Prof. Dr. Carlos Rodríguez Osorio, Tutor y Director de la presente tesis.

- Se adjuntan los Certificados de Capacitación en las Categorías “C” y “D” del Prof. Dr. Carlos Rodríguez Osorio.

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