TESE DE DOUTORAMENTO

TRANSCRIPTOMIC STUDY OF *PHOTOBACTERIUM DAMSELAE* SUBSP. *DAMSELAE*: ROLES OF TEMPERATURE AND OF THE TWO-COMPONENT SYSTEM RSTAB AS REGULATORS OF PHYSIOLOGY AND VIRULENCE

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D./Dna. **Xosé Manuel Matanza Fente**

Título da tese: **Transcriptomic study of *Photobacterium damselae* subsp. *damselae*: roles of temperature and of the two-component system RstAB as regulators of physiology and virulence**

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"The important thing is not to stop questioning. Curiosity has its own reason for existence" **Albert Einstein**

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PUBLICATIONS DERIVED FROM THE PRESENT THESIS

Article 1.

Matanza, X. M., & Osorio, C. R. (2018). Transcriptome changes in response to temperature in the fish pathogen *Photobacterium damselae* subsp. *damselae*: Clues to understand the emergence of disease outbreaks at increased seawater temperatures. *PLoS ONE, 13*(12), e0210118.

- **Contributions:** Xosé M. Matanza participated in the conceptualisation and design of the study. He performed the experimental work and the design and presentation of the figures. Furthermore, Xosé M. Matanza wrote the original draft that was finally revised and edited by Carlos R. Osorio.
- **Impact factor:** 2.776 (Q2, Multidisciplinary Sciences).
- **Authorisation from the journal to include this paper in the thesis:** articles published in PLOS ONE are under an open access license called Creative Commons CC_BY license, and therefore it is allowed to use and reproduce material, including the whole article.
- **Reproductions of the article in the present thesis:** this thesis contains reproductions of this article in Methodology and Results sections.

Article 2.

Matanza, X. M., & Osorio, C. R. (2020). Exposure of the opportunistic marine pathogen *Photobacterium damselae* subsp. *damselae* to human body temperature is a stressful condition that shapes the transcriptome, viability, cell morphology, and virulence. *Frontiers in Microbiology, 11*, 1771.

- **Contributions:** Xosé M. Matanza carried out the experimental work and designed figures. He also participated along with Carlos

R. Osorio in the design of the study, the analysis of the data, and the writing of the manuscript.

- **Impact factor (2019):** 4.236 (Q1, Microbiology).
- **Authorisation from the journal to include this paper in the thesis:** articles published in Frontiers in Microbiology are under an open access license called Creative Commons CC_BY license, and therefore it is allowed to use and reproduce material, including the whole article.
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These publications are included in the appendix section.

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ABBREVIATIONS & ACRONYMS

Amp: ampicillin
ATP: adenosine triphosphate
CFU: colony forming unit
ChrI, ChrII: chromosome I, chromosome II
Cm: chloramphenicol
CPS: capsular polysaccharides
DAP: diaminopimelic acid
DEG: differentially expressed gene
DNA: deoxyribonucleic acid
ECP: extracellular product
EDTA: ethylenediamine tetraacetic acid
Em: erythromycin
EPS: extracellular polysaccharides
FAME: fatty acid methyl esters
FC: Fold Change
FPKM: Fragments Per Kilobase per Million fragments mapped
Gal-1-P: galactose-1-phosphate
GT: glycosyltransferase
HaCat: cultured human keratinocyte cells
HMDS: hexamethyldisilazane
IPTG: isopropyl β-D-1-thiogalactopyranoside
kb: kilobase
kDa: kilodalton
Kdo: β-linked 3-deoxy-D-manno-oct-2-ulosonic acid
Km: kanamycin
LB: Luria Bertani
LPS: lipopolysaccharide
ºC: degree Celsius
OD_{600}: optical density at 600 nm
ONPG: *ortho*-Nitrophenyl-β-galactoside
ORF: Open Reading Frame
PAMPs: pathogen-associated molecular patterns
PBS: phosphate-buffered saline
PCR: polymerase chain reaction

PGT: phosphoglycosyltransferase
PHB: Poly-β-hydroxybutyrate
PLA, PLB, PLC, PLD: phospholipases A, B, C and D
RNA: ribonucleic acidsalyers
RNA-seq: RNA sequencing
SDS: sodium dodecyl sulfate
T2SS: type II Secretion System
T6SS: type VI Secretion System
Tc: tetracycline
TCBS: Thiosulfate Citrate Bile Sucrose
TCS: two-component system
TLR: toll-like receptor
Tm: melting temperature
TSA: Tryptic Soy Agar
TSA-1: Tryptic Soy Agar supplemented with NaCl 1%
TSB: Tryptic Soy Broth
TSB-1: Tryptic Soy Broth supplemented with NaCl 1%
UDP-gal: uridine diphospho galactose
UMP: uridine monophosphate
Und-P: undecaprenyl phosphate
wt: wild type

RESUMO

A familia *Vibrionaceae* inclúe un gran número de especies bacterianas acuáticas que representan unha ameaza para a saúde animal e humana. Durante os últimos anos produciuse un aumento gradual dos reportes de infeccións causadas por estas bacterias, probablemente ligado ao efecto do quecemento global. Dito incremento da temperatura das augas mariñas proporcionou as condición ideais para a expansión das poboacións de *Vibrionaceae*, o que incrementa o risco de infección; actualmente, o seu impacto na saúde a nivel global está no punto de mira e requírese de investigacións multidisciplinares que permitan o desenvolvemento de novas estratexias para facerlle fronte a esta problemática. Incluído nesta familia *Photobacterium damselae* subsp. *damselae*, un patóxeno heteroxéneo e de amplo rango de hospedeiros, é causante de infeccións severas nunha gran variedade de animais mariños e nos humanos. Non obstante, esta bacteria tamén foi illada en esteiros, auga de mar, sedimentos e como parte da microbiota comensal de organismos mariños. A pesar de que as condicións de catividade non son un requisito para o desenvolvemento da enfermidade (xa que esta bacteria causa tamén mortalidade en poboacións salvaxes), compromete gravemente a crianza de numerosas especies mariñas de gran importancia na industria da acuicultura. Un trazo característico da maior parte dos gromos infecciosos nos que *P. damselae* subsp. *damselae* se illa como axente causante en granxas é que ocorren principalmente nos meses de verán, ou en picos cálidos que acontecen ao longo do ano (nos que a temperatura da auga ronda os 25ºC). Esta bacteria é considerada un patóxeno emerxente na acuicultura que recentemente experimentou unha perigosa expansión, tanto xeograficamente coma no seu rango de hospedeiros; ademais, é un dos principais axentes zoonóticos que poden transmitirse de peixes a humanos. Nestes últimos, *P. damselae* subsp. *damselae* pode causar fascite necrosante e septicemias fatais; en moitos casos a necrose tisular é imparable a pesar do tratamento con antibióticos, sendo a amputación a única solución posible en moitos casos. Os principais factores de virulencia producidos por esta bacteria son citotoxinas, como a Dly e a PhlyP, codificadas no plásmido pPHDD1, ou PhlyC e PlpV, codificadas no cromosoma. Os gromos infecciosos non son causados por clons

especialmente ben adaptados, senón por unha poboación multiclonal composta por cepas portadoras e non portadoras de plásmido, o que indica que *P. damselae* subsp. *damselae* é un patóxeno altamente heteroxéneo.

Esta tese céntrase en aspectos importantes que gobernan a patoxenicidade e a *fitness* de *P. damselae* subsp. *damselae*, tanto no caso das cepas portadoras do plásmido pPHDD1 como das non portadoras. Concretamente, neste traballo tratamos de identificar puntos débiles da patobioloxía de *P. damselae* subsp. *damselae* que poidan ser empregados para mitigar o impacto das infeccións. A primeira parte da tese lidia coa resposta desta bacteria a temperaturas clave relacionadas coa *fitness* no medio mariño (15ºC) así como coa *fitness* e a virulencia dentro dos peixes e do ser humano (25ºC e 37ºC, respectivamente). Na segunda parte, o obxectivo foi desentrañar a rede xenética baixo o control do Sistema de Dous Compoñentes (TCS) RstAB, un importante regulador de virulencia descuberto recentemente. Adicionalmente, revelamos a existencia dunha cápsula polisacarídica descoñecida ata o momento, cuxa expresión é RstAB-dependente e xoga un papel esencial na virulencia e *fitness* dentro dos hospedeiros.

Estudo da virulencia e da *fitness* de *P. damselae* subsp. *damselae*: o papel da temperatura na modulación do perfil transcriptómico e do fenotipo

Na presente tese propuxémonos, primeiramente, descifrar a resposta de *P. damselae* subsp. *damselae* ás temperaturas clave para o desenvolvemento da enfermidade e/ou para a *fitness* dentro e fóra dos hospedeiros. Abordamos o impacto da temperatura no transcriptoma e en fenotipos chave, establecendo dúas comparativas baseándonos na súa importancia biolóxica: inicialmente, centrámonos en comparar *P. damselae* subsp. *damselae* cultivada a 15ºC (imitando o estilo de vida libre desta bacteria en latitudes medias) e cultivada a 25ºC, temperatura que demostrou ser un factor de risco para a aparición de gromos producidos por esta bacteria na acuicultura nos meses de verán.

Demostramos que o crecemento a 25ºC permítelle a *P. damselae* subsp. *damselae* multiplicarse ata acadar elevados recontos celulares, inferidos a partires de valores de densidade óptica a 600 nm (OD_{600}) máis altos que aqueles obtidos no crecemento a 15ºC. A proliferación observada a 25ºC axuda a comprender como despois dun pico de temperatura na auga mariña, as poboacións de *P. damselae* subsp. *damselae* acadarían un número que, inevitablemente, podería levar á aparición de episodios infecciosos en granxas acuícolas. Para afondar nesta comparativa, analizamos o transcriptoma da cepa RM-71 de *P. damselae* subsp. *damselae* a ambas temperaturas mediante secuenciación do ARN (RNA-seq); este constitúe o primeiro estudo transcriptómico que aborda a expresión xenética global de *P. damselae* subsp. *damselae*. En dita comparativa, aqueles xenes diferencialmente expresados (DEGs) cunha maior expresión a 25ºC foron designados como *upregulated*, termo empregado de xeito rutineiro para denominar aqueles xenes cuxos niveis de expresión están potenciados nunha determinada condición; así mesmo, *downregulated* aplicouse aos xenes menos expresados na condición de estudo, é dicir 25ºC. O crecemento a 25ºC incrementou a expresión de xenes relacionados coa adquisición de nutrientes, como os das porinas, permeases e transportadores. Adicionalmente, xenes implicados na síntese, reparación e tradución de ácidos nucleicos tamén aparecen maiormente expresados a dita temperatura. Estes niveis de expresión elevados poderían proporcionar precursores necesarios para o acentuado crecemento desta bacteria a 25ºC.

Contrariamente ao esperado, os niveis de expresión das citotoxinas non están incrementados de xeito notable a 25ºC; unicamente PhlyP amosou un leve aumento da expresión, nos límites de ser considerado DEG. En vista do seu papel fundamental na virulencia, abordamos a abundancia dos seus transcritos dentro do transcriptoma a estas dúas temperaturas a través da análise por Lectura dos Fragmentos por Kilobase de Transcrito por Millón de Lecturas Mapeadas (FPKM). Atopamos que os xenes codificadores das hemolisinas Dly, PhlyP e PhlyC están entre os máis expresados de todo o transcriptoma producido en ambas condicións; particularmente, o xene *dly* foi o noveno xene máis expresado a 15ºC, con valores comparables aos dos

xenes de proteínas ribosómicas e xenes *housekeeping* altamente expresados. Este achado suxire que a produción de citotoxinas non é un factor limitante para a aparición de gromos nas granxas de acuicultura cando as temperaturas son menores que as dos meses estivais. Porén, moitos outros elementos relacionados coa virulencia de *P. damselae* subsp. *damselae* amosan maiores niveis de expresión a 25ºC, incluíndo gran parte dos DEGs atopados no plásmido de virulencia pPHDD1; este feito reforza a idea de que este plásmido é un trazo distintivo dos illados altamente virulentos. Ditos xenes inclúen potenciais factores de virulencia non caracterizados ata o momento: a proteína de resistencia ao soro Vep07, o receptor de transferrina Vep20, a proteína de membrana externa OmpU, o complexo de secreción de toxinas TolC-AcrAB e proteínas que participan na defensa contra organismos competidores polos mesmos recursos ou que están relacionadas co sistema de secreción de tipo VI (T6SS; A0J47_18110-A0J47_18120). Alén dos anteriores, xenes que codifican para proteínas pertencentes ao sistema de secreción de tipo II (T2SS), implicado na secreción das principais citotoxinas, tamén presentan maiores niveis de expresión, así como moitos xenes implicados na motilidade e quimiotaxe e varias chaperonas e proteínas que actúan contra o estrés oxidativo. Finalmente, unha protease DegP cuxo homólogo en *V. cholerae* afecta á formación de biofilm, á colonización e á secreción, aparece tamén como *upregulated*. Estes datos suxiren que a maior probabilidade dos gromos nos meses de verán explícase pola regulación positiva doutros factores de virulencia, da motilidade, da quimiotaxe e da potenciación do crecemento, e non no aumento da expresión das citotoxinas.

Neste traballo atopamos tamén un desequilibrio entre o número de DEGs *upregulated* e *downregulated* no cromosoma II, cunha marcada predominancia dos xenes *downregulated*. Isto suxire que o cromosoma II de *P. damselae* subsp. *damselae* xoga un papel importante no crecemento a baixas temperaturas. Xenes *downregulated* a 25ºC (e por tanto máis expresados a 15ºC) inclúen o factor sigma alternativo RpoS, un regulador central da resposta ao estrés e o sistema metionina sulfóxido reductase MsrPQ, que participa na reparación do dano oxidativo. A betaína glicina é usada por certas bacterias para a adaptación a baixas temperaturas e no noso caso un transportador da

mesma aparece entre os xenes máis *downregulated* a 25ºC. O sistema da oligopéptido permease OppABCDEF (cuxa función podería estar relacionada coa nutrición) e xenes que participan no ciclo de Krebs e na fixación do nitróxeno tamén se atopan entre os xenes cunha maior diminución da súa expresión. Dentro dos valores máis altos de *downregulation*, moitas proteínas comparten homoloxía con outras implicadas na homeostase da parede celular. As proteínas aquí reveladas son merecedoras de estudos máis polo miúdo e desvelarán características descoñecidas do crecemento de *P. damselae* subsp. *damselae* a baixas temperaturas.

Seguidamente, deseñamos unha nova comparativa para abordar a resposta de *P. damselae* subsp. *damselae* cando é cultivada á temperatura do corpo humano, é dicir, 37ºC. Como condición control escollemos 25ºC, simulando augas mariñas cálidas nun escenario de quecemento global. O crecemento a 37ºC é unha das características clave que diferencia *P. damselae* subsp. *damselae* da outra subespecie *P. damselae* subsp. *piscicida*, permitíndolle colonizar e causar enfermidade nos humanos, aínda que a información dispoñible en canto a como evolucionou *P. damselae* subsp. *damselae* para infectar ao ser humano é escasa. Esta bacteria pode ser o axente causante de infeccións en feridas abertas e de fascite necrosante e, a diferencia doutros Vibrios que causan diarrea, esta especie non volve ao mar trala infección. Achamos que o crecemento a 37ºC activa unha proliferación inicial de *P. damselae* subsp. *damselae* cunha entrada temperá na fase exponencial, en comparación co crecemento observado a 25ºC. Ademais, contrariamente co que acontece cando é cultivada a 25ºC, unha exposición prolongada a 37ºC leva a unha caída da OD_{600}. Seguidamente, analizamos se esta caída da densidade óptica podería deberse a unha perda da viabilidade celular e atopamos que, logo de 6 horas de incubación, o número de células recuperadas dos cultivos mantidos a 37ºC era menor. Logo de 30 h de cultivo a 37ºC non se recuperaban células viables, mentres que alícuotas tomadas ao mesmo tempo de cultivos a 25ºC presentaban os maiores recontos. Para afondar no proceso que estaba tendo lugar, analizamos a morfoloxía celular de cultivos en crecemento exponencial mantidos a ambas temperaturas. A microscopía electrónica de varrido (SEM) revelou que as células que

medraron a 25ºC exhibían a morfoloxía bacilar e o tamaño normais, mentres que a 37ºC observouse que as células medran máis alongadas e forman estruturas catenais que suxiren defectos na separación das células fillas. Ademais, 37ºC supuxo un obstáculo para a resistencia natural a antibióticos de *P. damselae* subsp. *damselae*, xa que cando esta foi cultivada a dita temperatura amosou unha maior susceptibilidade á bencilpenicilina, un antibiótico que inhibe a síntese da parede celular. Unha das principais estratexias bacterianas para soportar cambios drásticos de temperatura é modular a composición dos ácidos graxos de membrana; a través dunha análise de ésteres metílicos de ácidos graxos (FAME), demostramos que *P. damselae* subsp. *damselae* aumenta a proporción de ácidos graxos saturados a medida que aumenta a temperatura de cultivo, o que podería axudar a manter a *fitness* en distintos ambientes.

Usando unha colección de illados de peixe e humanos de *P. damselae* subsp. *damselae*, estudamos se existe unha relación entre ser illado humano e presentar unha vantaxe á hora de medrar á temperatura corporal deste. A análise das curvas de crecemento amosou un patrón similar para illados de peixes e de humanos, indicando que estes últimos non presentan unha mellor adaptación.

Nesta tese presentamos, por primeira vez, o xenoma preliminar de dous illados humanos (CDC-2227-81 e 80077637), que nos permitiron conducir unha comparativa xenómica entre eles e os illados de peixes. As cepas illadas de humanos non son xeneticamente máis similares entre si do que o son ás illadas de peixes; se ben hai un número considerable de xenes específicos de cepa, non aparecen marcadores xenéticos exclusivos para as cepas humanas que estean ausentes nas illadas de peixes. Estes achados suxiren que calquera xenotipo do medio mariño pode causar infección en humanos, reforzando a idea de que *P. damselae* subsp. *damselae* é un patóxeno non clonal.

Coa fin de obter mutantes defectivos no crecemento a 37ºC que presentaran un crecemento normal a 25ºC, creamos unha libraría de mutantes por transposón mini-Tn*10*; isto permitiríanos identificar dianas moleculares para mitigar o impacto das infeccións humanas causadas por *P. damselae* subsp. *damselae*. Posto que non foi posible

acadar este propósito, suxerimos que a habilidade para medrar á temperatura do corpo humano podería constituír unha propiedade multixénica, no canto de deberse á presenza dun único xene. Mediante a técnica do RNA-seq, analizamos os cambios na expresión que experimenta a cepa RM-71 de *P. damselae* subsp. *damselae* cando medra a 37ºC, en comparación con 25ºC. Aqueles DEGs con maior expresión denomináronse *upregulated* e aqueles con menor expresión, *downregulated*. En xeral, unha temperatura de 37ºC aumenta a expresión de xenes relacionados coa resposta a choque térmico, defensa contra especies reactivas de osíxeno e xenes metabólicos que aportan o subministro necesario de biomoléculas e enerxía que sosteñen o rápido crecemento inicial. Un dos achados máis sorprendentes deste traballo foi que, á temperatura do corpo humano, moitas funcións relacionadas coa virulencia están *downregulated*. No que atinxe ás hemolisinas, a damselisina Dly presenta unha marcada baixada de expresión a 37ºC; aínda que, de forma similar ao que ocorre cando *P. damselae* subsp. *damselae* medra a 15 e 25ºC, a análise FPKM mostra que, medrando a 37ºC, as hemolisinas están dentro dos 150 xenes máis expresados de todo o transcriptoma. Estas observacións, sumadas aos resultados obtidos nos ensaios de crecemento e viabilidade, coinciden con estudos previos nos que se recollen as dificultades experimentadas á hora de illar *P. damselae* subsp. *damselae* de tecidos humanos infectados, pero si observando hemólise en ausencia de crecemento nas placas de agar sangue. Adicionalmente, xenes que codifican porinas, proteínas que forman o T2SS, que participan na captación de ferro, na motilidade e na quimiotaxe están tamén *downregulated*.

Os achados presentados nesta sección evidencian que a temperatura é un factor ambiental que goberna funcións fisiolóxicas e relacionadas coa virulencia en *P. damselae* subsp. *damselae*, sendo 25ºC unha temperatura próxima á óptima para desencadear un perfil virulento, mentres que 37ºC constitúe unha condición estresante que fai oportunistas as infeccións humanas, no canto de estar causadas por cepas ben adaptadas.

Estudo da virulencia e da *fitness* de *P. damselae* subsp. *damselae*: regulón completo do sistema RstAB e caracterización xenética dunha cápsula polisacarídica descoñecida ata o momento.

Considerando a alta heteroxeneidade de *P. damselae* subsp. *damselae*, é de especial relevancia identificar e caracterizar os mecanismos ubicuos que controlan a súa patoxenicidade. Continuando co estudo dos procesos implicados na *fitness* e na virulencia, a segunda parte desta tese céntrase no estudo do sistema de dous compoñentes RstAB, recentemente identificado, formado pola histidina quinase RstB e o regulador de resposta RstA. Os sistemas de dous compoñentes bacterianos detectan e responden a sinais ambientais, axustando a fisioloxía celular e a expresión xenética; xenes homólogos codificantes para o sistema están amplamente distribuídos na familia *Vibrionaceae*, pero os estudos funcionais dispoñibles son limitados. Investigacións previas demostraron que o sistema RstAB regula positivamente a expresión das citotoxinas e da virulencia en *P. damselae* subsp. *damselae*. Adicionalmente, mutantes en calquera dos dous xenes do sistema amosan unha secreción reducida de numerosas proteínas dependentes do T2SS. A pesar do coñecemento actual, o regulón do RstAB aínda non foi completamente dilucidado.

Empregamos a técnica do RNA-seq para comparar o perfil transcriptómico da cepa RM-71 de *P. damselae* subsp. *damselae* co dos mutantes por deleción Δ*rstA* and Δ*rstB*. Esta análise revelou que o sistema RstAB pode ser considerado un regulador positivo da expresión xénica: xenes *downregulated* nos mutantes mostran un alto grao de expresión diferencial, mentres que xenes *upregulated* presentan un nivel moi baixo (próximo ao límite de clasificación como diferencialmente expresados). Como é de agardar para un par cognato, RstA e RstB superpóñense na regulación positiva de 128 xenes (é dicir, *downregulated* en ambos mutantes Δ*rstA* and Δ*rstB*). Focalizamos este estudo en ditos xenes porque representan as funcións alteradas resultantes da inactivación do sistema. En concordancia con datos publicados recentemente, amosamos que o RstAB regula positivamente as citotoxinas Dly, PhlyP e PhlyC e proteínas das que se sabe que son secretadas polo T2SS. Así mesmo, as proteínas constituíntes do T2SS

tamén aparecen como *downregulated*. Moitos outros xenes non caracterizados con posibles implicacións na virulencia saíron á luz a raíz deste estudo transcriptómico; ditos xenes clasificáronse en diferentes categorías en base á súa potencial función: resistencia a axentes antimicrobianos e supervivencia nos hospedeiros, proteínas de membrana externa, síntese de polisacáridos extracelulares (EPS) e outros. É interesante mencionar que esta tese deu a coñecer un bo número de xenes regulados positivamente por RstAB e por unha temperatura de 25ºC (PhlyP, o T2SS, OmpU, TolC-AcrAB, DegP, A0J47_18110-A0J47_18120), reforzando a idea de que ambos factores son chave para entender a patoxenicidade de *P. damselae* subsp. *damselae*.

Un dos resultados máis impactantes que emerxeu entre os nosos datos transcriptómicos foi atopar, nos mutantes do sistema RstAB, un forte descenso na expresión de 21 xenes, distribuídos en 2 clusters, presumiblemente implicados na síntese de EPS e polisacáridos capsulares (CPS). O *cluster* I contén os xenes *wza*, *wzb*, *wzc* e glicotransferases entre outras, mentres que o *cluster* II contén os xenes *yjbEFGH*. *wza* codifica unha proteína de membrana externa implicada na exportación de CPS que traballan en conxunto coa tirosina quinase *wzc* e a súa fosfatase cognata *wzb*. Por outra banda, existe pouca información en canto á función de *yjbEFGH*, pero algúns estudos indican un posible papel na produción dun polisacárido extracelular en *E. coli*. Posto que ata o momento non se levou a cabo ningún tipo de investigación referente á habilidade de *P. damselae* subsp. *damselae* de producir unha cápsula polisacarídica, dita forte diminución de expresión levounos a afondar en tal función. A presencia dunha cápsula demostrouse por microscopía electrónica de transmisión (TEM); en liña cos datos transcriptómicos, os mutantes RstAB mostraron un fenotipo acapsular. Co obxectivo de demostrar que a síntese e exportación da capa capsular lévase a cabo mediante os xenes anteriormente mencionados, decidimos construír mutantes por deleción mediante intercambio alélico en xenes pertencentes a ambos *clusters*: *wza* e *wzc* no *cluster* I, e *yjbH* no *cluster* II, que se transcribe de xeito diverxente. A mutación de *wza* e *wzc* eliminou completamente a síntese da cápsula, mentres que a mutación de *yjbH* resultou nunha produción dunha capa

capsular significativamente máis fina que a observada na cepa salvaxe. O principal cambio macroscópico observado nos mutantes acapsulares Δ*wza* e Δ*wzc* foi un maior grao de translucidez respecto á cepa parental. A produción de biofilm foi notablemente maior nestes mutantes respecto a RM-71 e ao mutante Δ*yjbH*, o que suxire que outras moléculas, situadas por debaixo da cápsula, participan no tipo de adhesión e agregación necesario para formar biofilm. O crecemento, a motilidade e o fenotipo hemolítico mantivéronse inalterados nos tres mutantes; sen embargo, cando estes foron inxectados en rodaballo e dourada, a maior parte dos animais inxectados cos mutantes Δ*wza* e Δ*wzc* sobreviviron. Neste traballo tamén mostramos que estes mutantes acapsulares presentan unha redución significativa na *fitness* cando se cultivan en medio de cultivo suplementado con soro ou moco de rodaballo.

Os resultados aquí presentados indican claramente que a síntese da cápsula polisacarídica é esencial para a máxima virulencia de *P. damselae* subsp. *damselae*. A redución desta virulencia observada nos mutantes sen cápsula é independente do crecemento normal, da motilidade e da produción de hemolisinas. Neste traballo tamén amosamos que esta cápsula permite a evasión das defensas do hospedeiro; así mesmo, a expresión dos xenes *wza* e *wzc* aparece potenciada a 25ºC, contribuíndo á idea de que esta temperatura constitúe un forte sinal para desencadear o perfil virulento en *P. damselae* subsp. *damselae*.

RESUMEN

La familia *Vibrionaceae* incluye un gran número de especies bacterianas acuáticas que representan una amenaza para la salud animal y humana. En los últimos años se ha registrado un aumento gradual en el número de infecciones causadas por estas bacterias, probablemente ligadas a los efectos del calentamiento global. El aumento de la temperatura del agua marina ha proporcionado las condiciones ideales para la expansión de las poblaciones de *Vibrionaceae*, hecho que aumenta el riesgo de infección. Actualmente, su impacto en la salud a nivel global está en el punto de mira y se requiere de investigaciones multidisciplinares que permitan el desarrollo de nuevas estrategias para hacer frente a esta problemática. Incluido en esta familia, *Photobacterium damselae* subsp. *damselae*, un patógeno heterogéneo con un amplio rango de hospedadores, es un agente causal de infecciones severas en una gran variedad de animales marinos y en el ser humano. Sin embargo, esta bacteria también ha sido aislada en estuarios, agua de mar, sedimentos y como parte de la microbiota comensal de organismos marinos. A pesar de que las condiciones de cautividad no son un requisito indispensable para el desarrollo de la enfermedad (ya que esta bacteria causa mortalidad en poblaciones salvajes), compromete gravemente la crianza de numerosas especies marinas de gran importancia en la industria de la acuicultura. Un rasgo característico de la mayor parte de los brotes infecciosos en los que *P. damselae* subps. *damselae* se aísla como agente causal en granjas es que ocurren principalmente en los meses de verano o en picos cálidos alcanzados a lo largo del año, en los que la temperatura del agua ronda los 25ºC. Esta bacteria es considerada un patógeno emergente en acuicultura que ha experimentado una peligrosa expansión recientemente, tanto geográficamente como en su rango de hospedadores; además, es uno de los principales agentes zoonóticos que pueden transmitirse de peces a humanos. En este último, *P. damselae* subps. *damselae* puede causar fascitis necrosante y septicemias fatales; en muchos casos la necrosis tisular es imparable a pesar del tratamiento con antibióticos, siendo la amputación la única solución posible en muchos casos. Los principales factores de virulencia producidos por esta bacteria son citotoxinas, como la Dly y la PhlyP, codificadas en el

plásmido pPHDD1, o PhlyC y PlpV, codificadas en el cromosoma. Los brotes infecciosos no son causados por clones especialmente bien adaptados, sino por una población multiclonal compuesta por cepas portadoras y no portadoras de plásmido, lo que indica que *P. damselae* subsp. *damselae* es un patógeno altamente heterogéneo.

Esta tesis se centra en los aspectos importantes que gobiernan la patogenicidad y la *fitness* de *P. damselae* subsp. *damselae*, tanto en el caso de las cepas portadoras del plásmido pPHDD1 como de las no portadoras. Concretamente, este trabajo trata de identificar puntos débiles de la patobiología de *P. damselae* subsp. *damselae* que puedan ser utilizados para mitigar el impacto de las infecciones. La primera parte de la tesis se centra en la respuesta de esta bacteria a temperaturas clave relacionadas con la *fitness* en el medio marino (15ºC) así como con la fitness y la virulencia dentro de los peces y del ser humano (25 y 37ºC, respectivamente). En la segunda parte, el objetivo ha sido desentrañar la red genética bajo el control del Sistema de Dos Componentes (TCS) RstAB, un importante regulador de virulencia fundamentales descubierto recientemente. Adicionalmente, revelamos la existencia de una cápsula polisacarídica desconocida hasta la fecha, cuya expresión es RstAB-dependiente y juega un papel esencial en la virulencia y la *fitness* dentro de los hospedadores.

Estudio de la virulencia y de la *fitness* de *P. damselae* subsp. *damselae*: el papel de la temperatura en la modulación del perfil transcriptómico y del fenotipo

En la presente tesis nos propusimos, en primer lugar, descifrar la respuesta de *P. damselae* subsp. *damselae* a las temperaturas clave para el desarrollo de la enfermedad y/o para la *fitness* dentro y fuera de los hospedadores. Abordamos el impacto de la temperatura en el transcriptoma y en fenotipos clave, estableciendo dos comparativas basadas en su importancia biológica: inicialmente, nos centramos en comparar *P. damselae* subsp. *damselae* cultivada a 15ºC (imitando el estilo de vida libre de esta bacteria en latitudes medias) y cultivada a 25ºC, temperatura que ha demostrado ser un factor de riesgo para la

aparición de brotes producidos por esta bacteria en acuicultura en los meses de verano.

Demostramos que el crecimiento a 25ºC permite a *P. damselae* subsp. *damselae* alcanzar elevados recuentos celulares, inferidos a partir de los valores de densidad óptica a 600 nm (OD_{600}) más altos que aquellos obtenidos en el crecimiento a 15ºC. La proliferación observada a 25ºC ayuda a comprender cómo después de un pico de temperatura en el agua, las poblaciones de *P. damselae* subsp. *damselae* alcanzarían un número que, inevitablemente, podría llevar a la aparición de episodios infecciosos en granjas acuícolas. Para profundizar en esta comparativa, analizamos el transcriptoma de la cepa RM-71 de *P. damselae* subsp. *damselae* a ambas temperaturas mediante secuenciación de ARN (RNA-seq), éste constituye el primer estudio transcriptómico que aborda la expresión genética global de *P. damselae* subsp. *damselae*. En dicha comparativa, aquellos genes diferencialmente expresados (DEGs) con una mayor expresión a 25ºC se designaron como *upregulated*, término empleado de forma rutinaria para denominar aquellos genes cuya expresión está potenciada en una determinada condición; por otro lado, los genes menos expresados en la condición de estudio, es decir 25ºC, se designaron como *downregulated*. El crecimiento a 25ºC aumentó la expresión de genes relacionados con la adquisición de nutrientes como los de las porinas, permeasas y transportadores. Adicionalmente, genes implicados en la síntesis, reparación y traducción de ácidos nucleicos también aparecen más expresados a esta temperatura. Estos niveles de expresión podrían proporcionar precursores necesarios para el acentuado crecimiento de esta bacteria a 25ºC.

Contrariamente a lo esperado, los niveles de expresión de las citotoxinas no se ven incrementados de manera notable a 25ºC; únicamente PhlyP muestra un leve aumento de la expresión, en los límites de ser considerado DEG. En vista de su papel fundamental en la virulencia, abordamos la abundancia de sus transcritos dentro del transcriptoma a estas dos temperaturas mediante el análisis por Lectura de los Fragmentos por Kilobase de Transcrito por Millón de Lecturas Mapeadas (FPKM). Encontramos que los genes codificadores de las

hemolisinas Dly, PhlyP e PhlyC están entre los más expresados de todo el transcriptoma producido en ambas condiciones; particularmente, el gen *dly* es el noveno gen más expresado a 15ºC, con valores comparables a los presentados por genes de proteínas ribosómicas y genes *housekeeping* altamente expresados. Este hallazgo sugiere que la producción de citotoxinas no es un factor limitante para la aparición de brotes en las plantas de acuicultura cuando las temperaturas son menores que las típicas de los meses estivales. Sin embargo, muchos otros elementos relacionados con la virulencia de *P. damselae* subsp. *damselae* muestran mayores niveles de expresión a 25ºC, incluyendo a la mayor parte de los DEGs situados en el plásmido de virulencia pPHDD1; este hecho refuerza la idea de que este plásmido es un rasgo distintivo de los aislados altamente virulentos. Dichos genes incluyen potenciales factores de virulencia no caracterizados hasta el momento: la proteína de resistencia a suero Vep07, el receptor de transferrina Vep20, la proteína de membrana externa OmpU, el complejo de secreción de toxinas TolC-AcrAB y proteínas que participan en la defensa contra los organismos competidores por los mismos recursos o que están relacionadas con el sistema de secreción de tipo VI (T6SS; A0J47_18110-A0J47_18120). Mas allá de los anteriores, genes que codifican para proteínas pertenecientes al sistema de secreción de tipo II (T2SS), implicado en la secreción de las principales citotoxinas, también presentan mayores niveles de expresión, así como numerosos genes implicados en la motilidad y quimiotaxis, y varias chaperonas y proteínas que actúan contra el estrés oxidativo. Finalmente, una proteasa DegP cuyo homólogo en *V. cholerae* afecta a la formación de biofilm, a la colonización y a la secreción, aparece también como *upregulated*. Estos datos sugieren que la mayor probabilidad de los brotes en los meses de verano se explica por la regulación positiva de otros factores de virulencia, de motilidad, de quimiotaxis y de la potenciación del crecimiento, y no por un aumento de la expresión de citotoxinas.

En este trabajo encontramos, además, un desequilibrio entre el número de DEGs *upregulated* y *downregulated* en el cromosoma II, con una marcada predominancia de genes *downregulated*. Esto sugiere que el cromosoma II de *P. damselae* subsp. *damselae* juega un papel

importante en el crecimiento a bajas temperaturas. Genes *downregulated* a 25ºC (y por tanto más expresados a 15ºC) incluyen el factor sigma alternativo RpoS, un regulador central de la respuesta a estrés y el sistema metionina sulfóxido reductasa MsrPQ, que participa en la reparación del daño oxidativo. La betaína glicina es usada por ciertas bacterias para la adaptación a bajas temperaturas, y en nuestro caso, un transportador de la misma aparece entre los genes más *downregulated* a 25ºC. El sistema de la oligopéptido permeasa OppABCDEF (cuya función podría estar relacionada con la nutrición) y genes que participan en el ciclo de Krebs y en la fijación de nitrógeno también se encuentran entre los genes con una mayor disminución de la expresión. Dentro de los valores más altos de *downregulation*, muchas proteínas comparten homología con otras implicadas en la homeostasis de la pared celular. Las proteínas aquí reveladas son merecedoras de estudios más detallados y desvelarán características desconocidas del crecimiento de *P. damselae* subsp. *damselae* a bajas temperaturas.

Seguidamente, diseñamos una nueva comparativa para abordar la respuesta de *P. damselae* subsp. *damselae* cuando se cultiva a la temperatura del cuerpo humano, es decir, 37ºC; como condición control elegimos 25ºC, a modo de simulación de las aguas marinas cálidas en un escenario de calentamiento global. El crecimiento a 37ºC es una de las características clave que diferencia *P. damselae* subsp. *damselae* de la subespecie *P. damselae* subsp. *piscicida*, permitiéndole colonizar y causar enfermedad en los humanos, aunque la información disponible en cuanto a cómo ha evolucionado *P. damselae* subsp. *damselae* para infectar al ser humano es escasa. Esta bacteria puede ser el agente causal de infecciones en heridas abiertas y de fascitis necrosante y, a diferencia de otros Vibrios que causan diarrea, esta especie no vuelve al mar tras la infección. Hallamos que el crecimiento a 37ºC activa una proliferación inicial de *P. damselae* subsp. *damselae* con una entrada temprana en la fase exponencial, en comparación con el crecimiento observado a 25ºC. Además, contrariamente a lo que ocurre cuando se cultiva a 25ºC, una exposición prolongada a 37ºC lleva a una caída de la OD_{600}. Seguidamente, analizamos si esta caída de la densidad óptica podría deberse a una pérdida de la viabilidad celular y encontramos que, después de 6 horas de incubación, el número de células recuperadas en

los cultivos mantenidos a 37ºC era menor. Después de 30 h de cultivo a 37ºC no se recuperan células viables, mientras que alícuotas tomadas al mismo tiempo a 25ºC presentan los mayores recuentos. Para profundizar en el proceso que estaba teniendo lugar, analizamos la morfología celular de cultivos en crecimiento exponencial mantenidos a ambas temperaturas. La microscopía electrónica de barrido (SEM) reveló que las células que crecían a 25ºC exhibían una morfología bacilar y un tamaño normales, mientras que a 37ºC las células crecen más alargadas y forman estructuras en cadena que sugieren defectos en la separación de las células hijas. Además, 37ºC supuso un obstáculo para la resistencia natural a antibióticos de *P. damselae* subsp. *damselae*, ya que cuando ésta es cultivada a dicha temperatura muestra una mayor susceptibilidad a la bencilpenicilina, un antibiótico que inhibe la síntesis de la pared celular. Una de las principales estrategias bacterianas para soportar cambios drásticos de temperatura es modular la composición de los ácidos grasos de membrana; a través de un análisis de ésteres metílicos de ácidos grasos (FAME), demostramos que *P. damselae* subsp. *damselae* aumenta la proporción de ácidos grasos saturados a medida que aumenta la temperatura de cultivo, lo que podría ayudar a mantener la *fitness* en distintos ambientes.

Usando una colección de aislados de peces y humanos de *P. damselae* subsp. *damselae*, estudiamos si existe una relación entre ser aislado humano y presentar una ventaja a la hora de crecer a la temperatura corporal del mismo; el análisis de las curvas de crecimiento mostró un patrón similar para aislados de peces y humanos, indicando que estos últimos no presentan una mejor adaptación. En esta tesis presentamos, por primera vez, el genoma preliminar de dos aislados humanos (CDC-2227-81 y 80077637), que nos han permitido conducir una comparativa genómica entre ellos y los aislados de peces. Las cepas aisladas de humanos no son genéticamente más similares entre si de lo que lo son las aisladas de peces; si bien hay un número considerable de genes específicos de cepa, no aparecen marcadores genéticos exclusivos para las cepas humanas que estén ausentes en las aisladas de peces. Estos hallazgos sugieren que cualquier tipo de genotipo del medio marino puede causar infección en humanos, reforzando la idea de que *P. damselae* subsp. *damselae* es un patógeno no clonal.

Con el fin de obtener mutantes defectivos en el crecimiento a 37ºC que presentasen un crecimiento normal a 25ºC, creamos una librería de mutantes por transposón mini-Tn*10*; esto nos permitiría identificar dianas moleculares para mitigar el impacto de las infecciones humanas causadas por *P. damselae* subsp. *damselae*. Puesto que no fue posible conseguir este propósito, sugerimos que la habilidad para crecer a la temperatura del cuerpo humano podría constituir una propiedad multigénica, en vez de deberse a la presencia de un único gen. Mediante la técnica de RNA-seq analizamos los cambios en la expresión que experimenta la cepa RM-71 de *P. damselae* subsp. *damselae* cuando crece a 37ºC, en comparación con 25ºC. Aquellos DEGs con mayor expresión se denominaron *upregulated* y aquellos con menor expresión, *downregulated*. En general, una temperatura de 37ºC aumenta la expresión de genes relacionados con la respuesta a choque térmico, defensa contra especies reactivas de oxígeno y genes metabólicos que aportan el suministro necesario de biomoléculas y energía que sostienen el rápido crecimiento inicial. Uno de los hallazgos más sorprendentes de este trabajo fue que, a la temperatura del cuerpo humano, muchas de las funciones relacionadas con la virulencia están *downregulated*. En lo que concierne a las hemolisinas, la damselisina Dly presenta una marcada bajada de expresión a 37ºC; sin embargo, de forma similar a lo que ocurre cuando *P. damselae* subsp. *damselae* crece a 15 y 25ºC, el análisis FPKM muestra que, creciendo a 37ºC, las hemolisinas están dentro de los 150 genes más expresados de todo el transcriptoma. Estas observaciones, sumadas a los resultados obtenidos en los ensayos de crecimiento y viabilidad, coinciden con estudios previos en los que se recogen las dificultades experimentadas a la hora de aislar *P. damselae* subsp. *damselae* de tejidos humanos infectados, aún observando hemólisis en ausencia de crecimiento en placas de agar sangre. Adicionalmente, genes que codifican porinas, proteínas que forman el T2SS, que participan en la captación de hierro, en la motilidad y en la quimiotaxis están también *downregulated*.

Los hallazgos presentados en esta sección evidencian que la temperatura es un factor ambiental que gobierna funciones fisiológicas y relacionadas con la virulencia en *P. damselae* subsp. *damselae*, siendo 25ºC una temperatura próxima a la óptima para desencadenar un perfil

virulento, mientras que a 37ºC constituye una condición estresante que hace oportunistas las infecciones humanas en lugar de estar causadas por cepas bien adaptadas.

Estudio de la virulencia y de la *fitness* de *P. damselae* subsp. *damselae*: regulón completo del sistema RstAB y caracterización genética de una cápsula polisacarídica desconocida hasta el momento.

Considerando la alta heterogeneidad de *P. damselae* subsp. *damselae*, es de especial relevancia identificar y caracterizar los mecanismos ubicuos que controlan su patogenicidad. Continuando con el estudio de los procesos implicados en la *fitness* y en la virulencia, la segunda parte de esta tesis se centra en el estudio del sistema de dos componentes RstAB, recientemente identificado, formado por la histidina kinasa RstB y su regulador de respuesta RstA. Los sistemas de dos componentes bacterianos detectan y responden a señales ambientales, ajustando la fisiología celular y la expresión genética; genes homólogos codificantes para el sistema están ampliamente distribuidos en la familia *Vibrionaceae*, pero los estudios funcionales disponibles son limitados. Investigaciones previas demostraron que el sistema RstAB regula positivamente la expresión de las citotoxinas y de la virulencia en *P. damselae* subsp. *damselae*. Adicionalmente, mutantes en cualquiera de los dos genes del sistema muestran una secreción reducida de numerosas proteínas dependientes del T2SS. A pesar del conocimiento actual, el regulón del RstAB aún no ha sido completamente dilucidado.

Usamos la técnica de RNA-seq para comparar el perfil transcriptómico de la cepa RM-71 de *P. damselae* subsp. *damselae* con el de los mutantes por deleción Δ*rstA* y Δ*rstB*. Dicho análisis reveló que el sistema RstAB puede ser considerado un regulador generalmente positivo de la expresión génica: genes *downregulated* en los mutantes muestran un alto grado de expresión diferencial, mientras que genes *upregulated* presentan un nivel muy bajo (próximo al límite de clasificación como diferencialmente expresados). Como es de esperar

para un par cognato, RstA y RstB se superponen en la regulación positiva de 128 genes (es decir, *downregulated* en ambos mutantes Δ*rstA* y Δ*rstB*). Focalizamos este estudio en dichos genes, que representan las funciones alteradas de la inactivación del sistema. En concordancia con los datos publicados recientemente, mostramos que el RstAB regula positivamente las citotoxinas Dly, PhlyP y PhlyC y proteínas de las que se sabe que son secretadas por el T2SS; por otro lado, las proteínas constituyentes del T2SS también aparecen como *downregulated*. Muchos de los genes no caracterizados con posibles implicaciones en la virulencia han salido a la luz a raíz de este estudio transcriptómico; dichos genes se clasificaron en diferentes categorías en base a su potencial función: resistencia a agentes antimicrobianos y supervivencia en los hospedadores, proteínas de membrana externa, síntesis de polisacáridos extracelulares (EPS) y otros. Es interesante mencionar que esta tesis ha dado a conocer un gran número de genes regulados positivamente por RstAB y la temperatura de 25ºC (PhlyP, T2SS, OmpU, TolC-AcrAB, DegP, A0J47_18110-A0J47_18120), reforzando la idea de que ambos factores son clave para entender la patogenicidad de *P. damselae* subsp. *damselae*.

Uno de los resultados más impactantes que ha emergido entre nuestros datos transcriptómicos ha sido encontrar en los mutantes del sistema RstAB, un fuerte descenso en la expresión de 21 genes, distribuidos en 2 clústeres presumiblemente implicados en la síntesis de EPS y polisacáridos capsulares (CPS). El clúster I contiene los genes *wza*, *wzb*, *wzc* y glucotransferasas entre otras, mientras que el clúster II contiene los genes *yjbEFGH*. *wza* codifica una proteína de membrana externa implicada en la exportación de CPS que trabajan en conjunto con la tirosina kinasa *wzc* y su fosfatasa cognata *wzb*. Por otro lado, existe poca información en cuanto a la función de *yjbEFGH*, pero algunos estudios apuntan a un posible papel en la producción de un polisacárido extracelular en *E. coli*. Puesto que hasta el momento no se había llevado a cabo ningún tipo de investigación referente a la habilidad de *P. damselae* subsp. *damselae* para producir una cápsula polisacarídica, dicha disminución de la expresión fue el primer paso que nos llevó a profundizar en esta función. La presencia de una cápsula se demostró por microscopía electrónica de transmisión (TEM). En línea

con los datos transcriptómicos, los mutantes RstAB mostraron un fenotipo acapsular. Con el objetivo de demostrar que la síntesis y exportación de la capa capsular se lleva a cabo mediante los genes mencionados anteriormente, decidimos construir mutantes por deleción mediante intercambio alélico en genes pertenecientes a ambos clústeres: *wza* y *wzc* en el clúster I, e *yjbH* en el clúster II, que se transcribe de manera divergente. La mutación de *wza* y *wzc* eliminó completamente la síntesis de cápsula, mientras que la mutación de *yjbH* dio como resultado la producción de una capa capsular significativamente más fina que la observada en la cepa salvaje. El principal cambio macroscópico observado en los mutantes acapsulares Δ*wza* y Δ*wzc* fue un mayor grado de translucidez respecto a la cepa parental. La producción de biofilm fue notablemente mayor en estos mutantes respecto a RM-71 y al mutante Δ*yjbH*, lo que sugiere que otras moléculas, situadas por debajo de la cápsula, participan en el tipo de adhesión y agregación necesario para formar biofilm. El crecimiento, la motilidad y el fenotipo hemolítico se mantuvieron inalterados en los tres mutantes; sin embargo, cuando éstos fueron inyectados en rodaballo y dorada, la mayor parte de los animales inoculados con los mutantes Δ*wza* y Δ*wzc* sobrevivieron. En este trabajo mostramos, además, que estos mutantes acapsulares presentan una reducción significativa en la *fitness* cuando se cultivan en medio suplementado con suero o moco de rodaballo.

Los resultados aquí presentados indican claramente que la síntesis de la cápsula polisacarídica es esencial para la máxima virulencia de *P. damselae* subsp. *damselae*. La reducción de esta virulencia observada en los mutantes sin cápsula es independiente del crecimiento normal, de la motilidad y de la producción de hemolisinas. En este trabajo también mostramos que esta cápsula permite la evasión de las defensas del hospedador; asimismo, la expresión de los genes *wza* y *wzc* aparece potenciada a 25ºC, sumándose a la idea de que esta temperatura constituye una fuerte señal para desencadenar el perfil virulento de *P. damselae* subsp. *damselae*.

SUMMARY

The family *Vibrionaceae* comprises a large number of aquatic bacterial species that represent a menace for both animal and human welfare. There has been a gradual increase in the number of reported infections caused by these bacteria during the past years, likely linked to the effect of global warming. Such increase in seawater temperatures has provided ideal conditions for the thriving expansion of *Vibrionaceae* populations, a phenomenon that augments the risk of infection. Currently on the limelight and requiring multidisciplinary research, novel strategies are needed to tackle the impact of this problematic on global health. Included in the family, *Photobacterium damselae* subsp. *damselae* is a heterogeneous and broad host-range pathogen that has been the causative agent of life-threatening infections in a great diversity of marine animals and in humans. However, it has also been isolated from estuaries, seawater, sediments and as part of the commensal microbiota of marine organisms. Despite the fact that captivity conditions are not a requisite for the development of disease since this bacterium causes mortalities of wild populations, it severely compromises the rearing of many marine species with importance for the aquaculture industry. A common trait of most disease outbreaks in which *P. damselae* subsp. *damselae* is isolated as the causative agent in farms, is that they occur predominantly in summer months or in unusually warm peaks throughout the year (with seawater temperatures around 25ºC). This bacterium is considered an emerging pathogen in aquaculture, with a hazardous lately expansion of its host and geographical range. Furthermore, it is acknowledged as one of the main zoonotic agents that can be transmitted from fish to humans. In humans, *P. damselae* subsp. *damselae* can cause fulminant septicaemias and necrotising fasciitis; in some clinical cases, tissue necrosis cannot be stopped despite antibiotic administration, and frequently amputation is the only solution. The main virulence factors produced by this bacterium are cytotoxins Dly and PhlyP encoded by the pPHDD1 plasmid and PhlyC and PlpV by the chromosome. Outbreaks are not caused by well-adapted clones but by a multiclonal population of plasmid harbouring and plasmidless strains, which points at *P. damselae* subsp. *damselae* as a highly heterogeneous pathogen.

This thesis is focused on meaningful aspects that govern pathogenicity and fitness of *P. damselae* subsp. *damselae* strains of any type i.e., pPHDD1 harbouring and plasmidless strains. We aim to reveal weak points of *P. damselae* subsp. *damselae* pathobiology that can be targeted for mitigating the impact of infections. The first part of the thesis deals with the response of this bacterium to key temperatures related to fitness in the marine environment (15ºC), and to virulence and fitness inside fish and human hosts (25 and 37ºC, respectively). In the second part, we aimed to unravel the genetic network under the control of the Two Component System (TCS) RstAB, a recently-discovered major regulator of virulence. We revealed the existence of a hitherto unknown polysaccharidic capsule whose expression is RstAB-dependent and which plays an essential role in virulence and fitness inside hosts.

Study of *P. damselae* subsp. *damselae* virulence and fitness: role of temperature in modulating transcriptomic profile and phenotype

In the present thesis, we first aimed at deciphering the response of *P. damselae* subsp. *damselae* to temperatures that are key for the development of disease and/or in fitness inside/outside hosts. We assessed how temperature impacts on transcriptome and key phenotypes, designing two comparisons on account of their biological importance. First, we focused on the comparison between *P. damselae* subsp. *damselae* cultured at 15ºC, mimicking the free-swimming lifestyle of this bacterium at mid latitudes, vs at 25ºC which has been proven to be a warm and risky temperature for the development of aquaculture outbreaks produced by this bacterium in summer months.

We demonstrated that growth at 25ºC allows *P. damselae* subsp. *damselae* to multiply until achieving a higher number of cells, inferred from higher optical density at 600 nm (OD_{600}) values, than growth at 15ºC. The observed proliferation at 25ºC aids to understand how after a peak in seawater temperature, *P. damselae* subsp. *damselae* populations might achieve a sufficient number that unavoidably leads to the occurrence of disease episodes in fish farms. To dive further into

this comparison, we analysed by RNA sequencing (RNA-seq) the transcriptome produced by *P. damselae* subsp. *damselae* at both temperatures. This constituted the first transcriptomic study that tackled global genetic expression in *P. damselae* subsp. *damselae*. In this comparison, those differentially expressed genes (DEGs) with a greater expression at 25ºC were tagged as "upregulated" and those with lower as "downregulated". Growth at 25ºC upregulated mainly the expression of genes involved in nutrient acquisition such as porins, permeases and transporters. As well, genes related to synthesis, repair and translation of nucleic acids were upregulated. This greater expression might supply precursors for the above-mentioned enhanced growth revealed by the growth analysis.

Contrary to expectations, cytotoxins were not found to be highly upregulated at 25ºC, only PhlyP showed a slight upregulation very close to the threshold for being considered a DEG. In view of their central role in virulence, we assessed their transcript abundance in the transcriptome produced at these two temperatures through the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) analysis. We found that genes encoding haemolysins Dly, PhlyP and PhlyC were among the most expressed of the whole transcriptome produced at both conditions. Particularly remarkable, the gene *dly* was the ninth most expressed at 15ºC, with values comparable to ribosomal proteins and highly expressed housekeeping genes. This finding suggests that the production of cytotoxins is not a constraining factor for the emergence of outbreaks in aquaculture farms when temperatures are lower than those typical of summer months. Nevertheless, many other aspects of *P. damselae* subsp. *damselae* related to virulence were upregulated at 25ºC. Of all DEGs found in the virulence plasmid pPHDD1, most of them were upregulated, a fact that reinforces the idea of this plasmid as a distinctive feature of highly virulent isolates. This list includes several uncharacterised potential virulence factors: the serum resistance protein Vep07, the transferrin receptor Vep20, the outer membrane protein OmpU, the multidrug and toxin secretion complex TolC-AcrAB, and proteins that participate in the defence against organisms that compete for the same resources or are related to the type VI secretion system (T6SS; A0J47_18110-A0J47_18120).

Also, genes encoding for proteins that belong to the type II secretion system (T2SS) which is known to participate in the secretion of major cytotoxins were upregulated. Contributing to a better adhesion and colonisation of hosts at warm seawater temperatures, many motility and chemotaxis-related genes were more expressed at 25ºC. A number of chaperones and proteins that act against oxidative stress appeared among the upregulated genes. As well, a DegP protease whose homologue in *V. cholerae* affect biofilm, colonisation and secretion appeared to be upregulated. These data suggest that rather than an upregulation of cytotoxins, the increased likelihood of outbreaks in summer months lies in the upregulation of other virulence factors, motility, chemotaxis and an enhanced growth.

We also found an imbalance between the number of DEGs that were upregulated and downregulated in chromosome II (ChrII), with a marked predominance of downregulated genes. This suggests that *P. damselae* subsp. *damselae* ChrII plays an important role in growth at low temperatures. Genes downregulated at 25ºC (and therefore more expressed at 15ºC) include the alternative sigma factor RpoS, a major regulator of the stress response, and the methionine sulfoxide reductase system MsrPQ that participate in repair of oxidative damage. Betaine glycine is used by some bacteria for adaptation to low temperatures; a betaine glycine transporter was also found among the most downregulated genes. The oligopeptide permease system OppABCDEF, which function may be nutritional and genes that participate in the Krebs cycle and in nitrogen fixation were also found among the top downregulated genes. Presenting the strongest downregulation values, several proteins share homology with others involved in cell wall homeostasis. The proteins revealed here deserve further study and will surely unveil unknown features of *P. damselae* subsp. *damselae* growing at low temperatures.

Secondly, we designed another comparison to address how *P. damselae* subsp. *damselae* responds to cultivation at human body temperature, i.e, 37°C. As a control condition, we chose 25ºC simulating warm waters from a global warming scenario. Growth at 37ºC is one of the key assets that differentiate *P. damselae* subsp.

damselae from the other subspecies *P. damselae* subsp. *piscicida* and thus, allows subsp. *damselae* to colonise and cause disease in humans. Little is known about how *P. damselae* subsp. *damselae* evolved into colonising humans. This bacterium can be the causative agent of wounds and necrotizing fasciitis, and unlike other Vibrios that cause diarrhoea, it does not return to the marine environment after the infection. We found that growth at 37ºC activates an initial proliferation of *P. damselae* subsp. *damselae* with an earlier entry into the exponential phase if we compare it with growth at 25ºC. However, contrary to what happens when this bacterium is grown at 25ºC, exposure to 37ºC leads to a drop in the OD_{600}. We then analysed whether this drop could be an indicative of loss of cell viability and found that after 6 h cultivation, a lower number of cells was recovered from cultures maintained at 37ºC. Viable cells were no longer present after 30 h of cultivation whereas at 25°C, aliquots from the same time yielded the highest counts of viable cells. In order to gain more insight into the process taking place, we analysed cell morphology of exponentially growing cultures maintained at both temperatures. Scanning electron microscopy (SEM) revealed that cells grown at 25ºC exhibited normal rod morphology and size, whereas a temperature of 37ºC made cells grow longer and form chain-like structures that suggest defects in daughter cell separation. 37ºC also impose a burden into the natural resistance of *P. damselae* subsp. *damselae* to antibiotics, since when cultivated at this temperature it showed a higher susceptibility to benzylpenicillin, an antibiotic that inhibits cell wall synthesis. One of the main bacterial strategies to overcome drastic changes in temperature is modulating the composition of membrane fatty acids. By a fatty acid methyl esters (FAME) analysis, we demonstrated that *P. damselae* subsp. *damselae* increases the proportion of saturated fatty acids as temperature increases, which may act in enhancing fitness in different environments.

Using a collection of fish and human isolates of *P. damselae* subsp. *damselae*, we assessed whether there is a link between human body as a source of isolation and an advantage growing at human body temperature. The analysis of growth curves showed a similar pattern in fish and human isolates, so the latter do not present a better adaptation.

In this thesis, we present for the first time the draft genome of two human isolates (CDC-2227-81 and 80077637), which allowed us to conduct a genomic comparison between them and fish isolates. Strains isolated from humans are no more genetically similar to each other than they are to strains isolated from fish. Despite presenting a considerable number of strain-specific genes, there are no genetic markers exclusive for human strains and absent in those isolated from fish. These findings suggests that any genotype living in the marine environment can ultimately cause a human infection and reinforces the idea that *P. damselae* subsp. *damselae* is a non-clonal pathogen.

We created a mini-Tn*10* transposon mutant library in order to obtain defective mutants in growth at 37ºC presenting normal growth at 25ºC. This would allow us to identify molecular targets for mitigating the impact of human infections caused by *P. damselae* subsp. *damselae*. Given we were unable to fulfil this purpose, it is suggested that the ability to grow at human body temperature might rely not on the presence of a single gene but might constitute a multigenic property. Using an RNA-seq approach, we analysed expression changes that this bacterium undergoes when it grows at 37ºC in comparison to 25ºC. Those DEGs with a greater expression at 37ºC were tagged as "upregulated" and those with lower as "downregulated". In general, a temperature of 37ºC upregulates the expression of genes related to heat-shock response, defence against reactive oxygen species and metabolic genes that yield the appropriate supply of biomolecules and energy that sustain the rapid initial growth. One of the most striking findings of this work was to find that many functions related to virulence were downregulated at human body temperature. As far as haemolysins are concerned, damselysin Dly presents a marked downregulation at 37ºC. Nevertheless, similarly to what happens when *P. damselae* subsp. *damselae* grows at 15 and 25ºC, FPKM analysis shows that when growing at 37ºC, haemolysins are among the top 150 highest expressed genes of the whole transcriptome. Together with the results obtained in the growth and viability assays, these observations fit with earlier studies that reported difficulties in isolating *P. damselae* subsp. *damselae* from human infected tissues, but managed to see haemolysis in absence of growth on blood agar plates. According to the previous

transcriptomic study, genes encoding for porins, proteins that form the T2SS, and those that participate in iron uptake, motility and chemotaxis were also downregulated at 37ºC, reinforcing their relevant function at 25ºC.

The findings presented in this section evidence that temperature is an environmental factor that govern physiological and virulence-related functions in *P. damselae* subsp. *damselae*, being 25ºC around the optimum to trigger a virulent profile while 37ºC constituting a stressful condition that makes human infections opportunistic rather than caused by well-adapted strains.

Study of *P. damselae* subsp. *damselae* virulence and fitness: complete regulon of the RstAB system and genetic characterisation of a hitherto unknown polysaccharidic capsule

Considering *P. damselae* subsp. *damselae* high genetic heterogeneity, it is of particular relevance to identify and characterise ubiquitous mechanisms that control its pathogenicity. Continuing with the study of processes implicated in fitness and virulence, the second part of this thesis deals with the study of the recently identified TCS RstAB, formed by the histidine kinase RstB and the response regulator RstA. Bacterial TCSs sense and respond to environmental signals adjusting cellular physiology and gene expression. Homologous genes encoding for the system are widely spread across the family *Vibrionaceae*, but functional studies are limited. Previous research demonstrated that the RstAB system positively regulates the expression of cytotoxins and virulence in *P. damselae* subsp. *damselae*. As well, mutants in either gene of the system showed a reduced secretion of several T2SS-dependent proteins. Despite the current state of knowledge, the RstAB regulon has not been thoroughly revealed up to date.

We used RNA-seq to compare the transcriptomic profile of *P. damselae* subsp. *damselae* strain RM-71 with that of deletion mutants Δ*rstA* and Δ*rstB*. This analysis revealed that the RstAB system can be considered a generally positive regulator of genetic expression; downregulated genes in mutants showed a high degree of differential

expression, while upregulated genes showed a very low degree (closer to the threshold for being classified as differentially expressed). In addition, as expected for a cognate pair, mutants in RstA and RstB overlap with the downregulation of 128 genes. We focused this study in those downregulated genes, which represent the impaired functions that result from the inactivation of the system. In accordance with recently published data, we show that RstAB positively regulates cytotoxins Dly, PhlyP and PhlyC and proteins known to be secreted via the T2SS. As well, proteins that constitute the T2SS were found to be downregulated. Many other currently uncharacterised genes with potential roles in virulence came to light in this transcriptomic study. Such genes were classified according to their putative function in different categories: resistance to antimicrobial agents and survival within hosts, outer membrane proteins, synthesis of extracellular polysaccharides (EPS) and others. Interestingly, this thesis brough to the front a great number of genes positively regulated by the RstAB and a temperature of 25ºC (PhlyP, the T2SS, OmpU, TolC-AcrAB, DegP, A0J47_18110-A0J47_18120), reinforcing the idea that both factors are key for understanding pathogenicity of *P. damselae* subsp. *damselae*.

One of the most striking results to emerge from our transcriptomic data was to find that RstAB mutants show a strong downregulation of 21 genes, distributed in two clusters, predictably involved in the synthesis of EPS and capsular polysaccharides (CPS). Cluster I contains genes *wza*, *wzb*, *wzc* and sugar transferases among others; while cluster II contains genes *yjbEFGH*. *wza* codifies for an outer membrane protein involved in the exportation of CPS that work together with tyrosine kinase *wzc* and its cognate phosphatase *wzb*. Little is known about *yjbEFGH* function but some studies point at its role in production of an extracellular polysaccharide in *E. coli*. Since no research has been conducted on the ability of *P. damselae* subsp. *damselae* to produce a polysaccharidic capsular layer, such strong downregulation prompt us to shed light on that unknown function. The presence of a capsule around the wt strain RM-71 cells was demonstrated by transmission electron microscopy (TEM). In line with transcriptomic data, RstAB mutants showed acapsular phenotypes. In order to demonstrate that the synthesis and exportation of the capsular layer was carried out by the

above-mentioned downregulated genes, we decided to construct deletion mutants by allelic exchange in genes belonging to both clusters: *wza* and *wzc* in cluster I, and *yjbH* in the divergently-transcribed cluster II. Mutation of *wza* and *wzc* abolished completely the synthesis of the capsular layer while mutation of *yjbH* resulted in the production of a significantly thinner capsule. The main macroscopic change observed in acapsular mutants (Δ*wza* and Δ*wzc*) is an increase in translucency with respect to the parental strain. Notably, biofilm formation was much higher in capsule-deficient mutants (Δ*wza* and Δ*wzc*) than in the parental strain and the Δ*yjbH* mutant. This suggests that other molecules underneath the capsular layer participate in the type of adhesion and aggregation necessary for biofilm formation. Growth, motility and haemolytic phenotype was unaltered in mutants *wza*, *wzc* and *yjbH*; however, when these mutants were injected in turbot and sea bream, most Δ*wza* and Δ*wzc*-injected animals survived. We also show that acapsular mutants (Δ*wza* and Δ*wzc*) presented a significant reduction of fitness when growing in media supplemented with either turbot serum or mucus.

The results presented here clearly indicate that the synthesis of a polysaccharidic capsular layer is essential for full virulence in *P. damselae* subsp. *damselae*. The reduction in virulence of acapsular mutants proved to be independent of a normal growth, motility and production of haemolysins. We also showed that CPS are used by *P. damselae* subsp. *damselae* to evade host defences. Likewise, the expression of genes *wza* and *wzc*, was found to be upregulated at 25ºC adding to the idea that this temperature constitutes a strong signal for triggering a virulent profile in *P. damselae* subsp. *damselae*.

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1. INTRODUCTION

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1.1. BACTERIAL PATHOGENS IN AQUACULTURE

Global fish production is reported to have reached 179 million tonnes in 2018 (Fig. 1.1), 46 percent corresponding to aquaculture production valued at USD 250 billion. Aquaculture has a key role in employment and livelihood across the world and remarkably, local economic growth in developing countries. This activity has evolved into a key food source and in accordance, the management of animal health is an urgent concern for the sustainability of the sector (FAO, 2020).

Owing to commercial and production reasons, farmed fish are kept at high densities that unavoidably rise their stress levels and thus, make them more susceptible to disease. Infectious diseases are the result of susceptible organisms exposed to virulent pathogens under certain environmental stress conditions (Snieszko, 1974; Austin and Austin 2016). Aquatic animal disease constitutes one of the most serious constraining factors for expansion and development of sustainable aquaculture (FAO, 2020). Despite progress made in identification, diagnosis and management, numerous studies indicate that some diseases can still put the future of the industry at stake (Stentiford *et al.*, 2017). Extensive use of antibiotic prophylaxis in animal and human health has led to the emergence and the spread of antibiotic-resistant bacteria (Cabello *et al.*, 2013). The aquaculture industry is not immune to these ongoing threats. Antibiotics have been extensively utilised to reduce the impact of disease on fish and shellfish farms, so complementary approaches are also needed. The use of vaccines, prebiotics, probiotics, bacteriophages and the interference of quorum sensing communication among other factors are in constant investigation (Pérez-Sanchez *et al.*, 2018). Therefore, the study of aquaculture pathogens remains essential to guarantee the future of the sector as well as human and animal welfare.

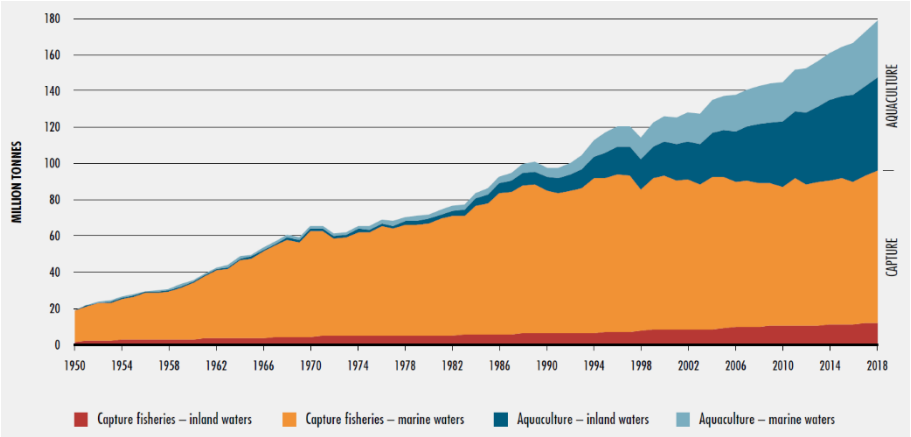

Figure 1.1: Global million tonnes derived from capture fisheries and aquaculture production (1950-2018; Image taken from FAO, 2020).

Concerning with bacterial causative agents of disease in aquaculture, major Gram-positive bacteria are included in the following genera: *Lactococcus, Nocardia, Renibacterium, Staphylococcus, Streptococcus, Vagococcus* and *Weissella.* On the other hand, most representative Gram-negative species are within the genera *Aeromonas, Chryseobacterium, Edwarsiella, Flavobacterium, Francisella, Moritella, Photobacterium, Piscirickettsia, Pseudomonas, Tenacibaculum, Vibrio* and *Yersinia* (Pridgeon and Klesius 2012). Several species of the family *Vibrionaceae* have been related to health problems of aquatic animals, including the species *Photobacterium damselae*. This organism has two subspecies; *P. damselae* subsp. *piscicida*, the causative agent of pasteurellosis or pseudotuberculosis in marine fish (Romalde, 2002) and *P. damselae* subsp. *damselae*, an emerging pathogen for a broad range of marine animals and a causative agent of opportunistic infections in humans (Osorio *et al.*, 2018).

1.2.*PHOTOBACTERIUM DAMSELAE* SUBSP. *DAMSELAE*

1.2.1.Taxonomy

In the 1970s, *P. damselae* subsp. *damselae* was recovered from human illnesses in which the origin of the infection was water-exposed wounds (Morris *et al.*, 1982). As well in that period, this bacterium was

recovered from ulcerative lesions on the skin of the blacksmith damselfish (*Chromis punctipinnis*) being assigned to the genus *Vibrio* (family *Vibrionaceae*) as *Vibrio damsela* (Love *et al.*, 1981). Based on the comparison of the 5S rRNA sequence of several *Vibrionaceae* strains, MacDonell and Colwell (1984) proposed the new genus *Listonella* which would include *Vibrio damsela* and *Vibrio anguillarum* renamed as *Listonella damsela* and *Listonella anguillara*. Phenotypic characteristics, such as the absence of flagellar sheath and the accumulation of poly-β-hydroxybutirate (PHB), led to the reassignment of *Listonella damsela* to the genus *Photobacterium* under the name *Photobacterium damsela* (Smith *et al.*, 1991). In 1995, the 16S rRNA sequence analysis and DNA-DNA hybridisation data confirmed a close relationship between the causative agent of pasteurellosis in fish, formerly named as *Pasteurella piscicida* (family *Pasteurellaceae*), with *Photobacterium damsela*. Therefore, the two above-mentioned bacteria were given the same species epithet *Photobacterium damselae* and named *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*, respectively (Gauthier *et al.*, 1995).

1.2.2.Morphological and biochemical characteristics

P. damselae subsp. *damselae* is a Gram-negative bacterium that belongs to the subgroup gamma-3 of the phylum Proteobacteria. This bacterium is motile by a polar flagellum and has rod but pleomorphic morphology (0.3-0.5 × 1.4-2.6 μm; Fig. 1.2). The biochemical characteristics of this pathogen are: oxidase positive, facultative anaerobic, sensitivity to the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropylpteridine), positive for the methyl red test (Voges-Proskauer), and growth in the selective and differential medium Thiosulfate Citrate Bile Sucrose (TCBS). Furthermore, it is positive for the decarboxylation of arginine and negative for the decarboxylation of lysine and ornithine. It ferments maltose, mannose and D-glucose which goes hand in hand with the production of gas. This organism requires salt for growth at a concentration that ranges from 1 to 5% NaCl but can resist up to 6 or 8% (Fouz *et al.*, 1992; Wu *et al.*, 2006; Abdel-Aziz *et al.*, 2013). *P. damselae* subsp. *damselae* strains grow over a wide temperature range from 15 to 37°C (Fouz *et al.*, 1992) but

some isolates can even grow at 4 and 42°C (Botella *et al.*, 2002; Richards *et al.*, 2008). The analysis of the lipopolysaccharide (LPS) content of different strains reveals a great variability that leads to the existence of at least 4 serogroups (Fouz *et al.*, 1992). The study of outer membrane proteins showed diverse patterns with molecular weights of 20-90 kilodalton (kDa; Fouz *et al.*, 1997).

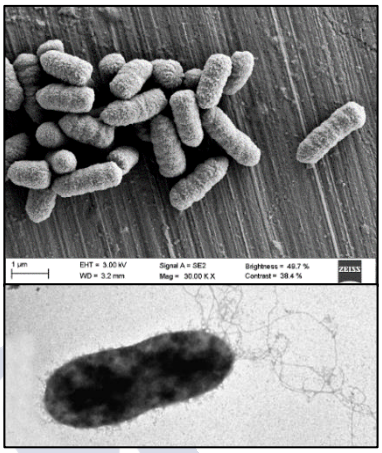

Figure 1.2: Observation of *P. damselae* subsp. *damselae* by electron microscopy: scanning electron microcopy (top panel) and transmission electron microscopy (bottom panel).

P. damselae subsp. *damselae* encodes a number of degradative enzymes: catalase (Fouz *et al.*, 1992), urease (Thyssen *et al.*, 1998; Botella *et al.*, 2002), gelatinase (Vera *et al.*, 1991; Fouz *et al.*, 1993; Pedersen *et al.*, 1997, Zhang *et al.*, 2011, Labella *et al.*, 2010a), amilase (Labella *et al.*, 2010a), lipase (Pedersen *et al.*, 1997; Labella *et al.*, 2010a; Zhang *et al.*, 2011), phospholipase (Vences *et al.*, 2017), acetylcholinesterase (Pérez *et al.*, 1998), caseinase (Khouadja *et al.*, 2014) and haemolysins (Osorio *et al.*, 2000b; Rivas *et al.*, 2011; Rivas *et al.*, 2013; Rivas *et al.*, 2014; Rivas *et al.*, 2015a). The presence of these enzymes varies depending on the strain.

There are numerous phenotypic characteristics that can differentiate between the two subspecies *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* (Table 1.1). Of interest, and positive exclusively for subsp. *damselae*, the list includes motility by flagella, urease activity, nitrate reduction, haemolysis of lamb erythrocytes, and the ability to grow at 37ºC. The latter is of special concern as it allows *P. damselae* subsp. *damselae* to colonise and cause disease in homeotherm organisms, for instance, humans.

Table 1.1. Main differential phenotypic traits between *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*.

Character	*P. damselae* subsp. *damselae*	*P. damselae* subsp. *piscicida*
Flagellar motility	+	-
Bipolar stain	-	+
Growth in TCBS	+ (green colonies)	-
Nitrate reductase	+	-
Lipase	+	-
Amilase	+	-
Growth at 5% NaCl	+	-
Maltose acid production	+	-
Growth at 37ºC	+	-
Haemolysis against lamb erithrocytes	+	-
Haemolysis against trout erithrocytes	+	-
Host	fish molluscs crustaceans mammals reptiles humans	fish
Serological diversity	+	-

1.2.3.Pathogenicity

Pathogenicity refers to the capability of a pathogen to cause damage to a host. Early on, the focus on disease causality was mainly on the microbe and its virulence factors; however, this could not explain the different outcomes of infection (health or disease) of different individuals infected with the same strain. Thus, pathogenesis must be understood within the damage-response framework that analyses the outcome of unique host-microbe interactions, basing on host damage and taking into account genetic and physiological features of both the pathogen and the host (Casadevall and Pirofski, 1999; Casadevall and Pirofski, 2003). Recently, the attributes that define host susceptibility were reviewed and proposed to be of key importance in host-microbe

interaction: microbiome, inoculum, sex, temperature, environment, age, chance, history, immunity, nutrition, and genetics (Casadevall and Pirofski, 2018).

P. damselae subsp. *damselae* grows in aquatic ecosystems such as estuaries, seawater, sediments, and has been isolated from seafood, plankton and apparently healthy animals (Ghinsberg *et al.*, 1995; Montanari *et al.*, 1999; Croci *et al.*, 2006; Acuña *et al.*, 2011; Sugita *et al.*, 2012; Klein *et al.*, 2014; Nigro and Steward, 2015). In fact, it is part of the normal microbiota characteristic of carcharhinid sharks (Grimes *et al.*, 1985). On the other hand, this organism is best known for being a primary pathogen of numerous animals encountered in the sea environment, which includes fish, molluscs, crustaceans, reptiles, sharks and cetaceans. It can also cause opportunistic infections that put human life at risk (Osorio *et al.*, 2018). Its long-time maintenance and survival in sea water and sediments maintaining infectivity potential has been demonstrated (Fouz *et al.*, 1998; Fouz *et al.*, 2000). Thus, data suggest that *P. damselae* subsp. *damselae* constitutes a generalist pathogen, capable of living as a free-swimming bacterium or a pathogen for a broad range of animal phyla.

1.2.3.1.P. damselae *subsp.* damselae *as a pathogen for poikilotherm animals*

P. damselae subsp. *damselae* has been isolated from apparently healthy both wild and cultured fish (Sugita *et al.*, 2000; Botella *et al.*, 2002; Pujalte *et al.*, 2003; Cantas *et al.*, 2012; Bjornsdottir-Butler *et al.*, 2015; Alikunhi *et al.*, 2017; Trevisani *et al.*, 2017), crustaceans (Yalcinkaya *et al.*, 2003; Krishnika and Ramasamy, 2013; Givens *et al.*, 2013) and bivalves (Lozano-León *et al.*, 2003; Richards *et al.*, 2008). However, this bacterium is best known for being a primary pathogen for marine animals. First *P. damselae* subsp. *damselae* isolation from a pathogenic process in fish took place in the coast of the United States when it caused skin ulcers and lesions over the skin of damselfish (Love *et al.*, 1981). In the wildlife, such is not the only report of diseased fish presenting this microorganism isolated as the causative agent. For instance, Eissa *et al.*, 2018 reported the infection from wild sea bass (*Dicentrarchus labrax*) and sea bream

(*Sparus aurata*), and McMurtrie *et al.*, 2019 reported it from the ballan wrasse (*Labrus bergylta*). This demonstrates captivity conditions are not an essential requirement for the development of disease produced by *P. damselae* subsp. *damselae*. Nonetheless, its constant isolation from fish species of economic value in farming portrays this bacterium as a serious threat for the aquaculture sector worldwide. Reared species whose populations have been threatened by this bacterium include Japanese amberjack (*Seriola quinqueradiata*; Sakata *et al.*, 1989), turbot (*Psetta maxima*) in Galicia (Spain; Fouz *et al.*, 1991; Fouz *et al.*, 1992), gilt-head sea bream in Spanish Mediterranean coast, Egypt and Tunisia (*Sparus aurata*; Vera *et al.*, 1991; Pujalte *et al.*, 2003; Labella *et al.*, 2010a; Abdel-Aziz *et al.*, 2013; Khouadja *et al.*, 2014; Essam *et al.*, 2016), eel (*Anguila reinhardtii*) in Australia (Ketterer and Eaves, 1992), rainbow trout (*Oncorhynchus mykiss*) in Denmark and Turkey (Pedersen *et al.*, 1997; Pedersen *et al.*, 2009; Capkin *et al.*, 2017), common dentex (*Dentex dentex*) in Mallorca (Spain; Company *et al.*, 1999), amberjack (*Seriola dumerili*) in Murcia (Spain; Alcaide, 2003), snapper (*Pagrus auratus*) in Australia (Stephens *et al.*, 2006), redbanded seabream (*Pagrus auriga*) and white seabream (*Diplodus sargus*) in Southern Spain (García-Rosado *et al.*, 2007; Labella *et al.*, 2006; Labella *et al.*, 2010a), Asian sea bass (*Lates calcarifer*) in Thailand and Malaysia (Kanchanopas-Barnette *et al.*, 2009; Mohamad *et al.*, 2019), ovate pompano (*Trachinotus ovatus*) in Guangdong (China; Zhao *et al.*, 2009), European seabass (*Dicentrarchus labrax*) in Southern Spain, Egypt, Tunisia, Turkey and Greece (Labella *et al.*, 2010a; Abdel-Aziz *et al.*, 2013; Khouadja *et al.*, 2014; Uzun and Ogut, 2015; Bellos *et al.*, 2015; Essam *et al.*, 2016), meagre (*Argyrosomus regius*) in Southern Spain (Labella *et al.*, 2010a), southern bluefin tuna (*Thunnus macoyii*) in Australia (Valdenegro-Vega *et al.*, 2013), olive flounder (*Paralichthys olivaceus*) in Korea (Kim *et al.*, 2014), cobia (*Rachycentron canadum*) in Karwar (India; Sharma *et al.*, 2017), silver pomfret (*Pampus argenteus*) in Eastern China (Tao *et al.*, 2018), red snapper (*Lutjanus spp*.) in Malaysia, hybrid grouper (*Epinephelus spp*.) in Malaysia (Mohamad *et al.*, 2019), and the half-smooth tongue sole (*Cynoglossus semilaevis*) in the Bohai Bay (China; Shao *et al.*, 2018) and rockfish (*Sebastes schlegeli*) in Daqing Island, China (Zhang *et al.*,

2019). It is considered the main pathogenic bacterium isolated from mortalities in fish of the sparids group in Southern Spain farms (Labella *et al.*, 2011). As well, it constitutes an emerging pathogen repeatedly isolated from outbreaks in recently introduced fish species in Asian aquaculture (Tao *et al.*, 2018; Zhang *et al.*, 2019; Shao *et al.*, 2018). Geographical expansion of this pathogen in the Mediterranean basin is confirmed by the outbreaks in gilthead seabream and European seabass in Egypt (Essam *et al.*, 2016) and Tunissia (Khouadja *et al.*, 2014). Its influence has reached the Black Sea, having been isolated from mortalities in sea bass in Turkey (Terceti *et al.*, 2016).

This bacterium is not only circumscribed to pathogenicity in fish aquaculture. With respect to cultivated invertebrate organisms, *P. damselae* subsp. *damselae* was first reported as a pathogen for shrimp in Taiwan in 1993, causing high mortalities of cultured tiger shrimp (*Penaeus monodon*; Song *et al.*, 1993). For the same species, *P. damselae* subsp. *damselae* would be later isolated from wild diseased individuals (Vaseeharan *et al.*, 2007). Recent reports, confirm its character as a present-day danger for the culture of the cleaner shrimp (*Lysmata amboinensis*) in South Korea (Choi *et al.*, 2018) and the Pacific white shrimp (*Litopenaeus vannamei*) in México (Aguilera-Rivera *et al.*, 2019), the United States (Bachand *et al.*, 2020) and China (Wang *et al.*, 2020). Moreover, the role as a pathogen for bivalves was reported in Australia when *P. damselae* subsp. *damselae* caused mortalities in giant clam larvae (*Tridacna gigas*; Sutton and Garrick, 1993).

As a generalist pathogen *P. damselae* subsp. *damselae* has also compromised the health of other poikilotherm animals of diverse phyla. Such cases are isolations from sharks (*Carcharhinus plumbeus* and *Squalus acanthias*; Grimes *et al.*, 1984a; Grimes *et al.*, 1984b), octopus (*Octopus joubini* and *O. briareus*; Hanlon *et al.*, 1984), leatherback turtle (*Dermochelys coriacea*; Obendorf *et al.*, 1987; Oliver-Guimerá *et al.*, 2019), green turtle (*Chelonia mydas*; Aguirre *et al.*, 1994) and loggerhead sea turtle (*Caretta caretta*; Alba *et al.*, 2016).

The portal of entry of *P. damselae* subsp. *damselae* seems to be the skin given the ability of the pathogen to adhere to mucus. Previous studies have also provided strong evidence that seawater transmits the

disease and the expansion of this bacterium is highly determined by water temperature (Fouz *et al.*, 2000). Even though symptoms in diseased fish are variable among species and individuals, the most common external symptoms are: haemorrhages around the mouth, anus, eyes, base of the fins, abdominal distension and cutaneous ulcers. Internal signs include a large quantity of peritoneal fluid or, from time to time, pale liver with abundant haemorrhagic petechiae (Fouz *et al.*, 1991; Fouz *et al.*, 1992; Fig. 1.3). In shrimp, symptoms include anorexia, poor growth rate, rough shell and milky musculature (Song *et al.*, 1993).

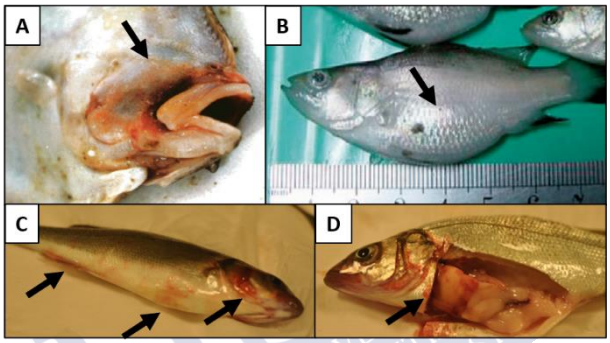

Figure 1.3: Symptoms in diseased fish infected with *P. damselae* subsp. *damselae*. Black arrows point at different manifestations. (A) Turbot showing haemorrhages around the mouth (Image taken with permission from Fouz *et al.*, 1992). (B) Asian sea bass showing abdominal distension (Image taken from with permission from Kanchanopas-Barnette *et al.*, 2009). (C) European sea bass showing haemorrhagic areas around the anus, mouth and in the base of fins. (D) European sea bass showing pale liver with haemorrhagic petechiae.

1.2.3.2.P. damselae *subsp.* damselae *as a pathogen for homeotherms*

The ability of some *P. damselae* subsp. *damselae* strains to grow at 37ºC allows it to colonise and establish an infection in homeotherm animals. In marine mammals, this bacterium was reported as a dolphin pathogen in 1988 for the first time, recovered from the injury of a bottlenose dolphin (*Tursiops truncatus;* Fujioka *et al.*, 1988).

This was not an unusual case, since more infections were documented in healthy and diseased dolphins (Buck *et al.*, 1991, 2006), and other cetaceans like the Bryde's whale (*Balaenoptera brydei*; Buck *et al.*, 1991) and the Risso's dolphin (*Grampus griseus*; Elad *et al.*, 2011). While the earliest incidents in cetaceans were concentrated on the coast of the United States (Fujioka *et al.*, 1988; O'Shea *et al.*, 1991; Buck *et al.*, 1991, 2006), over the last decade a number of cases have occurred in Europe (Keck *et al.*, 2010; Casalone *et al.*, 2014; Alba *et al.*, 2016; Godoy-Vitorino *et al.*, 2017).

Human diseases caused by *P. damselae* subsp. *damselae* have their origin mainly in wounds infected through exposition to brackish or salt water (Morris *et al.*, 1982; Dryden *et al.*, 1989; Nakamura *et al.*, 2008; Aigbivhalu and Maraqa, 2009; Hundenborn *et al.*, 2013; Chochlakis *et al.*, 2019) and wounds inflicted during fish handling (Clarridge and Zighelboim-Daum, 1985; Perez-Tirse *et al.*, 1993; Yuen *et al.*, 1993; Fraser *et al.*, 1997; Tang and Wong, 1999; Barber and Swygert, 2000; Yamane *et al.*, 2004; Akram *et al.*, 2015; Collins *et al.*, 2017). In fact, this bacterium is acknowledged as an important pathogen with zoonotic potential in infections acquired from fish (Lehane and Rawlin, 2000; Austin, 2010). On the other hand, there are other unusual cases of infection either through the urinary tract (Morris *et al.*, 1982; Álvarez *et al.*, 2006) or by ingestion of raw seafood (Kim *et al.*, 2009). This bacterium may cause an extremely aggressive necrotising fasciitis that can only be solved through debridement or amputation (Coffey *et al.* 1986; Barber and Swygert, 2000; Nakamura *et al.*, 2008; Collins *et al.*, 2017) when it does not lead to an unavoidable fatal outcome (Yuen *et al.*, 1993; Tang and Wong, 1999; Goodell *et al.*, 2004; Yamane *et al.*, 2004). Prompt antibiotic administration has been reported unsuccessful to ameliorate the disease in some cases (Yuen *et al.*, 1993; Tang and Wong, 1999; Yamane *et al.*, 2004; Goodell *et al.*, 2004; Alhemairi *et al.*, 2015). Common manifestations in human infections are shown in Fig. 1.4. Other common manifestations of *P. damselae* subsp. *damselae* infections are open wounds, edema, eritema, cellulitis, haemorrhagic bullous lesions or septicemia (Dryden *et al.* 1989; Perez-Tirse *et al.*, 1993; Hundenborn *et al.*, 2013; Chochlakis *et al.*, 2019; Sahu *et al.*, 2020). People in higher peril are those with prehistory of disease or

immunocompromise (Clarridge and Zighelboim-Daum, 1985; Shin *et al.*, 1996; Fraser *et al.*, 1997; Yamane *et al.*, 2004; Knight-Madden *et al.*, 2005; Nakamura *et al.*, 2008; Kim *et al.*, 2009; Collins *et al.*, 2017) but it is not a requisite since healthy individuals have been reported to undergo complications upon infection with *P. damselae* subsp. *damselae* that were fatal in a number of cases (Perez-Tirse *et al.*, 1993; Yuen *et al.*, 1993; Tang and Wong, 1999; Barber and Swygert, 2000; Yamane *et al.*, 2004; Chochlakis *et al.*, 2019). Most cases were found in the coastal areas of the United States, Japan and Australia, but there is a recent concern about the emergence of first European infections in Greece (Chochlakis *et al.*, 2019), Portugal (Guimaraes *et al.*, 2020) and Spain (Schröttner *et al.*, 2020). Chochlakis *et al.*, 2019 constitutes as well the third study that reported a coisolation of *P. damselae* subsp. *damselae* with *Vibrio harveyi* from a human infection (Hundenborn *et al.*, 2013; Akram *et al.*, 2015). Interestingly, this coisolation of the two species was also observed in aquaculture facilities that rear sea bream (*Sparus aurata*) and snapper (*Pagrus auratus*; Pujalte *et al.*, 2003; Stephens *et al.*, 2006), and cobia (*Rachycentron canadum*; Sulumane Ramachandra *et al.*, 2021).

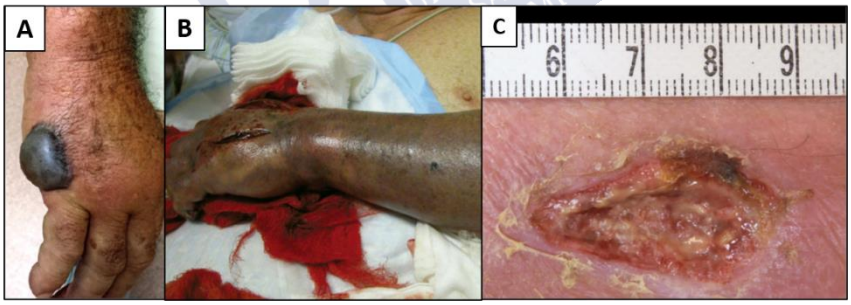

Figure 1.4: Common clinical manifestations in *P. damselae* subsp. *damselae* human infections. (A) Haemorrhagic bullous lesion (image taken with permission from Akram et al., 2015); (B) Necrotising fasciitis located in the arm and the hand (image taken with permission from Goodell *et al.*, 2004); (C) Infected wound after treatment with antibiotics and debridement (Image taken with permission from Hundenborn *et al.*, 2013).

1.3. THE ROLE OF WATER TEMPERATURE IN *VIBRIONACEAE*-RELATED DISEASES

1.3.1. Global warming as a drive for the expansion of *Vibrionaceae* populations and associated diseases

Global warming, one of the major consequences of climate change, has produced a gradual increase in average air and ocean temperatures particularly accentuated in recent years. Roemmich *et al.*, 2012 estimated that 90% of the surplus heat gathered on the Earth surface over the last decades, has been accumulated in oceans. Prokaryotes represent the richest living biomass found in the ocean (Whitman *et al.*, 1998) and owing to their implication in animal and human health, it is of significant importance to understand how they adapt to changing environments. Vibrios thrive in marine and estuarine habitats with ideal temperatures over 15ºC and moderate salinity below 25‰ (Froelich and Daines, 2020). In fact, the increase in water temperature is the environmental factor that most accounts for a greater *Vibrio* abundance (Takemura *et al.*, 2014; Bellos *et al.*, 2015). *Vibrionaceae*-related human infections occur mainly through aquatic exposure or ingestion of contaminated water and seafood, but some can be acquired from wounds inflicted through fish handling (Austin, 2010; Baker-Austin *et al.*, 2018). The above-mentioned increase in sea surface temperatures, may provide a favourable scenario for *Vibrio* and potential host (either human or animal) interactions, hypothesis that gained support as new related publications appeared.

European regional seas temperature increased over the last years (Reid *et al.*, 2011), and consistently the prevalence of human infections caused by *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* in European countries has augmented considerably (Baker-Austin *et al.*, 2013). Current expansion of Vibrios go dangerously beyond typical regions affected by associated diseases, reaching non-endemic areas like temperate and cold regions of northern latitudes (Vezzulli *et al.*, 2013). This is the case of the summer of 2014, when a large number of *Vibrio* infections were reported in Sweden and Finland (Baker-Austin *et al.*, 2017). Also, during the heatwave of the summer of 2018, the abundance of major *Vibrio* pathogens in a UK estuarine was

significantly greater (Ford *et al.*, 2020). Vezzulli *et al.*, 2012, 2016 carried out long-term studies showing an increased presence of plankton-associated Vibrios in the North Atlantic and North Sea during warm periods. In the same line, a very recent study published by Davis *et al.*, 2021 showed an overall positive association between water temperature and *V. parahaemolyticus* abundance in selected Washington coastal bays and consequently, a greater number of infections. They also showed that warmer air induces growth of this organism by raising temperatures in shellfish tissues. *Vibrio*-related infections are likely to rise in the near future due to global warming, constant population growth (especially in developing countries), long-term wars and reduced availability of safe and well-sanitised water, among others (Baker-Austin *et al.*, 2018). For that reason, new measures should be taken in order to protect those on the line such as the very young, the elderly, fishermen, aquaculture workers, and people living in seaside areas impacted by inundations or severe storms (Froelich and Daines, 2020).

Regarding animal infections, similar patterns were also evidenced. Current rising temperatures pose an undeniable and global threat to the viability of aquaculture, since they are correlated with an increase in the number of disease outbreaks caused by Vibrios. An unusually warm period in 2004 was the scenario of a high-mortality episode caused by *Vibrio ponticus* in cultured Japanese sea bass (*Lateolabrax japonicus*) in China (Xie *et al.*, 2007). Likewise, Korun *et al.*, 2013 described a *Vibrio alginolyticus* isolation from diseased European sea bass (*Dicentrarchus labrax*) farmed in Turkey when sea water temperature reached 27ºC. Mass mortalities caused by several *Vibrio* species are being constantly reported in the salmonid industry and they were the cause of a recent devastation of oyster beds in France (Le-Roux *et al.*, 2015). Large coral mortalities produced by *V. harveyi, V. splendidus and V. coralliilyticus* were concurrent with prolonged high sea surface temperatures in the Mediterranean Sea (Vezzulli *et al.*, 2010). Actually, there is evidence that an anomalously warm environment damages corals by reducing their resistance to infection by *V. harveyi* (Luna *et al.*, 2010; Krediet *et al.*, 2013). As for *V. parahaemolyticus* some studies showed that high bacterial densities within seafood were

characteristic in summer months in Spain (Martinez-Urtaza *et al.*, 2008), Chile (Fuenzalida *et al.*, 2007), and the United States (DePaola *et al.*, 2000). This spread of diseases along marine organisms may also increase the chances of human infection (Newton *et al.*, 2012).

Future research needs to take many factors into account. Not only is the expansion of these bacterial populations a problem, but the increase of *Vibrionaceae* infections may allow contacts with the human microbiome that can result in the transfer of mobile genetic elements that provide a source for antimicrobial resistance (Froelich and Daines, 2020). On the other hand, information is needed on how temperature affects physiology and virulence at a molecular level in marine life-threatening human and animal pathogens. These data will aid to forecast outbreaks and design new prevention and control strategies.

1.3.2. Seasonal aquaculture outbreaks produced by *P. damselae* subsp. *damselae*

Fouz *et al.*, 2000 demonstrated that the disease caused by *P. damselae* subsp. *damselae* is transmitted through water, being its salinity and temperature (22-25ºC) of key importance. First studies on *P. damselae* subsp. *damselae* isolation from aquaculture outbreaks mentioned that high sea water temperatures accompany infections. Vera *et al.*, 1991 reported the isolation of this microorganism from sea bream (*Sparus aurata*), when a water temperature of 26ºC occasioned stressful conditions in the Mediterranean coast of Spain. Fouz *et al.*, 1992, described turbot (*Psetta maxima*) outbreaks produced by this pathogen during the summers of 1987 and 1989, characterised by the abrupt increase of sea water temperature (from 18 to 22-24ºC) in the Galician coast. Pedersen *et al.*, 1997 reported that during the summer of 1994 and 1995, water temperatures were around 5°C above average in Denmark, coinciding with the isolation of *P. damselae* subsp. *damselae* in farms rearing rainbow trout (*Oncorhynchus mykiss*).

A number of researchers have highlighted the association between warm temperatures, typical from summer months, and the emergence of aquaculture outbreaks caused by this bacterium in Southern Spain. Pujalte *et al*., 2003, described the recurrent isolation of this bacterium from *Sparus aurata*. García-Rosado *et al.*, 2007 reported an outbreak in

white seabream (*Diplodus sargus*) in which mortality achieved 94% in August 2005. Labella *et al.*, 2011 showed a correlation between dramatic mortality peaks in summer months that affected different sparid fish species. Data collection from 2003-2006 outbreaks in cultivated sparid fish, showed mortalities over 80% between May and August in comparison with those of winter months around 20%.

There are many other similar examples of reported *P. damselae* subsp. *damselae* outbreaks associated to high temperatures in gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) farms in other locations: mortalities registered in a Tunisian farm when water temperature increased from 20 to 25ºC (Khouadja *et al.*, 2014), mortalities in Egypt during summer months (Essam *et al.*, 2016; Mahmoud *et al.*, 2017), outbreaks in Greece in May-August (temperature around 19-24ºC; Bellos *et al.*, 2015), mortalities in the Black Sea (Turkey) when the temperature increased from 22.9ºC to 25.8ºC (Terceti *et al.*, 2016), etc.

Recent evidence suggests that this dangerous pattern is observed in Asia affecting not only fish but also shrimp farms. *P. damselae* subsp. *damselae* was identified causing a disease outbreak for the first time in farmed cobia (*Rachycentron canadum*) in India in May 2013, when the temperature was significantly higher than in previous months (Sharma *et al.*, 2017). In June 2015, temperature of water was between 23 and 26ºC when a disease outbreak occurred in the Silver pomfret (*Pampus argenteus*) reared in Eastern China (Tao *et al.*, 2018). Also, in China, disease of net-cage cultured black rockfish (*Sebastes schlegeli*) was detected twice when seawater temperature was unusually high in July 2016 and August 2017 (Zhang *et al.*, 2019). *Litopenaeus vannamei* industry in this country has been recently threatened by *P. damselae* subsp. *damselae* as massive mortalities occurred in a local farm in Hainan Province (Wang *et al.*, 2020).

While there have been numerous studies that show how outbreaks produced by this pathogen in aquaculture farms have a strong seasonal distribution within the year, increasing their incidence during summer months or periods of unusual high temperatures, little is known about the response mechanisms of *P. damselae* subsp. *damselae* to rising temperatures.

1.4. CHARACTERISED VIRULENCE FACTORS AND THEIR REGULATION IN *PHOTOBACTERIUM DAMSELAE* SUBSP. *DAMSELAE*

The degree or measure of pathogenicity is called virulence, a quantitative character that defines the relative ability of a pathogen to cause damage. Pathogenicity and virulence are meaningful concepts in the context of host-pathogen interactions and are not inherent qualities of pathogens (Casadevall and Pirofski *et al.*, 1999, 2001).

Virulence factors are genetic, biochemical or structural features related to the ability of the pathogen to cause damage. Virulence factors can be classified as requisite, when they confer the ability to cause disease or they can be contributory if they modify the magnitude of the disease (Casadevall and Pirofski *et al.*, 2001). Attending to their function, virulence factors can promote: colonisation and invasion, direct damage by toxicity, indirect damage by inducing an inflammatory response or evasion and modulation of the immune system of the host (Salyers *et al.*, 1994; Johnson, 2018). Gram-negative bacteria virulence factors include: adhesins, extracellular polysaccharides (capsule), secretion systems, iron acquisition systems, enzymes, exotoxins, endotoxins, LPS, flagella, pili and virulence plasmids among others.

Even though there are still many aspects of *P. damselae* subsp. *damselae* virulence that remain unknown, research has provided information about those factors produced by this pathogen that mediate harm to such a great variety of hosts. Up to date characterised virulence factors and their regulation are described below and depicted in Fig. 1.5.

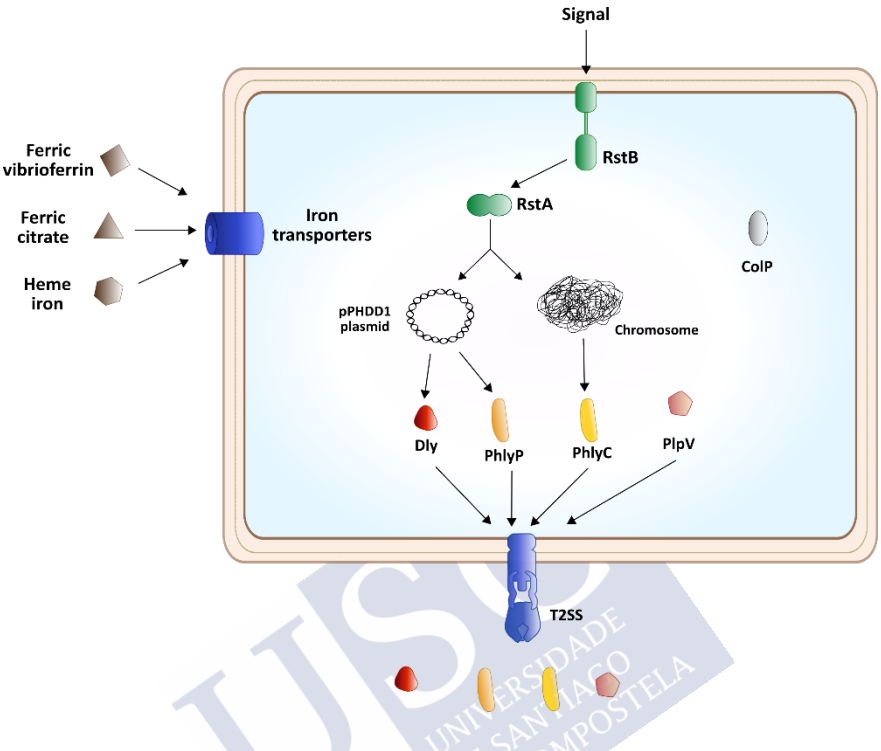

Figure 1.5: Virulence factors of *P. damselae* subsp. *damselae* reported to date. Depicted are mechanisms to obtain iron, the four main cytotoxins (Dly, PhlyP, PhlyC and PlpV) that are secreted via the T2SS, the collagenase ColP and the two-component regulatory system RstAB which controls the expression of genes located in the chromosome and the pPHDD1 plasmid.

1.4.1. Iron acquisition systems

Iron is an indispensable micronutrient for metabolic needs of most bacteria; however, its availability within the host is very low. Iron can be found in its reduced form as ferrous ion (Fe^{2+}) or in its oxidised form as ferric ion (Fe^{3+}). Free ferrous iron can be toxic by producing hydroxyl radicals that damage lipids, proteins and nucleic acids, through the Fenton Reaction ($Fe^{+2} + H_2O_2 = Fe^{+3} + OH^- + OH\cdot$). In neutral-pH and aerobic conditions ferrous iron is rapidly oxidised to ferric iron that forms insoluble ferric hydroxide precipitates. Within the host, iron is mostly found extracellularly bound to transferrin or

intracellularly bound to ferritin, haemosiderin or haeme-containing proteins such as haemoglobin or myoglobin (Andrews *et al.*, 2003; Johnson and Beck., 2018).

Low iron concentration resulting from the generation of ferric deposits and host iron sequestering strategies, made bacteria evolve iron acquisition systems in order to guarantee survival. By and large, the processes by which bacteria sequester iron from hosts comprise the subtraction of haeme-iron from haemoproteins (with the aid of secreted proteins or receptors located on the cell surface), the acquisition of transferrin and iron attached to lactoferrin (with the help of siderophores or specific receptors), and lastly, the uptake of free inorganic iron (by the production of ferric iron reductases and associated ferrous iron permeases; Sheldon *et al.*, 2016).

Fouz *et al.*, 1994 showed that *P. damselae* subsp. *damselae* has the ability to utilise haeme, haemoglobin and ferric ammonium citrate as only iron sources. Furthermore, they demonstrated the key role of iron in *P. damselae* subsp. *damselae* virulence, showing an increase in susceptibility to infection in fish and mice previously injected with an iron source. The sequence of 10 genes encoding a haeme utilization system was described by Río *et al.*, 2005, and cloning the sequence of the whole system into *Escherichia coli* demonstrated its role in the utilisation of haeme iron from haemin and haemoglobin. These genes encode for the proteins HutZ, HutX and HutW, the TonB system(TonB, ExbB and ExbD) and the membrane receptor HutA. Fouz *et al.*, 1997 demonstrated that *P. damselae* subsp. *damselae* takes the haeme group directly by interaction cell-haemoglobin, ruling out the possibility of proteolytic degradation of haemoglobin. In addition, this pathogen produces haemolysins when iron concentration is high, a trait that provides a source of haemoglobin. This is correlated with the high expression of the haeme receptor in low and high iron concentrations (Fouz *et al.*, 1994). The haeme group receptor gene *hutA* has been found to be present in fish and human isolates, with 97% nucleotide sequence identity between the haeme group uptake operons in both subspecies *damselae* and *piscicida* (Rio *et al.*, 2005). Up to date, the biological role of haeme uptake genes in the subsp. *damselae* has not been unravelled, however, these genes are essential for haeme utilisation in the subsp.

piscicida, and two haemin ABC transporter genes were proved to be expressed in tissues from experimentally infected fish (Osorio *et al.*, 2010). Early studies also showed that this pathogen produced a hydroxamate-type siderophore (Fouz *et al.*, 1997). Later, it was found that some *P. damselae* subsp. *damselae* isolates synthesise vibrioferrin as a siderophore under iron deprivation. Those genes involved in vibrioferrin synthesis and transportation may be located within a genomic island incorporated by horizontal transfer (Puentes *et al.*, 2017; Balado *et al.*, 2017; Osorio, 2019). The majority of isolates secrete autogenous citrate in iron limiting conditions, which indicates that citrate is not only a molecule that takes part in the structure of some siderophores, but it can also be utilised for the uptake of iron directly by itself (Balado *et al.*, 2017).

1.4.2. Cytotoxins with haemolytic activity and type II secretion systems (T2SS)

Previous studies (Kreger, 1984) reported the correlation between the capacity of various *P. damselae* subsp. *damselae* strains to cause disease in mice and produce a great amount of a heat-labile toxin in vitro. The haemolytic activity of the toxin was tested against erythrocytes from 16 animal species, showing a great activity against those of rat and mouse. A year later, Kothary and Kreger, 1985 purified this toxin and determined its molecular weight of 69 kDa. Later on, this major toxin was dubbed damselysin (Dly) and described as a haemolytic toxin with phospholipase-D activity against sphingomyelin, cleaving the polar sphingomyelin choline heads (Kreger *et al.*, 1987). The aforementioned study also evidenced for the first time that Dly acts synergistically with other haemolysins, as it increased the haemolytic activity of the delta-toxin of *Staphylococcus aureus* against sheep erythrocytes (Kreger *et al.*, 1987). Soon after, the *dly* gene was cloned and sequenced, and its presence was correlated with strains with high haemolytic activity. These authors demonstrated that some strains could lose the gene hence diminishing the haemolytic activity, so they hypothesised that this gene might be encoded within a prophage (Cutter and Kreger, 1990). Fouz *et al.*, 1993 showed the implication of extracellular products (ECP) of this organism in fish disease and again,

corroborated the different degree of haemolytic activity produced in different strains. Osorio *et al.*, 2000a showed that presence of *dly* is neither a requisite for haemolytic activity (negative strains for the *dly* gene showed haemolytic phenotypes) nor pathogenicity for mice and fish (*dly* negative strains caused disease). Later on, negative strains for *dly* gene would prove toxic to homeotherm and poikilotherm cell lines (Labella *et al.*, 2010a). Later on, Labella *et al.*, 2010a proved that strains that were negative for the *dly* gene showed toxicity against cell lines from homeotherms and poikilotherms.

Genetic context and location of the *dly* gene was unknown until Rivas *et al.*, 2011 characterised a 150 kilobase (kb) plasmid named pPHDD1, carried by both fish and human isolates. Sequence analysis of the plasmid showed 171 open reading frames (ORFs) with 5 gene modules: partition module (*par* genes), conjugation module (*tra* genes), replication module (*rep* genes), adhesion module (*tad* genes), and a haemolysis module. The latter consists of the *dly* gene, and the *hlyApl* gene encoding a pore-forming haemolysin, and the two genes are transcribed contiguous but from divergent strands. The experiments conducted with single and double *dly* and *hlyApl* mutants demonstrated that Dly is not the only responsible for the complete haemolytic phenotype, since the single *dly* mutant did not impair haemolysis completely. Furthermore, the mutation of *hlyApl* caused a smaller reduction of the haemolytic activity and the double mutant still produced small haloes. These findings suggested that Dly was the major driver of haemolysis but given the small haloes (mimicking those from plasmidless strains) produced by the double mutant, additional haemolysins non-pPHDD1 encoded still needed to be discovered (Rivas *et al.*, 2011). The toxin HlyApl was renamed as Phobalysin P (PhlyP) from "Photobacterial lysin encoded on a plasmid" (Rivas *et al* 2015a). Those strains lacking pPHDD1 still produced haemolytic activity and this feature led to the discovery of chromosome I (ChrI)-located *hlyAch* gene that encodes for another pore-forming toxin (Rivas *et al.*, 2013) renamed afterwards as Phobalysin C (PhlyC), which has a 92% amino acid identity with PhlyP (Rivas *et al.*, 2014). Thus, strains harbouring pPHDD1 plasmid encode three haemolysins, Dly, PhlyP and PhlyC, but plasmidless strains only PhlyC.

Rivas *et al.*, 2013 carried out experiments with different mutant combinations in haemolysin genes with the aim of shedding light on their specific contribution to haemolysis and virulence for mice and fish. They concluded that albeit Dly is unable to produce strong haemolysis in sheep blood when alone, it produces synergistic effects with both Phobalysins, so at least one of the latter is needed to produce detectable haemolysis. To reach maximum haemolytic values, synergistic CAMP effect of Dly with Phobalysins is necessary. Unlike in sheep blood, Dly alone can cause haemolysis of mouse and turbot blood. PhlyP or PhlyC alone can cause haemolysis of sheep, mice and turbot. In addition, the combined action of PhlyP and PhlyC was demonstrated to be additive. The evaluation of the pathogenic potential revealed that virulence for mice of pPHDD1 harbouring strains belongs mainly to Dly and PhlyP as deletion mutants in PhlyC were not less virulent. As for virulence for fish, they found that double mutants producing only one of the Phobalysins did not produce mortalities in turbot, so the actions of either Dly or the other Phobalysin proved essential to cause death.

In conclusion, up to that point it was known that the large haemolytic halo of pPHDD1-harbouring strains was due to the production of the three haemolysins combined with the synergistic effect between Dly and Phobalysins (PhlyP and PhlyC). On the other hand, strains lacking pPHDD1 produce small haemolytic haloes because of the secretion of PhlyC. The intermediate haemolytic haloes observed in some pPHDD1 harbouring strains isolated from fish are probably due to the effect of point mutations impairing the activity of one or more haemolysins (Rivas *et al.*, 2011; Rivas *et al.*, 2013; Rivas *et al.*, 2014).

PhlyP has the ability to form oligomers, probably pentamers, that bind to the erythrocyte membrane and create pore complexes. This mechanism has been proved to be cholesterol-dependent. Molecular mechanisms by which this pore-forming toxin PhlyP causes cell damage are loss of intracellular adenosine triphosphate (ATP) and K^+ ions, which attenuates translation (by inactivating mTORC1 complex and augmenting phosphorylation of translation initiation factor EIF2α) that produce cell death. As well, PhlyP causes morphological changes

related to actin and cytokeratin filaments that impair cell integrity (Rivas et al., 2015b).

A percentage of isolates from aquaculture outbreaks lack plasmid pPHDD1 and thus, damselysin Dly and phobalysin PhlyP (Rivas *et al.*, 2014, Terceti *et al.*, 2016). Interestingly, a recent study carried out by Vences *et al.*, 2017 confirmed the major role of *hlyAch* (encoding PhlyC) in cell toxicity and virulence for fish of strains lacking the plasmid pPHDD1. In addition, this research led to the identification of the phospholipase PlpV, haemolytic against fish erythrocytes (Vences *et al.*, 2017).

The mechanisms by which these toxins are secreted remained unknown for a long time. Rivas *et al.*, 2015a found that mutation of the *epsL* and the *pilD* genes, components of a type II secretion system (T2SS) impaired haemolysis in both plasmidless and pPHDD1 harbouring strains. The mutation in the *pilD* (prepilin peptidase) gene affected haemolysis even more severely than *epsL* mutation. Evaluation of promoter expression by β-galactosidase assays (cloning promoters of haemolysin genes in vector pHRP309 upstream of a promoterless *lacZ* gene) indicated that a deficiency of haemolysin secretion in *epsL* and *pilD* mutants might lead to a reduced expression of haemolysins and T2SS genes. Moreover, virulence challenges carried out in mice showed a dramatic decrease in virulence of above-mentioned mutants for mice. Rivas *et al.*, 2015b investigated whether haemolysins promoted bacterial adhesion to human epithelial cells (cultured human keratinocyte cells; HaCat cells). In coculture, bacterial cells lead to rapid haemolysin-dependent membrane permeabilization and haemolysins promoted the association of *P. damselae* subsp. *damselae* cells to epithelial cells since in comparison fewer mutant cells had the ability to adhere. In this regard, von Hoven *et al.*, 2018 showed that at low salinity, disruption of the chemotaxis-related gene *cheA* decreased association with epithelial cells and production of PhlyP so this field of study still requires further research.

1.4.3. Phospholipases and collagenases

Phospholipids are central components of cellular membranes that have two fatty acids in the sn-1 and sn-2 positions, joined in a glycerol molecule. They also contain a hydrophilic phosphate head group (primarily choline, ethanolamine, serine, and inositol) anchored to the sn-3 position. The latter determines the phospholipid group; i.e., phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol. Bacteria harbour phospholipases that may have a role in nutrition, but also participate in the pathogenic process acting as virulence factors. These enzymes are a group of lipolytic esterases which can be classified into 4 groups, based on the targeted site of attack against the phospholipid: phospholipases A (PLA), B (PLB), C (PLC) and D (PLD; Schmiel and Miller, 1999). They can be released by bacterial cells and can damage the host not only in a direct manner by cytolytic activity, but also by generating secondary products that may be a signal molecule that impacts the physiology of the damaged cells (Schmiel and Miller, 1999; Flores-Diaz *et al.*, 2016). There are numerous reports about phospholipases that are secreted by Gram-negative bacteria, including the SseJ of *Salmonella typhimurium* (Upton and Buckley, 1995; Lossi *et al.*, 2008), the cytotoxin ExoU of *Pseudomonas aeruginosa* (Sato and Frank, 2004, Diaz and Hauser, 2008; Howell *et al.*, 2013) and the haemolysin VHH of *Vibrio harveyi* (Sun *et al.*, 2007).

An important number of *P. damselae* subsp. *damselae* strains isolated from fish outbreaks lack the virulence plasmid pPHDD1 (Labella *et al.*, 2010a; Rivas *et al.*, 2014; Terceti *et al.*, 2016). Previous studies had already shown that the ECPs of this pathogen possessed phospholipase activity (Fouz *et al.*, 1993; Labella *et al.*, 2010a) and Vences *et al.*, 2017 deciphered its genetic basis. These authors identified the ChrI-encoded phospholipase PlpV (Phospholipase of *Vibrionaceae*) present in a collection of 34 *P. damselae* subsp. *damselae* strains. They found that strains encoding Dly had a high phospholipase activity in plates supplemented with egg-yolk and when the *dly* gene was deleted, the activity was similar to that of plasmidless strains. A strain that lacks the pPHDD1 plasmid in which the gene *plpV* was deleted, showed an abolishment of phospholipase activity. These

findings indicated that phospholipase activity in strains carrying the pPHDD1 plasmid relies primarily on the action of Dly but also PlpV, whereas for plasmidless strains, the only responsible is PlpV. Several experiments were carried out in order to check the role of PlpV in virulence. Firstly, the *hlyAch* mutant in a plasmidless strain exhibited haemolytic activity against fish erythrocytes but when *plpV* was deleted the strain gave no haemolysis. Thus, PhlyC constitutes the main causative of haemolysis of sheep erythrocytes while PlpV accounts for haemolysis of fish erythrocytes in plasmidless strains. As for virulence, *plpV* and *hlyAch* mutants were attenuated with respect to the wild type. Deletion of both genes led to the survival of the totality of fish. It was also demonstrated that PlpV is secreted via the T2SS since mutants in *epsL* and *pilD* genes of a plasmidless strain had null phospholipase activity (Vences *et al.*, 2017).

In addition to lipolytic enzymes, proteolytic enzymes have also been found to be involved in pathogenicity in other marine pathogenic bacteria. Such is the case of proteases of *Aeromonas salmonicida* (Gunnlaugsdóttir and Gudmundsdóttir, 1997) and *Vibrio anguillarum* (Denkin and Nelson, 2004), which are considered virulence factors involved in the degradation of fish tissues. In *P. damselae* subsp. *damselae* several strains were positive for proteolytic degradation (Pedersen *et al.*, 1997; Pedersen *et al.*, 2009; Vences *et al.*, 2017) although it cannot be considered a ubiquitous trait because of the existence of negative strains (Labella *et al.*, 2010a). The comparative genomics analyses carried out by Vences *et al.*, 2017, led to the identification of *colP* gene (named for collagenase of *Photobacterium*) present in some strains. Basing on the MEROPS database that classifies collagenolytic enzymes, ColP is a class II member of the M9 family that includes *Vibrio* and *Clostridium* metalloproteinases that present a putative collagenolytic activity (Rawlings *et al.*, 2016). Collagen can be used nutritionally by marine microorganisms and its decomposition adds molecules to the nitrogen and carbon cycles (Duarte *et al.*, 2014). A plasmidless strain in which the *colP* gene was deleted, lost the ability to degrade collagen, confirming that such ability depends on the action of ColP. Moreover, it was also found that the single deletion of *colP* had a slight impact on virulence for fish. When gelatin degradation

ability was checked in *epsL* or *pilD* mutants, it was shown that this degradation was impaired without being eliminated, which suggests that the T2SS participates partially in ColP secretion (Vences *et al.*, 2017).

1.4.4. Two-component regulatory systems (TCS)

To adapt to a wide range of environmental niches, bacteria must sense and respond to different signals. Two-component systems (TCS) are a family of signal transduction proteins existing in all domains of life. Present in the majority of bacterial genomes, they constitute the principal mechanism bacteria use to sense and respond to the environment adjusting their cellular physiology and gene expression (Stock *et al.*, 2000).

Classical two-component systems comprise a sensor histidine kinase that transduces a specific physical or chemical signal through the phosphorylation of a cognate response regulator (Groisman, 2016). Sensors utilise mostly ATP for carrying out their autophosphorylation when they receive the appropriate stimulus (Sureka *et al.*, 2007). Phosphorylated sensors then transfer the phosphoryl group specifically to an aspartic acid residue located in the regulator. Most sensors also present phosphatase activity for the control of the phosphorylation status of the associated regulator (Groisman, 2016). When regulators are phosphorylated, they suffer a conformational change that allows them to bind DNA, and change the expression of certain genes in an organism through activation and/or repression (Zwir *et al.*, 2012). The recognition surfaces responsible for the specificity between sensor and regulator, and the biochemical characteristics of the proteins that belong to the TCS, are highly conserved across systems and species (Laub and Goulian, 2007). Several regulators cannot bind DNA, so they make contact directly with protein (Hengge, 2008) or RNA (Shu and Zhulin, 2002) targets. Input signals and regulated genes differ among two-component systems, even homologous systems of closely related bacterial species (Mascher *et al.*, 2006; Pontes *et al.*, 2011; Chen and Groisman, 2013). There are some variants from the classical type. Hybrid TCS have their sensor and regulator domains located together as part of a single polypeptide (Raghavan and Groisman, 2010).

Another type is the so-called phosphorelay system which involves more steps. In this variant, a sensor autophosphorylates at a conserved histidine residue that, in turn, phosphorylates an aspartate residue located in a protein (or a domain belonging to the same protein). The latter transfers the phosphoryl group to a third protein or domain at a histidine residue and ultimately phosphorylates a regulatory protein or domain at a conserved aspartate residue (Burbulys *et al.*, 1991). The existence of intermediates makes possible to stop the phosphotransfer process in response to additional inputs (Perego and Brannigan, 2001).

Most TCS harbour a sensor that just phosphorylates its cognate regulator, but certain regulators are phosphorylated from cognate and noncognate sensors (Laub and Goulian, 2007). As well, a particular sensor may act as a phosphodonor for different regulators, a phenomenon that expands the array of genes, proteins, and functions controlled by a signal activating a particular sensor (Mika and Hengge, 2005).

Several studies have reported the involvement of TCS in regulating toxins and other virulence factors in *Vibrionaceae*. For example, in *V. cholerae* ToxRS and VarS/VarA TCS control the expression of ToxT, the transcriptional activator of the cholera toxin (DiRita and Mekalanos, 1991; Jang *et al.*, 2011). As well in this bacterium, the VprA-VprB TCS regulates virulence by modifying the lipid A of the LPS (Herrera *et al.*, 2014). In addition, *V. parahaemolyticus* ToxRS participates in virulence regulation by controlling effector proteins of the type III secretion system (Whitaker *et al.*, 2012). In order to shed light on cytotoxin regulation in *P. damselae* subsp. *damselae*, Terceti *et al.*, 2017 identified by transposon mutagenesis a clone with a severely impaired haemolytic phenotype whose insertion was in the *rstB* gene, which encodes a histidine kinase gene that belongs to a TCS. The authors named the system as RstAB, like the homologous system that was first characterised in *E. coli*. The *rstB* deletion mutant showed a high reduction in the promoter expression of the three haemolysins in β-galactosidase assays, suggesting that this gene encoded in the chromosome controls the expression of both chromosomal and pPHDD1 encoded genes. Also, virulence was attenuated when this mutant was inoculated in sea bass. Terceti *et al.*, 2019 identified the

cognate cytoplasmic regulator RstA of the RstAB system. These authors showed that the mutation of *rstA* and *rstB* genes in both pPHDD1-harbouring as well as plasmidless strains attenuates virulence for fish and impairs haemolytic activity (Terceti *et al.*, 2017, 2019). Moreover, they unveiled many phenotypic traits related to the RstAB system, because mutants in *rstA* and *rstB* showed defects in size, shape, motility and resistance to benzylpenicillin under low osmolarity. As well, deletion of *rstA* or *rstB* heavily impaired the secretion of cytotoxins and other uncharacterised proteins secreted by the T2SS that deserve further study.

Taken together, the aforementioned results showed a major role of the RstAB system in the virulence and physiology of *P. damselae* subsp. *damselae*, opening new horizons in the research of the RstAB regulon.

1.5. THE CELL SURFACE OF GRAM-NEGATIVE BACTERIA

The cell surface of Gram-negative bacteria contains several compartmentalised and generally well-differentiated structures (Fig. 1.6). Starting from the most internal part, the cytoplasmic membrane constitutes a phospholipid bilayer with embedded proteins, that surrounds the cytoplasm and thus, separates the inside of the bacterial cell from the environment. Its major functions include the transport of substances such as nutrients or waste products, either across the membrane itself (by permeability) or through transport proteins. Furthermore, given its energised state and the presence of attached proteins involved in metabolism, this structure establishes a site for energy conservation and consumption. Around the cytoplasmic membrane there can be found a polysaccharidic structure known as the peptidoglycan, which forms a layer (not as thick as in Gram-positive bacteria) that confers strength and protection from osmotic stress (Madigan *et al.*, 2019). Externally to the peptidoglycan, Gram-negative bacteria show a second lipid bilayer, the outer membrane or the lipopolysaccharide (LPS), which is formed not only by phospholipids and proteins but additionally by polysaccharides (Putker *et al.*, 2015). This layer provides some structural strength, not as much as peptidoglycan, but works as a barrier against external compounds that

can potentially penetrate it, for example, lipophilic antibiotics (Nikaido, 2003).

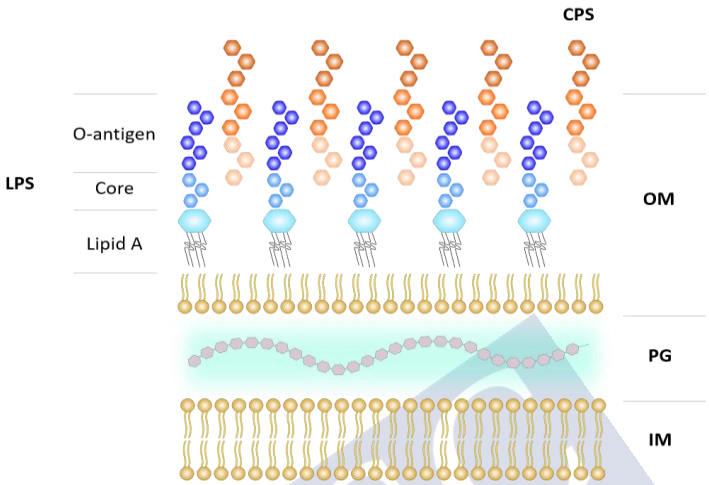

Figure 1.6: Schematic diagram of the Gram-negative bacteria cell envelope. IM, inner membrane; PG, peptidoglycan; OM, outer membrane; LPS, lipopolysaccharide (which includes lipid A, core and O-antigen; CPS, capsular polysaccharides.

Between the outer membrane and the cytoplasmic membrane, the periplasmic space also contains a great variety of proteins and enzymes involved in different processes like import and export of macromolecules (Whitfield and Valvano, 1993). Some bacteria can present other external polysaccharides known as extracellular polysaccharides (EPS), which include capsular polysaccharides (CPS). CPS form extensive hydrophilic layers called capsules that determine K-antigens employed in bacterial serotyping (Whitfield, 2006).

1.5.1. Capsular polysaccharides (CPS)

1.5.1.1.*Structure and classification*

Bacterial extracellular polysaccharides (EPS) comprise capsular polysaccharides (CPS, or the capsule for short) when the polysaccharide is closely attached to the cell surface, and slime

polysaccharides if they are not associated to the cell and easily shed off (Whitfield, 1988). The differentiation between CPS and EPS is often arbitrary since most polymers usually fall between the two categories, as CPS can be sometimes released from the cell like a slime polysaccharide (Whitfield, 1988; Whitfield *et al.*, 2020). However, CPS are typically retained on the cell surface after centrifugation and form a microscopically-identifiable structure around the cell (Whitfield *et al.*, 2020). As well, CPS may be confused with other cell surface polysaccharides such as the O-antigen of the LPS due to their external location. Despite the fact that CPS can be associated with the cell lacking a membrane anchor, they often do it through covalent bonds to phospholipids or lipid A molecules (Whitfield, 1988; Whitfield and Valvano, 1993).

As for CPS glycan nature, they are classified as homopolymers or heteropolymers formed by repeating monosaccharide units linked by glycosidic bonds (Roberts, 1996). Diversity relies on variations in monosaccharide components, anomeric configuration, linkage positions and the addition of noncarbohydrate substituents (Whitfield *et al.*, 2020). New CPS can be generated through mutation, gene duplication, lateral gene transfer or processes related to host defences; yet the main source of diversity is selective pressure by bacteriophages that use capsules as receptors (Mostowy *et al.*, 2018). The most predominant components of CPS and EPS are pentoses (ribose and arabinose), hexoses (glucose, mannose, galactose and fructose), deoxysugars (fucose and rhamnose), uronic acids (glucuronic and galacturonic acids) and aminosugars (glucosamine and galactosamine, often N-acetylated; Cescutti, 2010).

CPS biosynthesis and export genetic loci are generally clustered together (Roberts, 1996). In most bacterial species, these large clusters show conserved genetic organisation and can be classified in 4 groups based on the pattern showed by the model bacterium *E. coli*. This organism produces over 80 structures that generate a corresponding number of serologically different K antigens (Whitfield, 2006). In spite of the structural diversity, 2 strategies are mainly used for CPS biosynthesis and assembly: groups 1 and 4 capsules follow Wzy-dependent pathways while groups 2 and 3 capsules are assembled via

those dependent on ABC transporters (Whitfield, 2006; Whitfield *et al.*, 2020).

The Wzy-dependent pathway is the most common for CPS synthesis (Whitfield *et al.*, 2020). This route starts when cytosolic nucleotide sugar substrates (activated forms of monosaccharides) are combined with the undecaprenyl phosphate (Und-P) lipid carrier (embedded in the inner membrane bilayer) by a polyprenol phosphate phosphoglycosyltransferase (PGT). WbaP, a well-known PGT in *E. coli*, catalyses the addition of galactose-1-phosphate sugar (Gal-1-P) from uridine diphospho galactose (UDP-Gal) to Und-P with associated liberation of uridine monophosphate (UMP; Patel *et al.*, 2010). Subsequently, the addition of other monosaccharides to Und-PP-units is carried out by other glycosyltransferases (such as WbaZ, WcaO and WcaN; Whitfield and Paiment, 2003; Whitney and Howell, 2013). The Und-PP-oligosaccharide units are transported across the inner membrane by an integral protein, the Wzx flippase. In the periplasmic region, these units act as the substrate for the Wzy polymerase which transfers the nascent glycan from the Und-PP carrier to the incoming Und-PP-repeat unit (Whitfield, 2006). A similar approach is used in the biosynthesis of some LPS O antigens, where the Wzx and Wzy designations were firstly proposed (Raetz and Whitfield, 2002). Thus, polymerisation needs a constant supply of Und-PP, which is required for peptidoglycan biosynthesis as well (Whitfield *et al.*, 2020). EPS chain-length and amount is controlled by the phosphorylated status of a PCP protein (Whitfield, 2006). While most Wzy-dependent EPS systems have PCP-2 proteins which harbour an additional cytosolic tyrosine P loop (BY) kinase, it is its absence which characterise PCP-1 proteins from O-antigen biosynthesis. PCP-2 protein prototype Wzc is an integral membrane tyrosine autokinase protein that works together with the cognate cytosolic phosphatase Wzb and participate in the synthesis of group 1 capsules and colanic acid (Cuthbertson *et al.*, 2009). Constant polymerisation of the nascent polysaccharide requires phosphorylation and dephosphorylation of C-terminal residues of Wzc (Whitfield, 2006). Then, the nascent CPS is translocated to the cell surface by outer membrane polysaccharide export (OPX) proteins with *E. coli* Wza as the prototype (Cuthbertson *et al.*, 2009). Wza is an

octameric protein, which allows the conformation of a wide lumen that facilitates the exportation of the CPS (Dong *et al.*, 2006). Wzc and Wza interaction has been demonstrated to be helpful for the latter opening and consequent CPS export (Reid and Whitfield, 2005). Finally, found in *E. coli* and *Klebsiella pneumoniae*, Wzi is an outer membrane protein found only in group 1 capsules that participates in organising the capsule structure and modulates surface association (Bushell *et al.*, 2013). The schematic representation of the Wzy-dependent pathway is shown in Fig 1.7.

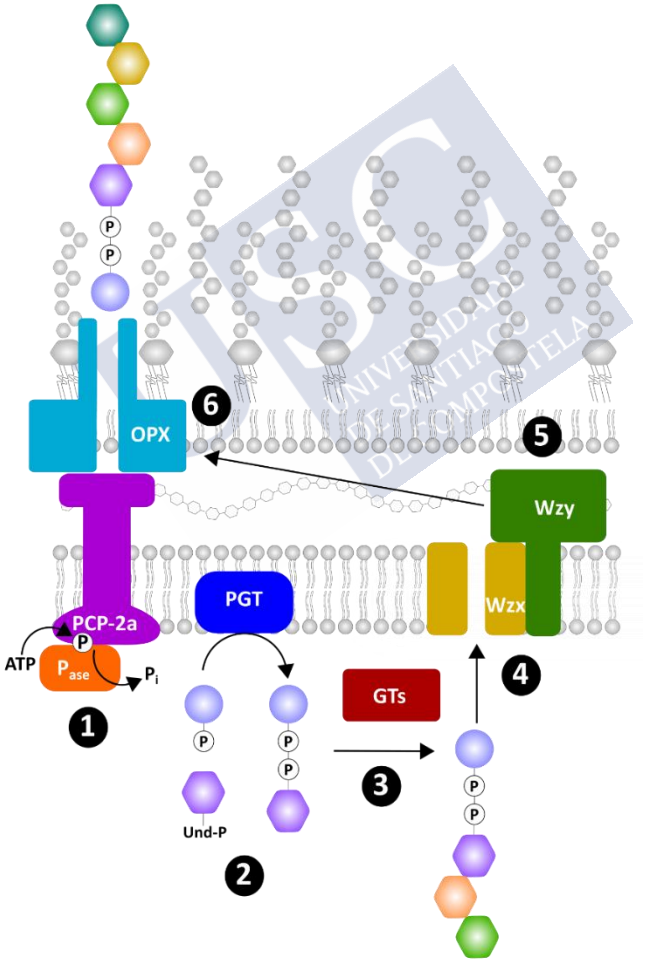

Figure 1.7: Wzy dependent pathway of capsule synthesis. (1) Polymerisation of exopolysaccharides requires autophosphorylation and dephosphorylation (by the cognate phosphatase; Pase) of PCP-2a. (2) A polyprenol phosphate phosphoglycosyltransferase (PGT) combine nucleotide sugar substrates with the undecaprenyl phosphate (Und-P) lipid carrier. (3) Other glycosyltransferases (GTs) transfer monosaccharides to Und-PP-units. (4) Und-PP-oligosaccharide units are transported across the inner membrane by the Wzx flippase. (5) Units are polymerised by the Wzy polymerase. (6) Translocation of the CPS the cell surface by the outer membrane polysaccharide export (OPX) protein.

On the other hand, group 2 and 3 capsules are assembled by ABC transporter-dependent pathways. In this via, phosphatidylglycerol is used as the acceptor for cytosolic polymerisation. β-linked 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) is constructed on phosphatidylglycerol with the aid of two CMP-Kdo-dependent GTs, KpsS and KpsC (Doyle *et al.*, 2019). Then, transfer of components to this β-Kdo oligosaccharide is carried out by various polymerising enzymes (nucleotide sugar-dependent polymerases and glycosyltransferases; Whitfield *et al.*, 2020). The glycan chain is exported across the inner membrane via the ABC transporter, KpsMT as the prototype from *E. coli*, which possesses two transmembrane domains (KpsM) and two nucleotide-binding domains (KpsT; Liston *et al.*, 2017). No specificity for the repeat-unit has been shown since the transporter is conserved among different capsule serotypes and even species (Willis and Whitfield, 2013). Similar to the Wzy-dependent pathway, translocation of the full-length polymer to the cell surface involves PCP and OPX proteins in ABC-transporter dependent pathways. Prototypical *E. coli* ABC-transporter dependent pathway OPX protein KpsD differs from Wza in several aspects, including an association to peptidoglycan through murein lipoprotein Lpp and a weaker association with the outer membrane with a strong presence in the periplasm (Arrecubieta *et al.*, 2001; Diao *et al.*, 2017; Sande *et al.*, 2019). In this pathway, PCP proteins belong to PCP-3 class which lack BY-kinase domains (Cuthbertson *et al.*, 2009). PCP partner prototype KpsE aids in locating KpsD in the outer membrane and proves to be essential for translocation of the final polymer (Arrecubieta *et al.*, 2001;

Whitfield, 2006). The schematic representation of the ABC transporter-dependent pathway is shown in Fig. 1.8.

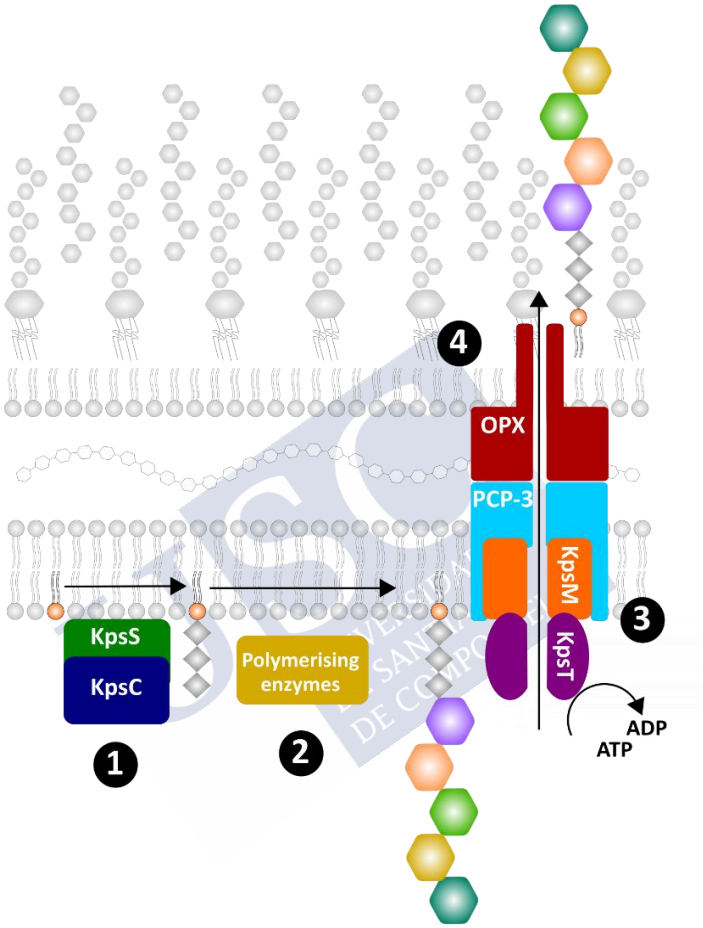

Figure 1.8: ABC transporter-dependent pathway. (1) Utilisation of phosphatidylglycerol as the acceptor to construct the ß-linked 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) by glycosyltransferases KpsS and KpsC. (2) Polymerising enzymes grow the nascent glycan chain. (3) The glycan chain is exported across the inner membrane via the ABC transporter. (4) Translocation of the full-length polymer to the cell surface by PCP and outer membrane polysaccharide export (OPX) proteins.

Although during the last decade CPS synthesis understanding has advanced significantly, the conservation status of proteins involved in above-mentioned routes and new variations of already established pathways will provide a deeper knowledge into CPS structure and organisation in bacterial species (Whitfield *et al.*, 2020).

1.5.1.2. *Biological functions of capsules*

Bacterial capsules have various functions generally summarised in relationship to virulence and survival inside and outside hosts. Capsules can form a hydrated gel around cells that may protect bacteria from desiccation (Roberson and Firestone, 1992), in fact, *E. coli* colanic acid capsule production increases in those conditions (Ophir and Gutnick, 1994). CPS and other EPS play an important role in adherence of bacteria to either surfaces or to each other, which make them capable of colonising different ecological niches and forming biofilms (Donlan and Costerton, 2002; Taylor and Roberts, 2005; Limoli *et al.*, 2015).

A virulence factor does not need to cause damage directly to host cells and tissues (as exotoxins do), it can as well be a molecule or structure that aids in adhesion to the host or protects the pathogen evading or modulating host defence systems to its replicative advantage. Examples of the latter include CPS and biofilm formation. Some capsules may be more effective in protecting bacteria from host immunity, aiding adherence and colonisation, or facilitating spread during infections (Wen and Zhang, 2015). Notably, certain capsular types contribute to virulence to a greater extent than others in the same bacterial species. Hsieh *et al.*, 2020 showed that those patients infected with *Acinetobacter baumannii* belonging to capsular types KL2, 10, 22, and 52 showed higher levels of pneumonia, severe infection, carbapenem resistance, and mortality in two medical centres in Taiwan between 2015 and 2017. *K. pneumoniae* strains that produce K1 or K2 capsules are particularly virulent on account of their strong resistance to host immune factors (Lin *et al.*, 2004). As well, certain *Streptococcus agalactiae* serotypes present a marked higher isolation from

aquaculture streptococcosis outbreaks depending on host species and geographical areas (Chideroli *et al.*, 2017).

Host defences are not insuperable obstacles, bacteria have evolved mechanisms to evade specific and non-specific immune factors. Bacterial capsules can totally surround all antigenic molecules found on the bacterial surface or can be expressed together with them (Saha, 2015). The complement system comprises a set of proteins which form a fundamental component of the innate immune system acting against microbial invasion (Heesterbeek *et al.*, 2018). Overall, bacterial capsules are typically weak complement activators, but they may mask underlying components like the O-antigen, which may be a strong activator (Merino and Tomás, 2015). In fact, non-encapsulated or low-capsulated strains belonging to various bacterial species are more sensitive to complement action. This has been demonstrated in human pathogens including *Streptococcus pneumoniae* (Hyams *et al.*, 2010) and *Salmonella enterica* (Wilson *et al.*, 2011), but also in aquaculture pathogens as for example *Vibrio vulnificus* (Williams *et al.*, 2014). Furthermore, masking of surface antigens can cause capsulated bacteria not to be opsonised by the complement and thus resistant to complement-mediated opsonophagocytosis in the absence of specific antibodies (Domenico *et al.*, 1994; Hyams *et al.*, 2010). Other structures that can be hidden by capsules are pathogen-associated molecular patterns (PAMPs) that if exposed would strongly activate the complement system or be recognised by Toll-like receptors (TLRs; Wilson *et al.*, 2008; Maue *et al.*, 2013). Capsules can also cause a reduction in the expression of proinflammatory cytokines, such as interleukins, that conditions the migration of antibodies and phagocytic cells to the sites of infection (Yoshida *et al.*, 2000; Merino and Tomás, 2015; Fang *et al.*, 2020).

As for the adaptative immune responses, CPS usually provoke an immune response, however, some are poorly immunogenic because of similarities with polysaccharides found in host tissues (Jann and Jann, 1990). The K5 CPS of *E. coli* shares an identical structure to N-acetylheparosan (Vann *et al.*, 1981). The capsule of *S. pyogenes* has the same structure with hyaluronic acid which is broadly distributed in multiple tissues (Wessels, 2006). CPS-based vaccines development

started in the 1920s when *S. pneumoniae* CPS were found to produce an immune response (Heidelberger and Avery, 1923, 1924). Following a series of studies with isolated polysaccharides, the first two hexavalent CPS-based vaccines to protect against *S. pneumoniae* were authorised in the USA in 1947 (Grabenstein and Klugman, 2012). Nevertheless, the discovery of antibiotics as a tool to combat bacterial infections meant that the investigation of carbohydrate-based vaccines was not taken up again until the appearance of antibiotic-resistant strains (Vliegenthart, 2006). Vaccines formulated with purified CPS have been proved successful against *Haemophilus influenzae*, *S. pneumoniae*, *Salmonella typhi*, and *N. meningitidis* (Wen and Zhang, 2015). Unfortunately, it was observed that CPS did not cause a sufficient immunogenic response to protect young children below the age of two (Barrett, 1985; Cadoz, 1998) so new approaches were needed. CPS are T-cell independent antigens, which can induce the production of low-affinity IgM antibody responses when inoculated alone. If conjugated with other proteins and polysaccharides, they may induce a stronger T-cell dependent immune response which leads to the production of IgG antibodies. CPS-conjugate vaccines have significantly contributed to a decrease in the prevalence of *H. influenzae* b, *S. pneumoniae* and *N. meningitidis* infections. Actually, the invasive disease caused by *H. influenzae* b is now near to eradication in developed countries after the implementation of a glycoconjugate vaccine (Mond and Kokai-Kun, 2008, Hutter and Lepenies, 2015).

1.5.2. Capsule production in *Photobacterium damselae*

Bonet *et al.*, 1994 demonstrated that *P. damselae* subsp. *piscicida* strains can synthesise a large amount of capsular material of a polysaccharidic nature. Later on, do Vale *et al.*, 2001 would show that its amount depends on the availability of iron and the growth phase of the culture. As far as resistance to host defences is concerned, the survival of strains of the subsp. *piscicida* to the bactericidal action of sea bream serum showed that those strains with capsules were more resistant to serum, persisted longer in the host and had lower lethal doses than acapsular strains. In addition, if capsule formation was induced in low-capsulated strains, the lethal doses were significantly

reduced (Magariños *et al.*, 1996). The role of the capsule in evasion of the immune system was also showed when the percentage of macrophage-mediated phagocytosis was reduced in capsulated strains (Arijo *et al.*, 1998; Acosta *et al.*, 2006). As for adherence, a thickening of the capsule caused a decrease in the adherence to the host tissues (Magariños *et al.*, 1996).

Nevertheless, there are many aspects that still need to be elucidated. One key point is that the production of a polysaccharide capsule in the subsp. *damselae* has not been investigated to date.

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2. JUSTIFICATION AND OBJECTIVES

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2. JUSTIFICATION AND OBJECTIVES

During the past decades, scientific knowledge regarding *P. damselae* subsp. *damselae* heterogeneity and pathogenicity has produced essential data to understand how this pathogen can cause disease in a variety of hosts. Nonetheless, there are subjacent aspects of virulence, ubiquitous of every *P. damselae* subsp. *damselae* isolate, whose roles still need to be elucidated. Global warming is providing ideal conditions for the expansion of *Vibrionaceae* populations. Although *P. damselae* subsp. *damselae* has a free-swimming lifestyle at temperatures around 15ºC in winter months, hazardous conditions for the development of outbreaks in aquaculture farms occur predominantly in summer months, when seawater temperature is around 25ºC. This bacterium can cause disease in human hosts because it can grow at human body temperature i.e., 37ºC. There are no data about how *P. damselae* subsp. *damselae* responds to temperatures that are key in its lifestyle both inside and outside hosts. Recently discovered, the RstAB system is a global regulator of virulence, and although it is known that it regulates the expression of cytotoxins and type II secretion system (T2SS)-dependent proteins, its thorough genetic regulon is still unknown.

Therefore, based on the above-mentioned description and data presented in the introduction, this thesis has the following objectives:

1. To study the transcriptomic and phenotypic responses of *P. damselae* subsp. *damselae* to temperatures of 15 vs 25ºC.
2. To study the transcriptomic and phenotypic responses of *P. damselae* subsp. *damselae* to temperatures of 25 vs 37ºC.
3. To unveil the genetic network under the control of the two-component system (TCS) RstAB, a major regulator of virulence.
4. To characterise RstAB-dependent factors with potential implication in virulence.

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3. MATERIALS AND METHODS

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3. MATERIALS AND METHODS

3.1. BACTERIAL STRAINS, CULTURE CONDITIONS AND VIABILITY ASSAYS

Strains and plasmids used in the present thesis are described in Table 3.1. *P. damselae* subsp. *damselae* strains were routinely grown at 25ºC on tryptic soy agar (TSA) or tryptic soy broth (TSB) supplemented with additional 0.5% NaCl so that the final salt concentration was 1% (TSA-1 and TSB-1, respectively). *E. coli* strains were cultured on Luria-Bertani (LB) agar or LB broth at 37ºC. All strains used throughout this study were stored at -80ºC in TSB-1 supplemented with 20% (vol/vol) glycerol. When required, antibiotics were added to media at the following concentrations: kanamycin (Km; NZYTech) at 50 µg/ml (stock solution in water at 50 mg/ml), ampicillin (Amp; NZYTech) at 50 µg/ml (stock solution in water at 50 mg/ml), gentamycin (Gm; NZYTech) at 15 µg/ml (stock solution in water at 15 mg/ml), chloramphenicol (Cm, NZYTech) at 12 or 20 µg/ml (stock solution in ethanol at 20 mg/ml). Prior to use, antibiotic solutions were filtered through 0.22 µm mixed cellulose esters membranes (Millipore) and stored at 4ºC.

For the analysis of growth curves, we used different combinations of temperature and salinity. These conditions were incubation at 15, 25 or 37ºC in TSB-1, or at 25ºC in TSB with a final NaCl concentration of 1, 3 or 5 %. Different temperature and salinity conditions were chosen because of their biological importance for *P. damselae* subsp. *damselae*. 15°C mimics the temperature of the free-swimming lifestyle of this bacterium at mid latitudes in winter months, 25°C is its optimal temperature and is typical from outbreaks in aquaculture in warm summer months, and 37ºC represents the human body temperature. The three assayed salinity conditions were selected in order to obtain a picture of *P. damselae* subsp. *damselae* growth at conditions that simulate the salinity of fish internal medium (1%), the planktonic

lifestyle in seawater (3%), and high-salinity potential habitats (5%). Strains were grown until obtaining exponentially growing precultures (optical density at 600nm, OD_{600}: 0.3) and then, 1:100 dilutions of each preculture were made in 100 µl TSB with the appropriate NaCl concentration in 96-well plates (Costar) and with three replicates per strain or condition. The optical density at 600 nm (OD_{600}) was measured at 2 h intervals using the spectrophotometer Epoch2 microplate reader (Biotek) during 24 or 48 h at the temperature of the experiment.

For drop-plating viability assays, precultures were grown under the same conditions used for growth curve analysis. Then 1:100 dilutions were made in 100 µl TSB-1 with three replicas per condition (incubation at 25 or 37ºC). 5 µl aliquots of ten-fold dilutions were drop-plated in TSA-1 plates after 6, 12, 24, 30, 36 and 48 h of incubation.

Table 3.1: List of bacterial strains and plasmids used in the present thesis.

Strain or plasmid	Description	Reference/ source
Strains		
P. damselae* subsp. *damselae		
RM-71	Isolated from turbot (*Psetta maxima*); pPHDD1-harbouring strain	Fouz *et al.*, 1992
A-162	Isolated from eel (*Anguilla anguilla*); plasmidless strain	Laboratory stock
LD-07	Isolated from seabream (*Sparus aurata*); plasmidless strain	Vera *et al.*, 1991
CDC-2227-81	Isolated from human; pPHDD1-harbouring strain	Kreger, 1984
80077637	Isolated from human; pPHDD1-harbouring strain	Hundenborn *et al.*, 2013
XMF288	RM-71 with insertion in *htpG* gene	This study
MT151	RM-71 with in-frame deletion of *rstB* gene	Terceti *et al.*, 2017
MT319	RM-71 with in-frame deletion of *rstA* gene	Terceti *et al.*, 2019
XMF215	RM-71 with in-frame deletion of *wza* gene	This study
XMF222	RM-71 with in-frame deletion of *wzc* gene	This study
XMF333	RM-71 with in-frame deletion of *yjbH* gene	This study

XMF363	XMF215 with pMRB24 + *wzabc* genes (complemented mutant)	This study
XMF324	XMF222 with pMRB24 + *wzabc* genes (complemented mutant)	This study
E. coli		
DH5α	Cloning strain	Laboratory stock
S17-1-λpir	RP4-2 (Km::Tn7, Tc::Mu-1) *pro*-82 λ*pir recA1 endA1 thiE1 hsdR17 creC510*	Herrero *et al.*, 1990
ß-3914	F− RP4-2-Tc::Mu Δ*dapA*::(*erm-pir*) *gyrA462 zei-298*::Tn*10* (Km^r Em^r Tc^r)	Le Roux *et al.*, 2007
Plasmids		
pWKS30	Low-copy-number cloning vector, Amp^r	Wang and Kushner, 1991
pNidKan	Suicide vector derived from pCVD442, Km^r	Mouriño *et al.*, 2004
pHRP309	*lacZ* reporter plasmid, Gm^r	Parales and Harwood, 1993
pUC118	Low-copy-number cloning vector, Amp^r	Vieira and Messing, 1987
pAJR45	*hlyAch* promoter fused to promoterless *lacZ* gene in pHRP309	Rivas *et al.*, 2013
pAJR51	*dly* promoter fused to promoterless *lacZ* gene in pHRP309	Rivas *et al.*, 2013
pAJR53	*hlyApl* promoter fused to promoterless *lacZ* gene in pHRP309	Rivas *et al.*, 2013
pMRB24	Cloning vector, *mob*, Cm^r	Le Roux *et al.*, 2011

Km^r, kanamycin resistance; Em^r, erythromycin resistance, Tc^r, tetracycline resistance, Amp^r, ampicillin resistance; Gm^r, gentamycin resistance.

3.2. GENOMIC AND PLASMID DNA EXTRACTION

When genomic DNA was used as a template for polymerase chain reaction (PCR) amplification, it was extracted using the Easy-DNA kit (Invitrogen). For genome sequencing of human strains CDC2227-81 and 80077637, high-purity genomic DNA was extracted using the G NOME DNA kit (MP Biomedicals). Plasmid DNA was purified by the use of the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). All extractions were performed following manufacturer's instructions.

3.3. POLYMERASE CHAIN REACTION (PCR) CONDITIONS

PCR reactions were carried out in volumes of 25 or 50 µl in the T100™ thermal cycler (Bio-Rad). For PCR amplification we used ready-to-use solutions containing DNA polymerase, dNTPs, buffer, and the optimal concentration of ions. Routinely, the NZY Taq II 2x Green Master Mix (NZYTech) was utilised, but for the amplification of fragments in the generation of allelic-exchange mutants, the NZYProof 2x Green Master Mix (NZYTech) was used for its higher accuracy (the containing polymerase exhibits 3'-5' exonuclease activity). Single reaction mixtures and PCR parameters are shown in Tables 3.2 and 3.3, respectively. An additional reaction with all components except the DNA template was added to every group of PCRs, constituting a negative control to ensure the absence of contaminating DNA.

PCR products were analysed through agarose gel electrophoresis. Gels were prepared with the appropriate agarose concentration (ranging from 0.7 to 2% depending on the size of the amplified DNA fragment) dissolved in buffer TAE 1X (Tris 40 mM, 0.5 mM EDTA, pH 8.0). A volume corresponding to a concentration of 0.5 µg/ml of Realsafe Nucleic Acid Staining Solution (Intron Biotechnology) was added to gels for the detection of DNA. For visualisation, gels were illuminated at a wavelength of 254 nm in a transilluminator (Gelprinter Plus). Purification of DNA fragments was carried out using the NZYGelpure kit (NZYtech).

Table 3.2: Components of a single PCR reaction (50 µl) using NZY Taq II 2x Green Master Mix or NZYProof 2x Green Master Mix

	NZY Taq II 2x Green Master Mix	NZYProof 2x Green Master Mix
Primers	0.2 – 0.5 µM	0.3 – 0.5 µM
Template DNA	5 pg – 0.5 µg	10 ng - 0.5 µg
Mix	25 µl	25 µl
Nuclease-free water	up to 50 µl	up to 50 µl

Table 3.3: PCR parameters using NZY Taq II 2x Green Master Mix or NZYProof 2x Green Master Mix

NZY Taq II 2x Green Master Mix			
Cycle step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95ºC	3 min	1
Denaturation	94ºC	30 – 50 s	25 - 35
Annealing	*	30 – 60 s	
Extension	72ºC	15 – 30 s/kb	
Final extension	72ºC	5 - 10 min	1
NZYProof 2x Green Master Mix			
Cycle step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95ºC	3 min	1
Denaturation	95ºC	30 s	20 - 40
Annealing	*	30 s	
Extension	72ºC	60 s/kb	
Final extension	72ºC	5 – 10 min	1

*, annealing temperature is optimised for each primer combination, calculated as primer melting temperature (Tm) – 5ºC.

3.4. CLONING OF DNA FRAGMENTS

In the generation of recombinant DNA, different plasmids were used, depending on the requirements of the methodology, to clone specific fragments (inserts). Inserts and plasmids were cut with the same restriction enzymes in order to generate compatible ends. In addition, when a single restriction enzyme was used, plasmids were dephosphorylated by the FastAP^{TM} Thermosensitive Alkaline Phosphatase (Thermo Fischer Scientific) to eliminate phosphate groups from the 5' end, avoiding self-ligation and facilitating the union with the insert. Inserts and plasmids, in different proportions depending on the case, were joined together with T4 DNA ligase (Thermo Fischer Scientific). Ligations were incubated from 2 h to overnight at 25 or 4ºC respectively. Next, ligations were dialysed for 15 min in 0.025 µm membrane filters (Millipore) prior to electroporation.

3.5. PLASMID ELECTROPORATION IN *ESCHERICHIA COLI*

To obtain electrocompetent cells, a 500 ml-culture of *E. coli* (strain DH5α or S17-1-λpir) in LB was incubated under agitation until it achieved an OD_{600} of 0.2 - 0.4. Cells were centrifuged at 5000 rpm at 4ºC and washed several times with sterile cold water. Last wash was

performed with sterile 10% glycerol. Finally, cells were resuspended with 1 ml sterile 10% glycerol and divided into 50 µl aliquots that were stored until used in 1.5 ml Eppendorf tubes at -80ºC.

For transformation by electroporation, 20 µl of a previously dialysed ligation was added to a 50 µl vial of electrocompetent cells in a 0.2 cm electroporation cuvette (Bio-Rad). The transformation was carried out in a Gene Pulser electroporator (Bio-Rad) with the following parameters: voltage of 2.5 kv, resistance of 200 Ω, capacitance of 25 µF, with pulses of 5 ms duration. Subsequently, 1 ml of LB was incorporated to the cuvette in order to collect the electroporated cells. The culture was transferred to a 1.5 ml Eppendorf and incubated under continuous shaking at 150 rpm for 1h at 37 °C. Finally, cells were pelleted, resuspended in 100 µl of LB and seeded onto LB plates supplemented with the appropriate antibiotics.

3.6. FATTY ACID METHYL ESTER (FAME) ANALYSIS

Fatty acid composition profiles were determined by a fatty acid methyl ester (FAME) analysis using MIDI Sherlock ® Microbial Identification System (MIDI, Inc) and following manufacturer's recommendations. The analysis was performed by extracting and preparing FAME from *P. damselae* subsp. *damselae* strain RM-71 cultures incubated at 15, 25 or 37ºC on TSA-1 for 24 h. Cells were harvested from late log phase-plate quadrants as their fatty acid compositions are most stable. Subsequently, cells were lysed to liberate fatty acids from the cellular lipids through saponification with methanol and NaOH. Then, FAME are formed by methylation with methanol and HCl, extracted with hexane and methyl tert-butyl ether, and washed with NaOH prior to identification through chromatographic analysis.

3.7. E-TEST ASSAY

To determine the susceptibility to benzylpenicillin, *P. damselae* subsp. *damselae* strain RM-71 was grown at a temperature of 25ºC in 10 ml TSB-1 in 100 ml flasks, and collected when cultures reached a sharp OD_{600} of 0.5. Aliquots of 100 µl were spread onto TSA-1 plates in the presence of a benzylpenicillin E-test gradient strip (bioMérieux). Plates were incubated for 24 h at 25 and 37ºC.

3.8. SEQUENCING TECHNIQUES AND COMPARATIVE GENOMICS

3.8.1. Genome sequencing

For genome sequencing of the two human isolates CDC-2227-81 (Kreger *et al*., 1984) and 80077637 (Hundenborn *et al.*, 2013), the G NOME DNA kit (MP Biomedicals) was used for high-purity genomic DNA extraction. Libraries were created using the TruSeq DNA PCR-free kit (Illumina) and then subjected to sequencing with the Illumina HiSeq 2000 platform (2×100-bp paired-end reads). The genome sequences were annotated using RAST (Aziz *et al.*, 2008). The draft genome sequences were deposited at GenBank/EMBL/DDBJ under accession numbers VZUQ00000000 (CDC-2227-81) and WAEO00000000 (80077637).

3.8.2. Comparative genomics

Sequence similarity analysis by comparison of orthologous fragments between pairs of genomes was conducted with OrthoAni, an algorithm that uses an average nucleotide identity value of 95-96 % as cut-off for species distinction (Lee *et al.*, 2016). Core genome and unique genes were calculated using RAST (Aziz *et al.*, 2008). The genomic BLAST files of *Photobacterium damselae* subsp. *damselae* strains 80077637, CDC-2227-81, RM-71, CIP102761, A-162 and LD-07 were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/genome/675), and the dendrogram was visualized by Interactive Tree of Life (iTOL v5; Letunic and Bork, 2019).

3.8.3. RNA sequencing (RNA-seq)

A diagram illustrating the entire RNA-sequencing (RNA-seq) process is shown in Fig. 3.1. Cells from three independent cultures of the wild type (wt) strain RM-71 at each temperature condition (15, 25 and 37ºC) and of deletion mutants Δ*rstA* and Δ*rstB* at 25ºC were cultivated in 10 ml TSB-1 in 100 ml flasks until they achieved an OD_{600} of 0.55. Cultures were instantly subjected to a treatment with the RNAprotect Bacteria Reagent (Qiagen) for RNA stabilisation. Next, a

resuspension of pelleted cells in TE buffer (30 mM Tris·HCl, 1 mM EDTA, pH 8.0) containing 10 µl lysozyme (15 mg/ml; Sigma Aldrich) and 15 µl Proteinase K (20 mg/ml; Qiagen) was performed. For RNA extraction, we employed the RNeasy Mini Kit (Qiagen) with a following DNase I treatment using the on-column kit RNase-free DNase (Qiagen) for the elimination of genomic DNA contamination. RNA was eluted in nuclease-free water. A Bioanalyzer 2100 (RNA 6000 Nano chip assay; Agilent Technologies) and a Qubit 3.0 (Quant-It dsRNA BR Assay; Invitrogen) were used to assess quality and quantity of RNA.

rRNA contamination was eliminated with the Ribo-Zero rRNA Removal Kit (Gram Negative Bacteria; Illumina) and cDNA libraries were produced accordingly to Illumina's recommendations. rRNA-depleted RNA was first chemically fragmented and subjected to reverse transcription for cDNA formation. The cDNA fragments underwent an end reparative process, an addition of a single 'A' base to the 3' end and then ligation of adapters. Lastly, the products were cleaned and enriched by PCR to generate the indexed final double stranded cDNA library. The library set was sequenced with an Illumina HiSeq 2500 sequencer.

FastQC[http://www.bioinformatics.babraham.ac.uk/projects/fastqc/] program was used for the quality control of raw reads. The raw pair-end reads were mapped against the reference genome of the *P. damselae* subsp. *damselae* type strain CIP102761 (GenBank accession number NZ_ADBS00000000.1) to provide better localisation of each gene in either chromosomes or plasmids, as this type strain genome is entirely closed. Reads not mapping to the type strain genome, corresponding to unique genes of RM-71 strain with respect to CIP102761, were mapped against the sketched genome sequence of strain RM-71 (GenBank Acc. No. NZ_LYBT00000000.1) via Bowtie2 (Langmead and Salzberg, 2012) v2.2.6 algorithm. A number of quality control steps were conducted. Very low-quality reads were excluded by Samtools (Li *et al.*, 2009) and Picard Tools softwares [http://broadinstitute.github.io/picard/]. Furthermore, one key determinant for sequencing processes is the GC content of samples, which was found to be normal (distribution between 40-60%) in our

assay. The distribution of duplicates was also assessed to confirm their normal small proportion. The genetic quantification process was accomplished by the HTSeq (Anders *et al.*, 2015) software (0.6.1 version).

Concordance between each condition or strain samples was assessed using the statistics program R through a correlation and distance study, taking the whole transcriptome normalised by the size of the library. Differential expression analysis was conducted by DESeq2 (Love *et al.*, 2014) method (1.18.1 version). The analysis of Differentially Expressed Genes (DEGs) was made by statistical packages developed by Python and R, using the DESseq2 (Love *et al.*, 2014) algorithm which applies a differential negative binomial distribution for the statistics significance. A Python script designed at Sistemas Genómicos (Valencia, Spain) was applied to create a data matrix for each group (defined by condition or strain) with the counts derived from HTSeq count for each sample. Those genes with Fold Change (FC) values lower than -1.5 or higher than 1.5 and a *P*-value corrected by False Discovery Rate (FDR) ≤ 0.05 were regarded as differentially expressed.

FPKM (Fragments Per Kilobase per Million fragments mapped) values determined with Cufflinks v2.2.1 (Langmead and Salzberg, 2012) were used to obtain the expression levels of each specific gene.

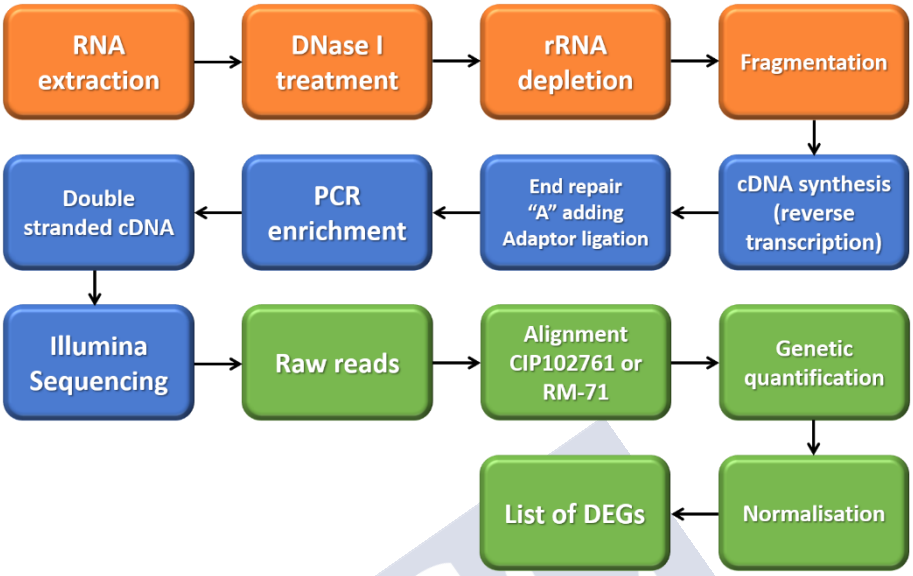

Figure 3.1: RNA-sequencing workflow. The diagram shows the main phases from RNA extraction to the obtention of the list of DEGs. 3 phases can be differentiated in the process depending on their nature: RNA processing (orange), cDNA processing (blue) and Bioinformatics (green). DEGs, differentially expressed genes.

3.9. B GALACTOSIDASE ASSAYS

The low-copy-number plasmid pHRP309 (Parales and Harwood, 1993; Fig. 3.2) is frequently used for transcriptional fusions. This plasmid presents a promoterless *lacZ* gene adjacent to the multicloning site. Therefore, when a promoter sequence is cloned upstream the *lacZ* gene (encoding a β-galactosidase), the expression of the latter is under the control of the former.

RM-71 strain derivatives carrying the *dly*, $hlyA_{pl}$ and $hlyA_{ch}$ haemolysin gene promoters fused to a promotorless *lacZ* gene in this reporter plasmid were obtained in a previous work (Rivas *et al.*, 2011). These strains were grown in TSB-1 at 25 and 37ºC, and β-galactosidase activities dependent on promoter activity, were measured following the Miller method (Miller *et al.*, 1992).

A volume (V) of 500 µl of bacterial culture, adjusted to an OD_{600} value of 0.3-0.4, was mixed with 500 µl of buffer Z ($Na_2HPO_4 \cdot 2H_2O$ 60 mM; $NaH_2PO_4 \cdot H_2O$ 40 mM; KCl 10 mM; $MgSO_4 \cdot 7H_2O$ 1 mM; β-mercaptoetanol 50 mM; pH 7,0). Subsequently, 20 µl of chloroform and 10 µl of 0.1% sodium dodecyl sulfate (SDS) were incorporated into the mixture. Samples were vortexed and incubated during 5 minutes at 37ºC. The addition of 100 µl of *ortho*-Nitrophenyl-β-galactoside (ONPG; 4 mg ml^{-1} dissolved in buffer Z) starts the reaction, and time (T) is counted until the colour of the mixture turns yellow. Immediately after, the reaction was blocked by the addition of 450 µl of Na_2CO_3 1 M and the absorbance of the mixture at 420 nm was measured. Miller units are calculated following the next formula: β galactosidase units = Abs_{420} x 1000/T (s) x V (ml) x OD_{600}. Three independent experiments with 3 replicates each were conducted. The statistical analysis of the expression data was carried out with the Student's t-test (adjusted P values <0.05). Mann Whitney test was used for non-parametric comparison of the mean values.

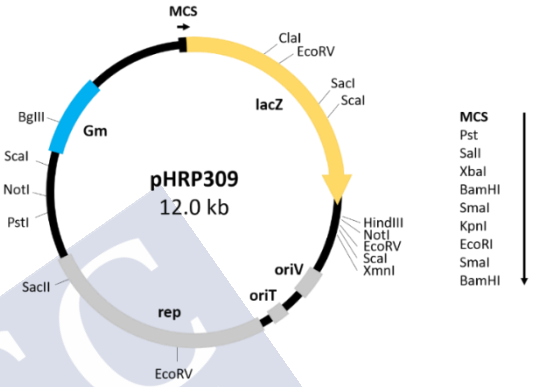

Figure 3.2: pHRP309 plasmid map. This plasmid is used for transcriptional fusions with *lacZ* as a reporter gene. MCS, multicloning site.

3.10. MUTAGENESIS

3.10.1. Construction and screening of mini-Tn*10* transposon insertional libraries

Transposon mutagenesis with the mini-Tn*10* commences with the transfer of the suicidal plasmid pLOFKm to a target bacterial strain. This plasmid contains the transposase IS10R gene under the control of the Isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced promoter Ptac-, and a Km resistance gene flanked by mobile elements (Herrero *et al.*, 1990; Fig. 3.3).

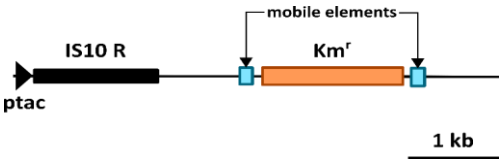

Figure 3.3: section of the plasmid pLOFKm which carries the mini-Tn*10* transposon.

E. coli strain β-3914 (diaminopimelic acid (DAP) auxotrophic) was utilised as the plasmid donor and *P. damselae* subsp. *damselae* RM-71 as the receptor strain. *E. coli* cells were grown in LB supplemented with DAP at 37ºC, while *P. damselae* subsp. *damselae* cells were grown in TSB-1 at 25ºC. When both strains achieved the exponential growth phase, 100 µl of each culture were mixed together in a 1.5 ml Eppendorf tube. The mix was drop-plated for conjugation in a TSA plate made with seawater in which DAP and IPTG (to guarantee *E. coli* survival and induce the expression of the transposase) had been previously added. After an incubation of 48 h, the conjugation was recovered and resuspended in 1 ml TSB-1. Serial dilutions were plated in TSB-1 without DAP (to eliminate the *E. coli* background) and supplemented with Km at 50 µg ml-1 (in order to select only *P. damselae* susbp. *damselae* mutants). Those clones were subsequently replicated in two plates, one incubated at 25 and the other at 37ºC. We aimed to isolate mutants capable of growing at 25ºC but unable to grow at 37ºC.

As well, we used a previously generated RM-71 mini-Tn*10* transposon mutant library of approximately 2,000 clones (Terceti *et al.*, 2017). The library, which consisted of clones maintained frozen at -80ºC in 96-well plates, was used in the present thesis for the screening of putative mutant clones exhibiting impaired growth at 37ºC. To this

aim, each 96-well plate was carefully thawed, and clones were replicated into 96-well plates containing fresh TSB-1 medium. Two replicas per plate were incubated at 25 and at 37ºC respectively, and bacterial growth was monitored for 24 h using the spectrophotometer Epoch2 microplate reader (Biotek).

3.10.2. Construction of insertional mutants

For insertional mutation of *clpB* (VDA_001325), *groEL* (VDA_003059), and *htpG* (VDA_002523) genes encoding heat-shock proteins, an internal fragment of each gene was PCR amplified using the NZYProof 2x Green Master Mix and cloned into the suicide vector pNidKan (Mouriño *et al.*, 2004; Fig 3.4).

Primers used in the generation of insertional mutants are listed in Table 3.4. The mutant allele constructions were mobilised from *E. coli* S17-1-λpir into the *P. damselae* subsp. *damselae* parental strain RM-71. Insertions of the suicide vectors into the chromosome by a single crossover would result in a Km-resistant phenotype and disruption of the coding sequence. The insertional mutants were selected on Thiosulfate Citrate Bile Sucrose (TCBS) agar plates containing Km (50 µg ml^{-1}). Mutants were grown in parallel with the parental strain RM-71 at 25 and 37ºC to assess differences in growth, using the Biotek spectrophotometer Epoch2 microplate reader.

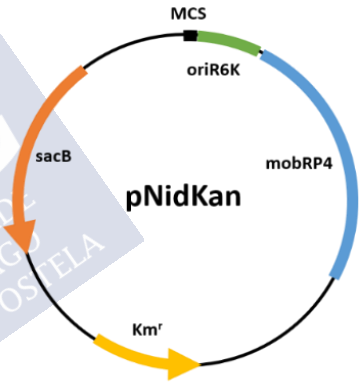

Figure 3.4: pNidKan plasmid map. It contains the sucrose sensitivity gene *sacB* and the pir-dependent R6K origin of replication. MCS, multicloning site.

Table 3.4: Primers used to construct of insertional mutants.

Primer	Sequence (5'-3')*
Mutation of gene *clpB*	
mut_ins_ClpB_F_XbaI	GCTCTAGATAACGATGAGACAGTGTTGC
mut_ins_ClpB_R_XbaI	GCTCTAGACTCTCGTCTTGCTGAAATGT
Mutation of gene *groEL*	
mut_ins_GroEL_F_XbaI	GCTCTAGATTTAATAACGGCAACACCGC

mut_ins_GroEL_R_XbaI	GCTCTAGACTCAAGCAAACGATGCTGCA
Mutation of gene *htpG*	
mut_ins_HtpG_F_XbaI	GCTCTAGACTTGGCCTTCTTTCATACGC
mut_ins_HtpG_R_XbaI	GCTCTAGAGACCCGAGAAGATGTGATTG

* Restriction sites are highlighted in primer sequences.

3.10.3. Allelic-exchange deletion mutant construction

The deletion mutants for *rstA* (VDA_000600) and *rstB* (VDA_000601) genes were constructed in two previous studies (Terceti *et al.*, 2017; Terceti *et al.*, 2019). In this thesis, we have constructed non-polar deletion mutants for the capsular genes *wza* (VDA_001508), *wzc* (VDA_001510) and *yjbH* (VDA_001504) in *P. damselae* subsp. *damselae* strain RM-71, following an allelic exchange methodology. Through this technique, the native copy of a particular gene can be exchanged for a copy containing a deletion of the central region. Approximately, 2 kilobase DNA fragments upstream and downstream of the target gene were obtained by PCR amplification using the NZYProof 2x Green Master Mix. Primers used in the generation of allelic-exchange deletion mutants are listed in Table 3.5. The appropriate restriction sites were added to the primers used for amplification (named as 1, 2, 3 and 4), in order to guarantee the correct orientation of fragments. Subsequently, those fragments were ligated (primers 2 and 3 present the same restriction site) so that the reconstructed allele underwent an in-frame deletion of >90% of its coding sequence.

Table 3.5: Primers used to construct allelic exchange deletion mutants.

Primer	Sequence (5'-3')*	Function
Mutation of gene *wza*		
Mut_wza_1_SalI	GCGTCGACCGTAATGCATAAGGGTTGTC	Mutant construction
Mut_wza_2_EcoRI	GCGAATTCGCCATCTACACTCAAATTTG	
Mut_wza_3_EcoRI	GCGAATTCGGCTTTAATGAGTTGACGGA	
Mut_wza_4_BamHI	GCGGATCCTTCACGTTGCGTCTTTGGTA	
Mut_wza_int_5'	AAATGCACCTAGTTGCGTGT	Checking
Mut_wza_int_3'	AAGTAGCGAGTAGCGAGTAG	
Mutation of gene *wzc*		
Mut_wzc_1_SalI	GCGTCGACATTACTGCATTGGCTTCGAC	Mutant construction
Mut_wzc_2_EcoRI	GCGAATTCTTGGCTCGTGGGATTATTTG	
Mut_wzc_3_EcoRI	GCGAATTCGCAAGTGGTTATTACGGTCA	

Mut_wzc_4_BamHI	GCGGATCCGCCTCATCAAATTCAACCAC	
Mut_wzc_int_5'	GAAGCTTGGGCGAAGAAGTT	Checking
Mut_wzc_int_3'	GACAGTTATTCTGCTATACG	
Mutation of *yjbH*		
mut_yjbH_1_SalI	GCGTCGACAGCGCGACCTTCTGCAGTAT	Mutant construction
mut_yjbH_2_EcoRI	GCGAATTCCCTCTAACTCGTGATGGTGG	
mut_yjbH_3_EcoRI	GCGAATTCGGCGGTAAAGCTAGATAGCA	
mut_yjbH_4_BamHI	GCGGATCCCAGTTGTAGTAGGTGCAGCA	
mut_yjbH_int5	GAGTCGCAATCGATAATCGA	Checking
mut_yjbH_int3	TGCAACTCTCTATGTTCACT	

* Restriction sites are highlighted in primer sequences.

Upstream and downstream fragments of each gene were sequentially cloned in plasmid pWKS30 (Fig. 3.5) using *E. coli* strain DH5α. The genetic construction was then liberated from pWKS30 (using restriction enzymes *Apa*I and *Not*I) and ligated in the Km-resistant suicide vector pNidKan (Mouriño *et al.*, 2004; Fig. 3.4) previously cut with the same enzymes. pNidKan bears the sucrose sensitivity gene *sacB* and the *pir*-dependent R6K origin of replication, so plasmid constructs carrying deleted alleles were electroporated in the *E. coli* strain S17-1-λpir (having the λpir gene). The genetic constructions were moved from *E. coli* S17-1-λpir into *P. damselae* subsp. *damselae* RM-71 by conjugation during 48 h on TSA plates made with seawater. Cells were suspended in TSB-1, and 100-µl aliquots of serial decimal dilutions were seeded on TCBS agar plates containing Km to force a first recombination event. The key feature of this plasmid is its inability to replicate in *P. damselae* subsp. *damselae* (because it lacks the λpir

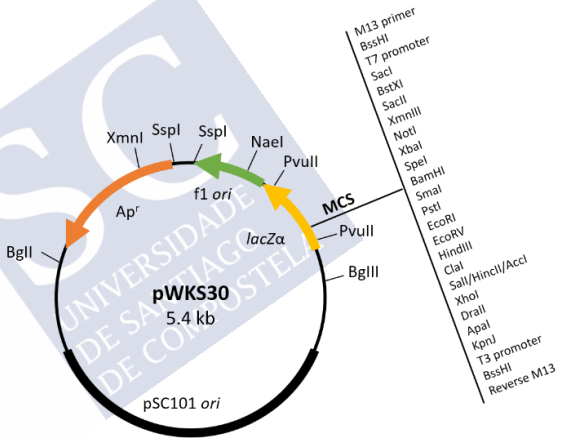

Figure 3.5: pWKS30 plasmid map. Low-copy number plasmid used for cloning. MCS, multicloning site.

gene), so it will only perpetuate when integrated into the bacterial genome by recombination between homologous sequences. Next, Km resistant colonies (first recombinants) were grown in TSB-1 (without selective pressure for resistance of the suicide plasmid) and after some passages, dilutions were plated on TSB-1 plates with 15% sucrose to force a second recombination event. Once the construct is integrated into the chromosome, in 50% of cases the second recombination will occur in the same region, and in these cases the suicide plasmid is released along with the deleted copy. On the other 50% of cases, recombination will occur in the other fragment, and the plasmid will carry the intact copy of the gene, leaving the deleted copy in the chromosome. Cells able to form colonies are those that have lost the suicide plasmid and thus the *sacB* gene. Checking primers are used in PCR reactions to identify the mutant colonies. These primers amplify the region around deleted sites, so mutants will produce smaller PCR products while revertant clones will show sizes as large as the native sites of the gene. Finally, the genome region involved in the deletion of each gene was subjected to DNA sequencing to verify that deletions were nonpolar. The mechanism for obtaining mutants by allelic exchange is schematised in Fig. 3.6.

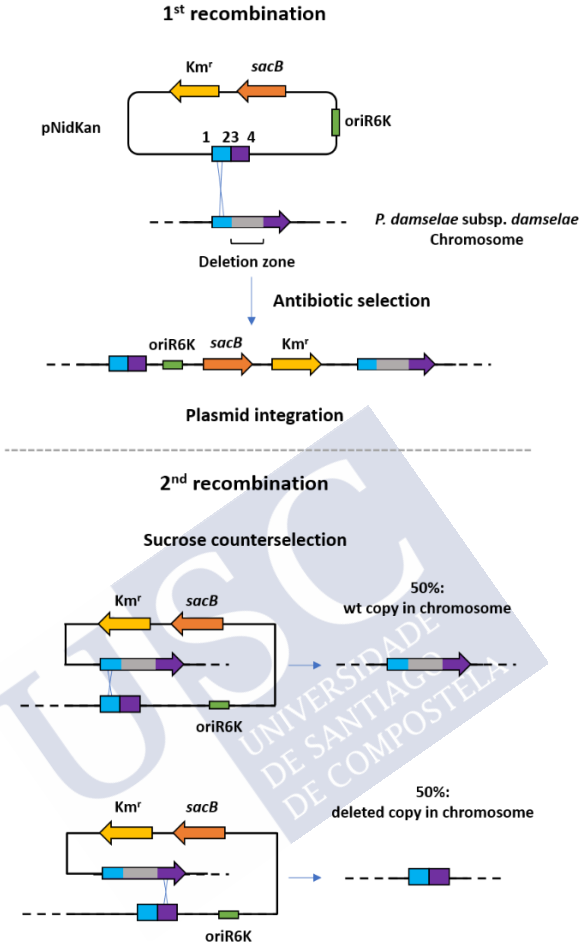

Figure 3.6: Schematic representation of mutagenesis by allelic exchange methodology. Upstream (1-2) and downstream (3-4) regions of the target gene are amplified and subsequently cloned in the suicide plasmid pNidKan that harbours a kanamycin resistance gene (Km^r), the sucrose sensitivity sacB gene and the origin of replication R6K (that requires the λpir gene to replicate). Once the plasmid is transferred to *P. damselae* susbp. *damselae* by conjugation, it is forced to integrate in the chromosome through homologous recombination. By antibiotic selection, Km-resistant *P. damselae* subsp. *damselae* first recombinants are generated. A second event of recombination by sucrose counterselection leads to two possible scenarios. In 50% of the cases, if the recombination occurs in the same site, the suicide plasmid will be liberated and carry the deleted copy. However, in the other 50%, the plasmid carries the wild type (wt) copy and leave the deleted allele in the chromosome.

3.10.4. Complementation of mutants with the wild-type gene

The pMRB24 vector (Le Roux *et al*., 2011; Fig. 3.7) was used to complement the allelic exchange mutants. The region including the entire gene and promoter to be complemented were amplified and cloned into the vector, adding a *Bam*HI restriction site to primer sequences. The recombinant DNA was transformed into the *E. coli* S17-1-λpir strain and cells were plated onto LB supplemented with Cm at a concentration of 20 µg/ml. After conjugation for 48 h in TSA made with seawater, complemented cells were selected on TCBS agar plates containing Cm (12 µg/ml). The presence of the plasmid in *P. damselae* subsp. *damselae* strains was confirmed by PCR. Primers used in the complementation process are shown in Table 3.6.

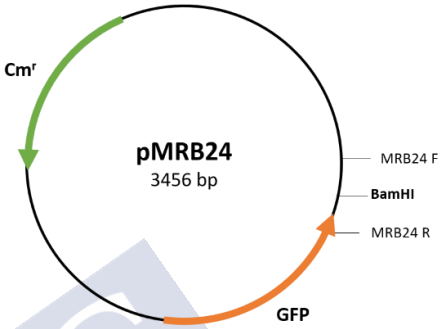

Figure 3.7: pMRB24 plasmid map. This plasmid is used for complementation.

Table 3.6: Primers used to complement allelic exchange deletion mutants.

Primer	Sequence (5'-3')*	Function
5' complem wza BamHI (bis)	GCGGATCCGCAAATAGTGCAGCAATAGC	Complementation
3' complem wzc BamHI	GCGGATCCCACCACCAGTAACGAGAATT	
MRB24_F	CGCCTGCTATATGCTTGCAT	Checking cloned sequence
MRB24_R	GCTGCTGGGATTACACATGG	

* Restriction sites are highlighted in primer sequences.

3.11. MICROSCOPY

3.11.1. Stereo microscopy

For the visualisation of colony morphology, a single colony of each *P. damselae* subsp. *damselae* strain grown on a TSA-1 plate was collected with the tip of a rounded wooden spike and seeded on a new TSA-1 plate where Congo red was added at a concentration of 0.01%

[wt/vol] for facilitating visualisation. After incubation at 25ºC during 24 h, colonies were observed under a stereo microscope (Leica M205FA) equipped with a digital camera (Leica 7000T) with 12.5x of magnification. Images were taken with bright field (BF) diascopic illumination at an exposure time of 8 ms.

3.11.2. Scanning electron microscopy (SEM)

For scanning electron microscopy (SEM), exponentially growing cultures of *P. damselae* subsp. *damselae* RM-71 in TSB-1 at 25 and 37ºC were stopped when they reached an OD_{600} of 0.55. Cells were carefully pelleted down by centrifugation (4000 *g*) and fixed for 3 h at 4ºC in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and postfixed for 1.5 h in 1% osmium tetroxide in the same buffer. Samples were washed three times in distilled water, dehydrated using a series of graded ethyl alcohols, chemically dried using hexamethyldisilazane (HMDS, Sigma), sputter-coated with iridium, and viewed and photographed in an Ultra Plus ZEISS scanning electron microscope.

3.11.3. Transmission electron microscopy (TEM)

For transmission electron microscopy (TEM), *P. damselae* subsp. *damselae* RM-71 and derivative strains were grown in TSA-1 for 24 h at 25ºC. Then a colony was suspended in TSB-1 and the culture was let to grow until an OD_{600} of approximately 0.5. Serial dilutions were spread on TSA-1 plates and grown overnight at 25º in order to obtain isolated colonies. 10 ml of a fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde and 0.075% ruthenium red in 0.1 M cacodylate buffer (pH 7.4) were poured on each plate and after 3 h at room temperature, colonies were gently detached from the agar, washed three times with 0.1 M cacodylate buffer (pH 7.4) containing 0.075% ruthenium red and fixed for 1 h with 1% osmium tetroxide, 0.075% ruthenium red in 0.1 M cacodylate buffer (pH 7.4). Colonies were washed with 0.1 M cacodylate buffer (pH 7.4) containing 0.075% ruthenium red, dehydrated through a graded series of ethanol and embedded in Epon resin. Ultrathin sections (40–60 nm thickness) were obtained on an RMC Ultramicrotome (PowerTome) using Diatome

diamond knifes, contrasted with 2% uranyl acetate for 5 min and observed under a JEOL JEM 1400 TEM. Images were digitally recorded using a CCD digital camera Orius 1100 W (Gatan Inc.). Capsule thickness of the RM-71 wt and Δ*yjbH* strains was determined by measuring the capsule of 30 cells per strain using the Fiji software (ImageJ version 1.51n; Schindelin *et al.*, 2012). For each individual cell, the capsule was measured at six different points and the capsule thickness for each cell was calculated as the average of the six measurements. Data were subjected to an unpaired t test with Welch's correction.

3.12. MOTILITY ASSAY

Motility was measured by a swim migration assay (Adler *et al.*, 1973). In this test, bacteria move because of an amino acid gradient generated by their own metabolism. Overnight cultures of each strain were diluted to an OD_{600} of 0.15 to inoculate 4 µl into the middle of a semi-solid TSA-1 plate (with a volume of 25 ml) containing 0.25% agar by vertically stabbing the agar half-way through and then gently releasing the culture while pulling the pipette tip out. Plates were incubated for 24 h at 25ºC and diameter of growth haloes were measured. Data from 15 independent measurements per strain were analysed with an ordinary One-way ANOVA test.

3.13. BIOFILM FORMATION

P. damselae subsp. *damselae* wt strain RM-71 and mutant strains were grown overnight at 25ºC in M9 minimal medium (Miller and Miller., 1972) supplemented with 0.2% (wt/vol) Casamino Acids (Bacto; CM9), 0.5% glucose (wt/vol) and NaCl adjusted to a final concentration of 1%. These overnight cultures were diluted to an OD_{600} of 0.3 and aliquots of 100 µl of each strain were allowed to grow statically in 96-well plates (Deltalab) for 24 h. Subsequently, the cultures were neatly removed and each well was washed thrice with sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, KH_2PO_4 2 mM, pH 7.4) to remove any poorly adherent cells. Biofilms were stained by adding crystal violet at a concentration of 0.1% (wt/vol; Panreac) followed by incubation at room temperature for 30 min.

Biofilms were quantified by solubilisation with ethanol and measurement of absorbance at 570 nm (A570). Experiments were carried out three times with 6 replicas per strain per time. One-way ANOVA for non-parametric data (Kruskall-Wallis test) was used to analyse the data.

3.14. ASSAYS OF HAEMOLYSIS AND PHOSPHOLIPASE ACTIVITIES

In *P. damselae* subsp. *damselae*, haemolytic activity is attributable to the synergistic and additive effects of the cytotoxins Dly, PhlyP and PhlyC. Phospholipase activity is mainly attributable to Dly toxin (a phospholipase D) with a minor contribution of phospholipase PlpV (Rivas *et al.*, 2013; Vences *et al.*, 2017). For the agar plate haemolysis assays, a single colony of each strain grown on a TSA-1 plate was collected with the tip of a rounded wooden spike and seeded on sheep blood agar plates (Oxoid), incubated at 25ºC and photographed after 24 h (Fig. 3.8). For phospholipase activity assays, the same procedure was followed with the modification that colonies were instead seeded on TSA-1 agar plates supplemented with 3% egg yolk extract (Oxoid; Fig. 3.8).

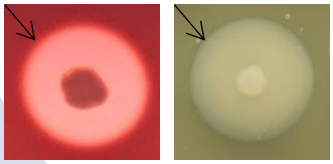

Figure 3.8: *P. damselae* subsp. *damselae* strain with haemolytic activity in blood agar (left) and bacterial strain with phospholipase activity in TSA-1 supplemented with 3% egg yolk extract (right).

3.15. SURVIVAL IN FISH SERUM AND MUCUS

The sensitivity of the different *P. damselae* subsp. *damselae* strains to turbot (*P. maxima*) serum or mucus was assessed with bacteria grown in microtiter plates, as described in previous studies (Sanjuán and Amaro, 2004), with modifications. Mucus was collected from 250 g fish by gently scraping the skin and subsequently centrifuged for 5 min (2655 *g*) to remove particulate material. The supernatant was dissolved in sterile saline solution (0.85%) in a ratio 1:1, filter-sterilised onto 0.22 µm and stored at -20ºC before use. Serum was aseptically obtained by blood extraction through a caudal vein puncture and was allowed to clot overnight at 4ºC. Serum was stored at -20ºC until use.

In each well of microtiter plates, 50 µl of turbot serum or mucus suspension were mixed with 50 µl of each bacterial suspension (10^3-10^4 colony forming units (CFU)/ml) in TSB-1. The assays were performed in triplicate, and samples were taken after 0, 1.5, 3 and 4.5 h of incubation at 25ºC. Viable counts were calculated by drop plating of 5 µl aliquots of 10-fold dilutions on TSA-1. The results were subjected to a Two-way ANOVA with an alpha value of 0.05 for the analysis.

3.16. VIRULENCE CHALLENGE

To test the impact of *wza*, *wzc* and *yjbH* deletions in virulence of *P. damselae* subsp. *damselae* for fish, *in vivo* assays were carried out using turbot (*P. maxima*; weighing 3.8 ± 1.10 g) and gilthead sea bream (*S. aurata*; weighing 8.8 ± 1.40 g). As a control, the *rstA* deletion mutant, which was previously demonstrated to be strongly attenuated in virulence (Terceti *et al.*, 2019), was also inoculated. Groups of 10 fish per strain tested were acclimated in 100 l aquaria at 24ºC for 2 days before performing the assays. Fish were inoculated intraperitoneally with 0.1 ml of bacterial suspensions of each strain in 0.85% NaCl solution at sharply adjusted doses of 2×10^7 (for gilthead sea bream) and 2×10^6 (for turbot) CFU/fish. A control group of 10 fish of each species was inoculated with 0.1 ml of sterile 0.85% NaCl solution. Fish mortality was recorded daily for 10 days after inoculation. Reisolation on TSA-1 and TCBS agar plates and identification of the bacteria from the kidney of dead fish were performed. Colonies were confirmed by the subsp. *damselae*-specific *ureD* gene PCR test as previously described (Osorio *et al.*, 2000). The protocols of animal experimentation used in this study have been reviewed and approved by the Animal Ethic Committee of the Universidade de Santiago de Compostela.

Table 3.7: Primers used to check the presence of *P. damselae* subsp. *damselae* in kidney of fish experimentally infected.

Primer	Sequence (5'-3')*	Function
ureD_F	TCATACGTATTTACTGCATC	Checking *P. damselae* subsp. *damselae* presence
ureD_R	AGATAAAGTGATTCAAGAGA	

4. RESULTS

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4. RESULTS

4.1. STUDY OF THE TRANSCRIPTOMIC AND PHENOTYPIC RESPONSES OF *P. DAMSELAE* SUBSP. *DAMSELAE* CULTURED AT 25 IN COMPARISON TO 15ºC

In the history of *P. damselae* subsp. *damselae* as a causative agent of disease in reared marine animals, sudden increases of seawater temperature, often during summer months, have been frequently reported to be associated to the emerging of outbreaks (Fouz *et al.*, 1992; Pedersen *et al.*, 1997; Eissa *et al.*, 2018; Aguilera-Rivera *et al.*, 2019; Zhang *et al.*, 2019). Previous studies have highlighted the fact that the disease caused by this pathogen is spread through water but only when it is warm enough, that is from temperatures around 22ºC (Fouz *et al.*, 2000). On the other hand, *P. damselae* subsp. *damselae* isolates maintained at 14 to 22ºC, even under starvation conditions, conserve their infectivity potential. At a temperature as low as 5ºC, cells were not culturable but they recovered when the temperature was risen to 22ºC (Fouz *et al.*, 1998).

Evidence suggests that marine outbreaks occur when a heterogenous population of *P. damselae* subsp. *damselae* takes advantage of favourable environmental conditions to cause disease (Terceti *et al.*, 2016, 2018). Despite the importance of temperature in *P. damselae* subsp. *damselae* biology, little is known about how it modulates transcriptome, fitness, and pathogenicity. In this study, we aim to reveal differential aspects and changes this bacterium undergoes when growing at a temperature typical from winter months in mid latitudes (15ºC), comparing to the optimal and closer to the temperature at which outbreaks develop (25ºC).

4.1.1.Comparative analysis of *P. damselae* subsp. *damselae* growth curves at 15 and 25ºC

Growth of *P. damselae* subsp. *damselae* strain RM-71 was analysed in 48-h continuous cultures at 15 and 25 ºC in TSB-1 medium. The two assayed temperatures simulated an *a priori* non-risky condition (15ºC) and warm water episodes that trigger aquaculture outbreaks (25ºC). The beginning of the exponential phase was delayed at 15ºC compared to growth at 25ºC and there was a great difference between optical density at 600 nm (OD_{600}) after 15 h of cultivation at 15ºC (OD_{600}: 0.129) and 25ºC (OD_{600}: 0.527) (Fig. 4.1). These observations suggest that 25ºC is closer to the optimal growth temperature of *P. damselae* subsp. *damselae* than 15ºC.

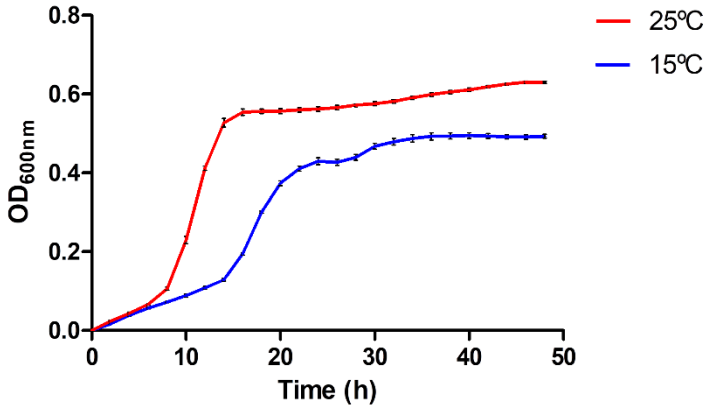

Figure 4.1: The influence of temperature cultivation in growth dynamics of *P. damselae* subsp. *damselae*. Growth of RM-71 strain was assayed at 15ºC and 25ºC in TSB-1 for 48 h. Vertical error bars represent standard deviation of biological triplicates.

4.1.2.Analysis of fatty acid methyl esters (FAMEs) produced at 15 and 25ºC

Bacteria are able to cope with fluctuations in environmental temperature through modifying the ratio of saturated and unsaturated fatty acids in membranes (Marr and Ingraham, 1962). We evaluated membrane fatty acid profiles in *P. damselae* subsp. *damselae* cultured at 15ºC (Table 4.1) and 25ºC (Table 4.2) by a fatty acid methyl esters

(FAME) analysis. As it can be seen from the tables, the profile of fatty acids encountered in membranes from cultures at 25ºC is more diverse than that of cultures at 15ºC, and includes a great number of unique species. The percentage of saturated fatty acids is slightly higher at 25ºC, yet most predominant fatty acids (16:1 w7c and 16:1 w6c) are the same when *P. damselae* subsp. *damselae* is cultured at 15 or 25ºC.

Table 4.1: Fatty acid composition of *P. damselae* subsp. *damselae* strain RM-71 cultured at 15ºC.

Fatty acid	Percentage
10:0 3OH	0.11
12:0	3.37
12:0 2OH	0.14
12:0 3OH	2.75
14:0	2.80
Sum In Feature 2 (14:0 3OH/16:1 iso I)	3.03
Sum In Feature 3 (16:1 w7c/16:1 w6c)	43.77
16:1 w5c	0.41
16:0	14.15
17:0	0.23
18:1 w9c	1.55
Sum In Feature 8 (18:1 w7c)	25.67
18:1 w5c	0.52
18:0	1.52

Table 4.2: Fatty acid composition of *P. damselae* subsp. *damselae* strain RM-71 cultured at 25°C.

Fatty acid	Percentage
Sum In Feature 2 (unknown 10.928)	0.09
10:0 3OH	0.07
12:0	3.16
11:0 3OH	0.17
13:0	0.05
12:0 2OH	0.1
12:0 3OH	2.58
14:0	3.2
Sum In Feature 1 (13:0 3OH/15:1 i H)	0.17
15:1 w8c	0.13
Sum In Feature 2 (14:0 3OH/16:1 iso I)	2.67
Sum In Feature 3 (16:1 w7c/16:1 w6c)	38.83
16:1 w5c	0.3
16:0	17.6
17:1 w8c	0.7
17:1 w6c	0.34
17:0	0.92
18:1 w9c	0.38
Sum In Feature 8 (18:1 w7c)	25.76
18:1 w5c	0.26
18:0	1.71
18:1 w7c 11-methyl	0.68
20:1 w7c	0.16
Summed In Feature 2 (12:0 aldehyde)	2.76

4.1.3.Comparative transcriptomic study of *P. damselae* subsp. *damselae* cultured at 15 and 25ºC: genes upregulated at 25ºC

P. damselae subsp. *damselae* strain RM-71 was grown at 15 and 25ºC, and cDNA prepared from mRNA that was isolated from cultures at the two different temperatures was subjected to Illumina sequencing. Growth at 15ºC was defined as the control condition. The comparative analyses of the transcriptomes at 15 and 25ºC resulted in a total of 1195 differentially expressed genes (DEGs): 641 genes with lower expression at 25ºC (FC lower than -1.5) and 554 genes with higher expression at 25ºC (FC higher than 1.5; Fig. 4.2).

The complete list of DEGs can be found in the following links: genes mapped to CIP102761 and genes mapped to RM-71.

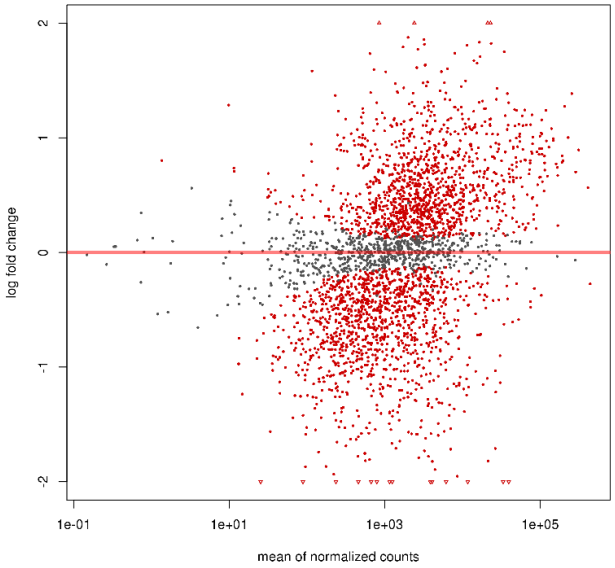

Figure 4.2: Smear plot of differentially expressed genes (DEGs) in *P. damselae* subsp. *damselae* RM-71 exposed to two temperatures, 15 and 25ºC. The smear plot shows the relationship between the log Fold Change (FC) and mean of normalised counts. Grey points represent genes with non-significant changes in expression, whereas red points represent genes that are significantly differentially expressed.

Similar to other members of the *Vibrionaceae* family, *P. damselae* subsp. *damselae* RM-71 contains two chromosomes. In addition, this strain harbours the virulence plasmid pPHDD1 (Rivas *et al.*, 2011). Using type strain CIP102761 complete sequences of chromosomes I and II (ChrI and ChrII), and the complete pPHDD1 plasmid sequence of strain RM-71 (GenBank Acc. No. NC_014653) as references, we distributed the DEGs into each replicon. Notably, we observed an imbalance in the number of DEGs between the two chromosomes (Fig. 4.3). In ChrI similar numbers of DEGs are upregulated and downregulated at 25ºC. However, ChrII contains 204 downregulated genes and only 77 upregulated genes at 25ºC. Interestingly, among the 31 DEGs in the virulence plasmid pPHDD1, 24 corresponded to genes whose expression is upregulated at 25ºC.

Growth at 25ºC resulted in the upregulation of 533 genes that mapped to the genome of the type strain (genes mapped to CIP102761) and of 21 additional genes unique to strain RM-71 (genes mapped to RM-71). A list of the 50 top DEGs upregulated at 25ºC plus additional selected genes is shown in Table 4.3. Main functions upregulated at 25ºC are described in the following sections.

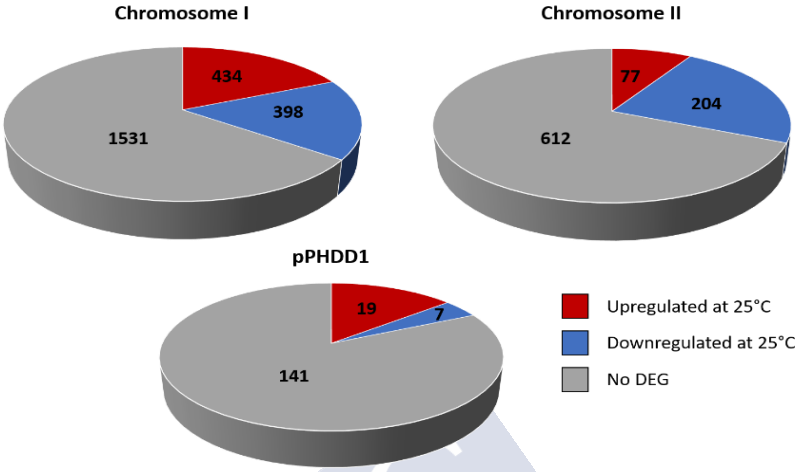

Figure 4.3: Graphical representation of DEGs distribution among the two *P. damselae* subsp. *damselae* chromosomes and pPHDD1 virulence plasmid. Numbers denote differentially expressed genes (DEGs) upregulated at 25°C (red), downregulated at 25°C (blue), and genes not differentially regulated (grey).

Table 4.3: List of selected differentially expressed genes (DEGs) with enhanced expression at 25°C including the 50 top upregulated genes at 25°C. Enhanced expression at 25°C is denoted by positive Fold Change (FC) values. Genes with VDA codes correspond to the annotation in the CIP102761 genome, and genes with A0J47 codes correspond to the annotation in the RM-71 genome.

Gene ID	Product/Function	Fold Change	p-value	Location
Nutrient acquisition/metabolism				
VDA_001789	Nucleoside permease NupC	5.1	2.2182E -122	ChrI
VDA_001833	GPR1/FUN34/yaaH putative acetate transporter	3.6	5.07132 E-17	ChrI
VDA_002532	Porin	5.1	1.08929 E-77	ChrI
VDA_003254	Porin	3.0	3.53973 E-49	ChrI
VDA_001005	Porin	2.5	3.2873E -69	ChrII
VDA_003133	Glutamine synthetase type I	4.7	2.8174E -108	ChrI
VDA_000463	L-asparaginase	3.3	8.16042	ChrII

			E-85	
VDA_003226	Glucosamine fructose-6-phosphate aminotransferase	3.5	3.71313 E-35	ChrI
VDA_001560	Aspartate/tyrosine/aromatic aminotransferase	2.8	1.19637 E-65	ChrI
A0J47_09785	Putative trypsin superfamily protein	4.3	4.6629E -132	ChrI
VDA_002568	Long-chain fatty acid transport protein	2.5	4.75483 E-39	ChrI
VDA_000298	Arginine decarboxylase catabolic	2.6	7.62289 E-83	ChrII
VDA_002194	Manganese-dependent inorganic pyrophosphatase	2.5	2.24757 E-83	ChrI
VDA_003183	Oligopeptidase A	2.7	2.38104 E-73	ChrI
VDA_003148	Vitamin uptake transporter	2.6	6.42707 E-28	ChrI
DNA synthesis and repair				
VDA_002372	Ribonucleotide reductase of class Ia (aerobic) alpha subunit	3.5	1.03533 E-70	ChrI
VDA_001894	Ribonucleotide reductase of class II (coenzyme B12-dependent)	3.1	6.75604 E-90	ChrI
VDA_003108	Outer membrane vitamin B12 receptor BtuB	3.0	9.66178 E-20	ChrI
VDA_001405	Uracil phosphoribosyltransferase	3.0	1.51133 E-50	ChrI
Translation				
VDA_003390	LSU ribosomal protein L31p	2.7	1.98928 E-09	ChrI
VDA_003099	Translation elongation factor Tu	2.6	3.07515 E-36	ChrI
VDA_002952	SSU ribosomal protein S21p	2.6	1.9713E -24	ChrI
Virulence and antimicrobial resistance				
VDA_000110	Serum resistance protein Vep07-like	2.0	5.76819 E-33	pPHDD1

VDA_000111	Transferrin receptor Vep20-like	3.0	5.67092 E-76	pPHDD1
VDA_000113	OmpU	2.5	4.19858 E-66	pPHDD1
VDA_000794	TonB-dependent siderophore receptor	2.8	5.7247E -111	ChrII
VDA_000157	TolC	2.8	2.115E-103	pPHDD1
VDA_000158	AcrA/MacA-like membrane fusion protein	3.2	7.9453E -112	pPHDD1
A0J47_18110	Unknown protein related to T6SS	2.8	1.0749E -24	pPHDD1
A0J47_18115	RNase toxin Ntox44	2.7	9.41221 E-55	pPHDD1
A0J47_18120	Proline-alanine-alanine-arginine (PAAR) domain protein	2.9	1.3511E -60	pPHDD1
Motility and chemotaxis				
VDA_003029	Flagellar protein MotX	2.6	9.54565 E-60	ChrI
VDA_002607	Flagellin protein FlaB	3.6	1.0743E -118	ChrI
VDA_002671	Flagellar motor rotation protein MotA	2.8	5.26275 E-79	ChrI
VDA_002604	Flagellar biosynthesis protein FliS	2.7	6.88724 E-93	ChrI
VDA_003044	Methyl-accepting chemotaxis protein	3.1	7.48432 E-68	ChrI
VDA_001198	Methyl-accepting chemotaxis protein	2.7	1.10855 E-85	ChrI
Stress response and defence mechanisms				
VDA_003059	Chaperonin complex GroEL-GroES	3.1	2.1437E -116	ChrI
VDA_003060	Chaperonin complex GroEL-GroES	3.5	1.517E-130	ChrI
VDA_002771	DnaK chaperonin	3.4	7.7384E -130	ChrI
VDA_001553	Peptidyl-prolyl cis-trans isomerase PpiD	2.5	3.30237 E-65	ChrI
VDA_002523	HtpG chaperonin	2.8	3.0531E -107	ChrI
VDA_001325	ClpB chaperonin	2.5	3.25923 E-77	ChrI
VDA_003124	Ribosome associated heat shock protein	3.0	1.98913 E-71	ChrI

VDA_003529	Protease DegP	3.3	1.13294 E-15	ChrI
VDA_003386	ATP-dependent protease HslV	3.1	4.0286E -132	ChrI
VDA_001154	Peroxidase	4.9	6.6194E -111	ChrII
VDA_000806	Peroxidase	3.5	5.64336 E-61	ChrII
VDA_000771	Iron-sulfur cluster assembly protein SufB	2.6	1.95494 E-63	ChrII
Transcriptional regulation and signalling				
VDA_003227	DeoR family transcriptional regulator	3.3	1.4446E -35	ChrI
VDA_001088	XRE family regulator	2.6	7.86116 E-25	ChrII
VDA_002825	Cyclic-di-GMP phosphodiesterase A	3.7	6.3346E -128	ChrI
Cell wall/membrane/envelope biogenesis				
VDA_003228	N-acetylglucosamine-1-phosphate uridyltransferase GlmU	2.7	4.3747E -86	ChrI
Hypothetical proteins of unknown function				
VDA_003431	Hypothetical protein	3.4	3.96176 E-86	ChrI
VDA_000943	Hypothetical protein	2.6	3.53416 E-24	ChrII
VDA_000598	Hypothetical protein	2.5	8.47329 E-75	ChrII

4.1.3.1. *Nutrient acquisition and metabolism*

Genes encoding membrane proteins, nutrient transporters and porins were upregulated at 25ºC (Table 4.3). The nucleoside permease NupC (VDA_001789) was the most upregulated gene at 25ºC. Growth at 25ºC upregulated two ribonucleotide reductases belonging to class Ia and II, one of them, VDA_001894, is coenzyme B12-dependent. In accordance, the vitamin B12 receptor BtuB was also upregulated (Table 4.3). Upregulation of the uracyl phosphoribosyltransferase VDA_001405, an enzyme necessary for the synthesis of precursors of all pyrimidine nucleotides, was also observed. Among upregulated amino acid biosynthesis enzymes and aminotransferases was the

glutamine synthetase type I, which has a central role in amino acid biosynthesis. The putative trypsin superfamily protein encoded by A0J47_09785, which has a possible function in peptide degradation, as well as a number of ribosomal and translation-related proteins were upregulated suggesting that growth at 25ºC enhances protein synthesis and renovation.

P. damselae subsp. *damselae* degrades extracellular lipids, which may serve as carbon and energy sources (Fouz *et al.*, 1992; Pedersen *et al.*, 1997; Vences *et al.*, 2017). An operon encoding an extracellular lipase (VDA_001610) and a fatty acid transporter FadL (VDA_001611) were 2-fold upregulated at 25ºC, and so was the long-chain fatty acid transporter VDA_002568 (Table 4.3).

Iron acquisition plays a role in *P. damselae subsp. damselae* virulence for fish (Fouz *et al.*, 1994; Fouz *et al.*, 1997). Our analysis unveiled upregulation at 25ºC of a pPHDD1 plasmid-borne gene that encodes a putative transferrin binding protein (VDA_000111), and of VDA_000794 encoding a TonB-dependent siderophore receptor.

4.1.3.2.*Motility and chemotaxis related genes*

Motility and tissue colonisation constitute important factors in *P. damselae* subsp. *damselae* pathogenicity (Fouz *et al.*, 1998, 2000). Four flagellum-related genes were found among the 50 most upregulated genes (Table 1). Additional upregulated genes included flagellar hook protein FlgE (VDA_002616), flagellar motor rotation protein MotB (VDA_002670), flagellar hook-associated protein FlgK (VDA_002609) and flagellar basal-body rod modification protein FlgD (VDA_002617). Notably, two chemotaxis-related genes were found among the 50 most upregulated genes at 25ºC and correspond to the methyl-accepting chemotaxis proteins VDA_003044 and VDA_001198 (Table 4.3).

4.1.3.3. *Genes involved in stress responses*

A number of chaperones, heat shock and stress-related proteins were listed among the top 50 DEGs (Table 4.3), suggesting that in comparison to growth at 15ºC, growth at 25ºC constitutes a heat stress condition. Upregulation was also found for DnaJ chaperonin

(VDA_002770), heat shock protein GrpE (VDA_002772) and heat-shock chaperonin (VDA_003125). Upregulated proteases included the outer membrane stress sensor protease DegP (VDA_003529) with high identity to *Vibrio cholerae* protease DegP (VC0566) which affects biofilm formation, intestinal colonisation and correct function of the type II secretion system (T2SS; Altindis *et al.*, 2014). Also among this set of genes, ATP-dependent protease HslV, two peroxidases and the iron-sulfur cluster assembly protein SufB were included. SufB synthesizes Fe-S clusters that act as cofactors in cellular processes under conditions of iron starvation or oxidative stress in *E. coli* (Outten *et al.*, 2004).

4.1.3.4.*Transcriptional regulators and intracellular signalling*

A DeoR family transcriptional regulator and a XRE family regulator were listed within the top-50 upregulated DEGs. Some DeoR regulators are involved in bacterial intracellular growth (Haghjoo and Galan, 2007; Morris *et al.*, 2013). Cyclic-di-GMP is an intracellular second messenger involved in environmental signalling that regulates a number of phenotypes in bacteria, such as motility and biofilm (Ayala *et al.*, 2015; Pursley *et al.*, 2018). VDA_002825, encoding a cyclic-di-GMP phosphodiesterase A, was 3.67-fold upregulated at 25 ºC. The two-component system (TCS) RstAB, a major positive regulator of virulence in *P. damselae* subsp. *damselae* (Terceti *et al.*, 2017; Terceti *et al.*, 2019), was not significantly affected by temperature.

4.1.3.5. *Type II secretion system (T2SS)*

The complete cluster of *eps* (extracellular protein secretion) genes (VDA_003114-VDA_003123), encoding part of the T2SS machinery, which is proved to be involved in cytotoxin secretion (Rivas *et al.*, 2015a; Vences *et al.*, 2017) was moderately upregulated at 25ºC relative to 15ºC.

4.1.3.6.*pPHDD1 uncharacterised potential virulence factors*

The analysis of the differential gene expression profiles along the pPHDD1 plasmid at 15 and 25ºC brought to the forefront a collection of genes upregulated at 25ºC plasmid-encoded genes which

have putative roles in virulence and will surely deserve special attention in future studies (Table 4.4). A schematic representation of these genes and their organisation in plasmid regions is shown in Fig. 4.4.

Table 4.4: List of Differentially Expressed Genes (DEGs) upregulated at 25ºC within the virulence plasmid pPHDD1. Genes with VDA codes correspond to the annotation in the CIP102761 genome, and genes with A0J47 codes correspond to the annotation in the RM-71 genome.

ORF	FC	Predicted function	Characterised homologues	Reference
VDA_000108	1.66	kinase phosphorylase	-	-
VDA_000110	1.95	putative toxin with a role in virulence	*Vibrio vulnificus* Vep07 (36% id)	Roig and Amaro, 2009
VDA_000111	2.98	TonB-dependent hemin ferrichrome receptor with a role in virulence	*Vibrio vulnificus* Vep20 (60% id)	Pajuelo *et al.*, 2015
VDA_000112	2.45	hypothetical protein	-	-
VDA_000113	2.47	putative virulence factor porin OmpU	*Vibrio tasmaniensis* OmpU (30% id)	Duperthuy *et al.*, 2010, 2011
VDA_000118	1.73	outer membrane porin	-	-
VDA_000121	2.17	hypothetical protein	-	-
VDA_000138	1.64	protein for plasmid conjugative trasfer, TraI	*Vibrio tapetis* TraI (40% id)	Erauso *et al.*, 2011
VDA_000142	1.92	DNA-binding protein for plasmid replication	-	-
VDA_000143	1.79	DNA-binding protein H-NS for transcriptional regulation	*Yersinia enterocolitica* H-NS (57,8% id)	Baños *et al.*, 2008
VDA_000144	1.87	acetate/propionate kinase	-	-
VDA_000145	1.51	protein that contains a domain of unknown function DUF2913	-	-
VDA_000154	2.42	ATPase component of a tripartite	*E. coli* MacB (52% id)	Kobayashi *et al.*, 2001

		pump for multidrug efflux and toxin secretion, MacB		
VDA_000155	2.08	permease component of a tripartite pump for multidrug efflux and toxin secretion	-	-
VDA_000156	2.41	permease component of a tripartite pump for multidrug efflux and toxin secretion	-	-
VDA_000157	2.82	outer membrane protein of a tripartite pump for multidrug efflux and toxin secretion, TolC	*Vibrio vulnificus* TolC (69% id)	Lee *et al.*, 2013
VDA_000158	3.19	membrane-fusion protein of a tripartite pump for multidrug efflux and toxin secretion, MacA	*E. coli* MacA (22% id)	Modali and Zgurskaya, 2011
VDA_000160	1.59	cytolysin and hemolysin HlyA Pore-forming toxin	characterised in *P. damselae* subsp. *damselae*	Rivas *et al.*, 2015b
A0J47_18110	2.77	hypothetical protein involved in type 6 secretion system	-	-
A0J47_18115	2.67	RNase toxin with a putative role in virulence Ntox44	-	-
A0J47_18120	2.92	PAAR domain-containing protein of the type 6 secretion system	*Vibrio cholerae* PAAR protein (33% id)	Shneider *et al.*, 2013

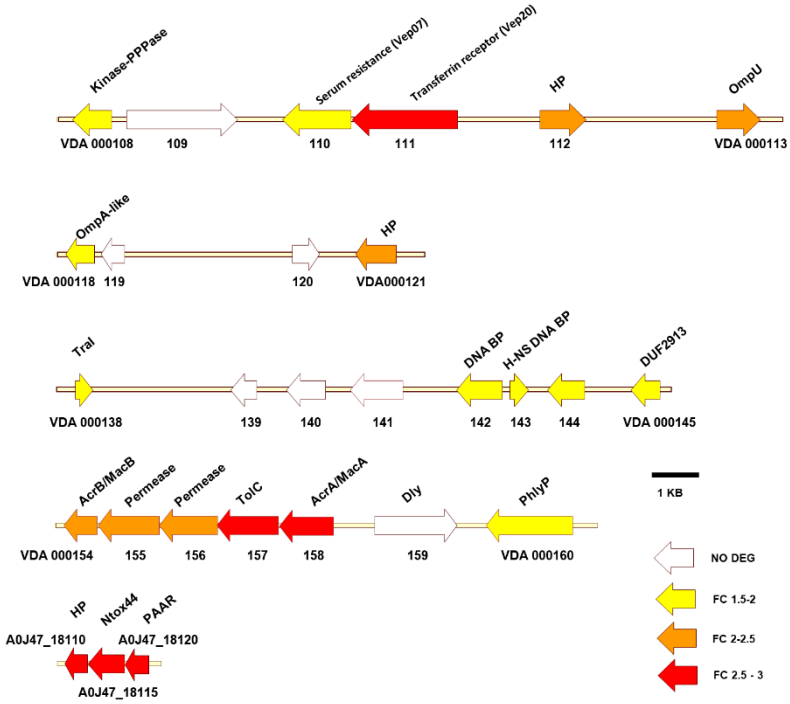

Figure 4.4: Potential virulence factors encoded within pPHDD1 plasmid are upregulated at 25˚C. Plasmid genes upregulated at 25˚C are mainly distributed along 5 plasmid regions. Numbers denote the VDA gene codes of the type strain CIP102761. Genes with A0J47 labels correspond to genes unique to RM-71. Colour codes of genes (represented as arrows) denote each of the three ranges of FC values, whereas white arrows denote genes that are not differentially expressed (NO DEG).

A cluster of 5 upregulated genes (VDA_000154 to VDA_000158) encode the TolC and AcrAB proteins, that form a tripartite multidrug and toxin secretion efflux pump, plus two additional proteins. VDA_000110 is 36% identical to *V. vulnificus* Vep07, an outer membrane lipoprotein that confers resistance to eel serum (Roig and Amaro, 2009). VDA_000113 is homologous to OmpU, a protein with an essential role in pathogenicity in several *Vibrio* species (Goo *et al.*, 2006; Duperthuy *et al.*, 2010, 2011). The above-mentioned

VDA_000111 shares 60% identity with the *V. vulnificus* Vep20 that acts as a transferrin and has a role in virulence (Pajuelo *et al.*, 2015).

The three genes A0J47_18120, A0J47_18115 and A0J47_18110 are exclusive of RM-71 and have no known homologues in other pPHDD1-containing strains of *P. damselae* subsp. *damselae* studied so far. However, the functions of homologues genes are related to survival and type VI secretion system (T6SS)-related functions (Zhang *et al.*, 2012).

4.1.4.Comparative transcriptomic study of *P. damselae* subsp. *damselae* cultured at 15 and 25ºC: genes downregulated at 25ºC

Growth at 25ºC resulted in the downregulation of 614 genes that mapped to the genome of the type strain (genes mapped to CIP102761) and of 27 additional genes unique to strain RM-71 (genes mapped to RM-71), whose changes in expression are denoted by a negative FC value. A list of the top-50 downregulated genes at 25ºC, plus additional genes, is shown in Table 4.5 and reveals an important number of loci organised in operons.

Table 4.5: List of the top differentially expressed genes (DEGs) in *P. damselae subsp. damselae* with lower expression at 25ºC than at 15ºC. Note that downregulated expression at 25ºC is denoted by negative Fold Change (FC) values.

Gene ID	Product/Function	Fold Change	p-value	Location
Cell wall/membrane/envelope biogenesis				
VDA_001578	Stage V sporulation protein SpoVR family	-13.6	9.67E-189	ChrI
VDA_001762	Lysophosphatidic acid acyltransferase PlsC	-3.2	1.26692E-50	ChrI
VDA_002031	LrgB-family protein	-3.1	1.3247E-117	ChrI
Various/unknown function				
VDA_001579	Domain of unknown function, 444 superfamily	-28.2	0.0	ChrI
VDA_001580	PrkA-family serine protein kinase	-24.1	6.1873E-268	ChrI
VDA_001764	DedA superfamily member	-5.4	3.85608E-13	ChrI
Glycine betaine transport				
VDA_002013	Glycine betaine	-7.8	1.07E-130	ChrI

	transporter			
Nutrient transport and metabolism				
VDA_001763	Putative cyanophicin synthetase	-7.1	2.98741E-37	ChrI
VDA_001723	Dipeptidase	-3.7	9.2245E-150	ChrI
VDA_000377	Metallopeptidase M24 family	-4.3	1.10747E-42	ChrII
VDA_001632	Oligopeptide ABC transporter OppA	-4.2	9.9638E-59	ChrI
VDA_001633	Oligopeptide ABC transporter OppB	-3.5	1.21575E-52	ChrI
VDA_001634	Oligopeptide ABC transporter OppC	-3.0	7.71823E-42	ChrI
VDA_001635	Oligopeptide ABC transporter OppD	-2.8	5.5165E-35	ChrI
VDA_001636	Oligopeptide ABC transporter OppF	-3.3	6.69827E-54	ChrI
VDA_001382	Methionine ABC transporter, substrate binding component	-3.3	4.52395E-44	ChrI
VDA_001251	Iron-molybdenum cluster-binding protein with NifB/NifX domain	-6.1	1.2384E-133	ChrI
VDA_001252	NADH:quinone oxidoreductase NqrM	-3.0	2.51284E-19	ChrI
VDA_002257	Alanine dehydrogenase	-4.3	7.76228E-76	ChrI
VDA_001997	Agmatinase	-3.9	9.89728E-95	ChrI
VDA_001605	Aspartate-semialdehyde dehydrogenase	-2.1	3.49578E-26	ChrI
VDA_002504	Citrate synthase	-3.5	5.08558E-45	ChrI
VDA_002724	Malate synthase	-3.1	8.95602E-40	ChrI
VDA_002723	Isocitrate lyase	-3.0	3.72706E-32	ChrI
VDA_000495	Putative hydrolase, alkyl/aryl sulfatase	-4.1	2.35121E-79	ChrII
VDA_002425	Acetoacetyl-CoA-reductase	-3.7	4.67185E-25	ChrI
VDA_002349	Alpha acetolactate decarboxylase	-3.3	3.9868E-50	ChrI
VDA_001166	Alpha-1,2-	-3.2	1.30795E-	ChrII

	mannosidase		38	
Stress response and host defence				
VDA_003169	Cold-shock protein	-4.6	8.21889E-58	ChrI
VDA_000629	RpoS	-3.5	5.16984E-57	ChrII
VDA_001327	Glutamate decarboxylase	-3.3	9.17337E-45	ChrI
VDA_001328	Glutaminase	-3.4	2.2451E-42	ChrI
VDA_001329	Glutamate/GABA antiporter	-3.0	7.67865E-16	ChrI
VDA_001116	Multidrug resistance efflux pump	-3.5	6.15611E-72	ChrII
VDA_000570	Methionine sulfoxide reductase MsrQ	-3.1	9.25296E-34	ChrII
VDA_000571	Methionine sulfoxide reductase MsrP	-4.6	1.28296E-51	ChrII
VDA_001099	Cu-Zn Superoxide dismutase	-3.1	1.9361E-122	ChrII
Virulence factors				
VDA_002242	Phospholipase PlpV	-1.8	2.02363E-17	ChrI
Hypothetical proteins of unknown function				
VDA_000632	Hypothetical protein	-6.2	8.1218E-133	ChrII
VDA_001326	Hypothetical protein	-3.4	2.00678E-63	ChrI
VDA_000814	Hypothetical protein	-3.8	7.28892E-46	ChrII
VDA_000647	Hypothetical protein	-3.6	2.93637E-36	ChrII
VDA_001949	Hypothetical protein	-3.5	1.82412E-53	ChrI
VDA_001746	Hypothetical protein	-3.4	1.43464E-49	ChrI
VDA_002346	Conserved hypothetical membrane protein	-3.3	1.30302E-29	ChrI
VDA_001892	Putative transporter	-3.1	4.50201E-55	ChrI
VDA_001660	Hypothetical protein	-3.0	2.70017E-34	ChrI
VDA_003210	Hypothetical protein	-3.0	4.74045E-89	ChrI
VDA_000496	Hypothetical protein	-3.0	7.01092E-40	ChrII

VDA_003208	Hypothetical protein	-3.0	8.59014E-50	ChrI
VDA_000405	Hypothetical protein	-3.0	9.44929E-53	ChrII
VDA_002379	Hypothetical protein	-3.0	4.8787E-117	ChrI
VDA_000439	Hypothetical protein	-3.0	1.36579E-08	ChrII

4.1.4.1.*Genes related to the cell envelope*

The most important change is experienced by a putative operon constituted of VDA_001578, VDA_001579 and VDA_001580 (Table 4.5). VDA_001579 is the most differentially expressed gene in the whole transcriptome of this pathogen, and has no known homologues with a demonstrated function so far. VDA_001580 is a predicted serine protein kinase PrkA, a family of proteins involved in cell wall homeostasis (Pensinger *et al.*, 2016), saline stress, motility (Chen *et al.*, 2017) and virulence (Zhao *et al.*, 2016). VDA_001578 is predicted to be a member of the Stage V sporulation protein SpoVR family. SpoVR confers resistance to *Bacillus subtilis* spores and it has been hypothesized that homologues in other species might play a role in peptidoglycan synthesis regulation (Beall and Moran, 1994). Also related to the cell wall is VDA_002031, which encodes a LrgB-family protein, a group of enzymes responsible for modulation of murein hydrolase activity (Groicher *et al.*, 2000). VDA_001762 encodes lysophosphatidic acid acyltransferase PlsC, an integral membrane protein involved in phospholipid biosynthesis (Yao and Rock, 2013).

4.1.4.2.*Metabolic genes*

Growth at 25ºC downregulated the expression of peptidases, membrane transporters and metabolic enzymes (Table 4.5). Of note is the downregulation of the oligopeptide permease system *oppABCDF* whose main function is predicted to be nutritional (Braibant *et al.*, 2000).

VDA_001251 contains a NifB/NifX domain for synthesis of iron-molybdenum cofactors. These cofactors bind the active site of dinitrogenase enzyme which participates in nitrogen fixation (Rubio

and Ludden, 2008). Enzymes of amino acid metabolism were also downregulated at 25ºC.

Genes of the Krebs cycle were downregulated at 25ºC: citrate synthase, malate synthase and isocitrate lyase.

4.1.4.3.*Genes involved in stress responses*

Of note, a glycine betaine transporter showed a strong downregulation at 25ºC in our study (Table 4.5). Only one gene among the main 50 downregulated genes at 25ºC encoded a cold shock protein (VDA_003169).

The alternative sigma factor RpoS (VDA_000629) is downregulated at 25ºC. RpoS is a major regulator of the general stress response pathway in bacteria (Weber *et al.*, 2005). A three-gene operon encoding a glutamate decarboxylase, a glutaminase and a glutamate/GABA antiporter is potentially involved in acid resistance (Zhao and Houry, 2010). An operon which encodes the methionine sulfoxide reductase system MsrPQ was downregulated in *P. damselae* subsp. *damselae* at 25ºC. The MsrPQ systems participate in the repair of oxidative damage (Brot and Weissbach, 2004).

Based on transcriptomic data, we have constructed a diagrammatic summary of genes upregulated and downregulated at 25º, relative to 15ºC, in *P. damselae* subsp. *damselae* (Figure 4.5).

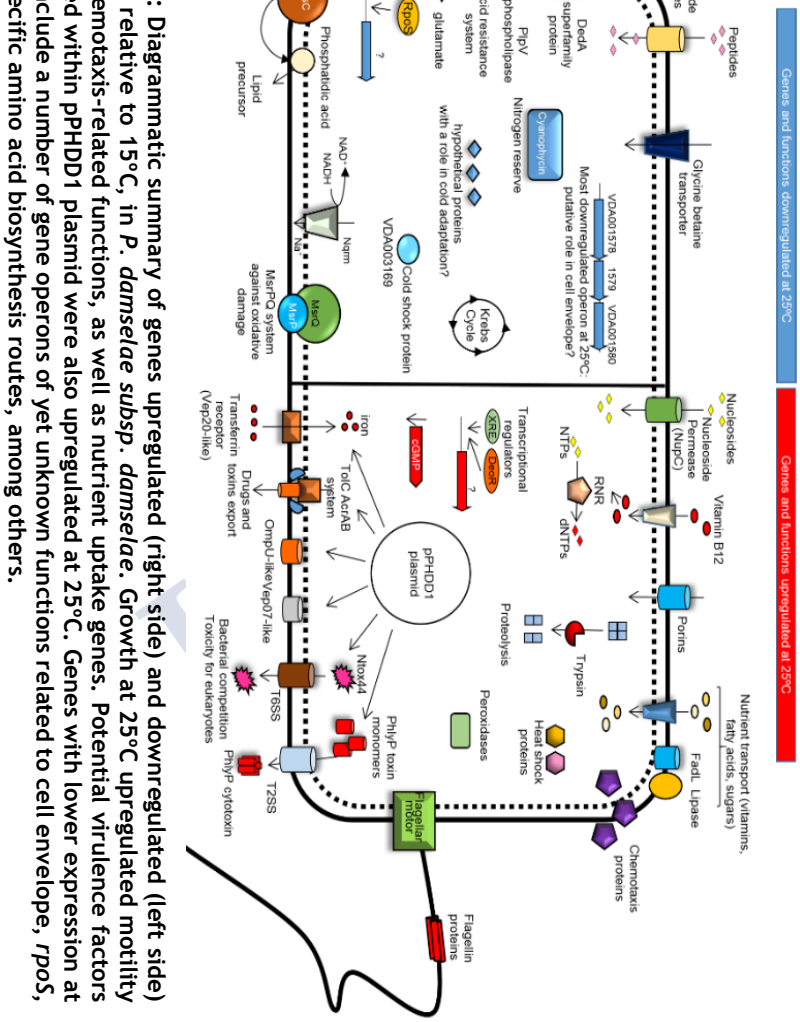

Fig 4.5: Diagrammatic summary of genes upregulated (right side) and downregulated (left side) at 25°, relative to 15°C, in *P. damselae subsp. damselae*. Growth at 25°C upregulated motility and chemotaxis-related functions, as well as nutrient uptake genes. Potential virulence factors encoded within pPHDD1 plasmid were also upregulated at 25°C. Genes with lower expression at 25°C include a number of gene operons of yet unknown functions related to cell envelope, *rpoS*, and specific amino acid biosynthesis routes, among others.

4.1.5.Transcript abundance of haemolysin genes in *P. damselae* subsp. *damselae*: quantification by the Fragments per Kilobase of transcript per Million mapped reads (FPKM) method

Considering their importance in the pathogenicity of *P. damselae* subsp. *damselae* for fish, expression of the cytotoxins Dly, PhlyP, PhlyC and PlpV would be expected to be upregulated at 25ºC. Unexpectedly, expression of Dly (VDA_000159) and PhlyC (VDA_002420) did not experience significant expression changes in growth at 25ºC compared to 15ºC, PhlyP (VDA_000160) was slightly upwnregulated and PlpV (VDA_002242) was slightly downregulated at 25ºC.

This observation prompted us to analyse the RNA sequencing (RNA-seq) data to identify which are the most expressed genes in the genome of *P. damselae* subsp. *damselae* at each temperature of the study. Transcript abundance was quantified by FPKM, a method that allows the comparison of transcripts abundance among samples and conditions. The list of FPKM values of each gene for each biological replicate at 15°C and 25°C is available here. Notably, the *dly* gene was the ninth most expressed at 15ºC (Table 4.6) with transcript abundance levels similar to genes of ribosomal proteins, which are among the most actively transcribed genes in fast-growing prokaryotic cells (Dennis and Bremer, 2008). Two cold shock proteins and the NAD-dependent glyceraldehyde-3-phosphate dehydrogenase were included within the 10 most expressed genes at 15ºC. The 10 most expressed genes at 25ºC all corresponded to ribosomal protein genes (Table 4.7). LSU ribosomal protein L24p (L26e) (VDA_003450) was the most expressed gene under both conditions.

Table 4.6: List of most expressed genes at 15ºC and their corresponding Fragments per Kilobase of transcript per Million mapped reads (FPKM) values. Damselysin (Dly) toxin is highlighted in bold. FPKM values shown correspond to mean values of the three biological replicates.

Locus tag	Protein	FPKM
VDA_003450	LSU ribosomal protein L24p (L26e)	14782
VDA_000346	Putative cold shock-like protein	14687
VDA_003244	LSU ribosomal protein L34p	14467
VDA_003169	Cold shock protein	12798

VDA_001583	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	12190
VDA_003449	LSU ribosomal protein L14p (L23e)	11923
VDA_003447	LSU ribosomal protein L29p (L35e)	11833
VDA_003446	LSU ribosomal protein L16p (L10e)	11690
VDA_000159	**Damselysin toxin (Dly)**	10938
VDA_003456	SSU ribosomal protein S5p (S2e)	10255

Table 4.7: List of most expressed genes at 25ºC and their corresponding FPKM values. FPKM values shown correspond to mean values of the three biological replicates.

Locus tag	Protein	FPKM
VDA_003450	LSU ribosomal protein L24p (L26e)	16486
VDA_003447	LSU ribosomal protein L29p (L35e)	15622
VDA_003093	LSU ribosomal protein L7/L12 (L23e)	15513
VDA_003446	LSU ribosomal protein L16p (L10e)	14870
VDA_003461	SSU ribosomal protein S13p (S18e)	13609
VDA_003444	LSU ribosomal protein L22p (L17e)	13577
VDA_003449	LSU ribosomal protein L14p (L23e)	13557
VDA_003456	SSU ribosomal protein S5p (S2e)	13188
VDA_003244	LSU ribosomal protein L34p	12838
VDA_003463	SSU ribosomal protein S4p (S9e)	12813

To illustrate the dominance of damselysin toxin transcripts, the FPKM values of *dly* and other virulence-related genes, as well as a selection of genes related with secretion systems and housekeeping cellular functions were compared (Fig. 4.6). Although far from the top 10 most expressed genes, the FPKM values of the mRNA levels for the two pore-forming toxins PhlyP and PhlyC were also higher than those of housekeeping genes *gyrB*, *recA*, *mreB* and *ftsZ*. Dly was the most highly expressed virulence factor at the two temperatures of the study, being particularly the case at 15ºC. The plasmid-encoded putative virulence factors OmpU, Vep07 and Vep20 showed transcript abundance levels largely inferior to Dly cytotoxin, again reinforcing the

dominance of Dly as the top expressed virulence factor in *P. damselae* subsp. *damselae*.

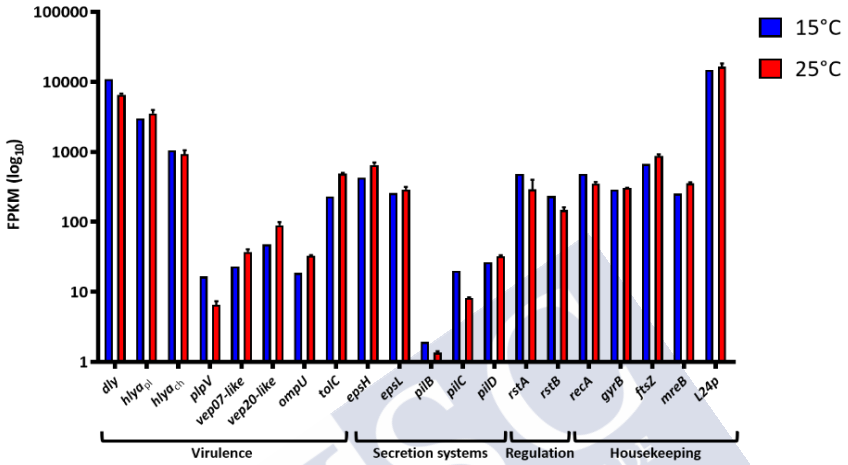

Figure 4.6: Damselysin toxin gene *dly* is one of the most highly expressed genes in *P. damselae subsp. damselae*. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values at the two assayed temperatures were obtained for a selection of virulence and regulatory genes, secretion system genes and housekeeping genes including the top-expressed gene encoding ribosomal protein L24p, and compared using a logarithmic scale. Vertical error bars represent standard deviation of biological triplicates.

4.2. STUDY OF THE TRANSCRIPTOMIC AND PHENOTYPIC RESPONSES OF *P. DAMSELAE* SUBSP. *DAMSELAE* CULTURED AT 37 IN COMPARISON TO 25ºC. COMPARISON BETWEEN FISH AND HUMAN ISOLATES

Continuing with the study of the impact that temperature has on *P. damselae* subsp. *damselae*, we wanted to evaluate its effect from a human pathogen perspective. The ability to grow at temperatures over 30ºC is indeed a differential phenotypic trait of *P. damselae* subsp. *damselae* in comparison to its sibling subspecies, the subsp. *piscicida*. There are no data about the putative specialisation of certain *P. damselae* subsp. *damselae* clones to infect a human host or how this bacterium evolved to replicate at temperatures near 37ºC. In many

human cases in which this bacterium was isolated, difficulties to recover the organism have been reported (Clarridge and Zighelboim-Daum, 1985; Coffey *et al.*, 1986; Goodell *et al.*, 2004). The severity of human infections is of special concern since a number of patients with no underlying conditions were not able to overcome the infection (Perez-Tirse *et al.*, 1993, Yuen *et al.*, 1993; Tang and Wong, 1999; Yamane *et al.*, 2004), and in other cases, the only solutions are amputation and debridement (Nakamura, 2008; Collins *et al.*, 2017; Guimaraes *et al.*, 2020).

By a comparison between cultures at 25 and 37ºC, we aimed at identifying genetic markers that can be targeted to ameliorate the impact of human *P. damselae* subsp. *damselae* infections, potential advantages of human-isolated clones, and revealing the transcriptomic changes this bacterium undergoes when growing at human body temperature (with respect to warm waters simulating a global warming scenario).

4.2.1.Comparative analysis of *P. damselae* subsp. *damselae* growth curves and viability at 37 and 25ºC

To assess the effect of temperature in *P. damselae* subsp. *damselae* growth, strain RM-71 was cultured at 25ºC and 37ºC for 48 h. Cultivation at 37ºC triggered an earlier entry into exponential phase than at 25ºC, but OD_{600} values dropped after 24 h suggesting a cell death scenario (Fig. 4.7).

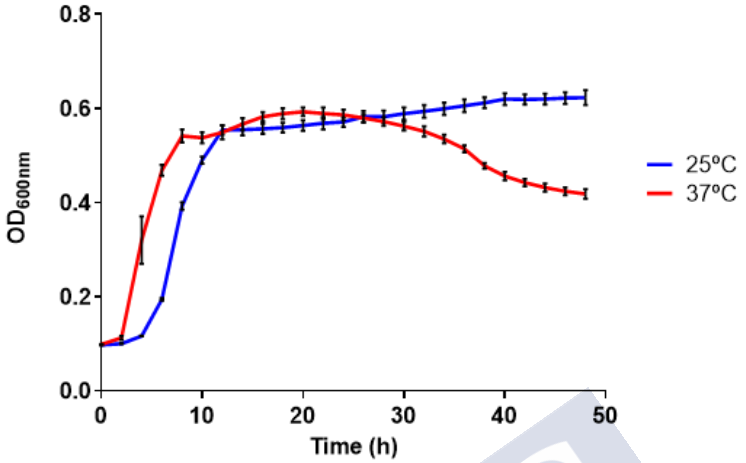

Figure 4.7: Growth curves of *P. damselae* subsp. *damselae* RM-71 at 25 and 37ºC in TSB-1. Mean data and standard deviation (vertical bars) of three independent experiments are shown.

In order to test this hypothesis, viable cells were quantified by drop-counting on agar plates. As a result, there was a >3.5-fold reduction in the number of colony forming units (CFU) at 12 h incubation in cultures at 37 in comparison to 25ºC. Notably, CFU were no longer detected from cultures at 37ºC after 30 h (Fig. 4.8A and 4.8B).

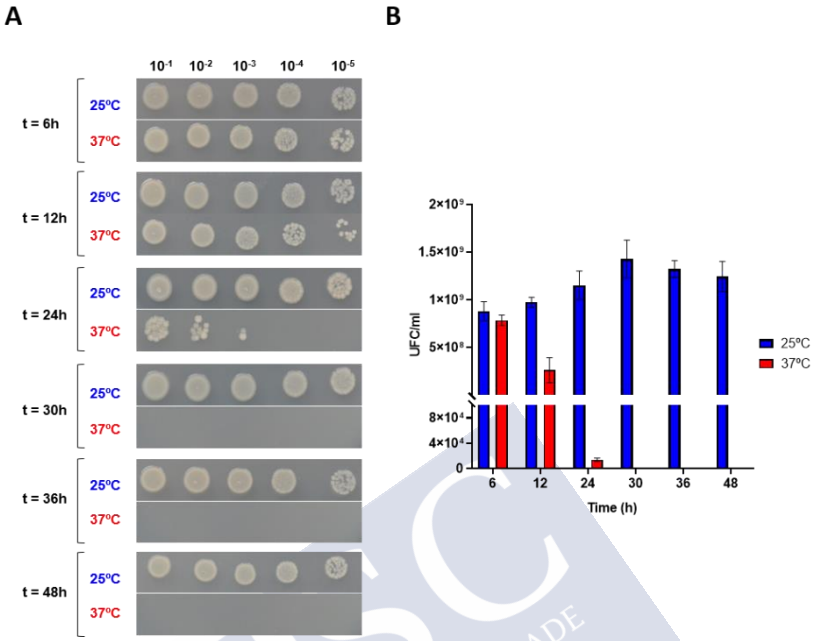

Figure 4.8: Growth at 37ºC impairs cell survival at 37ºC. (A) Drop-plate assays reveals that growth at 37ºC causes a drastic reduction in the colony forming units (CFU) numbers. (B) Quantitative representation of the drop-plate assay. Viable counts are zero after 30 h cultivation, whereas at 25ºC the number of CFU is still increasing.

4.2.2.Growth at 37ºC impairs *P. damselae* subsp. *damselae* cell morphology and resistance to benzylpenicillin

The observation of cell viability reduction at 37ºC prompted us to study cell morphology by scanning electron microscopy (SEM), in exponentially-growing (OD_{600} of 0.55) cultures at 25 and 37ºC. Remarkably, cells grown at 25ºC showed normal rod morphology while cells grown at 37ºC exhibited chain-like structures and aberrant long shapes suggesting a defect in daughter cell separation and in septum formation (Fig. 4.9A). Quantitatively, mean cell length values at 37ºC were significantly higher than at 25ºC (Fig. 4.9B).

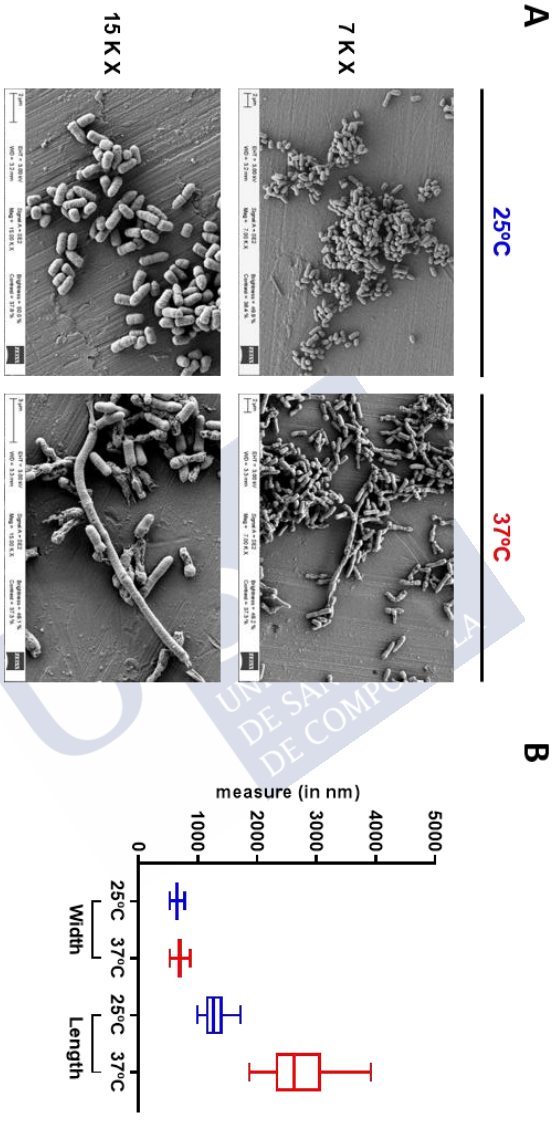

Figure 4.9: Growth at 37ºC impairs cell morphology in *P. damselae* subsp. *damselae*. (A) scanning electron microscopy (SEM) images showing the increase in cell length and the impairment in daughter cell separation when cells are grown at 37ºC with respect to the normal cell size at 25ºC. Pictures from two magnifications, 7,000 × (7 K X) and 15,000 × (15 K X) are shown. (B) Box plot graphs showing cell width and cell length values at 25ºC and at 37ºC. Whiskers indicate min and max values. Data correspond to the measurements of 50 independent cells per temperature assayed.

In addition, we wanted to check whether apart from the observed loss in cell viability and the impairment of cell morphology, the intrinsic tolerance of *P. damselae* subsp. *damselae* RM-71 to

benzylpenicillin may be affected. When cultured at 37ºC, *P. damselae* subsp. *damselae* showed an increase susceptibility to benzylpenicillin in comparison to 25ºC (Fig. 4.10).

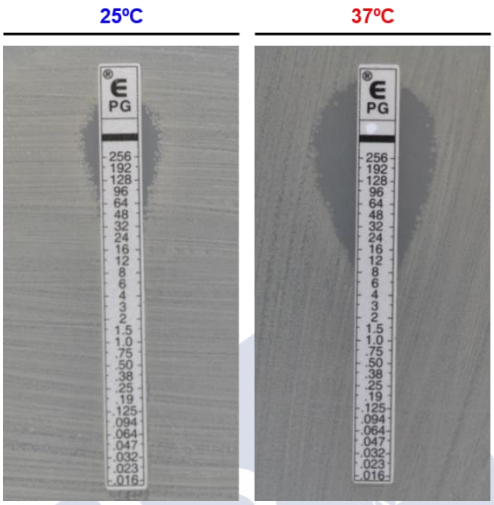

Figure 4.10: *E*-tests for benzylpenicillin of *P. damselae* subsp. *damselae* RM-71 cultivated at 25 and 37ºC in TSA-1, showing the decreased tolerance to this antimicrobial agent at 37ºC.

4.2.3.Analysis of fatty acid methyl esters (FAME) produced at 37ºC

As we have presented here for the comparison between temperatures of 15 and 25ºC, we carried out another analysis of FAME from *P. damselae* subsp. *damselae* RM-71 cultures at 37ºC. In this case, we observed that the percentage of saturated fatty acids in membranes increased abruptly at 37ºC (Table 4.7) with respect to that of cultures at 15 or 25ºC (Tables 4.1 and 4.2).

Table 4.7: Fatty acid composition of *P. damselae* subsp. *damselae* strain RM-71 cultured at 37ºC.

Fatty acid	Percentage
Sum In Feature 2 (unknown 10.928)	0.14
10:0 3OH	0.06
12:0	3.76
11:0 3OH	0.18
13:0	0.08
12:0 2OH	0.12
12:1 3OH	0.09
12:0 3OH	3.16
14:0	5.17
Sum In Feature 1 (13:0 3OH/15:1 i H)	0.34
15:1 w8c	0.14
16:1 w7c alcohol	0.16
Sum In Feature 2 (14:0 3OH/16:1 iso I)	3.89
16:0 N alcohol	0.3
16:0 iso	0.08
Sum In Feature 3 (16:1 w7c/16:1 w6c)	32.68
16:0	26.97
17:1 w8c	0.68
17:1 w6c	0.34
17:0	2.44
18:1 w9c	0.46
Sum In Feature 8 (18:1 w7c)	12.59
18:1 w5c	0.19
18:0	3.74
18:1 w7c 11-methyl	1.72
Sum In Feature 7 (un 18.846/19:1 w6c)	0.15
19:0 cyclo w8c	0.18
20:1 w7c	0.2
Summed Feature 2	4.02

4.2.4.Comparison between human and fish isolates of *P. damselae* subsp. *damselae*

4.2.4.1.*Human isolates do not present an advantage in growing at 37ºC with respect to fish isolates*

We selected two strains isolated from human clinical cases (CDC 2227-81 and 80077637), and three strains from diseased fish (RM-71, LD-07 and A-162), and growth of all the isolates was monitored at 25ºC (Fig. 4.11A) and 37 ºC (Fig. 4.11B). The ability to grow at 37ºC was not a differential trait of human isolates since all the strains showed similar dynamics: early exponential growth and a drop in the OD_{600} after approximately 24h (Fig. 4.11B). Of note, human strain 80077637 achieved the least OD_{600} values at the two temperatures.

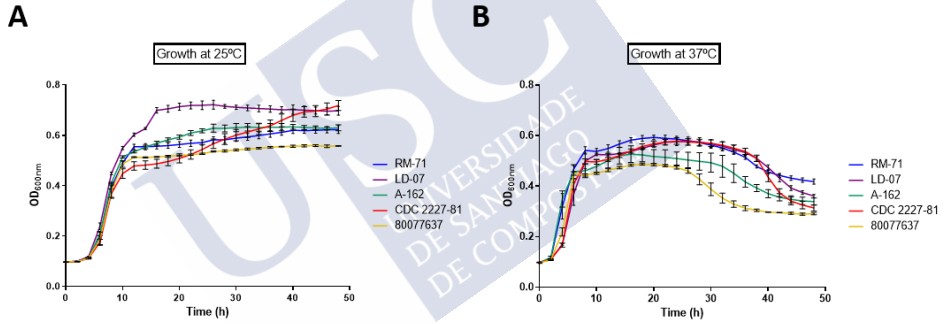

Figure 4.11: Growth curves of *P. damselae* subsp. *damselae* strains isolated from human (CDC 2227-81 and 80077637) and fish (RM-71, LD-07 and A-162), cultivated at 25ºC (A) and 37ºC (B). Note that human isolate 80077637 achieves the least OD600 values of all strains at the two temperatures of the assay.

4.2.4.2.*Comparative genomics does not reveal human-specific genetic markers*

To assess the existence of gene markers characteristic of human isolates, we here obtained for the first time the draft genome sequences of two *P. damselae* subsp. *damselae* strains isolated from humans, CDC-2227-81 (Kreger, 1984) and 80077637 (Hundenborn *et al.*, 2013), and a comparative genomics analysis was conducted

including the genomes of four fish isolated strains, RM-71, LD-07, A-162 and CIP102761. Ortho Average nucleotide identity (OrthoANI) values, which are derived from the pairwise comparison of strains taking into consideration the core genes shared by all the strains, ranged from 97.19 to 98.97 between strains (Fig. 4.12). Two genomes are considered the same species when the ANI value is higher than 95 to 96% (Lee *et al.*, 2016). The 4 strains harbouring the virulence plasmid pPHDD1 (80077637, RM-71, CDC-2227-81 and CIP102761) clustered together independently of their source of isolation, and human isolates were not more similar to each other than to the other genomes, when core genes where compared. In fact, the highest similarity value was found between RM-71 (fish isolate) and 80077637 (human isolate).

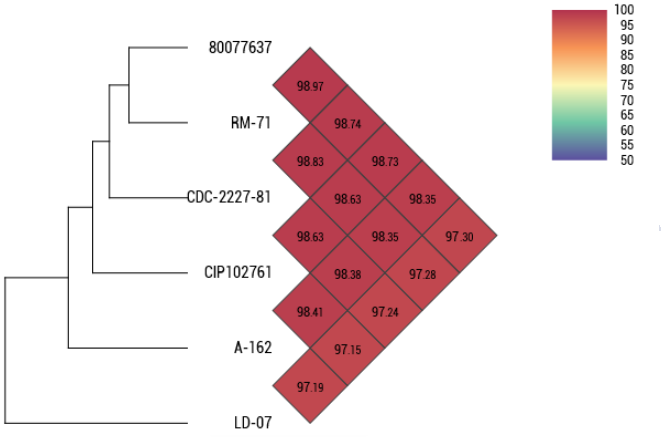

Figure 4.12: Heatmap generated by comparison of core genes of six *P. damselae* subsp. *damselae* strains with OrthoANI values calculated with the OAT software. It shows the absence of close phylogenetic association of human isolates (CDC-2227-81 and 80077637) vs fish isolates (RM-71, LD-07, A-162 and CIP102761).

Notably, genomics analysis unveiled a large number of strain-specific genes, with 265 genes unique to strain CDC-2227-81 and 132 genes unique to strain 80077637 (Fig. 4.13). Four genes were common to the human isolates and absent in the fish isolates. These genes encoded a racemase (locus F6450_06750), an HD domain-containing protein (F6450_11305), an MFS transporter (F6450_16290), and a multidrug transporter (F6450_07685; GenBank loci tags corresponding to strain CDC 2227-81). However, BLAST searches in GenBank database unveiled that three of these genes were also present in other *P. damselae* subsp. *damselae* strains isolated from fish, and the multidrug transporter F6450_07685 was found in a *P. damselae* subsp. *damselae* isolate from the porpoise *Neophocaena asiaeorientalis* and in non-pathogenic *Photobacterium* species.

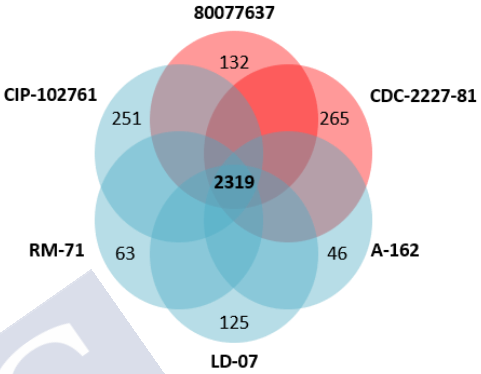

Figure 4.13: Venn diagram depicting the comparative genomics of six *P. damselae* subsp. *damselae* strains. The core genome was estimated in 2319 genes, and each isolate has a varying number of strain-unique genes, ranging from 46 unique genes of strain A-162 to the 265 unique genes to strain CDC-2227-81.

A phylogenetic tree constructed based on the genome alignments (including core and accessory genome) of *P. damselae* subsp. *damselae* strains revealed that human-isolated strains do not represent an independent evolutionary line with respect to the fish isolates, and human clinical strain 80077637 fell into the same clade with fish-isolated RM-71 (Fig. 4.14).

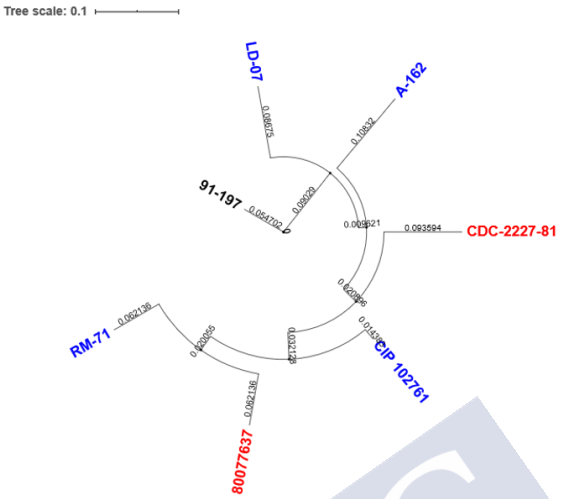

Figure 4.14: Dendrogram of *P. damselae* subsp. *damselae* strains based on genomic BLAST, showing that human clinical strains do not form an independent clade with respect to fish isolates. The genome of the sibling subspecies *P. damselae* subsp. *piscicida* strain 91-197 was included for comparative purposes. The genomic BLAST file was downloaded from the NCBI database and the tree was visualised by Interactive Tree Of Life (iTOL v5).

4.2.5.Identification of temperature sensitive mutants in P. *damselae* subsp. *damselae*. Construction and screening of mini-Tn*10* transposon insertional libraries

We postulated that there may be genes that give *P. damselae* subsp. *damselae* the ability to replicate at high temperatures like that of the human body. Lacking such genes could make this subspecies behave similarly to other members of the family *Vibrionaceae* that are not able to grow at those temperatures, for example, the subspecies *piscicida*. In order to identify mutants defective in growth at 37ºC in *P. damselae* subsp. *damselae*, we constructed a mutant library with the mini-Tn*10* transposon. Identification of such markers would be of interest for the

design of control strategies of human infections. Mutant colonies were picked in parallel on plates grown at both 25ºC and 37ºC. We also screened another Tn*10* transposon mutant library of *P. damselae* subsp. *damselae* RM-71 constructed in a previous study (Terceti *et al.*, 2017); each clone was grown in TSB-1 in 96-well plates per duplicate at 25 and at 37ºC. Despite analysing more than 4000 clones in total, all of them were able to grow at 37ºC without any evident signs of impairment. This result suggests that any potential transposon-insertional mutation that would prevent growth at 37ºC, would also be deleterious for growth at 25ºC (the temperature at which the transposon libraries were originally obtained).

4.2.6.Comparative transcriptomic study of *P. damselae* subsp. *damselae* cultured at 25 and 37ºC: genes upregulated at 37ºC

P. damselae subsp. *damselae* strain RM-71 was grown at 25 and 37ºC, and cDNA prepared from mRNA that was isolated from cultures at the two different temperatures was subjected to Illumina sequencing. Growth at 25ºC was defined as the control condition. The RNA-seq analysis of RM-71 resulted in 1607 DEGs: 804 upregulated (FC higher than 1.5) and 803 downregulated (FC lower than -1.5) at 37ºC with respect to 25ºC (Fig. 4.15).

The complete list of DEGs mapped to CIP102761 can be found in the following link: DEGs 25 vs 37.

A list of the top DEGs upregulated at 37ºC is shown in Table 4.8. Main functions upregulated at 37ºC are described in the following sections.

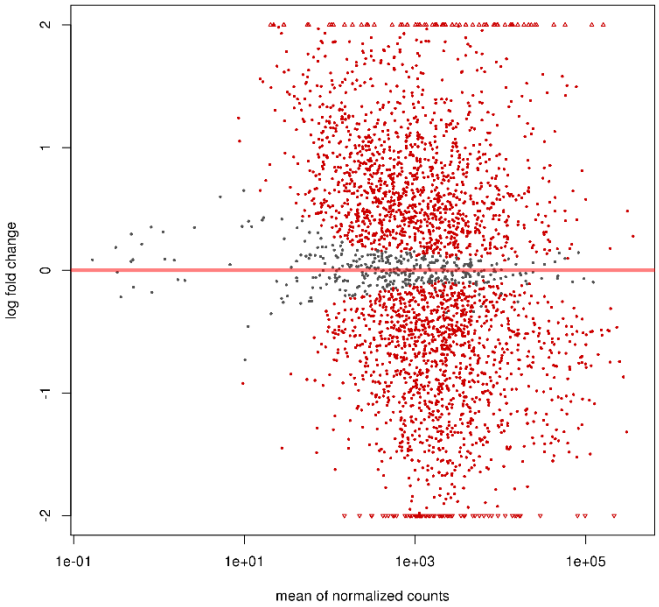

Figure 4.15: Smear plot of differentially expressed genes (DEGs) in *P. damselae* subsp. *damselae* RM-71 exposed to two temperatures, 25ºC and 37ºC. The smear plot shows the relationship between the log Fold Change (FC) and mean of normalised counts. Grey points represent genes with non-significant changes in expression, whereas red points represent genes that are significantly differentially expressed.

Table 4.8: List of selected top DEGs whose expression is upregulated at 37ºC. Enhanced expression at 37ºC is denoted by positive Fold change (FC) values. Genomic location of each DEG in either chromosome I (ChrI), chromosome II (ChrII) or in virulence plasmid pPHDD1 is detailed.

Gene ID	Product/Function	Fold Change	p-value	Location
Stress response and defence mechanisms				
VDA_001325	ClpB protein	12.1	0	ChrI
VDA_003060	heat shock protein 60 family co-chaperone GroES	10.4	0	ChrI
VDA_003059	heat shock protein 60 family chaperone GroEL	10.4	0	ChrI

VDA_002523	chaperone protein HtpG	10.1	0	ChrI
VDA_000709	heat shock protein A	7.3	0	ChrII
VDA_001813	phage shock protein A	7.1	8.97261E-76	ChrI
VDA_000710	chaperone protein DnaK	6.4	0	ChrII
VDA_001814	phage shock protein B	6.1	1.624E-197	ChrI
VDA_001815	hypothetical phage shock protein C	5.9	1.7736E-117	ChrI
VDA_002771	chaperone protein DnaK	5.9	0	ChrI
VDA_003125	heat-shock chaperonin	5.5	0	ChrI
VDA_000998	peptide methionine sulfoxide reductase MsrA	4.9	0	ChrII
VDA_001609	alkyl hydroperoxide reductase subunit C-like protein	4.4	0	ChrI
VDA_002772	heat shock protein GrpE	4.6	0	ChrI
VDA_000104	lipoprotein precursor OmpA family	5.4	5.73249E-76	pPHDD1
Metabolism				
VDA_002723	Glyoxylate cycle isocitrate lyase	4.0	1.1169E-114	ChrI
VDA_001102	ATP synthase gamma chain	4.1	8.8225E-67	ChrII
VDA_001103	ATP synthase alpha chain	3.5	1.55568E-69	ChrII
VDA_002810	phosphopentomutase	3.2	1.7648E-261	ChrI
VDA_002812	deoxyribose-phosphate aldolase	4.8	0	ChrI
VDA_002811	thymidine phosphorylase	3.6	7.7733E-277	ChrI
VDA_003271	thiamine biosynthesis ThiC	4.5	1.325E-176	ChrI
VDA_002967	thiamine ABC transporter	4.4	1.3878E-296	ChrI
VDA_002261	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	13.5	0	ChrI

VDA_003183	oligopeptidase A	5.5	0	ChrI
VDA_002812	deoxyribose-phosphate aldolase	4.8	0	ChrI
VDA_000814	hypothetical protein (DUF 4832)	12.5	1.2613E-200	ChrII
VDA_002964	3-isopropylmalate dehydratase large subunit	7.1	0	ChrI
VDA_002965	3-isopropylmalate dehydratase small subunit	7.1	0	ChrI
VDA_002963	3-isopropylmalate dehydrogenase	6.6	0	ChrI
VDA_001041	adenylosuccinate synthetase	6.5	1.6314E-128	ChrII
VDA_002962	2-isopropylmalate synthase	6.3	0	ChrI
VDA_002881	homoserine kinase	4.7	0	ChrI
VDA_002880	threonine synthase	4.7	0	ChrI
Transporters, adaptation and colonisation				
VDA_002633	Sulfate transporter CysZ	3.1	3.0492E-158	ChrI
VDA_003428	gluthatione-regulated potassium-efflux system protein KefB	3.4	4.4457E-183	ChrI
VDA_000372	putative choline-glycine betaine transporter	7.7	0	ChrII
VDA_002854	Na(+)/H(+) antiporter NhaA type	4.7	6.0472E-303	ChrI
VDA_000302	urease accessory protein UreG	3.2	2.30662E-44	ChrII
VDA_000303	urease accessory protein UreF	4.8	2.62317E-51	ChrII
VDA_000304	urease accessory protein UreE	4.8	2.62317E-51	ChrII
VDA_000305	urease subunit alpha UreC	3.5	4.26325E-17	ChrII
VDA_000306	urease gamma subunit UreA	3.3	1.4581E-58	ChrII
Lipases				
VDA_000412	hypothetical protein (patatin similar to Yjju protein)	10.6	0	ChrII
VDA_002140	putative	3.5	2.11351E-	ChrI

	lipase/esterase protein		81	
Pilus assembly				
VDA_000103	protein TadG associated with Flp pilus assembly	5.4	1.55701E-93	pPHDD1
VDA_000134	IncF plasmid conjugative transfer pilus assembly protein TraF	5.0	3.42729E-12	pPHDD1
VDA_000102	Flp pilus assembly protein TadD	4.9	1.09531E-19	pPHDD1
VDA_000142	mfp1 (mar binding filament-like protein 1)	4.9	0	pPHDD1
Transcriptional regulators and DNA-binding proteins				
VDA_001014	hypothetical transcriptional regulator	5.7	6.7055E-294	ChrII
VDA_000143	DNA-binding protein H-NS	5.0	0	pPHDD1
VDA_001042	transcriptional regulator LysR family	4.6	3.1515E-180	ChrII
VDA_000105	site-specific recombinase resolvase family	8.3	1.925E-296	pPHDD1
VDA_000152	chromosome segregation ATPase	5.0	9.68836E-64	pPHDD1

4.2.6.1.*Heat shock proteins and defence-related mechanisms*

To better illustrate the impact of human body temperature on gene expression, a heat map was generated with 32 selected DEGs that showed remarkable FC values (Fig. 4.16). Notably, heat-shock proteins and molecular chaperones were among the most upregulated genes at 37ºC (Table 4.8 and Fig. 4.16). The genes encoding ClpB, GroEl and HtpG heat-shock proteins were selected for construction of insertional mutants, using the suicide plasmid pNidKan (Mouriño et al., 2004) containing an internal fragment of the target gene. A *htpG* insertional mutant was successfully generated although it did not show any impairment in growth at 37ºC compared to 25ºC. Notably, we were unable to obtain insertional mutants for *clpB* and *groEL* despite numerous attempts, suggesting that these genes are essential, even for

growth at 25ºC (the temperature at which strains were incubated during the mutant construction process).

Genes involved in prevention of oxidative damage were found among the most upregulated at 37ºC (Table 4.8 and Fig. 4.16), including the peptide methionine sulfoxide reductase MsrA, alkyl hydroperoxide reductase subunit C-like protein and the thioredoxin peroxidase, among others. Together with the viability assays, this association between high temperatures and upregulation of stress and defence mechanisms supports the idea that 37ºC constitutes a stressful condition for *P. damselae* subsp. *damselae*.

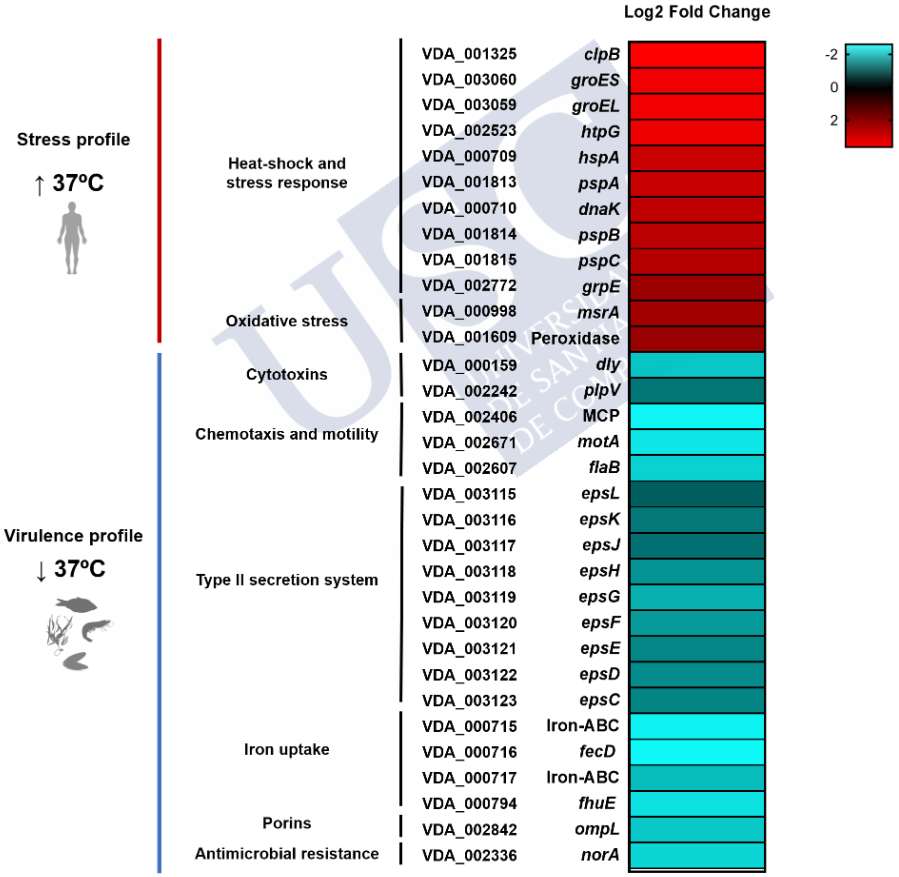

Figure 4.16: Heatmap of 32 selected DEGs of the *P. damselae* subsp. *damselae* transcriptome, including the main upregulated genes at 37ºC in the categories of heat-shock and stress response mechanisms, and the main downregulated genes related to virulence properties. Cultivation of *P. damselae* subsp. *damselae* at 37ºC triggers a strong stress response, indicating that human body temperature represents a stressful condition for this marine pathogen. Conversely, growth at 37ºC downregulates the virulence gene expression profile with respect to 25ºC, corroborating that 25ºC is the optimal temperature for the pathogenic lifestyle of this bacterium in the marine environment. VDA gene tags correspond to the complete genome sequence of type strain CIP 102761.

4.2.6.2. *Metabolic pathways and ATP generation*

Cultivation at 37ºC causes a rapid initial growth of *P. damselae* subsp. *damselae* (Fig. 4.7). In the RNA-seq analysis, the expression of genes encoding enzymes of catabolic and anabolic pathways were strongly upregulated at 37ºC (Table 4.8), being the glycolytic enzyme NAD-dependent glyceraldehyde-3-phosphate dehydrogenase one of the most strongly upregulated genes at 37ºC. Leucine, isoleucine, threonine and thiamine biosynthetic genes, enzymes of the phosphate pentose pathway and the purine/pyrimidine metabolism were upregulated at 37ºC, likely to supply precursors for proteins, nucleotides and coenzymes to sustain the rapid initial growth of *P. damselae* subsp. *damselae* observed at 37ºC. Accordingly, genes encoding the ATP-synthase subunits were upregulated.

4.2.6.3. *Transporters and nutrient acquisition*

A number of pH-dependent and independent transporters were among the most upregulated genes at 37ºC, including a Na^+/H^+ antiporter of NhaA-type. In *V. cholerae* this gene contributes to resistance to Na^+ at alkaline pH leading to improved fitness in the environment (Herz *et al.*, 2003). Also upregulated were several sulfate transporters, and the potassium-efflux system protein KefB. Of relevance, *kefB* belongs to the locus of heat resistance in *E. coli* (Mercer *et al.*, 2017) and it has been studied for its ability to acidify intracellular pH in response to electrophilic stress (Ferguson *et al.*, 1995).

P. damselae subsp. *damselae* is one of the few urease-producer species within the *Vibrionaceae* family, and genes encoding urease

subunits were upregulated at human body temperature (VDA_000302-VDA_000306).

Genes encoding degradative functions were upregulated, including several putative lipases (VDA_000412 and VDA_002140) and an operon for utilisation and transport of galactosamine and glycosaminoglycans (VDA_001072-VDA_001083). Glycosaminoglycans are major components in extracellular matrix of animals (Gandhi and Mancera, 2008). *P. damselae* subsp. *damselae* is considered a generalist pathogen of a wide range of animals (Osorio *et al.*, 2018) so genetic networks involved in the utilisation of different carbon sources may allow colonisation of different niches and hosts. At 37ºC, *P. damselae* subsp. *damselae* also upregulated genes of two pili structures encoded within the virulence plasmid pPHDD1, including the *tra* genes for conjugative pilus biogenesis, and the genes of the Flp/Tad pilus, with a potential role in adhesion and colonisation.

4.2.6.4.*DNA-binding protein H-NS*

How *P. damselae* subsp. *damselae* modulates genetic expression in response to external conditions remains largely unknown. Several LysR family transcriptional regulators were upregulated at 37ºC (VDA_001014, VDA_001042, VDA_000547 and VDA_001035). Of special relevance is the observation that the DNA-binding protein H-NS (VDA_000143), encoded within the virulence plasmid pPHDD1 showed an upregulation higher than 5-fold at 37ºC. In *V. cholerae*, this protein functions as a virulence repressor controlling motility and biofilm formation (Wang *et al.*, 2015). Accordingly, as detailed below, growth at 37ºC was found to downregulate virulence and motility functions in *P. damselae* subsp. *damselae*.

4.2.7.Comparative transcriptomic study of *P. damselae* subsp. *damselae* cultured at 25 and 37ºC: genes downregulated at 37ºC

Growth at 37ºC resulted in the downregulation of 803 genes that mapped to the genome of the type strain whose changes in expression are denoted by a negative FC value (DEGs 25 vs 37). A list of the top downregulated genes at 37ºC, is shown in Table 4.9.

Table 4.9: List of selected top DEGs whose expression is downregulated at 37°C. Note that downregulated expression at 37°C is denoted by negative Fold change values.

Gene ID	Product/Function	Fold Change	p-value	Location
Defence and virulence				
VDA_000717	iron ABC-transporter	-3.9	2.7907 E-177	ChrII
VDA_000716	iron (III) dicitrate transport system permease protein FecD	-6.1	6.4554 E-244	ChrII
VDA_000715	ABC type periplasmic iron siderophore/cobalamin binding protein	-5.6	0	ChrII
VDA_000794	ferrichrome-iron receptor	-5.1	0	ChrII
VDA_000159	damselysin	-4.2	8.6351 E-269	pPHDD1
VDA_002336	multidrug efflux protein NorA	-4.6	1.7265 E-164	ChrI
VDA_002842	ompL porin-like protein L precursor	-4.3	4.4961 E-185	ChrI
VDA_000341	ABC transporter periplasmic spermidine putrescine-binding protein PotD	-7.9	0	ChrII
VDA_001897	thiol-disulfide isomerase	-4.6	6.0694 E-145	ChrI
VDA_002117	ABC transporter periplasmic spermidine putrescine-binding protein PotD	-4.2	7.62485 E-75	ChrI
VDA_003028	flavohemoprotein	-4.2	0	ChrI
VDA_000342	oxidoreductase	-3.2	4.73193 E-72	ChrII
Cold response				
VDA_003169	cold shock protein	-16.3	0	ChrI
VDA_000346	putative Cold shock-like protein	-8.4	3.1036 E-252	ChrII
VDA_000863	cold-shock DEAD-box protein A	-5.3	9.3703 E-299	ChrII

Hypothetical proteins of unknown function				
VDA_002460	lipoprotein putative	-15.9	0	ChrI
VDA_002316	hypothetical protein (Helix-turn-helix domains)	-14.2	0	ChrI
VDA_001898	putative heat shock protein YegD	-7.3	7.2519 E-209	ChrI
Histone acetylation				
VDA_000822	histone acetyltransferase HPA2	-10.8	4.0491 E-204	ChrII
Flagellar motility and chemotaxis				
VDA_002406	N-acetylglucosamine regulated methyl-accepting chemotaxis protein	-6.0	5.4912 E-139	ChrI
VDA_002671	Flagellar motor rotation protein MotA	-5.4	1.0403 E-179	ChrI
VDA_001613	sodium-type flagellar protein MotY precursor	-4.9	1.5077 E-180	ChrI
VDA_003029	sodium-type polar flagellar protein MotX	-4.8	3.9208 E-150	ChrI
VDA_001198	putative methyl-accepting chemotaxis protein	-4.1	1.6095 E-186	ChrI
VDA_003044	methyl-accepting chemotaxis protein	-4.0	3.2241 E-137	ChrI
VDA_002619	chemotaxis protein CheV	-3.4	6.3682 E-259	ChrI
VDA_002670	Flagellar motor rotation protein MotB	-4.1	9.0774 E-129	ChrI
VDA_002607	flagellin protein flaB	-4.5	2.4193 E-132	ChrI
Porins, permeases and transporters				
VDA_001789	nucleoside permease NupC	-10.7	0	ChrI
VDA_001677	sulfate permease	-6.5	0	ChrI
VDA_002944	probable low-affinity inorganic phosphate transporter	-6.8	0	ChrI
VDA_002943	phosphate transport regulator	-7.4	0	ChrI
Transcriptional regulators				

VDA_003227	putative transcriptional regulator DeoR family protein	-3.4	1.2526 E-136	ChrI
VDA_001543	hypothetical response regulator	-3.7	2.486E-250	ChrI
VDA_002957	putative LuxZ	-4.6	0	ChrI
Metabolism				
VDA_002716	myo-inositol-1(or 4)-monophosphatase	-7.4	1.66767 E-53	ChrI
VDA_001806	orotidine 5'-phosphate decarboxylase	-5.1	2.4264 E-246	ChrI
VDA_003379	fructose-1.6-bisphosphatase GlpX type	-4.5	4.51805 E-64	ChrI
VDA_001717	cytochrome c-type protein torC	-4.3	3.762E-125	ChrI
VDA_000979	HNH nuclease	-10.9	3.2236 E-205	ChrII
VDA_002963	inosine-5'-monophosphate dehydrogenase	-6.6	0	ChrI
VDA_003340	orotate phosphoribosyltransferase	-4.1	1.4272 E-176	ChrI
Adjustment of membrane composition				
VDA_002169	phosphatidylglycerophosphatase B	-3.9	1.8426 E-114	ChrI
VDA_002932	1-acyl-sn-glycerol-3-phosphate acyltransferase	-3.5	3.0484 E-200	ChrI
VDA_002087	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase	-4.2	0	ChrI
VDA_002463	UDP-2.3-diacylglucosamine hydrolase	-4.1	6.7473 E-233	ChrI

4.2.7.1.*Virulence factors*

We demonstrated that many *P. damselae* subsp. *damselae* virulence factors were upregulated at 25ºC in comparison with 15ºC (Table 4.3). Of note, well-known virulence factors of *P. damselae*

subsp. *damselae* were downregulated at 37ºC, suggesting that infection of a human host is not a condition that has been favoured during the evolution of this marine pathogen (Table 4.9 and Fig. 4.16). Damselysin (Dly) toxin, a major virulence factor of *P. damselae* subsp. *damselae*, showed a 4-fold downregulation at 37ºC in the RNA-seq experiment. The two pore-forming toxins PhlyP and PhlyC did not show a differential expression at 37ºC, and the phospholipase PlpV showed downregulation at 37ºC. β-galactosidase assays of transcriptional fusions of the haemolysin gene promoters to *lacZ* gene validated the RNA-seq results (Fig. 4.17).

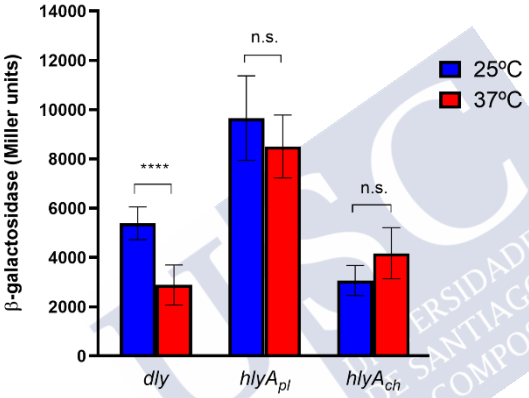

Figure 4.17: Transcription activities of the three major cytotoxin genes damselysin (*dly* gene), phobalysin P (*hlyA*$_{pl}$ gene) and phobalysin C (*hlyA*$_{ch}$ gene) under the temperature conditions of a human host (37ºC) in comparison to the temperature in warm seawaters (25ºC), as determined by transcriptional fusions of *dly*, *hlyA*$_{pl}$ and *hlyA*$_{ch}$ promoters to *lacZ* reporter gene. The ß-galactosidase values (expressed in Miller units) show that *dly* is significantly downregulated at 37ºC whereas expression of *hlyA*$_{pl}$ and *hlyA*$_{ch}$ is not significantly affected at 37ºC vs. 25ºC, thus validating the results of the transcriptomics analysis.

Despite the absence of upregulation, the three major cytotoxins Dly, PhlyP and PhlyC exhibited high FPKM values at 37ºC, within the top 150 most expressed genes. This is in accordance with their expression at 15 and 25ºC (FPKM values at 15 and 25ºC) and with a previous study that showed that toxins represent a major fraction of the *P. damselae* subsp. *damselae* secretome (Terceti *et al.*, 2019). Genes of the T2SS (*eps* genes, VDA_003115-VDA_003123) were downregulated at 37˚C (Fig. 4.16). The two-component system RstAB was not significantly affected by temperature.

Other virulence-related functions were downregulated at 37ºC (Table 4.9 and Fig. 4.16): an iron ABC-transporter (VDA_00715-VDA_000717), a TonB-dependent siderophore receptor (VDA_000794), and the *potABCD* operon (VDA_002114-VDA_002117, VDA_000341) encoding a polyamine (spermidine and putrescine) transport system. Polyamines are essential for normal growth and proliferation in bacteria (Shah and Swiatlo, 2008). In *Vibrio vulnificus* expression of *pot* genes is up-regulated in presence of human serum (Williams *et al.*, 2014) and in *V. cholerae* they regulate biofilm formation (McGinnis *et al.*, 2009).

Regarding porins that modulate pathogenicity of many *Vibrio* species, OmpU (VDA_002842) is downregulated at 37ºC Multidrug efflux protein VDA_002336 showed a down-regulation at 37ºC. It belongs to the multidrug and toxic-compound extrusion (MATE) proteins group, involved in protecting cells against antibiotics and other substances in human pathogenic *Vibrios* as *Vibrio parahaemolyticus* and *V. cholerae* (Begum *et al.*, 2005; Otsuka *et al.*, 2005).

This study revealed a large set of genes with a role in chemotaxis and flagellar motility which were downregulated at 37ºC (Table 4.9 and Fig. 4.16). This is in accordance to previously mentioned data in which motility and chemotaxis genes were also upregulated at 25 in comparison to 15ºC (Table 4.3).

4.2.7.2.*Genes encoding cold shock proteins*

As it might be expected, there was a significant downregulation of cold shock related genes at 37ºC. Included in this list we have found the cold shock protein VDA_003169, cold-shock

DEAD-box protein VDA_000863 and the putative cold shock protein VDA_000346. Moreover, 2 hypothetical proteins present a very high change in expression, so we speculate they might play a role in cold tolerance in *P. damselae* subsp. *damselae*: VDA_002460, VDA_002316.

4.2.7.3.*Nutrient transporters*

Phosphate, nucleoside and vitamin transporters, and permeases for inorganic ions were downregulated at 37ºC (Table 4.9). DNA can be utilised as a source of phosphate, carbon and nitrogen. Phosphate limitation is encountered by marine pathogens in either the environment or inside the host. We have found that a phosphate transport regulator (VDA_002943) and a low-affinity inorganic phosphate transporter (VDA_002944), which shares homology with *E. coli pitA* (identity 33%), are downregulated at 37ºC. As well, nucleoside permeases NupC (VDA_001789 and VDA_002313) were also down-regulated at 37ºC. We have also shown that NupC VDA_001789 presented a high upregulation at 25ºC compared to 15ºC (Table 4.3). These results suggest that *P. damselae* subsp. *damselae* takes advantage of the utilization of DNA as a source of biomolecules especially at 25ºC (temperature at which outbreaks in fish farms are favoured).

4.2.7.4.*Metabolic functions and adjustment of membrane composition*

The myo-inositol-1(or 4)-monophosphatase (VDA_002716), which participates in myo-inositol biosynthesis, showed a remarkable down-regulation at 37ºC. This function has been associated with a role in correct rRNA biosynthesis (Singh *et al.*, 2016). During anaerobic respiration, bacteria use alternative electron acceptors such as trimethylamine N-oxide to oxidise organic compounds. The cytochrome c-type protein TorC (VDA_001717) that takes part in this electronic transfer (Gon *et al.*, 2001) shows a downregulation at 37ºC. The association between anaerobic growth and temperature in *P. damselae* subsp. *damselae* has not been explored to date. Functions related to nucleotide and pyrimidine biosynthesis, and of carbohydrate metabolism were also downregulated.

Functions for the maintenance of lipid membranes, phospholipid biosynthesis, fatty acid biosynthesis and lipid IVA biosynthesis were markedly downregulated at 37ºC. These observations might correlate with the observed changes in membrane lipid composition in *P. damselae* subsp. *damselae* cultured at 25ºC vs. 37ºC as reported above (Table 4.2 and Table 4.7).

4.3. TRANSCRIPTOMIC ANALYSIS OF THE TWO-COMPONENT SYSTEM (TCS) RSTAB, A POSITIVE REGULATOR OF VIRULENCE IN *P. DAMSELAE* SUBSP. *DAMSELAE*

In two recent studies, it was reported that the TCS RstAB is a positive regulator of the three main cytotoxins (Dly, PhlyP and PhlyC) produced by *P. damselae* subsp. *damselae* strain RM-71, and mutants in *rstA* and *rstB* genes are strongly impaired in virulence in a fish infection model. The genes encoding the histidine kinase RstB (VDA_000601) and the response regulator RstA (VDA_000600) are linked in the *P. damselae* subsp. *damselae* genome and are predicted to constitute a cognate pair (Terceti *et al.*, 2017; Terceti *et al.*, 2019). This TCS constitutes a ubiquitous mechanism that control pathogenicity in both *P. damselae* subsp. *damselae* populations, those harbouring the pPHDDD1 virulence plasmid and plasmidless strains. Despite all the available data, the complete regulon under the control of the RstAB system has not been entirely disclosed up to date.

To gain a deeper insight into the RstAB regulon in this pathogen, we conducted an RNA-seq analysis of *P. damselae* subsp. *damselae* strain RM-71 (wild type, wt) and its derivative deletion mutants Δ*rstB* and Δ*rstA*. As in the other transcriptomic studies compiled in this thesis, genes exhibiting a FC value higher than 1.5 or lower than -1.5 were considered DEGs. From the comparison between the wt and the Δ*rstB* mutant, the number of genes that underwent a differential expression was 747 (257 downregulated and 490 upregulated relatively to the wt. Likewise, 397 genes were differentially expressed in the Δ*rstA* mutant (230 downregulated and 167 upregulated respect to the wt). There was a great difference in the range of FC values between downregulated and upregulated genes. As an example, the top 20 downregulated genes in the Δ*rstA* and Δ*rstB* mutants exhibited acute changes of gene

expression with respect to the wt, with FC values ranging between -100 and -1400 (Δ*rstA*), and between -41 and -1509 (Δ*rstB*). On the contrary, the FC value ranges of the top 20 upregulated genes in Δ*rstA* and Δ*rstB* mutants were as low as 4-10 and 4-6.8, respectively. Overall, more than 90% of the genes whose expression is upregulated in the Δ*rstA* and Δ*rstB* mutants exhibited very low degrees of differential expression with respect to the wt and the FC values were very close to the threshold for being considered as DEGs. These data clearly indicate that the RstAB system can be considered as a positive regulator of gene expression in *P. damselae* subsp. *damselae*, and its inactivation causes acute downregulation of diverse gene functions.

The number of DEGs exclusively downregulated in either the Δ*rstB* or the Δ*rstA* mutant was 129 and 102 genes, respectively. As expected for a putative cognate pair, there was a substantial overlap between the RstB and RstA regulons, represented by 128 genes downregulated in both mutants. These genes constitute the focus of the present study (Fig. 4.18).

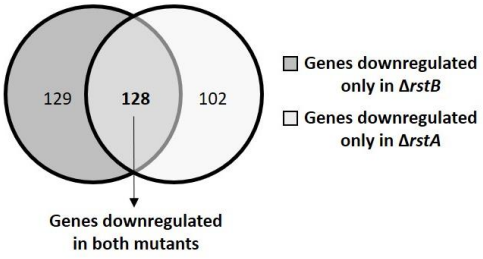

Figure 4.18: Graphical representation of the number of downregulated genes in Δ*rstA* and/or Δ*rstB* mutants, revealing the strong association between the histidine kinase RstB and the response regulator RstA.

Corroborating previous observations (Terceti *et al.*, 2019), the RstAB regulon included chromosome- and plasmid-encoded genes with either a demonstrated or a predicted role in virulence, indicating that RstAB is a major regulator of virulence in *P. damselae* subsp. *damselae*. The top downregulated genes are listed in Table 4.10, and the FC values correspond to the comparison between Δ*rstA* vs wt. These genes were classified into functional categories that include synthesis of cell envelope polysaccharides, cytotoxins, the T2SS, resistance to antimicrobial agents, survival within the host, outer membrane proteins and other functions with potential roles in virulence.

Table 4.10: List of top DEGs downregulated by the RstAB system. FC values correspond to the comparison between Δ*rstA* and wt (note that downregulated expression is denoted by negative FC values). Genes with VDA codes correspond to the annotation in the CIP102761 genome and genes with A0J47 codes correspond to the annotation in the RM-71 genome.

Gene ID	Product/function	Fold Change	*P*-value	Location
Virulence-related genes and proteins				
Capsule and exopolysaccharide synthesis				
VDA_001510	Wzc	-1299,9	7,88E-269	ChrI
VDA_001507	Hypothetical protein YjbE	-1239,58	0	ChrI
VDA_001518	UDP-glucose dehydrogenase	-1156,87	4,26E-207	ChrI
VDA_001508	Wza	-955,43	2,99E-268	ChrI
A0J47_19170	Glycosyltransferase group 1	-911,3	8,98E-214	ChrI
A0J47_19145	Aminotransferase	-862,95	2,01E-185	ChrI
A0J47_19190	EPS biosynthesis	-772,88	9,55E-169	ChrI
A0J47_19160	Glycosyltransferase	-673,59	1,20E-159	ChrI
VDA_001520	Glucose-1-phosphate thymidylyltransferase	-633,83	5,39E-179	ChrI
A0J47_19185	Glycosyltransferase	-588,63	9,49E-151	ChrI
A0J47_19165	Polysaccharide pyruvyl transferase	-541,87	5,24E-134	ChrI
A0J47_19170	Glycosyltransferase	-522,27	6,74E-143	ChrI
A0J47_19140	Acetyltransferase	-518,56	4,09E-141	ChrI
VDA_001504	YjbH	-488,95	4,19E-197	ChrI
VDA_001519	dTDP-glucose 4,6-dehydratase	-429,72	1,42E-171	ChrI
VDA_001509	Wzb	-396,09	8,41E-166	ChrI
VDA_001524	UTP-glucose-1-phosphate uridylyltransferase	-331,37	9,34E-146	ChrI
A0J47_19135	dTDP-6-deoxy-3,4-keto-hexulose isomerase	-301,71	1,93E-111	ChrI
VDA_001506	YjbF	-298,77	1,72E-139	ChrI
VDA_001505	YjbG	-232,8	1,76E-123	ChrI
A0J47_19155	O-antigen translocase	-64,72	2,08E-38	ChrI

Cytotoxins				
VDA_000159	Damselysin	-1408,26	0	pPHDD1
VDA_000160	PhlyP	-465,66	0	pPHDD1
VDA_002420	PhlyC	-102,4	0	ChrI
Type II secretion system				
VDA_003118	EpsH	-4,83	3,35E-136	ChrI
VDA_003119	EpsG	-4,69	1,30E-263	ChrI
VDA_003122	EpsD	-4,32	5,45E-179	ChrI
VDA_003120	EpsF	-4,16	2,28E-158	ChrI
VDA_003121	EpsE	-4,12	4,03E-203	ChrI
VDA_003123	EpsC	-3,54	1,92E-175	ChrI
VDA_003117	EpsJ	-3,44	1,27E-111	ChrI
VDA_003115	EpsL	-3,11	6,58E-24	ChrI
VDA_003116	EpsK	-3,07	3,92E-120	ChrI
Resistance to antimicrobial agents and survival within the host				
VDA_001878	DUF535, VirK-like	-159,9	0	ChrI
VDA_001124	DUF535, VirK-like	-135,39	0	ChrII
VDA_000158	AcrA/MacA-like membrane fusion protein	-115,36	0	pPHDD1
VDA_000599	Phosphoetanolamine lipid A transferase, EptA-like	-37,36	0	ChrII
VDA_000157	Outer membrane protein TolC	-36,94	0	pPHDD1
VDA_000156	ABC-type antimicrobial peptide transport system permease component	-24,9	0	pPHDD1
VDA_000155	ABC-type antimicrobial peptide transport system permease component	-21,67	0	pPHDD1
VDA_000154	Macrolide export ATP-binding/permease protein MacB	-15,09	0	pPHDD1
VDA_002435	TolA protein	-3,95	1,69E-170	ChrI

VDA_002436	MotA/TolQ/ExbB proton channel family protein	-3,59	1,80E-161	ChrI
VDA_002433	TolAB	-3,55	1,98E-121	ChrI
A0J47_18115	Bacterial toxin 44	-3,3	1,08E-69	pPHDD1
VDA_002432	TolAB	-3,22	1,07E-178	ChrI
VDA_002434	TolAB	-3,18	6,85E-170	ChrI
A0J47_18120	PAAR domain-containing protein	-3,07	1,00E-61	pPHDD1
A0J47_18110	hypothetical protein	-2,4	7,08E-17	pPHDD1
Outer membrane proteins and porins				
VDA_000284	Outer membrane protein	-338,06	0	ChrII
VDA_001503	OmpA	-125,49	1,40E-65	ChrI
VDA_000966	Outer membrane protein	-21,77	0	ChrII
A0J47_15910	Outer membrane protein	-11,91	1,85E-34	ChrII
VDA_000118	Outer membrane protein	-9,63	6,71E-32	pPHDD1
VDA_000113	OmpU	-2,83	4,49E-65	pPHDD1
Other genes with a putative role in virulence				
VDA_000358	Hypothetical protein, T2SS-dependent	-349,15	0	ChrII
VDA_002799	Delta endotoxin, T2SS-dependent	-206,86	0	ChrI
VDA_003529	Protease DegP	-12,73	2,23E-63	ChrI
A0J47_07530	Hypothetical protein, T2SS-dependent	-12,34	7,28E-17	ChrI
VDA_000112	Hypothetical protein, T2SS-dependent	-9	5,70E-223	pPHDD1
VDA_001571	CAAX protease self-immunity	-3,57	1,49E-125	ChrI
Other genes and proteins				
VDA_000598	Hypothetical protein	-108,43	1,56E-301	ChrII
VDA_001132	Hypothetical protein	-74,92	0	ChrII
A0J47_09785	Trypsin	-69,92	0	ChrII
VDA_002544	Gamma-Crystallin-like superfamily	-54,19	0	ChrI
VDA_000787	Sodium-dependent transporter	-10,21	0	ChrII

VDA_000563	Beta-ketoadipate enol-lactone hydrolase	-9,11	0	ChrII
VDA_000359	Thioredoxin	-8,7	0	ChrII
VDA_000351	Cysteine desulfurase	-8,1	3,19E-185	ChrII
VDA_000943	Hypothetical protein	-5,91	3,73E-30	ChrII
VDA_001932	Hypothetical protein	-5,38	2,72E-65	ChrI
VDA_000942	Mox-R like protein	-5,28	1,70E-23	ChrII
VDA_001051	Hypothetical protein	-5,27	3,39E-268	ChrII
VDA_003431	Hypothetical protein	-5	3,02E-175	ChrI
VDA_002368	Putative ATP-binding component of a transport system	-4,68	3,52E-33	ChrI
VDA_000564	Acetyltransferase	-4,48	4,50E-76	ChrII
VDA_000944	BatA protein	-4,16	1,65E-64	ChrII
VDA_001026	Hypothetical protein	-3,94	7,09E-106	ChrII
VDA_000520	Hypothetical protein	-3,94	6,91E-120	ChrII
VDA_001543	Hypothetical response regulator	-3,47	9,63E-184	ChrI
VDA_001621	Alpha-aspartyl dipeptidase	-3,39	6,66E-167	ChrI
VDA_000544	Heavy metal-binding domain-containing protein	-3,38	6,07E-60	ChrII
VDA_002181	Hypothetical protein	-3,34	3,92E-106	ChrI
VDA_000877	Hypothetical nitroreductase	-3,31	2,52E-190	ChrII
VDA_000979	HNH nuclease	-3,12	5,99E-60	ChrII
VDA_000757	Cyclic diguanylate phosphodiesterase	-3,05	2,61E-13	ChrII
VDA_002217	Hypothetical protein	-3,05	2,17E-182	ChrI
VDA_000575	DUF4344	-2,91	5,86E-09	ChrII
A0J47_02580	AraC family transcriptional regulator	-2,63	6,72E-38	ChrI
A0J47_01760	Hypothetical protein	-2,01	8,65E-20	ChrI
A0J47_00295	HNH endonuclease	-1,96	8,44E-13	ChrI

A heat map comparing expression in Δ*rstA* vs wt, Δ*rstB* vs wt and Δ*rstA* vs Δ*rstB* was constructed to illustrate the log_2 FC of selected virulence-related genes of the RstAB regulon (Fig. 4.19). In general, there is a common downregulation (represented by green colour) of virulence-related genes in the RstAB system mutants. The comparison between Δ*rstA* and Δ*rstB* regulons is also presented, showing that the vast majority of genes do not show differences (denoted with a black colour), indicative of a co-regulation of such genes by the cognate pair RstAB. The slight level of different regulation levels in a reduced number of genes between the two mutants (depicted in yellow colour) is largely explained by the very low expression levels of those genes in both mutants.

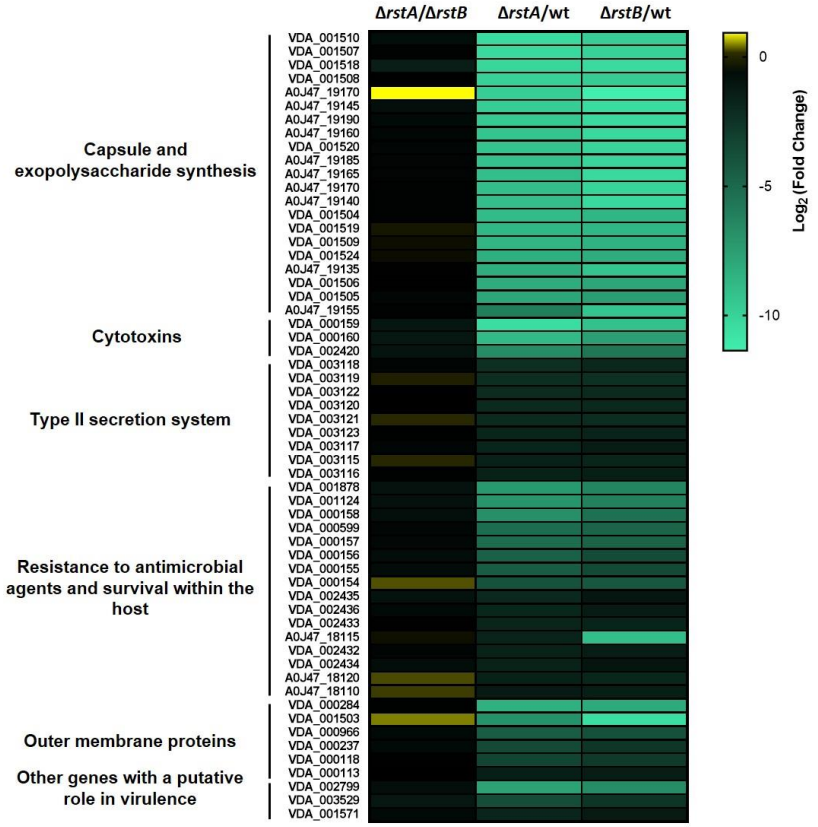

Figure 4.19: Heatmap showing the differential expression of virulence-related genes in pairwise comparisons of the wt, Δ*rstB* and Δ*rstA* mutants of *P. damselae* subsp. *damselae*. RNA-sequencing reveals the strong association between the histidine kinase RstB and the response regulator RstA in the regulation of numerous genes related to virulence. Gene expression is plotted as log2 Fold Change (FC). Genes were attributed to functional categories: capsule and exopolysaccharide synthesis, cytotoxins, type II secretion system, resistance to antimicrobial agents and survival within the host, outer membrane proteins, and other genes with a putative role in virulence. VDA codes correspond to the annotation in the genome of the type strain CIP102761 and A0J47 codes correspond to the annotation in the RM-71 genome.

4.3.1.Cytotoxin genes and additional type II secretion system (T2SS)-dependent proteins

The transcriptomic data showed a strong downregulation of the three cytotoxins Dly, PhlyP and PhlyC in *rstA* and *rstB* mutants. Notably, the *dly* gene (VDA_000159) encoding Dly toxin was the top most downregulated gene in the Δ*rstA* transcriptome, with a remarkable FC value of -1408 (Table 4.10). The two phobalysins PhlyP (VDA_000160) and PhlyC (VDA_002420) genes exhibited FC of -465 and -102, respectively (Table 4.10). These results corroborate data from previous studies that reported a downregulation of cytotoxin gene promoters in a *rstB* mutant background (Terceti *et al.*, 2017), as well as the impairment in secretion of the three cytotoxins in *rstA* and *rstB* mutants (Terceti *et al.*, 2019). In addition, it was here corroborated a strong downregulation in the *rstA* mutant, of five genes encoding proteins known to be secreted by the T2SS in *P. damselae* subsp. *damselae* RM-71 (Terceti *et al.*, 2019), and whose biological roles await further investigation. The two genes VDA_002799 and A0J47_07530 encode a predicted δ-endotoxin and a small protein of unknown function, respectively (Table 4.10). These two genes occur in a fraction of *P. damselae* subsp. *damselae* strains and are located in a region of the *P. damselae* subsp. *damselae* chromosome that exhibits a high genetic variability (Terceti *et al.*, 2018). The T2SS-dependent hypothetical proteins VDA_000112, VDA_000358 and trypsin-like A0J47_09785, are also under the control of RstAB (Table 4.10). As we have unveiled here; *dly*, $hlyA_{pl}$ and $hlyA_{ch}$ are among the most highly expressed genes of the whole *P. damselae* subsp. *damselae* transcriptome, so it can be concluded that the RstAB system is a major

regulator of the secretome in this pathogenic bacterium. The regulation of T2SS genes themselves remained largely uncharacterised so far. We here found that the expression of T2SS genes displays some level of downregulation in *rstA* and *rstB* mutants (Table 4.10). Of note, the enormous leap between the FC values of toxin genes (FC of -1408 in *dly* gene) and of structural T2SS genes (*eps* genes; FC values ranging from -3 to -4.8) further demonstrates that the absence of cytotoxin secretion in mutants for the RstAB system is mainly explained by the downregulation of cytotoxin genes themselves and not by the downregulation of the T2SS genes.

4.3.2.Outer membrane proteins and porins

The analysis of genes whose expression is downregulated in RstAB system mutants has brought to the forefront a set of genes not yet characterised with potential roles in virulence (Table 4.10). Amongst these genes are outer membrane proteins and porins, found to be strongly downregulated in RstAB system mutants. The most downregulated gene in this category, VDA_000284 (FC value of -338), encodes an outer membrane protein of unknown function. VDA_001503 and VDA_000966 encode two distinct OmpA-like outer membrane proteins, whose homologues constitute virulence factors in some species, being involved in invasion and immune evasion in *E. coli* (Weiser and Gotschlich, 1991), and in adherence to epithelia and biofilm formation in *Acinetobacter baumannii* (Gaddy *et al.*, 2009). In other *Photobacterium* species VDA_001503 homologous proteins are up-regulated under UVB radiation (Matallana-Surget *et al.*, 2012) and high pressure (Le Bihan *et al.*, 2013). pPHDD1-encoded OmpU (VDA_000113) was also this set of genes and its expression was also found to be enhanced at 25ºC (Table 4.3).

4.3.3.Genes with functions in resistance to antimicrobials and host defences

Genes related to survival within the host and resistance to antimicrobial agents are also under control of RstAB (Table 4.10). This is the case of the five-gene cluster (VDA_000154 to VDA_000158) encoded within the virulence plasmid pPHDD1 that encodes TolC

protein, AcrAB and two proteins of a complex for multidrug efflux pumping (Du *et al.*, 2014). Its role in the biology of *P. damselae subsp. damselae* remains uncharacterised, although we reveal in the present thesis that its expression is enhanced at 25ºC (when compared to 15ºC) and positively regulated by the RstAB. The Tol-Pal system (VDA_002432-VDA_002436), also downregulated in RstAB system mutants, is a periplasmic protein complex that has many functions in Gram-negative bacteria such as outer membrane integrity, colicin tolerance and survival within host cells (Gerding *et al.*, 2007; Hirakawa *et al.*, 2019). Our analysis also showed the downregulation of VDA_000599 homologous to phosphoethanolamine lipid A transferase EptA (VCA1102) of *V. cholerae* which contributes to polymyxin B resistance (Herrera *et al.*, 2017).

Also, in this set of downregulated genes in RstAB mutants, domain of unknown function (DUF) 535-containing proteins (VDA_001878, VDA_001124) were identified. Of note, the homologous protein in *Salmonella enterica*, dubbed VirK, was found to be a major virulence factor influencing long-term survival in the host (Spencer *et al.*, 2010), and the homologous protein in *V. cholerae* is induced during early infection (LaRocque *et al.*, 2005). In our data set, we also found VDA_003529 with high identity to *V. cholerae* protease DegP (VC0566), involved in biofilm formation and intestinal colonisation (Altindis *et al.*, 2014) which was also among upregulated genes at 25ºC. Furthermore, the protease encoded by VDA_001571 is homologous to an *E. coli* virulence factor that modulates host immune response (Jandu *et al.*, 2009). Again, the three RstAB-regulated, pPHDD1 plasmid-encoded genes related to the type 6 secretion system (T6SS) whose expression was found to be upregulated at 25 with respect to 15ºC (Table 4.4), was also found to be positively regulated by the RstAB system (Table 4.10).

4.3.4.Capsule biosynthesis genes

One of the most remarkably findings of this study was the observation that mutation of either *rstA* or *rstB* caused a drastic downregulation of 21 genes predicted to encode functions for synthesis of extracellular and capsular polysaccharides (EPS and CPS; Fig. 4.19).

Thirteen of these genes exhibited a higher than 500-fold downregulation in their expression in RstAB mutants compared to the parental strain, achieving dramatic FC values higher than 1000-fold downregulation in three genes (Table 4.10). These genes map to two divergently transcribed gene clusters located in a 25 kb contig of the draft genome sequence of *P. damselae* subsp. *damselae* RM-71 (Genbank Acc. No. LYBT01000063.1), and were here dubbed as cluster I and cluster II (Fig. 4.20). Recent studies have revealed that this genomic region is highly variable among *P. damselae* subsp. *damselae* isolates, containing many strain-specific genes (Osorio *et al.*, 2018; Terceti *et al.*, 2018).

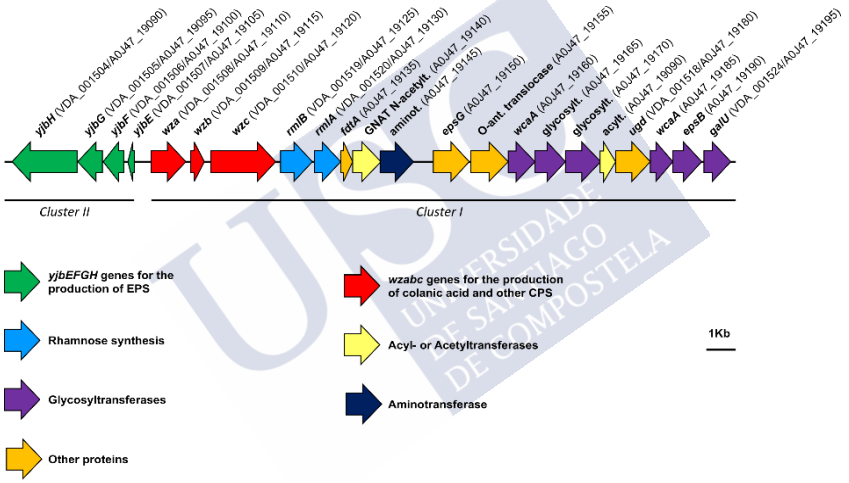

Figure 4.20: Scheme depicting the two divergently-transcribed gene clusters (cluster I and II) that encode functions related to synthesis of capsular and extracellular polysaccharides (CPS and EPS), and that exhibit a strong downregulation in RstAB system mutants. VDA codes correspond to the annotation of the type strain CIP102761 genome, and A0J47 codes correspond to the annotation of the RM-71 genome.

Similarity searches clearly suggest that the genes of these two clusters are involved in the synthesis and export of a polysaccharide capsule. The first three genes of cluster I, *wza*, *wzb*, and *wzc*, are homologous to three *E. coli* genes that encode the export system for biosynthesis of colanic acid and group 1 and 4 capsules (Whitfield, 2006). Wza is an outer membrane protein responsible for the export of

CPS from the periplasm to the cell surface (Dong *et al.*, 2006). Wzc is a tyrosine kinase while Wzb is the cognate phosphatase that regulates the phosphorylation state of Wzc (Temel *et al.*, 2013). In addition, a number of genes encoding sugar transferases for capsule and polysaccharide synthesis are found downstream *wza*, *wzb*, and *wzc* (Fig. 4.20). Interestingly the expression of *wza*, *wzb* and *wzc* is also slightly upregulated at 25ºC (DEGs 15 vs 25, DEGs 25 vs 37). The divergently-transcribed cluster II comprises genes *yjbEFGH*. Homologues of these genes have received scarce attention so far. Mutants for these genes have been reported to exhibit alterations of colony morphology and of the production of an extracellular polysaccharide in *E. coli* (Ferrières *et al.*, 2007), but their specific function remains unknown.

4.4. DESCRIPTION OF AN RSTAB-DEPENDENT AND HITHERTO UNKNOWN POLYSACCHARIDE CAPSULE IN *P. DAMSELAE* SUBSP. *DAMSELAE*: GENETIC CHARACTERISATION AND ROLE IN VIRULENCE

The transcriptomic characterisation of RstAB system mutants revealed a strong downregulation of many yet-uncharacterised genes with potential roles in virulence. Remarkably, the strong downregulation of the two putative capsular gene clusters prompted us to conduct functional studies that revealed the production of an unknown capsule in *P. damselae* subsp. *damselae* with a major role in virulence.

4.4.1. Mutation of *rstA* and *rstB* impair capsule production

To assess the presence of a capsule in *P. damselae* subsp. *damselae* RM-71, we first investigated the differences between wt and Δ*rstA* and Δ*rstB* mutant strains in colony morphology, as changes in colony morphology have frequently been related to alterations of cell-surface components such as capsular layers (Ferrières *et al.*, 2007; Petruzzi *et al.*, 2017). Parental strain RM-71 and Δ*rstA* and Δ*rstB* mutants were plated on TSA-1 supplemented with the polysaccharide-staining dye Congo Red (0.01% [wt/vol]) and differences between strains were evidenced; parental RM-71 colonies grew opaquer in comparison to Δ*rstA* and Δ*rstB* which were more translucent (Fig. 4.21).

Next, presence of capsule was analysed by transmission electron microscopy (TEM; Fig. 4.22). Ultrathin sections of the parental strain

RM-71 revealed the presence of an approximately 70 nm electron dense capsular layer outside the outer membrane. This layer was absent in mutants Δ*rstA* and Δ*rstB*. Interestingly, Δ*rstA* and Δ*rstB* mutants displayed small projections and vesicles at the cell surface or in the close vicinity of the cells.

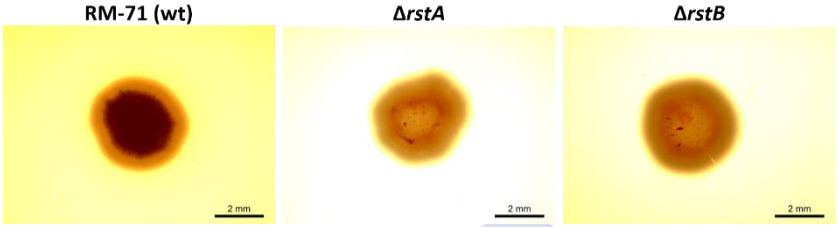

Figure 4.21: Observation of colony morphology in TSA-1 supplemented with Congo Red 0.01% [wt/vol] by stereo microscopy. *P. damselae* subsp. *damselae* RM-71 grows opaquer than deletion mutants Δ*rstA* and Δ*rstB* suggesting changes in cell envelope. Pictures were taken after cultures had been incubated for 24 h. Scale bar: 2 mm.

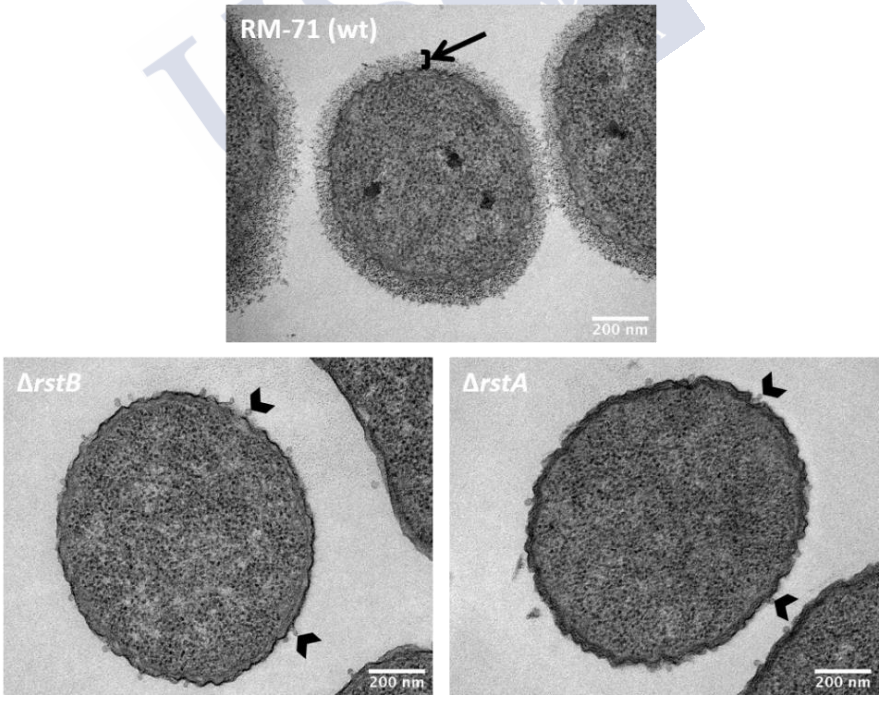

Figure 4.22: Observation of bacterial capsule by transmission electron microscopy (TEM). Evidence of capsule production in *P. damselae* subsp. *damselae* RM-71 wt, and of impaired capsule synthesis in Δ*rstA* and Δ*rstB* mutants. The black thin arrow shows the electron dense capsular layer around the surface of RM-71 wt. Black arrowheads point at small projections around the cell surface of acapsular mutants of the RstAB system (Δ*rstA*, Δ*rstB*).

4.4.2.Functional characterisation of exopolysaccharide synthesis genes *wza, wzc* and *yjbH*

4.4.2.1.*Mutants in genes* wza, wzc *and* yjbH *are impaired in capsule synthesis*

To demonstrate that the putative capsular gene clusters downregulated in RstAB mutants are involved in the production of the capsular layer that we revealed by TEM, we constructed in-frame deletion mutants by allelic exchange of three selected genes, *wza* and *wzc* in cluster I, and *yjbH* in the divergently-transcribed cluster II depicted in Fig. 4.20.

When plated in TSA-1 supplemented with Congo Red, we observed that Δ*yjbH* colonies grew opaque similarly to parental RM-71 strain. However, Δ*wza* and Δ*wzc* mutants exhibited a more translucent phenotype than the wt strain RM-71 (Fig. 4.23) and similar to that of deletion mutants Δ*rstA* and Δ*rstB* (Fig. 4.21).

When analysed by TEM, Δ*wza* and Δ*wzc* mutants were shown to be acapsulated (Fig. 4.24), whereas the Δ*yjbH* mutant exhibited a capsule, although thinner than that of the wt (Fig. 4.25). The small projections observed in Δ*rstA* and Δ*rstB* mutants at the cell surface were not observed in Δ*wza*, Δ*wzc* and Δ*yjbH* mutants. When acapsular mutant strains (Δ*wza* and Δ*wzc*) were complemented with wt genes, capsule synthesis was restored. Altogether, these results demonstrate that *wza* and *wzc* genes are essential for capsule biosynthesis in *P. damselae* subsp. *damselae*, and clearly suggest that the acute downregulation observed in these genes in RstAB system mutants causes, at least in part, the absence of capsule in Δ*rstA* and Δ*rstB* strains.

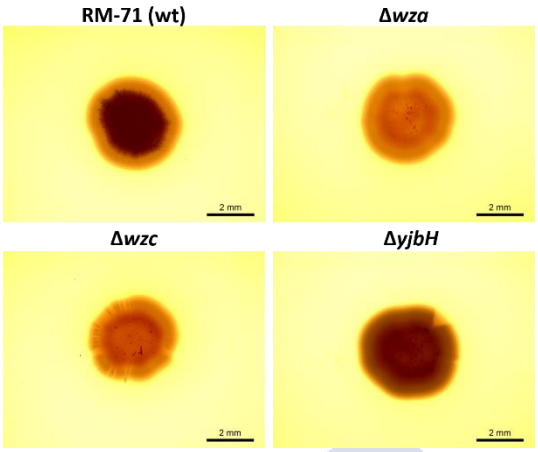

Figure 4.23: Observation of colony morphology in TSA-1 supplemented with Congo Red 0.01% [wt/vol] by stereo microscopy. *P. damselae* subsp. *damselae* RM-71 and deletion mutant Δ*yjbH* grow opaquer than deletion mutants Δ*wza* and Δ*wzc* that were translucent. Pictures were taken after cultures had been incubated for 24 h. This suggests changes in cell envelope. Scale bar: 2 mm.

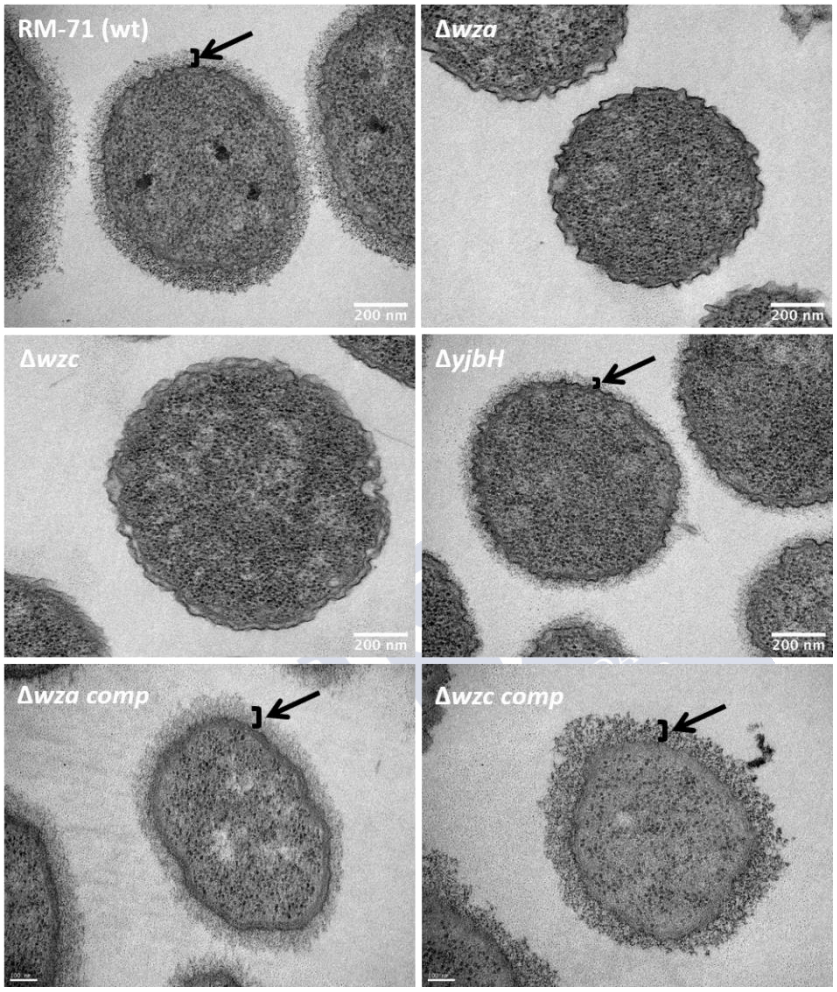

Figure 4.24: Observation of bacterial capsule by transmission electron microscopy. This shows an impaired capsule synthesis in Δ*wza*, Δ*wzc* and a thinner capsule in Δ*yjbH* mutant. Black thin arrows point at the electron dense capsular layer around the surface of RM-71 wt, Δ*yjbH*, and complemented strains Δ*wza* comp and Δ*wzc* comp.

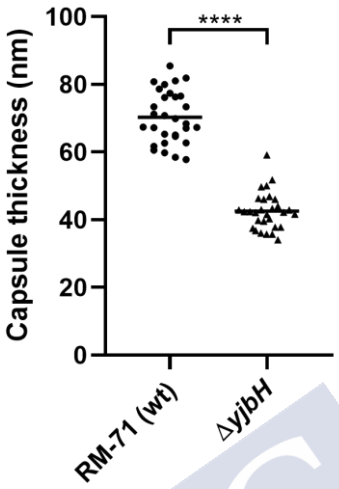

Figure 4.25: Analysis of capsule thickness in *P. damselae* subsp. *damselae* RM-71 wt and the respective Δ*yjbH* mutant. Capsule was significantly thinner in the Δ*yjbH* mutant (P-value <0.0001).

4.4.2.2. *Mutants in genes* wza, wzc *and* yjbH *do not exhibit growth defects*

We wanted to assess whether mutants defective in the synthesis of a capsule layer display different growth properties. Consequently, we analysed growth of strains wt RM-71, Δ*wza*, Δ*wzc* and Δ*yjbH* at 25ºC, which is around the optimal temperature for *P. damselae* subsp. *damselae* growth (Fouz *et al.*, 2000) at different salinity conditions: 1% NaCl (conditions that mimic the salinity of a fish internal medium), 3% NaCl (conditions that mimic the planctonic lifestyle in seawater) and 5% NaCl (abnormally high salinity for potential habitats). We also checked whether different strains had a different response to the human body temperature (37ºC). In general, analysis of the growth curves of RM-71, Δ*wza*, Δ*wzc* and Δ*yjbH* (Fig. 4.24) revealed no differences when strains were cultured in different

conditions of salinity and temperature as they all showed virtually identical dynamics.

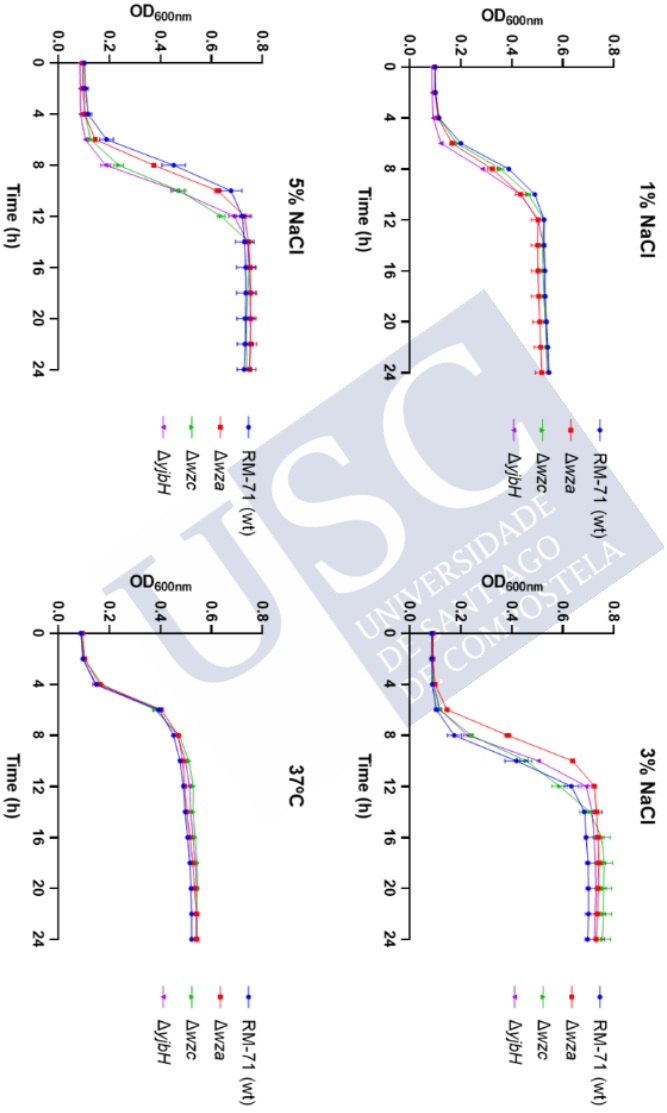

Figure 4.26: Growth of mutants in capsule synthesis genes Δ*wza*, Δ*wzc and* Δ*yjbH* is not impaired at different conditions of salinity and temperature. Mean data of three independent experiments are shown.

4.4.2.3. *Biofilm formation is higher in acapsular mutants* wza *and* wzc

Biofilms play a significant role in bacterial environmental survival and also in pathogenicity and antimicrobial resistance. Mutations in the genes evaluated here in other organisms, impair production of EPS and CPS and impact biofilm formation, either by increasing or by impairing its formation (Joseph and Wright, 2004; Wu *et al.*, 2011). The ability to form biofilm has been scarcely studied in *P. damselae* subsp. *damselae* so far. Here, the parental strain RM-71 and Δ*wza*, Δ*wzc* and Δ*yjbH* mutants were screened for their ability to form biofilm using a crystal violet assay. Notably, biofilm formation was inversely related to capsule production as capsule-deficient mutants (Δ*wza* and Δ*wzc*) presented a significantly greater capacity to form biofilm than the parental strain and Δ*yjbH* mutant (*P*-value <0.0001 in Kruskal-Wallis test; Fig. 4.27).

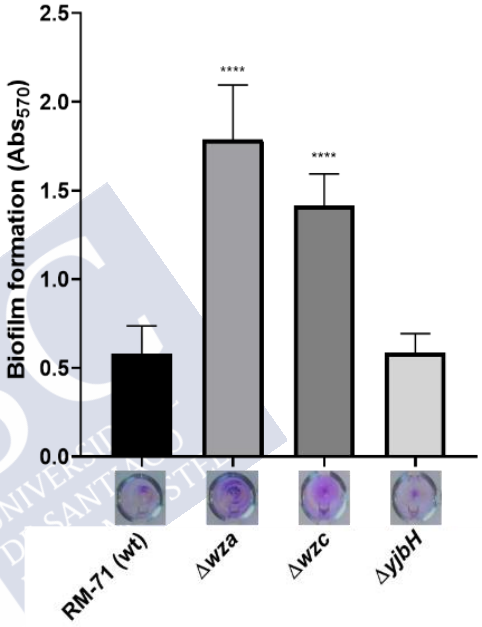

Figure 4.27: Biofilm formation of RM-71 wt strain and deletion mutants Δ*wza*, Δ*wzc* and Δ*yjbH*. Biofilm formation was quantified as crystal violet absorbance at 570 nm. The data summarize 3 independent experiments (** = p value < 0.0001). A representative image of biofilm formation on 96-well plates is shown.**

4.4.2.4.*Mutation of* wza, wzc *and* yjbH *does not affect haemolysin and phospholipase production*

As it has been mentioned haemolysins Dly, PhlyP and PhlyC are main virulence factors in *P. damselae* subsp. *damselae* pathogenicity (Rivas *et al.*, 2011, 2013; Vences *et al.*, 2017). In addition, phospholipase PlpV also contributes to full virulence (Vences *et al.*, 2017). Haemolytic activity is attributable to the synergistic and additive effects of cytotoxins Dly, PhlyP and PhlyC (Rivas *et al.*, 2013), whereas phospholipase activity is attributable to Dly and PlpV (Vences *et al.*, 2017).

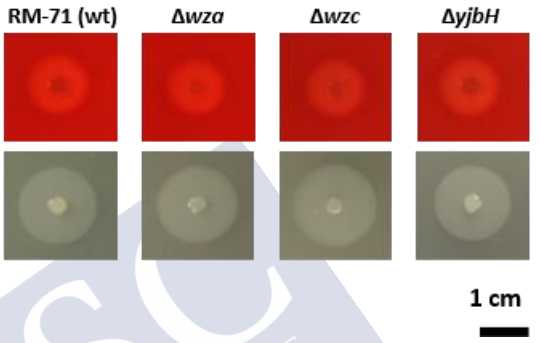

Figure 4.28: Haemolytic (top panel) and phospholipase (bottom panel) phenotypes of *P. damselae* subsp. *damselae* wt RM-71 and its derivative mutants Δ*wza*, Δ*wzc* *and* Δ*yjbH*. Scale bar, 1 cm. Note capsular gene mutants exhibit wt phenotypes.

We found that exopolysaccharide synthesis mutants Δ*wza*, Δ*wzc* and Δ*yjbH* show a normal haemolytic phenotype such as that of the wt strain RM-71 which indicates no defect in production or secretion of these cytotoxins (Fig. 4.28). As well, none of the mutants displayed any defect in phospholipase secretion (Fig. 4.28).

4.4.2.5. *Mutation of* wza, wzc *and* yjbH *does not affect swimming motility*

Swimming motility is thought to constitute an important factor in the pathogenicity of *P. damselae* subsp. *damselae* for fish as it has been demonstrated that seawater transmits the disease (Fouz *et al.*, 2000). We found that mutant strains (*wza*, *wzc* and *yjbH*) were not affected in swimming motility with respect to parental strain in motility agar (Fig. 4.29).

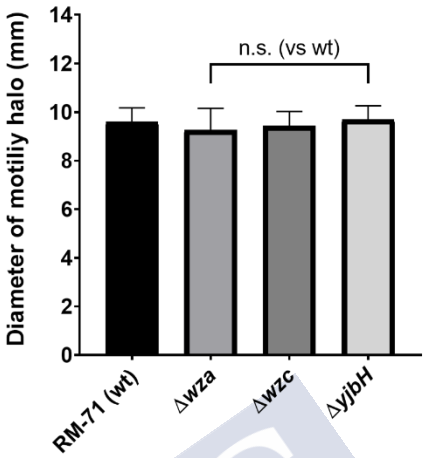

Figure 4.29: Quantitative measurements of swimming motility phenotypes of wt and mutant strains in motility agar. Haloes (in mm) were measured for 15 independent inocula per strain, and mean data with standard deviation are shown. A One-way ANOVA analysis revealed no significant differences among groups.

4.4.2.6.*Mutation of* wza *and* wzc *reduces growth in fish serum and mucus*

Next, to gain an insight into the role of the *P. damselae* subsp. *damselae* polysaccharide capsule in resistance against fish defensive mechanisms, survival assays of parental RM-71 and deletion mutants Δ*wza*, Δ*wzc* and Δ*yjbH* in turbot serum and mucus were performed. Results showed that parental and mutant strains were able to proliferate in presence of serum or mucus, however, the number of culturable cells of Δ*wza* and Δ*wzc* mutants was significantly lower than RM-71 and Δ*yjbH* (Fig. 4.30). During the incubation period (from 0 to 4.5 h), the number of CFU in turbot serum increased in a percentage of 1629% for RM-71, 1000% for Δ*wza*, 971% for Δ*wzc* and 1519% for Δ*yjbH*. After 3-h exposure, differences were evident between RM-71 and Δ*wza* and Δ*wzc* since RM-71 yielded 1.72-fold and 1.68-fold more culturable cells (*P*-value <0.0001) than mutants. These differences remained remarkable after 4.5 h, when RM-71 CFU numbers were 1.74-fold and 1.73-fold higher than Δ*wza* and Δ*wzc* (*P*-value <0.0001). In contrast,

culturability of Δ*yjbH* in turbot serum remained unaffected in comparison to the parental strain RM-71 in all the exposure times tested.

Concerning mucus survival, during the incubation period (4.5 h), the percentage of culturable cells increased a 1364 % for RM-71, 1051% for Δ*wza*, 539% for Δ*wzc* and 1606% for Δ*yjbH*. After 3 h, differences were found between strain RM-71 and Δ*wza* and Δ*wzc* given that RM-71 yielded 7-fold and 2-fold more CFU than mutants (*P*-value <0.0001 and *P*-value = 0.0009, respectively). These differences

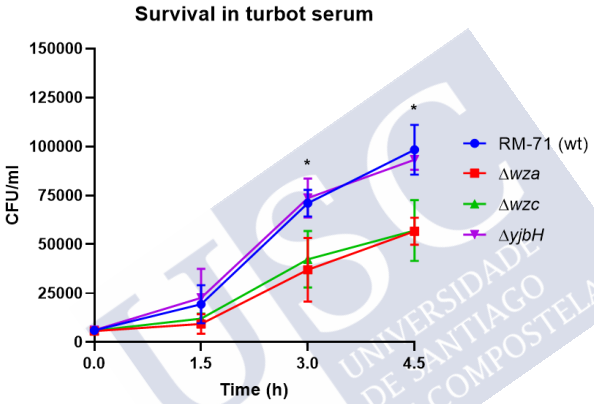

Figure 4.30: Deletion of *wza* and *wzc* impairs survival of *P. damselae* subsp. *damselae* in turbot serum. Data are shown as means ± standard deviation. *: significant differences between the wt and mutants Δ*wza* and Δ*wzc*. CFU, colony forming units.

remained also notable after 4.5 h, when RM-71 showed 1.66-fold and 2.1-fold more CFU than Δ*wza* and Δ*wzc*, respectively (*P*-value <0.0001). Similar to serum experiments, culturability of Δ*yjbH* in turbot mucus remained unaffected in comparison to RM-71 in all the exposure times tested (Fig. 4.31).

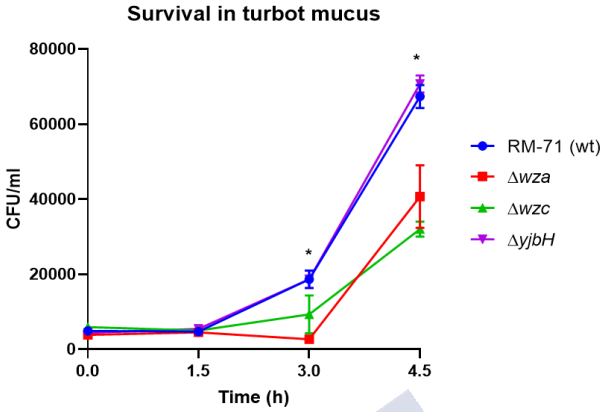

Figure 4.31: Deletion of *wza* and *wzc* impairs survival of *P. damselae* subsp. *damselae* in turbot mucus. Data are shown as means ± standard deviation. *: significant differences between the wt and mutants Δwza and Δwzc. CFU, colony forming units.

4.4.2.7. *Mutation of* wza *and* wzc *strongly reduces virulence for fish*

In order to study the impact of mutations in *wza*, *wzc* and *yjbH* genes and, hence, to assess the contribution of capsule to virulence of *P. damselae* subsp. *damselae* for fish, we conducted virulence tests using turbot (Fig. 4.32A), which is the natural source of isolation of the highly virulent strain RM-71 (Fouz *et al.*, 1992), and gilthead sea bream (Fig. 4.32B), a fish species that also constitutes a host for *P. damselae* subsp. *damselae* (Vera *et al.*, 1991).

Remarkably, the results showed a major impairment in virulence in the two mutants Δ*wza* and Δ*wzc* with respect to wt RM-71, whereas Δ*yjbH* mutant was not affected with respect to the wt. Collectively, these results provide strong evidence that the attenuation in virulence observed in Δ*wza* and Δ*wzc* mutants and their lower growth in serum and mucus, is attributable to the absence of capsule despite the production of wt levels of haemolysins, unaltered swimming motility and normal growth under different temperatures and NaCl concentrations.

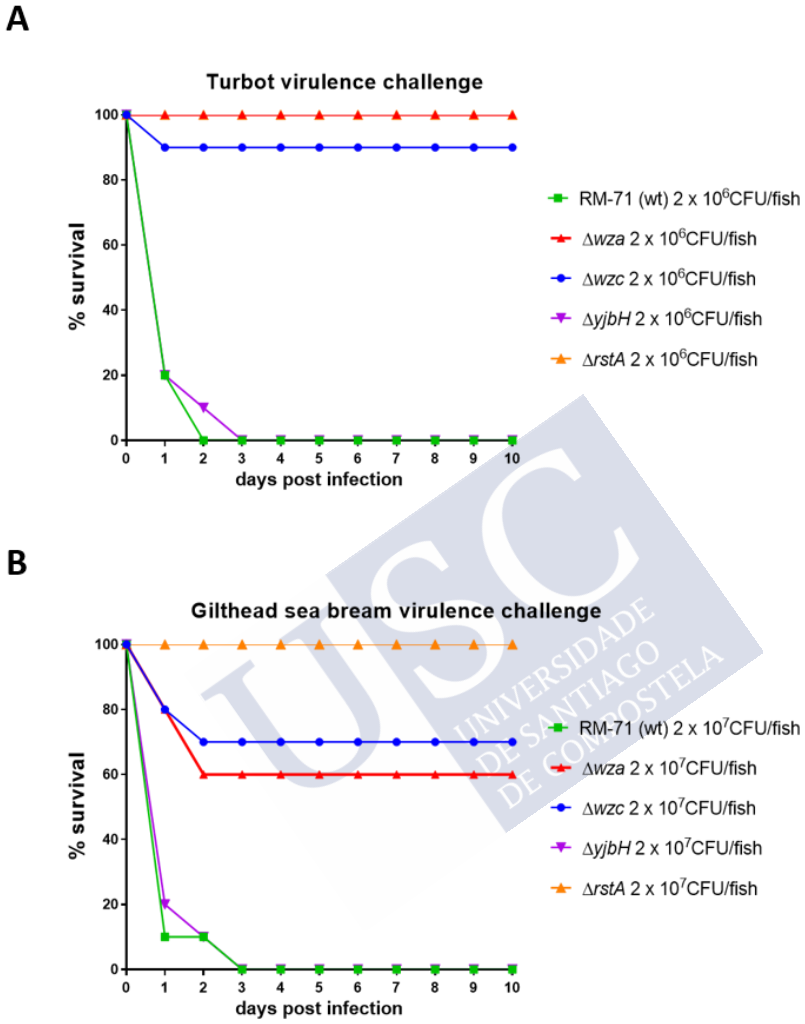

Figure 4.32: Survival (%) of turbot (A) and gilthead sea bream (B) fish after intraperitoneal injection of *P. damselae* subsp. *damselae* RM-71 and mutants. A total of 10 fish were inoculated per strain. The respective control fish group (10 fish inoculated with 0.1 ml of sterile 0.85% NaCl solution) did not register any mortalities (data not shown). Deletion of *rstA*, *wza* or *wzc* impaired virulence for fish.

5. DISCUSSION

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5. DISCUSSION

P. damselae subsp. *damselae*, a marine bacterium belonging to the family *Vibrionaceae*, is a life-threatening pathogen for a wide array of marine organisms and for humans. Given that Vibrios are favoured by warm (over 15ºC) and moderate saline waters, their spread and the expansion of associated diseases triggered by global warming came into the limelight as a serious health concern in recent times (Vezzulli *et al.*, 2012, 2013, 2016; Baker-Austin *et al.*, 2013, 2018; Le Roux *et al.*, 2015; Froelich and Daines, 2020). *P. damselae* subsp. *damselae* is recognised as an important menace for the aquaculture industry with a lately hazardous expansion of its geographical range (Osorio *et al.*, 2018). Increased water temperatures either because of unusual high peaks or warmer periods over the year, play a critical role in the development of outbreaks in farms (Vera *et al.*, 1991; Fouz *et al.*, 1992; Pedersen *et al.*, 1997; Abdel-Aziz *et al.*, 2013; Essam *et al.*, 2016; Sharma *et al.*, 2017; Tao *et al.*, 2018; Zhang *et al.*, 2019; Aguilera-Rivera *et al.*, 2019). Concerning human *P. damselae* subsp. *damselae* infections, they can evolve to fulminant septicaemias or necrotising fasciitis with fatal outcome, in spite of administered antibiotic treatment (Yuen *et al.*, 1993; Tang and Wong, 1999; Yamane *et al.*, 2004; Goodell *et al.*, 2004; Alhemairi *et al.*, 2015). This bacterium is acknowledged as one of the main zoonotic bacteria acquired from fish (Lehane and Rawlin, 2000; Austin, 2010). Over the last decades, most human infections concentrated along coastal areas of the United States, Japan and Australia (Love *et al.*, 1981; Dryden *et al.*, 1989; Fraser *et al.*, 1997; Goodell *et al.*, 2004; Alvarez *et al.*, 2006; Nakamura *et al.*, 2008; Hundenborn *et al.*, 2013, Akram *et al.*, 2015; Sahu *et al.*, 2020) but it is of notable relevance the very new European cases, namely in Greece, Portugal and Spain (Chochlakis *et al.*, 2019; Guimaraes *et al.*, 2020; Schrottner *et al.*, 2020). The ability of some *P. damselae* subsp. *damselae* strains to grow at human body temperature is key, however,

difficulties to recover the organism from infected tissues have been reported (Clarridge and Zighelboim-Daum, 1985; Coffey *et al.*, 1986; Goodell *et al.*, 2004).

The present thesis aims at analysing the role of aspects that modulate virulence and fitness of *P. damselae* subsp. *damselae*. As this bacterium is regarded as a non-clonal pathogen, we focused this study on characteristics subjacent to all strains, whether or not they harbour the pPHDD1 virulence plasmid: response to temperature and transcriptional regulation by the two-component system (TCS) RstAB.

Despite clear evidence of the impact of environmental temperature on the pathobiology of *P. damselae* subsp. *damselae* for marine cultivated animals and humans, the physiological and molecular changes this bacterium undergoes in response to this parameter have yet to be explored. This field of research is poorly studied in marine pathogenic bacteria and likewise, little is known about how environmental parameters regulate the expression of their virulence factors. Therefore, we first examined by RNA-sequencing (RNA-seq) the transcriptome produced by the *P. damselae* subsp. *damselae* highly virulent and pPHDD1 harbouring strain RM-71 cultured at two temperatures: 15ºC simulating life in cold waters during winter months and 25ºC mimicking warm summer months reproducing favouring conditions for the development of outbreaks in aquaculture. Next, we designed another RNA-seq approach combined with phenotypic assays to disclose the transcriptome changes experienced by *P. damselae* subsp. *damselae* when entering the human body. Hence, we compared expression and phenotypic traits at 25 and 37ºC, simulating warm waters derived from global warming and human body temperature, respectively.

There are numerous external and internal signals that may control gene expression in bacterial pathogens. Recently discovered, the TCS RstAB was recognised as a positive regulator of cytotoxin expression and virulence. As well, mutants in either gene of the system resulted in a weaker secretion of various type II secretion system (T2SS)-dependent proteins (Terceti *et al.*, 2017, 2019). This system is broadly present across main pathogens of the family *Vibrionaceae* but information about their whole regulon is scarce. Despite its strong

regulation of virulence in *P. damselae* subsp. *damselae*, the genetic network under the control of this key system has not been thoroughly elucidated so far. As the second part of this thesis, we investigated the genes regulated by the RstAB system and thus revealed new genetic functions in relation to virulence. We designed an RNA-seq study comparing the transcriptome produced by deletion mutants Δ*rstB* and Δ*rstA* with that of the wild type strain RM-71. This brought to the forefront a number of functions that still need to be studied in *P. damselae* subsp. *damselae* and, of particular relevance, the presence of a capsule that proved to be crucial for virulence.

In sections 5.1 and 5.2 we will discuss the obtained data.

5.1. STUDY OF *P. DAMSELAE* SUBSP. *DAMSELAE* VIRULENCE AND FITNESS: ROLE OF TEMPERATURE IN MODULATING TRANSCRIPTOMIC PROFILE AND PHENOTYPE

P. damselae subsp. *damselae* RM-71 was the strain selected for the majority of the experiments compiled in the present thesis because of its high virulence, strong haemolytic phenotype and for harbouring the virulence plasmid pPHDD1 (Rivas *et al.*, 2011). Interestingly, this strain was isolated during a disease outbreak in a turbot (*Psetta maxima*) farm in Galicia, when the temperature of the water rocketed from 18ºC to 22-24ºC in the summer of 1988 (Fouz *et al.*, 1992).

Following a rise in water temperature, Vibrios can quickly reproduce and switch from almost undetectability to predominance among bacterial populations (Gilbert *et al.*, 2012; Martin-Platero *et al.*, 2018). The concurrence of high temperatures and outbreaks of disease caused by *P. damselae* subsp. *damselae*, although repeatedly described worldwide, has not been the principal subject of research since Fouz *et al.*, 2000 evidenced that seawater transmits the disease and its spread largely depends on temperature. *P. damselae* subsp. *damselae* isolation was first reported from skin ulcers in damselfish (*Chromis punctipinnis*) during the summer and fall seasons in southern California, showing a seasonal pattern of infectivity (Love *et al.*, 1981). In the Spanish Mediterranean and Southern coast, the culture of commercially-important sparid species has been constantly compromised by this organism during summer months (Vera *et al.*, 1991; Botella *et al.*, 2002;

Pujalte *et al.*, 2003) and, of notice, García-Rosado *et al.*, 2007 reported its isolation from redbanded sea bream (*Pagrus auriga*) not only during the summer but also during an especially warm autumn in 2004. More recently, the continuous publication of reports describing disease episodes correlated with high temperatures in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) in Egypt, establishes the importance of this phenomenon in the area (Essam *et al.*, 2016; Eissa *et al.*, 2018). As well, *Litopennaeus vannamei* cultures in America and Asia were damaged in past summers (Aguilera-Rivera *et al.*, 2019; Wang *et al.*, 2020). With great significance, this phenomenon has been connected to outbreaks caused by both *P. damselae* subsp. *damselae* populations, i.e., strains harbouring the virulence plasmid pPHDD1 (thus presenting the two plasmid-encoded cytotoxins Dly and PhlyP plus the chromosome-encoded PhlyC) and those lacking it (encoding only the PhlyC; Fouz *et al.*, 1992; Terceti *et al.*, 2016). By growth curve analysis, we determined that under a temperature simulating warm waters typical from summer months (25ºC), *P. damselae* subsp. *damselae* undergoes a substantial increase in bacterial proliferation (evident from the very first hours). In contrast, growth at a control *a priori* non-hazardous condition for the development of aquaculture outbreaks (15ºC) revealed a lower and slower growth. At high temperatures, host defences can be likely compromised by the increased number of bacteria at the site of infection, being the complement and macrophage action deeply compromised (Lew *et al.*, 1980; Finlay and McFadden, 2006). This result evidences how increased water temperatures activate this pathogen multiplication and helps to understand why they represent a serious threat for the development of *P. damselae* subsp. *damselae* outbreaks in farms.

The present thesis includes the first report on the global transcriptomic characterisation of *P. damselae* subsp. *damselae*. We started with the comparison of the mRNA expressed at the same point of the growth curve, at 15ºC as a control condition vs 25ºC. Genes with enhanced expression at 25 compared to 15°C are labelled as "upregulated" and those with lower as "downregulated". Firstly, we found that genes with functions in nutrient acquisition and energy production were upregulated at 25ºC, being the nucleoside permease

NupC (VDA_001789) the most upregulated of the whole transcriptome. A *V. cholerae* mutant in which the *nupC* gene was deleted, showed an impairment in nucleoside acquisition and a fitness reduction in nutrient-limited environments (Gumpenberger *et al.*, 2016). Other genes involved in the acquisition and synthesis of nucleotides, vitamins and amino acids were also upregulated. Lipases and fatty acid transporters appeared also among that set of genes. Therefore, the genetic activation of processes related to synthesis and uptake of essential biomolecules at 25ºC may sustain the fast replication of this bacterium observed in the growth analysis.

The expression of virulence factors in response to temperature has been researched extensively in homeotherm infecting bacteria (Konkel and Tilly, 2000; Johansson and Cossart, 2003; Steinmann and Dersch, 2013). On the other hand, today's knowledge on such type of regulation in fish pathogenic bacteria is limited (Guijarro *et al.*, 2015). Unlike other marine pathogens, the optimal growth temperature of *P. damselae* subsp. *damselae* coincides with the typical temperatures at which aquaculture outbreaks occur. Considering their importance in the pathogenicity of *P. damselae* subsp. *damselae* for fish, our first premise was that cytotoxins would likely be upregulated at those temperatures at which outbreaks are favoured. Strikingly, growth at 25ºC does not upregulate the expression of Dly, PhlyC and PlpV and slightly upregulates that of PhlyP. Such discovery led us to analyse transcript abundance of cytotoxin encoding genes in the whole transcriptome context. Especially relevant was that we found that the expression of the *dly* gene was the ninth highest of the whole transcriptome produced at 15ºC; the expression of this cytotoxin was comparable to that of ribosomal proteins. RNA-seq data showed that Dly was the most expressed virulence factor at 15 and 25ºC. These observations fit with previous studies that presented a correlation between virulence and richness in the content of a cytolytic toxin in culture supernatants of several *P. damselae* subsp. *damselae* strains (Kreger, 1984). The two pore-forming toxins PhlyP and PhlyC exhibit great levels of expression (higher than those of housekeeping genes *gyrB*, *recA*, *mreB* and *ftsZ*) but unlike *dly*, they are not within the top 10. In light of these results, it is clear that the lower frequency of outbreaks caused by *P. damselae*

subsp. *damselae* occurring in fish farms at lower temperatures, around 15ºC, do not stem from a weak expression of cytotoxins. On the other hand, our study constitutes another evidence on how the presence of the virulence plasmid pPHDD1 made possible the evolution of highly virulent lineages of this bacterium.

A remarkable discovery emerging from this thesis was that, excluding cytotoxins, many other genes involved in pathogenic processes were upregulated at 25ºC. In *P. damselae* subsp. *damselae*, cytotoxins Dly, PhlyP, PhlyC and PlpV are secreted through the T2SS (Rivas *et al.*, 2015a; Vences *et al.*, 2017). The upregulation of the system indicates that the T2SS secretome play particularly relevant roles when *P. damselae* subsp. *damselae* grows at temperatures mimicking those of aquaculture outbreaks. Vences *et al.*, 2017 showed that a double deletion mutant in *dly* and $hlyA_{pl}$ genes caused more mortalities than an innately plasmidless strain in a fish model, which provided evidence about the presence of other uncharacterised pPHDD1-encoded genes involved in virulence. We observed that from the 31 differentially expressed genes (DEGs) of the virulence plasmid, 24 were upregulated at 25ºC. This finding contributes to the designation of pPHDD1 as a hallmark of highly virulent isolates. Potential pPHDD1-encoded virulence factors upregulated at 25ºC that were revealed in this thesis, will deserve special attention in future studies. Among those upregulated genes, a yet uncharacterised multidrug and toxin secretion efflux pump complex which include TolC and AcrAB proteins was found. Expression of *tolC* and *acrAB* in the fish pathogen *Yersinia ruckery* is higher at 28ºC with respect to 18ºC with a concomitant increased resistance to antibiotics and toxic substances such as acriflavine (Mendez *et al.*, 2018). Antibiotic resistance is a promising field of study in *P. damselae* subsp. *damselae*; recent studies showed that a number of multidrug resistant isolates harbour different variants of pAQU-family plasmids that are large, highly mobilisable and encode for several genes involved in antibiotic resistance (Vences *et al*., 2020). As well as a putative role in efflux of toxic substances, it cannot be ruled out that the TolC-AcrAB system might participate in the secretion of virulence factors in *P. damselae* subsp. *damselae*; TolC is part of the type I secretion system that exports haemolysins and other

factors in Gram-negative pathogens (Hinchliffe *et al.*, 2013). Other upregulated pPHDD1-encoded genes with known homologues in pathogenic Vibrios are the VDA_000113 (OmpU-like), VDA_000110 (Vep07-like) and VDA_000111 (Vep20-like). OmpU is involved in host-cell recognition and pathogenesis in several *Vibrio species* (Goo *et al.*, 2006; Nyholm *et al.*, 2009) and in particular, it is essential for *Vibrio tasmaniensis* adhesion and invasion of mollusc cells (Duperthuy *et al.*, 2011). *V. vulnificus* biotype 2 transferable plasmid (pVvbt2) encodes for Vep07 and Vep20, two proteins with a major role in virulence for eels. Vep07 is an outer membrane lipoprotein that participates in resisting the bactericidal action of eel serum (Amaro *et al.*, 2015) and Vep20 is a transferrin receptor (Pajuelo *et al.*, 2015). Iron uptake is key in *P. damselae* subsp. *damselae* virulence for fish and this bacterium can utilise transferrin as the only source of iron (Fouz *et al.*, 1994; Fouz *et al.*, 1997). Alongside with Vep20, a chromosome-encoded TonB-dependent siderophore receptor (VDA_000794) whose expression is enhanced under iron-limiting conditions (Puentes *et al.*, 2017) was also upregulated at 25ºC. Among this set of uncharacterised genes belonging to the pPHDD1 plasmid, we found that the expression of three genes only present in strain RM-71, that putatively encode for functions related to the type VI secretion system (T6SS), were upregulated at 25ºC. Homologues in *V. cholerae* and other species participate in defence against other bacterial cells that may contend for the same environments and resources (Zhang *et al.*, 2012; Shneider *et al.*, 2013). Also related to virulence, 25ºC acts as a signal for the upregulation of the expression of flagellar motility and chemotaxis genes. Previous studies have indicated the importance of motility in *P. damselae* subsp. *damselae* host tissue colonisation and pathogenicity (Fouz *et al.*, 1998; Fouz *et al.*, 2000). Chemotaxis may control flagellar motility through a signal cascade in which membrane chemoreceptors (methyl-accepting chemotaxis proteins) are involved. Recently, *P. damselae* subsp. *damselae* mutants in chemotaxis proteins proved to have unaltered motility phenotype but reduced PhlyP production and thus adhesion to cell cultures (Von Hoven *et al.*, 2018). In other members of the family *Vibrionaceae*, such as *V. anguillarum* and *V. fischeri*, mutation of chemotaxis proteins affects virulence and host tissue colonisation

(O'Toole *et al.*, 1996; DeLoney-Marino *et al.*, 2003). Our results suggest that enhancing chemotaxis and flagellum-dependent motility may favour adhesion to hosts and thus outbreaks in aquaculture farms. Transcriptional regulators and peroxidases whose functions are yet to be discovered were also found among the top upregulated genes. This is the case of a DeoR regulator whose homologues in *Shigella flexneri* and *Salmonella typhi* are key for intracellular growth and virulence (Haghjoo *et al.*, 2007; Morris *et al.*, 2013). Peroxidases constitute mechanisms to defend against reactive oxygen species produced by infected host cells (Miller and Britigan, 1997). Collectively, observations presented here suggest that contrary to expectations, the high likelihood of outbreaks occurring at warm water temperatures (around 25ºC) do not rely on a higher expression of cytotoxins, but on the upregulation of growth, motility, chemotaxis and the expression of other potential virulence factors, remarkably those encoded by the pPHDD1 plasmid. The characterisation of the latter will surely inspire future studies to broaden the understanding of the burden that *P. damselae* subsp. *damselae* represents for aquaculture.

Temperatures around the area of isolation of strain RM-71 in Galicia are known to vary between 13 and 15ºC in winter months (Martínez-Urtaza *et al.*, 2018). Before the heat wave that preceded the outbreak in which RM-71 was recovered, temperatures in the area were around 18ºC (Fouz *et al.*, 1992). Data point to the fact that this bacterium inhabits extensive marine ecosystems around the globe in which temperatures are below its optimum. To understand *P. damselae* subsp. *damselae* lifestyle and survival in cold temperatures, we also analysed DEGs downregulated at 25ºC, what is to say, with a higher expression at 15ºC with respect to 25ºC. As other members of the family *Vibrionaceae*, *P. damselae* subsp. *damselae* contains two chromosomes. Unlike chromosome I (ChrI) which encodes most essential genes, chromosome II (ChrII) presents a more flexible content and generally participates in environmental adaptations (Okada *et al.*, 2005; Dryselius *et al.*, 2007). As inferred from our data, *P. damselae* subsp. *damselae* ChrII might play a relevant role in cold adaptation for it presents 3 times more DEGs with a higher expression at 15ºC than at 25ºC. The operon with the highest expression at 15 in comparison to

25ºC has not been characterised up to date. It shares homology with genes that are involved in *B. subtilis* peptidoglycan synthesis and spore resistance (Beall *et al.*, 1994). The way in which the cell wall influences *P. damselae* subsp. *damselae* adaptation to low temperatures will deserve further attention. Other peptidases, membrane transporters and metabolic enzymes were also downregulated. Such set of genes includes the oligopeptide permease system *oppABCDF* whose expression has proved to be higher at temperatures as low as 4 and 15ºC in *V. alginolyticus* (Liu *et al.*, 2017). Although its main function is predicted to be nutritional (Braibant *et al.*, 2000), the contribution of the system to virulent processes such as adhesion, haemolytic activity or biofilm formation was also demonstrated (Liu *et al.*, 2017). The function of the *opp* operon in *P. damselae* subsp. *damselae* has not been studied so far, but evaluating its role in how this bacterium survives at low temperatures is a future field of study. The expression of genes belonging to the Krebs cycle (citrate synthase, malate synthase and isocitrate lyase) is also favoured at 15ºC. Interestingly, the Krebs cycle is linked to iron uptake in *P. damselae* subsp. *damselae* as citrate can be used as a sole source of iron (Fouz *et al.*, 1994) or as a siderophore that can be secreted to absorb iron from the environment (Balado *et al.*, 2017). More evidence is needed, but data presented here suggest that utilisation of citrate for iron acquisition at low temperatures, typical from some geographical areas in which *P. damselae* subsp. *damselae* lives, might be key for the survival of this bacterium in a free-swimming lifestyle outside hosts. Some members of the family *Vibrionaceae* have the ability to fix nitrogen (Tibbles and Rawlings, 1994) and although unknown in *P. damselae* subsp. *damselae*, we have revealed here a higher expression of genes related to this activity at 15º. Enzymes of the amino acid metabolism were also among this set of genes; this might be attributable to the need to provide optimal amino acid ratios for the different types of proteins produced at each temperature. As far as cold shock proteins are concerned, we only found one cold shock protein and a betaine glycine transporter among the 50 most expressed genes at 15ºC with respect to 25ºC. Other members of the family *Vibrionaceae* including *V. cholerae* and *V. parahaemolyticus* highly express cold shock proteins after a change to low temperatures around 15ºC (Yang

et al., 2009; Townsley *et al.*, 2016). Betaine glycine protects *Vibrio anguillarum* in acclimatisation to cold temperatures (Ma *et al.*, 2017). Other genes predicted to be involved in acid resistance functions (glutamine decarboxylase pathway; Zhao and Houry, 2010) and repair of oxidative damage (methionine sulfoxide reductase system MsrPQ; Brot and Weissbach, 2000) were also downregulated at 25ºC. These findings could indicate that growing at 15ºC does not abolish completely those processes related to virulence in *P. damselae* subsp. *damselae* as protection against reactive oxygen species produced by infected hosts is also activated. As well, the alternative sigma factor RpoS, a key regulator of bacterial stress responses (Weber *et al.*, 2005) and of host colonisation and virulence in *V. cholerae* and *V. parahaemolyticus* (Wurm *et al.*, 2017; Chang and Lee, 2018) presents an upregulation at 15ºC.

Continuing with the study of *P. damselae* subsp. *damselae* relationship with key temperatures in infective processes, we addressed how this bacterium responds to human body temperature. From an evolutionary perspective, the fact that *P. damselae* subsp. *damselae* has the ability to infect humans remains poorly understood. Common clinical manifestations of *P. damselae* subsp. *damselae* human infections include septicaemia, bullous lesions, wound infections and necrotising fasciitis (Pérez-Tirse *et al.*, 1993; Hundenborn *et al.*, 2013; Alhemairi *et al.*, 2015; Guimaraes *et al.*, 2020; Schrottner *et al.*, 2020). These symptoms differ from those caused by other members of the family *Vibrionaceae* (such as *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus*) that predominantly cause gastrointestinal problems and consequently, successful infective clones go back to the marine environment. For *P. damselae* subsp. *damselae*, no such specialisation seems plausible since human infections do not facilitate a return to marine waters. The above lines of evidence point at the potential of *P. damselae* subsp. *damselae* to infect humans as an anecdotal circumstance rather than an evolutionarily favoured characteristic.

In comparison to the optimal temperature of 25ºC, we show here that human body temperature (37ºC) decreases *P. damselae* subsp. *damselae* viability and significantly impairs growth, cell morphology and shape. Most human infections caused by this bacterium start with

wounds exposed to seawater or inflicted during the manipulation of fish (Pérez-Tirse *et al.*, 1993; Yuen *et al.*, 1993; Yamane *et al.*, 2004; Chochlakis *et al.*, 2019; Shröttner *et al.*, 2020). The review of the scientific literature reveals that wounds and lesions affect mainly upper and lower limbs (Goodell *et al.*, 2004; Yamane *et al.*, 2004; Hundenborn *et al.*, 2013; Akram *et al.*, 2015; Collins *et al.*, 2017; Guimaraes *et al.*, 2020) which are the coldest areas of the human body. This could be explained by the low viability of *P. damselae* subsp. *damselae* at 37ºC, so it is conceivable to believe that bacterial proliferation will concentrate in those regions of the body. Such lower viability at 37ºC observed in our results, fit with previous studies that reported difficulties in isolating this microorganism from infected human tissues. According to this idea, Coffey *et al.*, 1986 reported a rapid necrotising infection in which only cultures at the base of the finger yielded bacterial colonies, but those from more proximal parts of the arm were negative. Clarridge and Zighelboim-Daum, 1985 showed that culturing bullae fluid and blood barely produced colonies, but when cultures were streaked in blood agar plates, haemolytic activity was noticeable. Fraser *et al.*, 1997 suggested that rather than bacterial proliferation, the main cause behind a fatal human infection may have been a systemic toxin. These authors cultured blood and wound samples, but only the latter yielded colonies in plates. In addition, other authors reported that the microorganism was not detectable in Gram-stained preparations of infected tissues (Coffey *et al.*, 1986; Goodell *et al.*, 2004). Our observation of aberrant long cells of 2-3 µm after cultivation at 37ºC, agrees with earlier studies that reported the presence of large bacilli with similar dimensions in Gram-stained sections of infected tissues (Clarridge and Zighelboim-Daum, 1985; Coffey *et al.*, 1986, Goodell *et al.*, 2004). Altogether, our results suggest that a temperature of 37ºC is in itself a stressful condition for *P. damselae* subsp. *damselae*.

The analysis of *P. damselae* subsp. *damselae* human and fish isolates growth curves revealed similar dynamics; human isolates do not have an advantage in growing at human body temperature, which represents a stressful condition for all strains regardless their source of origin. As well, the genomic comparison did not reveal any genetic

marker exclusive to human isolates. The comparative genomic analysis also revealed the presence of the virulence plasmid pPHDD1 in the sequence of the human isolates presented here (80077637 and CDC-2227-81). However, Kreger, 1984, showed a collection of human isolates with haemolytic activities of different magnitude, suggesting that some of them lack the pPHDD1 plasmid. In fish isolates, the variable presence of this plasmid has already been demonstrated (Rivas *et al.*, 2011). Such patterns suggest that any genotype of *P. damselae* subsp. *damselae* living in marine waters may potentially cause an opportunistic infection in humans.

As other species of the genera *Vibrio* and *Photobacterium*, *P. damselae* subsp. *damselae* is naturally resistant to antibiotic agents that inhibit cell wall synthesis, for example, benzylpenicillin. Recently, Weaver *et al.*, 2018 reported that beta lactam tolerance in *Vibrio cholerae* is pleiotropic in nature, and genes that play a role in cell envelope synthesis and modification also serve an important function in beta lactam tolerance. In a recent study, mutants in the RstAB system showed aberrant morphological changes; mutant cells grew wider and longer (Terceti *et al.*, 2019), and in turn, they exhibited increased levels of susceptibility to benzylpenicillin (Terceti *et al.*, 2019). In the present study, we have found that a culture temperature of 37°C also causes significant morphological changes causing cells to grow longer. Likewise, natural benzylpenicillin resistance was reduced when *P. damselae* subsp. *damselae* was cultured at 37°C. Therefore, we conclude that certain conditions affecting cell shape may also affect natural penicillin resistance in *P. damselae* subsp. *damselae*.

A well-known bacterial mechanism to cope with environmental temperature variations is the adjustment of membrane fluidity by altering its lipid composition (Sinensky, 1974; Rock and Cronan, 1996; Mansilla *et al.*, 2004). The main strategy is to change the proportion of saturated fatty acids and unsaturated fatty acids in membranes (Marr and Ingraham, 1962; Russell, 1983; Zhang and Rock, 2008). Analysis of membrane fatty acids from cultures of *P. damselae* subsp. *damselae* RM-71 incubated at 15, 25 and 37ºC have shown that the amount of membrane saturated fatty acids is higher as temperature increases. In view of the results obtained, it can be assumed that *P. damselae* subsp.

damselae can inhabit such diverse environments (and such different hosts) due to, among other factors, its ability to reversibly modify membrane fatty acids.

Transposon mutagenesis has been widely used for constructing defective mutants and find genes that participate in a given bacterial phenotype. Using *P. damselae* subsp. *damselae* strain RM-71, we generated and screened transposon insertional libraries for the search of mutants with an impairment in growth at 37 with respect to 25ºC. Our goal was to identify molecular targets as a basis for drug design that mitigate the impact of future human infections caused by this bacterium. Nevertheless, after analysing more than 4000 clones we were unable to isolate any mutant showing the desired phenotype. Our results suggest that the ability to grow at 37°C is a multigenic trait rather than a monogenic characteristic.

To gain further insight into how *P. damselae* subsp. *damselae* responds to human body temperature, we designed a second transcriptomic study choosing 25ºC as a control condition (mimicking warm water temperatures derived from global warming) in comparison with 37ºC (human body temperature). Hereafter, those genes with enhanced expression at 37 compared to 25°C are labelled as "upregulated" and those with lower as "downregulated". *P. damselae* subsp. *damselae* growth at 37ºC is characterised by a fast initial proliferation that might be maintained by the enhanced expression of metabolic and ATP-producing functions. As well, we found some nutrient transporters among the upregulated genes at 37ºC. In addition to the previously described impaired growth, viability, shape and resistance to benzylpenicillin, a temperature of 37ºC upregulates the expression of numerous chaperones, heat-shock proteins and others that act in prevention of oxidative damage. Other members of the family *Vibrionaceae* (*V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*) that also cause disease in humans and marine species are regularly cultured at 37ºC as it does not constitute a stressful condition. Actually, in a recent transcriptomic study of *V. parahaemolyticus*, a temperature of 37ºC was chosen as the control condition because it shows the maximum number of genes with stable expression in comparison to temperatures of 4, 15, 20 and 42ºC (Urmersbach *et al.*, 2015). In order

to check whether mutation of these heat-shock proteins would prevent *P. damselae* subsp. *damselae* to grow at 37ºC, we tried to construct insertional mutants in *clpB*, *groEl* and *htpG*. The results were unsuccessful, reinforcing the idea of the ability of this bacterium to grow at 37ºC as a multigenic trait rather than a characteristic derived from the presence of a single gene marker. The analysis of transcriptomes produced at different temperatures shows how, at the temperature of the human body, *P. damselae* subsp. *damselae* activates a protective strategy by an upregulation of heat-shock proteins and other defensive strategies.

Many upregulated DEGs that were found in the present thesis are in wait for further research. The outer membrane protein (VDA_000104) shares homology to *V. cholerae* peptidoglycan-associated lipoprotein (VC1835) whose expression is under the control of ToxR, which also regulates the cholera toxin operon (Bina *et al.*, 2003). Also, the homologous protein in *V. parahaemolyticus* (VP1390) was suggested to play a role in antimicrobial activities (Ronholm *et al.*, 2016). VDA_000412, a protein of the patatin-like phospholipase superfamily with an unknown function in *P. damselae* subsp. *damselae* is upregulated at 37ºC. First characterised patatin-like protein, the *Pseudomonas aeruginosa* ExoU, participates in virulence and toxicity (Sato and Frank, 2014). We also found urease genes in the set of those upregulated at human body temperature. The role of urease in gastric pathogens has to do with survival in acidic environments; it contributes to the maintenance of periplasmic pH around neutrality (Sachs *et al.*, 2003). For urinary pathogens, urease plays a vital role in virulence (Nielubowicz and Mobley, 2010; de Paiva-Santos *et al.*, 2018). These genes have not been functionally characterised in *P. damselae* subsp. *damselae*, but their upregulation at 37ºC might account for the unusual case reported by Alvarez *et al.*, 2006 of a gravid woman suffering from a urinary tract infection caused by this bacterium.

One finding that stood out in the present thesis was the broad downregulation of virulence functions at 37ºC. Notably, the major virulence factor Dly, PlpV and genes that encode for the T2SS underwent a significant downregulation. When we analysed Fragments Per Kilobase per Million fragments mapped (FPKM) values at 15 and

25ºC, haemolysin genes were among the most highly expressed genes in *P. damselae* subsp. *damselae*. Likewise, despite the strong downregulation of Dly (analysed by RNA-seq and a β-galactosidase essay) at 37ºC, we also confirmed that the three major haemolysins, Dly, PhlyP and PhlyC exhibit high transcription levels at human body temperature being all of them within the top 150 most expressed genes of the whole transcriptome. Collectively, based on the results that we present here, our hypothesis is that a temperature of 37ºC decreases *P. damselae* subsp. *damselae* viability, yet permits the production of a large quantity of haemolysins in human tissues during initial infection, that may accumulate and be enough to produce harm in absence of bacterial growth. Iron acquisition systems, flagellar and chemotaxis related genes follow a similar pattern. These results are consistent with those from the previous transcriptomic comparison (15 vs 25ºC), which showed a marked enhanced expression of iron acquisition systems, flagellar and chemotaxis related proteins at 25ºC. These results reinforce the idea that the optimal temperature of *P. damselae* subsp. *damselae* for triggering a virulence expression profile is close to 25ºC. In addition, the *nupC* gene (VDA_001786) which is upregulated at 25 in comparison to 15ºC, also presents a downregulation at 37ºC (meaning a higher expression at 25ºC). The role of this gene awaits further investigation, but our transcriptomic data suggest that nucleoside acquisition in *P. damselae* subsp. *damselae* seems to be favoured at 25ºC. In general, it is expected that the transcriptomic data presented here will facilitate research into new strategies for the prevention and milder control of human infections caused by *P. damselae* subsp. *damselae*.

A schematic representation of main processes enhanced when *P. damselae* subsp. *damselae* grows at each temperature of this study are shown in Fig. 5.1.

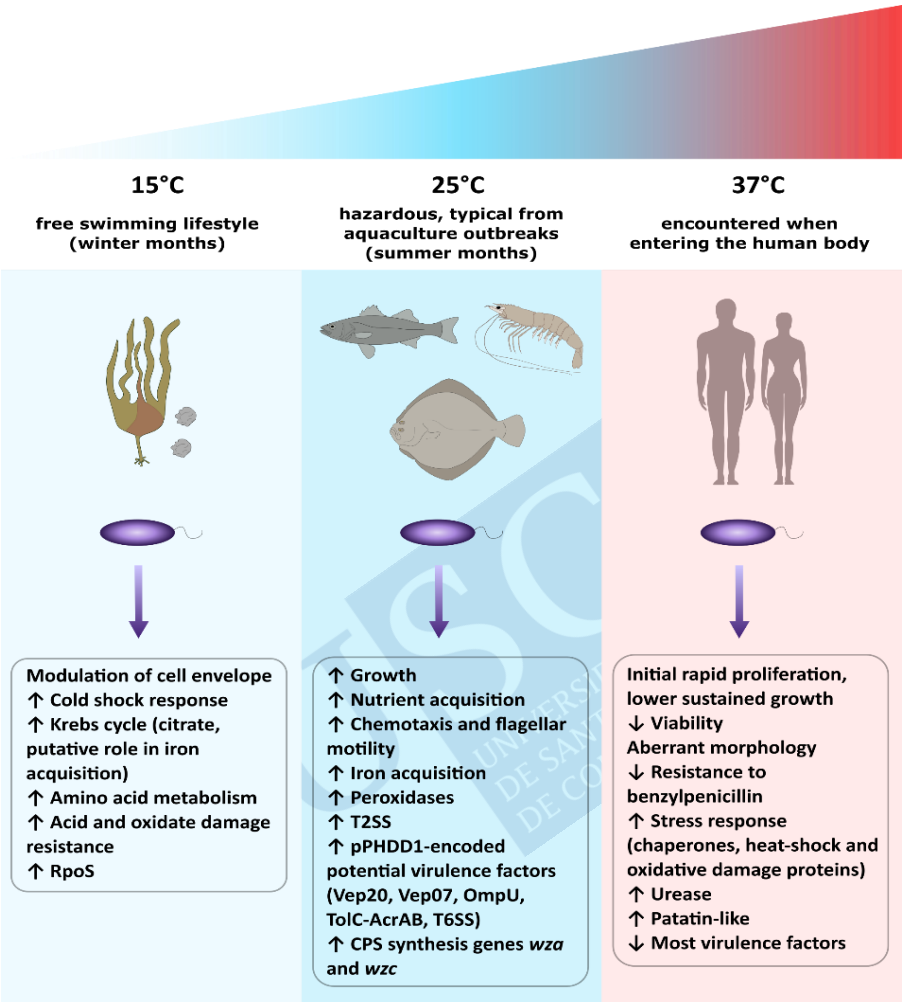

Figure 5.1: Main processes that are enhanced at important temperatures for the pathobiology of *P. damselae* subsp. *damselae*. 15°C represents the temperature of the free-swimming lifestyle of this bacterium in cold waters, 25°C is around the typical temperatures that precede outbreaks in aquaculture and 37°C is the temperature encountered when colonising a human body. Note that 25°C triggers growth and the virulent profile of this bacterium.

5.2. STUDY OF *P. DAMSELAE* SUBSP. *DAMSELAE* VIRULENCE AND FITNESS: COMPLETE REGULON OF THE RSTAB SYSTEM AND GENETIC CHARACTERISATION OF A HITHERTO UNKNOWN POLYSACCHARIDE CAPSULE

The characterisation of regulatory mechanisms is crucial to comprehend the environmental and host conditions that control fitness and virulence in pathogenic bacteria. Two-component systems (TCS) are key bacterial mechanisms involved in adaptation to environmental changes (Stock *et al.*, 2000). The RstAB is a TCS identified as a positive regulator of the expression of cytotoxin genes and virulence in *P. damselae* subsp. *damselae* (Terceti *et al.*, 2017; Terceti *et al.*, 2019). However, the complete genetic machinery under its control still needed to be elucidated. Given the importance of the system to understand the pathobiology of *P. damselae* subsp. *damselae*, in the present study we unveiled its whole regulon, demonstrating that it controls the expression of main virulence factors, including a hitherto unknown polysaccharide capsule.

It has been proposed that the histidine kinase RstB and the response regulator RstA function as a cognate pair (Terceti *et al.*, 2017; Terceti *et al.*, 2019). Nevertheless, we show here that there is no complete overlapping between RstA and RstB regulons. This happens with many other TCS cognate pairs, and it is partially accounted for by cross-regulation between noncognate partners of different TCSs (Rabin and Stewart, 1993; Matsubara *et al.*, 2000; Howell *et al.*, 2006; Gangaiah *et al.*, 2017; Guckes *et al.*, 2017).

Up to date, there is no data about the specific stimuli that activates RstB autophosphorylation. In *E. coli* and *Salmonella enterica*, *rstAB* genes expression is controlled by the Mg^{2+}-sensing TCS PhoPQ (Ogasawara et al., 2007; Pérez et al., 2009). *rstAB* homologues in *V. cholerae* (*casSR*) are negatively regulated by the concentration of Ca^{2+} (Bilecen and Yildiz, 2009). In the present study we have demonstrated that at temperatures of 15 vs 25 and 25 vs 37, the expression of *rstA* and *rstB* genes remain unaltered. Transcriptomic analyses of *P. damselae* subsp. *damselae* RstAB homologues are limited. As far as we know, these studies have only been carried out in *V. cholerae* (Bilecen and Yildiz, 2009) and *E. coli* (Oshima *et al.*, 2002; Liu *et al.*, 2019; Gao *et*

al., 2020). In order to identify RstAB-regulated genes, other approaches have been developed, such as studies of *rstAB* deletion or overexpression (Cabeza *et al.*, 2007; Ogasawara *et al.*, 2007). There are several reports that link the RstAB with virulence-related functions. RstAB controls the expression of functions related to iron acquisition, acid tolerance and biofilm formation in *E. coli* (Oshima *et al.*, 2002; Ogasawara *et al.*, 2007; Ogasawara *et al.*, 2010; Gao *et al.*, 2015; Liu *et al.*, 2019; Gao *et al.*, 2020). In *S. typhimurium*, RstAB also regulates acid tolerance and iron acquisition (Tran *et al.*, 2016; Lamberti *et al.*, 2019). RstAB inactivation leads to a reduction in the virulent properties of *Yersinia pseudotuberculosis* (Flamez *et al.*, 2008), *Edwardsiella ictaluri* (Menanteau-Ledouble and Lawrence, 2008) and avian pathogenic *E. coli* (Gao *et al.*, 2015). The TCS RstAB is present in most species belonging to genera *Vibrio* and *Photobacterium* (Terceti *et al.*, 2019). Nonetheless, in addition to the study presented here, only two other RstAB homologues in members of the family *Vibrionaceae* have received attention until now. This is the case of *V. cholerae* CarSR that negatively regulates biofilm formation and the expression of *vps* genes that participate in EPS synthesis (Bilecen and Yildiz, 2009), but positively regulates the expression of *almEFG* genes that modify lipid A and increase polymyxin B resistance (Herrera *et al.*, 2014; Bilecen *et al.*, 2015). Huang *et al.*, 2018 showed that *rstA* and *rstB* silencing in *V. alginolyticus* impair motility, biofilm, adhesion, haemolysis and virulence although they did not decipher the genetic regulon under the control of the system. Remarkably the expression of the *rstA* homologous gene in *V. vulnificus* (VV2_1414) is enhanced during human infection compared to *in vitro* conditions (Bisharat *et al.*, 2013), yet there is no available data about the RstAB regulon of this bacterium.

In comparison to the transcriptome produced by the wild type strain RM-71, deletion mutants in the histidine kinase RstB (Δ*rstB*) or its response regulator RstA (Δ*rsta*) showed a strong downregulation of key genes involved in virulence. Fig. 5.2 shows a schematic diagram of the whole RstAB regulon. We corroborate previous studies that found that mutation of *rstB* and *rstA* impaired the haemolytic activity and the expression of haemolysin promoters in this subspecies (Terceti *et al.*, 2017; Terceti *et al.*, 2018). In the same line, we found that T2SS-

encoding genes also showed a significant downregulation in RstAB mutants.

The present study revealed the RstAB positive control over the expression of numerous outer membrane proteins. Constituting a promising research area, their importance relies on the fact that homologous proteins have not only been characterised as important virulence factors (Weiser and Gotschlich, 1991), they have also been recently employed for the development of vaccines against aquaculture diseases caused by other marine bacteria, such as *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Edwarsiella tarda* (Li *et al.*, 2010; Cheng *et al.*, 2018). In addition, we show here that the expression of a number of genes potentially involved in the resistance to antimicrobial agents and survival within hosts, appear to be substantially less expressed in the TCS mutants. Remarkably, there is a common positive regulation of several genes potentially involved in virulence by both a temperature of 25°C (typical of aquaculture outbreaks) and the RstAB system. The list includes porin OmpU, the TolC-AcrAB system, protease DegP and genes belonging to the T2SS and T6SS. This fact reinforces the idea that both factors (a temperature of 25ºC and the RstAB system), central in the course of the present thesis, should be taken into account when studying the pathogenicity of *P. damselae* subsp. *damselae*. The characterisation of these genes constitutes an important issue for future research.

One of the most striking results obtained from this work was the strong downregulation of 2 divergently transcribed operons containing genes for capsular and extracellular polysaccharide (EPS) synthesis. This region was reported to present a high variability among *P. damselae* subsp. *damselae* strains (Terceti *et al.*, 2018; Osorio *et al.*, 2018). TEM assays presented here demonstrated for the first time the presence of a polysaccharide capsule around *P. damselae* subsp. *damselae* cells. We wanted to investigate the contribution of selected genes belonging to the above-mentioned operons, by constructing deletion mutants of *wza, wzc and yjbH*. Interestingly *wza* and *wzc* are also upregulated at a temperature of 25ºC, typical from outbreaks in aquaculture. Mutation of *wza* and *wzc* abolished the formation of the capsule while mutation of *yjbH* leads to the formation of a thinner

capsule. Wza, Wzb and Wzc work together as an exportation system for colanic acid and group 1 and 4 capsules (Whitfield *et al.*, 2006). Information about *yjbEFGH* genes is scarcer. They produce an extracellular polysaccharide in *Escherichia coli* that alters colony morphology (Ferrieres *et al*., 2007) and participates in adaptation to osmotic stress (Ionescu *et al*., 2008; Ionescu and Belkin, 2009). In *V. parahaemolyticus*, a deletion mutant in *yjbH* gene produce K-antigen polysaccharides overexpression (Chen *et al.*, 2010).

Several studies show that changes in colony morphology in agar plates are related to modifications in cell-surface structures (Hasman and Schembri, 2000; Schembri *et al.*, 2004; Ferrieres *et al.*, 2007; Petruzzi *et al.*, 2017). We showed here that mutants Δ*rstA*, Δ*rstB*, Δ*wza* and Δ*wzc* display translucent phenotypes in agar plates. By a TEM analysis we discovered a thick capsular layer around wt strain RM-71 cells that was absent from those translucent mutants. Mutant Δ*yjbH* showed a capsular layer that was thinner than that of the wt. These results suggest that translucency can be considered a macroscopic change that indicates a loss of capsular material, which is in accordance to what happen with other members of the family *Vibrionaceae* (Wright *et al*., 2001; Hsieh *et al*., 2003). We demonstrate that genes *wza*, *wzc* and *yjbH* participate in the synthesis of a *P. damselae* subsp. *damselae* capsular layer that was here described for the first time.

Biofilms consist of cells embedded in a matrix of polysaccharide nature, but also contains extracellular proteins and nucleic acids (Costerton *et al.*, 1995). Biofilms can help bacterial cells to avoid being attacked by the host immune system or antibacterial agents (Donlan and Costerton, 2002). Mutations of *wza* and *wzc* led to a greater capacity to form biofilm. Interestingly, other studies in *V. vulnificus* (Joseph and Wright, 2004) or *Riemerella anatipestifer* (Yi *et al*., 2017) showed that mutants in this transportation system had as well a greater capacity to form biofilms. On the other hand, mutants in these genes also present reduced biofilm formation in *Klebsiella pneumoniae* (Wu *et al.*, 2011). Our results suggest that capsular polysaccharides may inhibit biofilm formation by hiding adhesive molecules in the cell surface of *P. damselae* subsp. *damselae*.

Capsules are normally the outermost layer of bacterial cells. Therefore, they may facilitate the interaction with components of the immune system of the host (Moxon and Kroll, 1990). One of their main functions is hiding molecules that activate a strong immune response, preventing complement attack and phagocytosis, in order to facilitate bacterial survival inside hosts (Merino and Tomás, 2015). We wanted to test whether acapsular mutants had a defect in the response to immune components of the host. Thus, we tested growth of wt and mutant strains in turbot serum or mucus, characterised by their rich proportion in defensive components (complement, antimicrobial peptides, enzymes, etc). Δ*wza* and Δ*wzc* showed an impaired growth in serum in comparison to the wt and Δ*yjbH*. Accordingly, a Δ*wza* mutant in *V. vulnificus* also shows an impaired survival in human serum (Carda-Diéguez *et al.*, 2018). A similar pattern was observed for growth in mucus, with Δ*wza* and Δ*wzc* survival being lower than that of the wt and Δ*yjbH*. *P. damselae* subsp. *damselae* strain RM-71, used in the present study, was demonstrated to be unaffected by the antimicrobial action of fish mucus and it showed the ability to adhere to it (Fouz *et al.*, 2000). Altogether, the present study demonstrates that *P. damselae* subsp. *damselae* capsule play a role in evading the host immune system and therefore facilitates colonisation.

The mutation of genes *wza* and *wzc* produced a vast attenuation of the virulent profile of *P. damselae* subsp. *damselae* against turbot and sea bream. Similar results were obtained with mutants for these genes in other pathogenic bacteria, for instance *Acinetobacter baumannii* (Niu *et al*., 2020), *Klebsiella pneumoniae* (Lin *et al*., 2017), *Vibrio anguillarum* (Croxatto *et al*., 2007; Weber *et al*., 2010) and *Vibrio alginolyticus* (Hernández-Robles *et al*., 2016). In addition, the strong abolishment of virulence in fish cannot be attributed to a defect in growth, motility or haemolytic capacity since we demonstrated that all these phenotypes are normal in acapsular mutants. This project therefore provided for the best of our knowledge, the first experimental proof of the existence of a polysaccharide capsule in *P. damselae* subsp. *damselae* which is in turn key for full virulence.

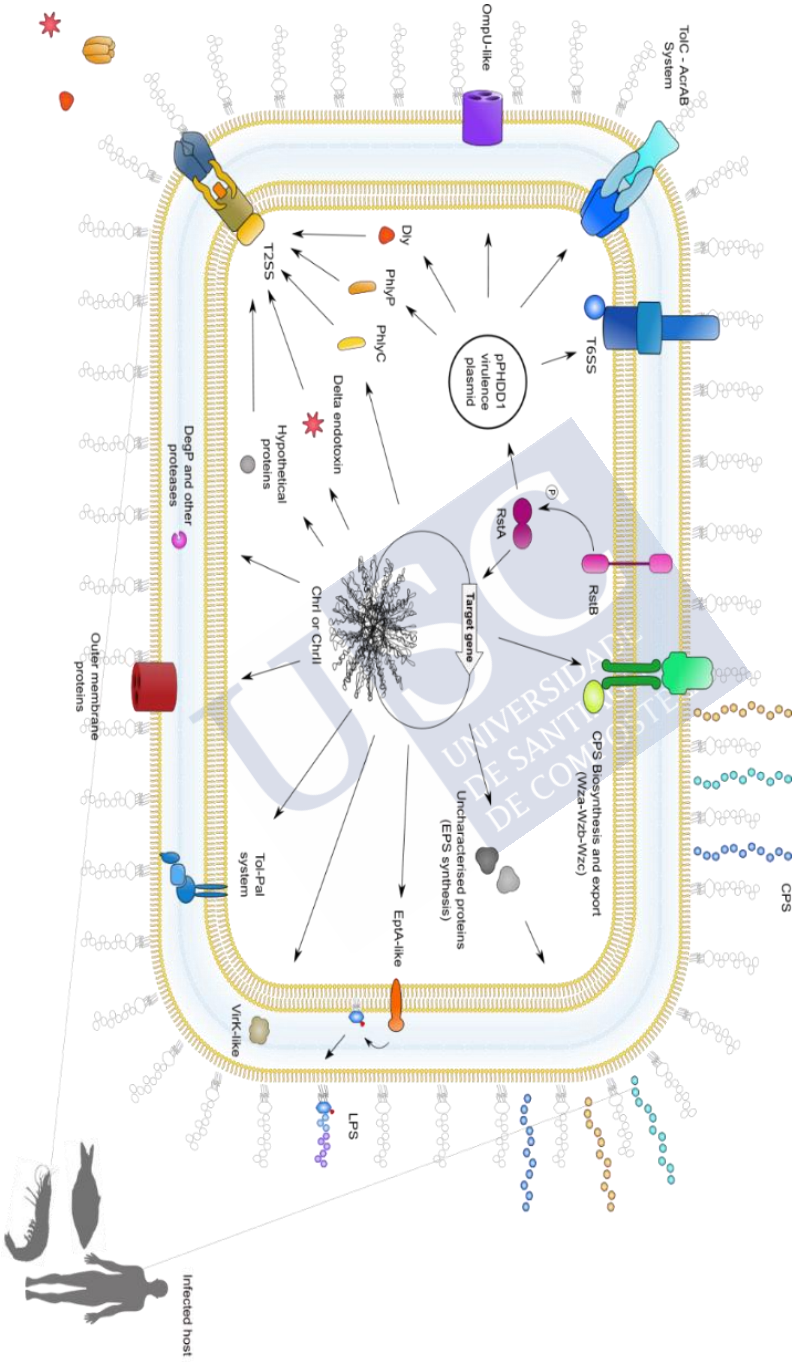
OmpU-like
TolC - AcrAB System
Dly
PhyP
PhyC
T2SS
T6SS
pPHDD1 virulence plasmid
Delta endotoxin
Hypothetical proteins
DegP and other proteases
RstA
RstB
Target gene
ChrI or ChrII
Outer membrane proteins
Tol-Pal system
EptA-like
Uncharacterised proteins (EPS synthesis)
CPS Biosynthesis and export (Wza-Wzb-Wzc)
CPS
VirK-like
LPS
Infected host

Figure 5.2: The two-component system RstAB is a major positive regulator of virulence-related functions in *P. damselae* subsp. *damselae*. The RstAB regulon comprises functions encoded within the two chromosomes and within the virulence plasmid pPHDD1. Regulated functions include the type II secretion system (T2SS)-dependent cytotoxins Dly, PhlyP and PhlyC and a putative δ-endotoxin; outer membrane proteins, type VI secretion system (T6SS), Tol-Pal system, TolC-AcrAB efflux pump for antimicrobial resistance, and lipid-A modification protein EptA among others. Notably, RstAB is a major regulator of functions related to synthesis and export of capsular polysaccharides with a major role in virulence.

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6. CONCLUSIONS

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6. CONCLUSIONS

The results presented here contribute to expand the understanding of infective processes in which *P. damselae* subsp. *damselae* is involved. This work has drawn the following conclusions:

1) An increase in temperature to 25ºC, in comparison to 15ºC, triggers a virulence profile in *P. damselae* subsp. *damselae*. Furthermore, a temperature of 25ºC helps to build a higher bacterial population, which may be key for the development of disease outbreaks.

2) Exposure of *P. damselae* subsp. *damselae* to 37ºC constitutes a stressful condition that impairs viability and cell morphology and triggers a heat-shock response.

3) Human isolates do not exhibit an advantage in growing at human body temperature, and they are not genetically more similar to each other than to fish isolates. Any clone in the marine environment might cause an opportunistic infection in humans, and the ability of this pathogen to grow at 37ºC appears to be a non-selected trait.

4) Haemolysins Dly, PhlyP and PhlyC are among the top highest expressed genes in the whole transcriptome at 15, 25 and 37ºC. Especially relevant, at 15ºC, *dly* is the ninth highest expressed gene, with expression levels comparable to ribosomal protein genes.

5) The RstAB TCS is a major positive regulator of *P. damselae* subsp. *damselae* gene expression and virulence. In addition to cytotoxins, it regulates the expression of porins, genes with functions in resistance to antimicrobial agents and host defences and, notably, genes that participate in capsule synthesis.

6) *P. damselae* subsp. *damselae* synthesises a hitherto uncharacterised polysaccharide capsule with a role in resistance against immune components of the host and essential for full virulence.

7. REFERENCES

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8. APPENDIX

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ETHICAL ASPECTS

- This thesis includes studies in which fish are used as experimental animal models, following RD 53/2013.
- The research project is entitled “Experimental inoculations of fish with pathogenic bacteria and bacterial proteins", in which Prof. Dr. Carlos Rodríguez Osorio, tutor and director of this doctoral thesis, is the responsible researcher.
- The authorisation number of this animal experimentation project is: 15004/14/003.
- The work was conducted at the Experimental Animal Facility of the Faculty of Biology of the Universidade de Santiago de Compostela, centre code: 15004AE: ES150780263301.
- Animal experiments were carried out by Prof. Dr. Carlos Rodríguez Osorio, tutor and director of the present thesis.

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RESEARCH ARTICLE

Transcriptome changes in response to temperature in the fish pathogen *Photobacterium damselae* subsp. *damselae*: Clues to understand the emergence of disease outbreaks at increased seawater temperatures

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Abstract

The marine bacterium *Photobacterium damselae* subsp. *damselae* (*Pdd*) is a generalist and facultative pathogen that causes disease in a wide range of marine animals including fish species of importance in aquaculture. Disease outbreaks in fish farms have been correlated with an increased water temperature during summer months. In this study, we have used RNA sequencing to analyze the transcriptome of *Pdd* RM-71 cultured at two different temperatures, which simulated temperature conditions experienced during free swimming lifestyle at mid latitudes in winter months (15˚C) and during outbreaks in aquaculture in warm summer months (25˚C). The enhanced bacterial growth of *Pdd* observed at 25˚C in comparison to 15˚C suggests that an elevated seawater temperature contributes to the build-up of a sufficient bacterial population to cause disease. In comparison to growth at 15˚C, growth at 25˚C resulted in the upregulation of genes involved in DNA synthesis, nutrient uptake, chemotaxis, flagellar motility, secretion systems and antimicrobial resistance. Plasmid-encoded virulence factors, which include a putative adhesin/invasin OmpU, a transferrin receptor and a serum resistance protein, were also upregulated. Transcription factor RpoS, genes involved in cold shock response, modulation of cell envelope and amino acid metabolism, as well as genes of yet unknown function were downregulated at 25˚C. Notably, the gene encoding damselysin cytotoxin (Dly) was among the most highly transcribed genes at the two assayed temperatures, at levels comparable to the most highly expressed housekeeping genes. This study contributes to our understanding of the regulatory networks and biology of a generalist marine bacterial pathogen, and provides evidence that temperature regulates multiple physiological and virulence-related functions in *Pdd*.

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Introduction

The *Vibrionaceae* family includes aquatic bacteria found in ocean environments and temperature and salinity are the main abiotic factors that shape their abundance and distribution [1, 2]. Rising ocean temperatures contribute to the increase in prevalence and severity of a wide range of Vibrio-related diseases in marine organisms and also in humans [3, 4]. *Vibrio* and *Photobacterium* infections have a marked seasonal distribution, and most cases occur during the warmer summer months [5].

Photobacterium damselae subsp. *damselae* (hereafter *Pdd*), a marine bacterium of the *Vibrionaceae* family, is a pathogen of a wide range of marine animals, including fish, molluscs, crustaceans and cetaceans. It is also an emerging pathogen in marine aquaculture systems that causes wound infections and septicaemia in economically important fish species [6–8]. In addition, it is an opportunistic human pathogen which causes severe wound infections that can have a fatal outcome [9].

Notably, disease outbreaks in aquaculture caused by *Pdd* have been associated with an increased seawater temperature during summer months. This has been the case for outbreaks in farms rearing turbot (*Scophthalmus maximus*) [10], rainbow trout (*Oncorhynchus mykiss*) [11, 12], seabass (*Dicentrarchus labrax*) [13–16], gilthead seabream (*Sparus aurata*) [14, 15, 17], cobia (*Rachycentron canadum*) [18] and silver pomfret (*Pampus argenteus*) [19]. There is increasing evidence that outbreaks of *Pdd* in fish farms are caused by genetically heterogeneous populations existing in the environment [7, 20]. It is believed that under advantageous environmental conditions these populations cause disease by taking advantage of stressed fish hosts.

The major reported virulence factors of *Pdd* are cytotoxins with hemolytic activity [8]. Highly virulent strains harbor the virulence plasmid pPHDD1 that carry the cytotoxin genes *dly* and $hlyA_{pl}$ [21]. *dly* encodes damselysin toxin (Dly), a phospholipase-D active against sphingomyelin [22], and $hlyA_{pl}$ encodes the pore-forming toxin phobalysin P (PhlyP) [23]. In addition, the pore-forming toxin phobalysin C (PhlyC), encoded by the $hlyA_{ch}$ gene located on chromosome I, and the phospholipase PlpV also contribute to virulence in fish and to cell toxicity [24, 25]. These four cytotoxins are secreted via a type II secretion system [26]. The two-component regulatory system RstAB positively regulates transcription of the hemolysin genes *dly*, $hlyA_{pl}$ and $hlyA_{ch}$, and its inactivation severely impairs virulence [27].

Despite the increasing evidence suggesting that outbreaks caused by *Pdd* in fish farms are triggered by rises in seawater temperature during summer months, the role of temperature in the physiology and gene regulation of this pathogen has not been studied so far. The aim of this study was to investigate the transcriptome of *Pdd* at two different temperatures, 15˚C and 25˚C, using an RNA-seq approach. We identified a number of genes upregulated at 25˚C—the temperature at which most outbreaks occur in aquaculture farms—that likely contribute to an increased bacterial growth rate and to an enhanced ability to colonize and survive in fish hosts. Additionally, we found genes with higher expression at 15˚C which might aid *Pdd* to adapt to life in colder waters during winter months. The global transcriptome data also shed light on the relative expression values of genes encoding virulence factors compared to housekeeping genes and demonstrated that the damselysin toxin gene is one of the most highly expressed genes in the cell. Finally, this study has brought to the forefront many previously overlooked genetic networks and gene clusters of this pathogen. Overall, the information generated in this study is expected to provide novel approaches for the prevention and control of vibriosis caused by *Pdd* in marine fish aquaculture.

Materials and methods

Growth analysis

Cells were routinely grown at 15 or 25˚C on tryptic soy agar (TSA) or in tryptic soy broth (TSB) supplemented with NaCl up to 1% (TSA-1 and TSB-1, respectively). For growth curves, three replicates for each temperature of the assay (15 and 25˚C) were grown in TSB-1 until obtaining exponentially growing precultures (OD_{600}: 0.3). Then, 1:100 dilutions of each preculture were grown in 100 μl of TSB-1 in 96 well plates and the optical density (OD_{600}) was measured during 48h using the spectrophotometer Epoch2 microplate reader (Biotek). Three replicates were measured for each temperature condition.

RNA-seq

Experimental design, RNA extraction and purification. As for RNA-seq approach, 3 biological replicates were performed for each condition. 15˚C was chosen as the control condition and is close to the temperature that this bacterium finds during free swimming lifestyle in mid latitudes, whilst 25˚C represents the higher temperature condition that usually precedes aquaculture outbreaks. For each temperature, three independent precultures were started and grown until an OD_{600}: 0.3. Then, 1:100 dilutions of each preculture were grown in 10 ml of TSB-1 in 100 ml flasks until they reached a sharp OD_{600} of 0.55. Cells were immediately treated with RNAprotect Bacteria Reagent (Qiagen) for stabilization of RNA following manufacturer's instructions. Pelleted cells were then carefully resuspended in TE buffer (30mM Tris·Cl, 1mM EDTA, pH 8.0) containing 15mg/ml lysozyme (Sigma Aldrich) and the appropriate volume of Proteinase K (Qiagen). RNA extraction was subsequently carried out using RNeasy Mini Kit (Qiagen) following manufacturers' instructions. An extra DNase I treatment was carried out using the on-column kit RNase-free DNase (Qiagen) to eliminate genomic DNA contamination. RNA was eluted using nuclease-free water. The quality and the quantity of the total RNA was determined using a Bioanalyzer 2100 (RNA 6000 Nano chip assay) and a Qubit 3.0 (Quant-It dsRNA BR Assay).

Libraries preparation and sequencing. Total RNA was rRNA-depleted using the Ribo-Zero rRNA Removal Kit (Gram Negative Bacteria) (Illumina) and cDNA libraries were obtained using the TruSeq RNA kit following Illumina´s recommendations. Briefly, rRNA-depleted RNA was chemically fragmented prior to reverse transcription and cDNA generation. The cDNA fragments then went through an end repair process, the addition of a single 'A' base to the 3' end and then ligation of the adapters. Finally, the products were purified and enriched by PCR to create the indexed final double stranded cDNA library. The pool of libraries was sequenced on an Illumina HiSeq 2500 sequencer.

Mapping and quantification of transcripts. The quality control of the raw data (raw reads) was performed using the FastQC [http://www.bioinformatics.babraham.ac.uk/projects/fastqc/] program. The raw pair-end reads were first mapped against the reference genome of the *Photobacterium damselae* subsp. *damselae* type strain CIP102761 (GenBank Acc. No. NZ_ADBS00000000.1). Reads that did not map to the reference genome (corresponding to genes of RM-71 strain not present in CIP102761) were subsequently mapped to the draft genome sequence of strain RM-71 (GenBank Acc. No. NZ_LYBT00000000.1). The two processes were completed using the Bowtie2 [28] v2.2.6 algorithm. Several quality control steps were performed. Reads displaying a very low quality were removed by using Samtools [29] and Picard Tools software [30]. Furthermore, one of the key factors that can condition the sequencing process is the GC content of samples which was checked as normal (distribution between 40–60%) in our experiment. Likewise, distribution of duplicates was evaluated to

confirm the normal small proportion. The process of genetic quantification was carried out by the HTSeq [31] software (0.6.1 version).

Comparison between samples. Concordance between samples of the same condition (replicates of each of the two assayed temperatures) was carried out by a study of correlation and distance considering the whole transcriptome normalized by the size of the library. This process was made using the statistics program R. Differential expression analysis was assessed using DESeq2 [32] method (1.18.1 version). The analysis of Differentially Expressed Genes (DEG) was done by using statistical packages designed by Python and R, using the DESseq2 [32] algorithm applying a differential negative binomial distribution for the statistics significance. Comparison between the two different conditions (25˚C vs. 15˚C) was set as fixed effect in DEseq2. A Python script developed at Sistemas Genómicos (Valencia, Spain) was employed to generate a data matrix for each group condition with the counts obtained from HTSeq count for each sample (each of the three replicates at each of the two temperatures). We considered differentially expressed genes those with Fold Change (FC) value lower than -1.5 or higher than 1.5 and a P value adjusted by False Discovery Rate (FDR) [33] $\leq$0.05. FPKM (Fragments per kilobase per million fragments mapped) values calculated with Cufflinks v2.2.1 [34] were used to represent the expression of each individual gene. FPKM is used for normalization of the data since it indicates the number of lectures of a given gene per kilobase (independently of the length of the gene), and per million reads (independently of the size of the library).

Results and discussion

Growth dynamics of *Pdd* at 15 and 25˚C

Pdd RM-71 was the strain selected for the present study. It was isolated during a disease outbreak in a turbot (*Scophthalmus maximus*) farm in Galicia (NW Spain), when the water temperature increased suddenly from 18˚C to 22–24˚C in the summer of 1988 [10]. It is a highly virulent, strongly hemolytic and cytotoxic strain and contains the pPHDD1 virulence plasmid [21]. Growth of RM-71 was analyzed in 48-h continuous cultures at 15 and 25˚C in TSB-1 medium (S1 Table). The two assayed temperatures simulated an *a priori* non-risky condition (15˚C) and warm water episodes that trigger aquaculture outbreaks (25˚C). The beginning of the exponential phase was delayed at 15˚C compared to growth at 25˚C and there was a great difference between optical density values after 15 h of cultivation at 15˚C (OD_{600}: 0.129) and 25˚C (OD_{600}: 0.527) (Fig 1). These observations suggest that 25˚C is closer to the optimal growth temperature of *Pdd* than 15˚C.

This substantial increase in bacterial proliferation at 25˚C with respect to 15˚C during the first 15 h of cultivation might contribute to the rapid progression of *Pdd* outbreaks when the sea water temperature increases. Proliferation of species of the *Vibrionaceae* family is favoured by warm (>15˚C) sea waters [35], and recent studies have demonstrated that following an increase in water temperature, Vibrios can go from barely detectable to being the predominant bacteria in a very short time [36, 37]. *Pdd* isolation was first reported from ulcers in damselfish (*Chromis punctipinnis*) during the summer and fall seasons in southern California and was shown to follow a seasonal pattern of infectivity. It was proposed that elevated water temperatures might allow the build-up of sufficient bacterial populations to cause disease in damselfish, hence the seasonal infectivity of *Pdd* [38]. Previous studies have provided sound evidence that seawater transmits the disease caused by *Pdd* and that the spread of this bacterium largely depends on water temperature [39]. Skin is suggested to be a potential route of penetration for this pathogen, which is able to specifically adhere to fish mucus [39]. Hence, even if fish are colonized by a small number of bacteria, fast proliferation enhanced by warm temperature will facilitate the infecting bacterial population to evade host immune responses by a variety of mechanisms [40]. High numbers of bacterial cells at the infection site might cause exhaustion

of complement components as well as of phagocytes [41] leading to systemic infection and fish death. Overall, the results of the growth dynamics analysis at 15 and 25˚C contribute to understand why increased water temperatures precede most outbreaks caused by this pathogen.

RNA sequencing results

Strain RM-71 was grown at 15 and 25˚C, and cDNA prepared from mRNA isolated from cultures at the two different temperatures was subjected to Illumina sequencing. Around 55 to 71 million reads were generated for each biological replicate (S2 Table). Growth at 15˚C was defined as the control condition. The comparative analyses of the transcriptomes at 15 and 25˚C resulted in a total of 1195 differentially expressed genes (DEGs): 641 genes with lower expression at 25˚C (FC lower than -1.5) and 554 genes with higher expression at 25˚C (FC higher than 1.5) (Fig 2, S3 and S4 Tables).

Similar to other members of the *Vibrionaceae* family, *Pdd* RM-71 contains two chromosomes. In addition, this strain harbors the virulence plasmid pPHDD1 [21]. Using the complete sequences of chromosome I and II of the type strain CIP102761, and the complete pPHDD1 plasmid sequence of strain RM-71 (GenBank Acc. No. NC_014653) as references, we distributed the DEGs into each replicon. Notably, we observed an imbalance in the number of DEGs between the two chromosomes (Fig 3). In chromosome I similar numbers of DEGs are upregulated and downregulated. However, chromosome II contains 204 downregulated genes and only 77 upregulated genes at 25˚C. The chromosome I of *Vibrionaceae* species contains most of the essential genes, whereas chromosome II has a more flexible gene content and is responsible for adaptation to environmental changes [42, 43]. Interestingly, among the 31 DEGs in the virulence plasmid pPHDD1, 24 corresponded to genes whose expression is upregulated at 25˚C, an observation of special interest since this plasmid constitutes a hallmark of highly virulent isolates [8]. Indeed, the present study has unveiled potential virulence factors

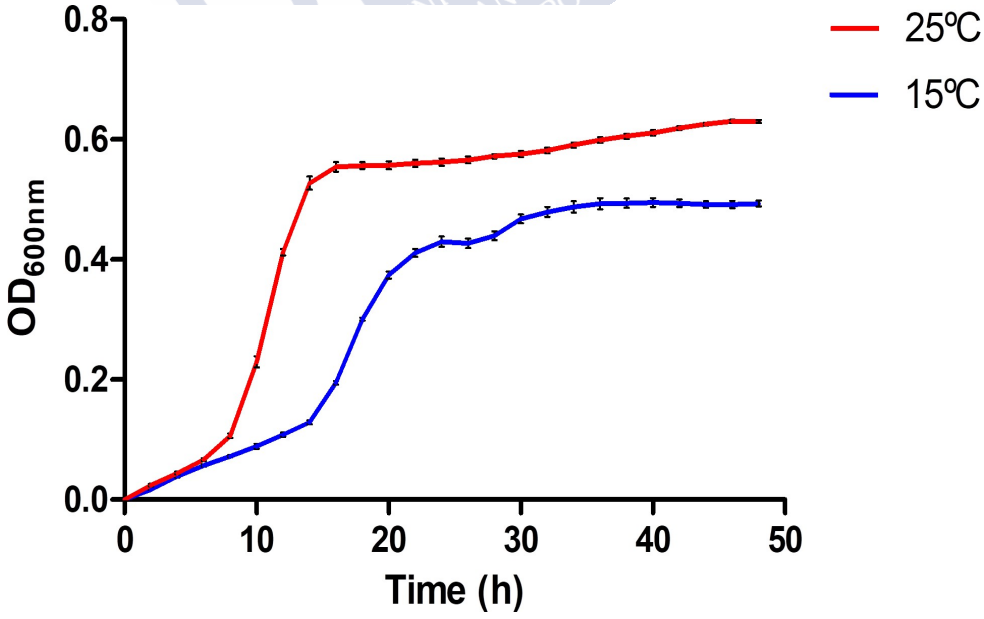

Fig 1. The influence of temperature cultivation in growth dynamics of *Pdd*. Growth of RM-71 strain was assayed at 15˚C and 25˚C in TSB-1 for 48 h. Vertical error bars represent standard deviation of biological triplicates.

https://doi.org/10.1371/journal.pone.0210118.g001

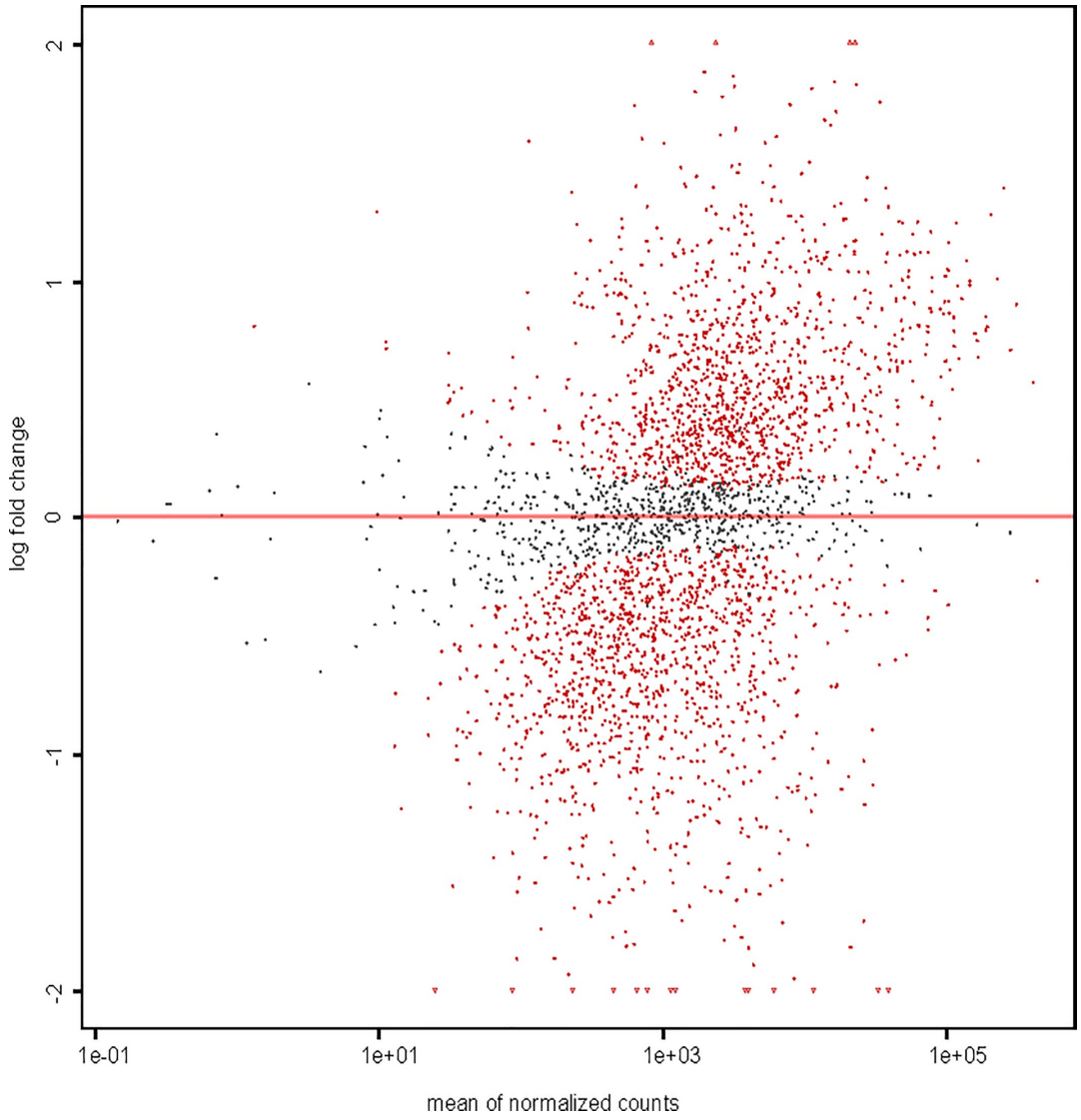

Fig 2. Smear plot of differentially expressed genes (DEGs) in *Pdd* RM-71 exposed to two temperatures, 15˚C and 25˚C. The smear plot shows the relationship between the log FC and mean of normalized counts. Grey points represent genes with non-significant changes in expression, whereas red points represent genes that are significantly differentially expressed.

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encoded by pPHDD1 plasmid among the group of genes upregulated at 25˚C (see below), thus highlighting the importance of this virulence plasmid in the pathobiology of *Pdd*.

Genes involved in growth and virulence are upregulated at 25˚C

Growth at 25˚C resulted in the upregulation of 533 genes that mapped to the genome of the type strain (S3 Table) and of 21 additional genes unique to strain RM-71 (S4 Table). A list of the 50 top DEGs upregulated at 25˚C plus additional selected genes is shown in Table 1.

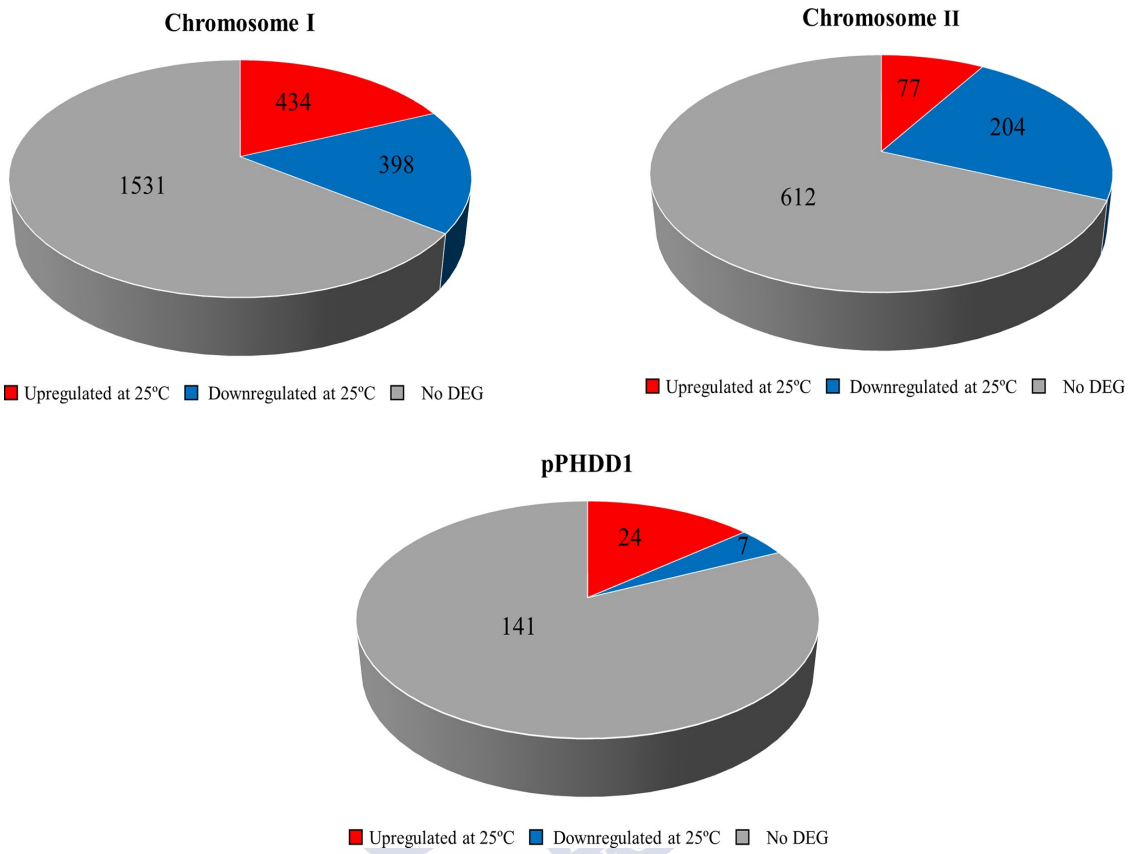

Fig 3. Graphical representation of DEGs distribution among the two *Pdd* chromosomes and pPHDD1 virulence plasmid. Numbers denote DEGs upregulated at 25˚C (red), downregulated at 25˚C (blue), and genes not differentially regulated (grey).

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Nutrient acquisition and metabolism. Genes encoding membrane proteins, nutrient transporters and porins were upregulated at 25˚C (Table 1). The nucleoside permease NupC (VDA_001789) was the most upregulated gene at 25˚C. A *V. cholerae nupC* deletion mutant was impaired for nucleoside acquisition leading to diminished fitness in nutrient-limited environments [44]. Growth at 25˚C upregulated two ribonucleotide reductases belonging to class Ia and II, one of them, VDA_001894, is coenzyme B12-dependent. In accordance, the vitamin B12 receptor BtuB was also upregulated (Table 1). Upregulation of the uracyl phosphoribosyltransferase VDA_001405, an enzyme necessary for the synthesis of precursors of all pyrimidine nucleotides, was also observed. Among upregulated amino acid biosynthesis enzymes and aminotransferases was the glutamine synthetase type I, which has a central role in amino acid biosynthesis. The putative trypsin superfamily protein encoded by A0J47_09785, which has a possible function in peptide degradation, as well as a number of ribosomal and translation-related proteins were upregulated suggesting that growth at 25˚C enhances protein synthesis and renovation.

Pdd degrades extracellular lipids, which may serve as carbon and energy sources [10, 11, 25]. An operon encoding an extracellular lipase (VDA_001610) and a fatty acid transporter

Table 1. List of selected DEGs with enhanced expression at 25˚C including the 50 top upregulated genes at 25˚C. Enhanced expression at 25˚C is denoted by positive FC values. Genes with VDA codes correspond to the annotation in the CIP102761 genome, and genes with A0J47 codes correspond to the annotation in the RM-71 genome.

Gene ID	Product/Function	Fold Change	p-value	Location
Nutrient acquisition/metabolism				
VDA_001789	Nucleoside permease NupC	5.1	2.2182E-122	ChrI
VDA_001833	GPR1/FUN34/yaaH putative acetate transporter	3.6	5.07132E-17	ChrI
VDA_002532	Porin	5.1	1.08929E-77	ChrI
VDA_003254	Porin	3.0	3.53973E-49	ChrI
VDA_001005	Porin	2.5	3.2873E-69	ChrII
VDA_003133	Glutamine synthetase type I	4.7	2.8174E-108	ChrI
VDA_000463	L-asparaginase	3.3	8.16042E-85	ChrII
VDA_003226	Glucosamine fructose-6-phosphate aminotransferase	3.5	3.71313E-35	ChrI
VDA_001560	Aspartate/tyrosine/aromatic aminotransferase	2.8	1.19637E-65	ChrI
A0J47_09785	Putative trypsin superfamily protein	4.3	4.6629E-132	ChrI
VDA_002568	Long-chain fatty acid transport protein	2.5	4.75483E-39	ChrI
VDA_000298	Arginine decarboxylase catabolic	2.6	7.62289E-83	ChrII
VDA_002194	Manganese-dependent inorganic pyrophosphatase	2.5	2.24757E-83	ChrI
VDA_003183	Oligopeptidase A	2.7	2.38104E-73	ChrI
VDA_003148	Vitamin uptake transporter	2.6	6.42707E-28	ChrI
DNA synthesis and repair				
VDA_002372	Ribonucleotide reductase of class Ia (aerobic) alpha subunit	3.5	1.03533E-70	ChrI
VDA_001894	Ribonucleotide reductase of class II (coenzyme B12-dependent)	3.1	6.75604E-90	ChrI
VDA_003108	Outer membrane vitamin B12 receptor BtuB	3.0	9.66178E-20	ChrI
VDA_001405	Uracil phosphoribosyltransferase	3.0	1.51133E-50	ChrI
Translation				
VDA_003390	LSU ribosomal protein L31p	2.7	1.98928E-09	ChrI
VDA_003099	Translation elongation factor Tu	2.6	3.07515E-36	ChrI
VDA_002952	SSU ribosomal protein S21p	2.6	1.9713E-24	ChrI
Virulence and antimicrobial resistance				
VDA_000110	Serum resistance protein Vep07-like	2.0	5.76819E-33	pPHDD1
VDA_000111	Transferrin receptor Vep20-like	3.0	5.67092E-76	pPHDD1
VDA_000113	OmpU	2.5	4.19858E-66	pPHDD1
VDA_000794	TonB-dependent siderophore receptor	2.8	5.7247E-111	ChrII
VDA_000157	TolC	2.8	2.115E-103	pPHDD1
VDA_000158	AcrA/MacA-like membrane fusion protein	3.2	7.9453E-112	pPHDD1
A0J47_18110	Unknown protein related to T6SS	2.8	1.0749E-24	pPHDD1
A0J47_18115	RNase toxin Ntox44	2.7	9.41221E-55	pPHDD1
A0J47_18120	Proline-alanine-alanine-arginine (PAAR) domain protein	2.9	1.3511E-60	pPHDD1
Motility and chemotaxis				
VDA_003029	Flagellar protein MotX	2.6	9.54565E-60	ChrI
VDA_002607	Flagellin protein FlaB	3.6	1.0743E-118	ChrI
VDA_002671	Flagellar motor rotation protein MotA	2.8	5.26275E-79	ChrI
VDA_002604	Flagellar biosynthesis protein FliS	2.7	6.88724E-93	ChrI
VDA_003044	Methyl-accepting chemotaxis protein	3.1	7.48432E-68	ChrI
VDA_001198	Methyl-accepting chemotaxis protein	2.7	1.10855E-85	ChrI
Stress response and defence mechanisms				
VDA_003059	Chaperonin complex GroEL-GroES	3.1	2.1437E-116	ChrI
VDA_003060	Chaperonin complex GroEL-GroES	3.5	1.517E-130	ChrI

(*Continued*)

Table 1. (Continued)

Gene ID	Product/Function	Fold Change	p-value	Location
VDA_002771	DnaK chaperonin	3.4	7.7384E-130	ChrI
VDA_001553	Peptidyl-prolyl cis-trans isomerase PpiD	2.5	3.30237E-65	ChrI
VDA_002523	HtpG chaperonin	2.8	3.0531E-107	ChrI
VDA_001325	ClpB chaperonin	2.5	3.25923E-77	ChrI
VDA_003124	Ribosome associated heat shock protein	3.0	1.98913E-71	ChrI
VDA_003529	Outer membrane stress sensor protease DegQ	3.3	1.13294E-15	ChrI
VDA_003386	ATP-dependent protease HslV	3.1	4.0286E-132	ChrI
VDA_001154	Peroxidase	4.9	6.6194E-111	ChrII
VDA_000806	Peroxidase	3.5	5.64336E-61	ChrII
VDA_000771	Iron-sulfur cluster assembly protein SufB	2.6	1.95494E-63	ChrII
Transcriptional regulation and signalling				
VDA_003227	DeoR family transcriptional regulator	3.3	1.4446E-35	ChrI
VDA_001088	XRE family regulator	2.6	7.86116E-25	ChrII
VDA_002825	Cyclic-di-GMP phosphodiesterase A	3.7	6.3346E-128	ChrI
Cell wall/membrane/envelope biogenesis				
VDA_003228	N-acetylglucosamine-1-phosphate uridyltransferase GlmU	2.7	4.3747E-86	ChrI
Hypothetical proteins of unknown function				
VDA_003431	Hypothetical protein	3.4	3.96176E-86	ChrI
VDA_000943	Hypothetical protein	2.6	3.53416E-24	ChrII
VDA_000598	Hypothetical protein	2.5	8.47329E-75	ChrII

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FadL (VDA_001611) were 2-fold upregulated at 25˚C, and so was the long-chain fatty acid transporter VDA_002568 (Table 1), suggesting that exogenous lipid degradation and uptake of fatty acids might constitute an advantage for *Pdd* fast replication at 25˚C.

Iron acquisition plays a role in *Pdd* virulence for fish [45–47]. Our analysis unveiled upregulation at 25˚C of a pPHDD1 plasmid-borne gene that encodes a putative transferrin binding protein (VDA_000111) (see below), and of VDA_000794 encoding a TonB-dependent siderophore receptor. The ligand(s) transported through VDA_000794 are unknown, but a recent study demonstrated that expression of this gene is enhanced in *Pdd* under iron-limitation conditions [48].

Motility and chemotaxis. Motility and tissue colonization constitute important factors in *Pdd* pathogenicity [39, 49]. Four flagellum-related genes were found among the 50 most upregulated genes (Table 1). Additional upregulated genes included flagellar hook protein FlgE (VDA_002616), flagellar motor rotation protein MotB (VDA_002670), flagellar hook-associated protein FlgK (VDA_002609) and flagellar basal-body rod modification protein FlgD (VDA_002617) (S3 Table). Previous studies have reported that *Pdd* can infect new hosts through seawater and that increased water temperature boosted infection by this route [49]. The upregulation of motility-related genes at 25˚C supports these previous observations and surely calls for further studies along these lines.

Chemotaxis is initiated by membrane chemoreceptors dubbed methyl-accepting chemotaxis proteins, which bind ligands and transduce a signal cascade that modulates flagellum activity. Notably, two chemotaxis-related genes were found among the 50 most upregulated genes at 25˚C and correspond to the methyl-accepting chemotaxis proteins VDA_003044 and VDA_001198 (Table 1). A recent study showed that mutants in chemotaxis genes are not only impaired in swimming motility in *Pdd* but also exhibit diminished production of the major virulence factor PhlyP and impaired adhesion to eukaryotic cells [50]. *Vibrio fischeri* and *V.*

anguillarum mutants in chemotaxis functions also display a high reduction of virulence in fish and the inability to colonize host tissues [51, 52]. Collectively, these results suggest that growth of *Pdd* at 25˚C could enhance chemotaxis and flagellum-dependent motility, contributing to access and adhesion to fish hosts and increasing chances of outbreaks in aquaculture farms during summer months.

Stress response. A number of chaperones, heat shock and stress-related proteins were listed among the 50-top DEGs (Table 1), suggesting that growth at 25˚C constitutes a heat stress condition. Upregulation was also found for DnaJ chaperonin (VDA_002770), heat shock protein GrpE (VDA_002772) and heat-shock chaperonin VDA_003125 (S3 Table). Upregulated proteases included the outer membrane stress sensor protease DegQ and the ATP-dependent protease HslV, two peroxidases and the iron-sulfur cluster assembly protein SufB. SufB synthesizes Fe-S clusters that act as cofactors in cellular processes under conditions of iron starvation or oxidative stress in *E. coli* [53]. Altogether, it is conceivable that an increase in temperature might constitute a signal for *Pdd* to activate its molecular machinery against reactive oxygen species formation by host cells, therefore linking temperature rise with virulence for fish hosts.

Transcriptional regulators and intracellular signalling. A DeoR family transcriptional regulator and a XRE family regulator were listed within the top-50 DEGs. Although studies about these transcriptional regulators are scarce, in *Shigella flexneri* and *Salmonella typhi* DeoR regulators are important for virulence and for intracellular growth [54, 55]. Thus, these two genes may regulate processes related to *Pdd* pathogenicity and further investigation into these regulators is required. Cyclic-di-GMP is an intracellular second messenger involved in environmental signalling and regulates a number of phenotypes in bacteria. VDA_002825, encoding a cyclic-di-GMP phosphodiesterase A, was 3.67-fold up-regulated at 25˚C. In *V. cholerae* cyclic-di-GMP regulates motility and biofilm formation [56, 57] and DNA repair [58], among other functions.

Secretion systems. The *Pdd* type II secretion system (T2SS) plays a major role in the secretion of the four cytotoxins Dly, PhlyP, PhlyC and PlpV [25, 26]. The complete cluster of *eps* (extracellular protein secretion) genes (VDA_003114-VDA_003123), encoding part of the T2SS machinery, was moderately upregulated at 25˚C relative to 15˚C (S3 Table), suggesting that the T2SS secretome serves important functions when this organism is growing at the temperature conditions that enhance outbreaks in aquaculture farms.

Potential virulence factors encoded within pPHDD1 plasmid are upregulated at 25˚C. A recent study reported that a double mutant of RM-71, with deletion of Dly and PhlyP-encoding genes, was still more virulent in a sea bass fish model than a naturally plasmid-less strain [25], suggesting that pPHDD1 encodes additional yet uncharacterized virulence genes. The analysis of the differential gene expression profiles along pPHDD1 at the two temperatures brought to the forefront a collection of plasmid-encoded genes which have putative roles in virulence and that surely will deserve special attention in future studies (Fig 4, S5 Table).

A cluster of 5 upregulated genes (VDA_000154 to VDA_000158) encode the TolC protein and AcrAB, plus two additional proteins. TolC and AcrAB form a tripartite multidrug and toxin secretion efflux pump. Expression of *tolC* and *acrAB* in the fish pathogen *Yersinia ruckery* is increased at 28˚C with respect to 18˚C with a concomitant increased resistance to antibiotics and toxic substances such as acriflavine [59]. The role of this system in the biology of *Pdd* remains unknown. In addition to a possible role in efflux of toxic substances, we cannot rule out the possibility that this system participates in the secretion of virulence factors, as TolC is part of the type I secretion system that exports hemolysins and other virulence factors in several Gram negative pathogens [60].

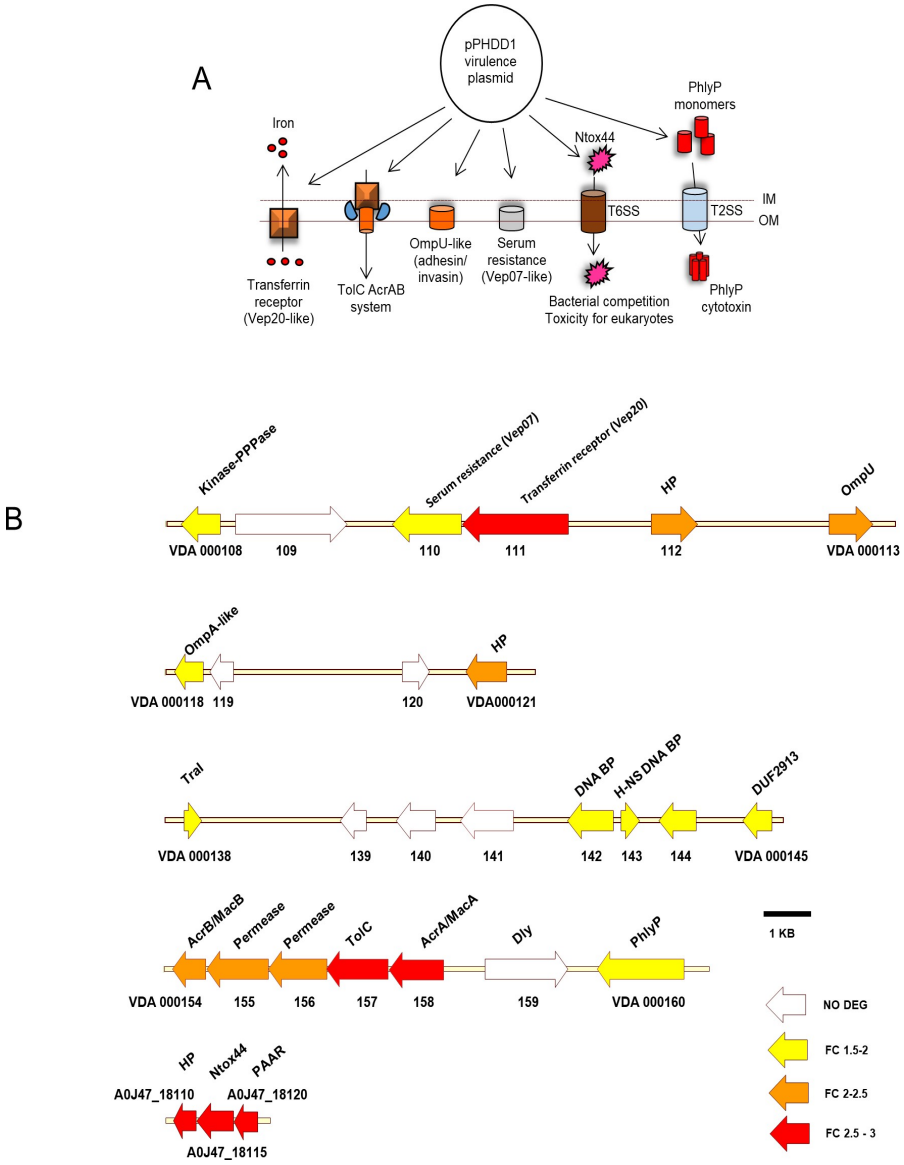

Fig 4. Virulence factors encoded within pPHDD1 plasmid are upregulated at 25˚C. (A) pPHDD1 encodes a series of membrane-associated proteins and toxins with demonstrated and potential roles for virulence in fish. (B) plasmid genes upregulated at 25˚C are mainly distributed along 5 plasmid regions. Numbers denote the VDA gene codes of the type strain CIP102761 (GenBank Acc. No. NZ_ADBS00000000.1). Genes with A0J47 labels correspond to genes unique to RM-71. Colour codes of genes (represented as arrows) denote each of the three ranges of FC values, whereas white arrows denote genes that are not differentially expressed (NO DEG).

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VDA_000110 is 36% identical to *V*. *vulnificus* Vep07, an outer membrane lipoprotein that confers resistance to eel serum. Mutation of *vep07* caused the loss of virulence in eels in *V*. *vulnificus* biotype 2 [61]. Of note, *V*. *vulnificus* Vep07 is encoded within pVvbt2, a transferable

virulence plasmid [62]. VDA_000113 is homologous to *Vibrio tasmaniensis* OmpU, a protein with an essential role in adhesion to and invasion of mollusc host cells [63]. OmpU homologues in other *Vibrio* species also play major roles in host-cell recognition and pathogenesis [64, 65]. Finally, the protein encoded by VDA_000111 shares 60% identity with the *V. vulnificus* plasmid-encoded Vep20 protein, a transferrin receptor with a major role for virulence in eels [66]. A previous study has demonstrated that *Pdd* strains can multiply in the presence of transferrin as the sole iron source [46]. Surely, the role of this gene deserves further investigation as it may play a main role in iron acquisition from host transferrin.

The three genes A0J47_18120, A0J47_18115 and A0J47_18110 are exclusive of RM-71 and have no known homologues in other pPHDD1-containing strains of *Pdd* studied so far. These genes are predicted to participate in defence against other bacteria which occupy the same ecological niches and compete for the same resources. A0J47_18120 is a proline-alanine-alanine-arginine (PAAR) domain-containing protein. Homologues of these proteins in *V. cholerae* are essential for secretion through the type VI secretion system (T6SS) and killing of target cells [67]. A0J47_18115 encodes a putative RNase toxin dubbed Ntox44 which is found in many bacterial groups and is predicted to be exported by the type II, type VI or type VII secretion systems [68].

Growth at 25˚C causes downregulation of functions related to cell envelope, metabolism and stress response

Albeit 15˚C being far from the optimal growth temperature (25˚C) of *Pdd* in laboratory conditions, it is conceivable that this bacterium lives in marine ecosystems in vast areas of the globe at temperatures below its optimum. Indeed, seawater temperatures during the summer of 1988 previous to the heat wave that caused a *Pdd* outbreak in a turbot farm in Galicia were around 18˚C [10], and seawater temperatures in the same geographical area during the winter months are known to fluctuate between 13 and 15˚C [69]. Growth at 25˚C resulted in the downregulation of 614 genes that mapped to the genome of the type strain (S3 Table) and of 27 additional genes unique to strain RM-71 (S4 Table), whose changes in expression are denoted by a negative FC value. A list of the top-50 downregulated genes at 25˚C, plus additional genes, is shown in Table 2 and reveals an important number of loci organized in operons.

The most important change is experienced by a putative operon constituted of VDA_001578, VDA_001579 and VDA_001580 (Table 2). VDA_001579 is the most differentially expressed gene in the whole transcriptome of this pathogen in this study, and has no known homologues with a demonstrated function so far. VDA_001580 is a predicted serine protein kinase PrkA, a family of proteins involved in cell wall homeostasis [70], saline stress, motility [71] and virulence [72]. VDA_001578 is predicted to be a member of the Stage V sporulation protein SpoVR family. SpoVR confers resistance to *Bacillus subtilis* spores and it has been hypothesized that homologues in other species might play a role in peptidoglycan synthesis regulation [73]. Also related to the cell wall is VDA_002031, which encodes a LrgB-family protein, a group of enzymes responsible for modulation of murein hydrolase activity [74]. VDA_001762 encodes lysophosphatidic acid acyltransferase PlsC, an integral membrane protein involved in phospholipid biosynthesis [75]. Adjustment of membrane composition is a conserved strategy that bacteria use to face variations in environmental parameters [76, 77]. The importance of the cell envelope in *Pdd* acclimatization to temperature changes surely will deserve further investigation.

Growth at 25˚C downregulated the expression of peptidases, membrane transporters and metabolic enzymes (Table 2). Of note is the downregulation of the oligopeptide permease

Table 2. List of the top DEGs in *Pdd* with lower expression at 25˚C than at 15˚C. Note that downregulated expression at 25˚C is denoted by negative FC values.

Gene ID	Product/Function	Fold Change	p-value	Location
Cell wall/membrane/envelope biogenesis				
VDA_001578	Stage V sporulation protein SpoVR family	-13.6	9.67E-189	ChrI
VDA_001762	Lysophosphatidic acid acyltransferase PlsC	-3.2	1.26692E-50	ChrI
VDA_002031	LrgB-family protein	-3.1	1.3247E-117	ChrI
Various/unknown function				
VDA_001579	Domain of unknown function, 444 superfamily	-28.2	0.0	ChrI
VDA_001580	PrkA-family serine protein kinase	-24.1	6.1873E-268	ChrI
VDA_001764	DedA superfamily member	-5.4	3.85608E-13	ChrI
Glycine betaine transport				
VDA_002013	Glycine betaine transporter	-7.8	1.07E-130	ChrI
Nutrient transport and metabolism				
VDA_001763	Putative cyanophicin synthetase	-7.1	2.98741E-37	ChrI
VDA_001723	Dipeptidase	-3.7	9.2245E-150	ChrI
VDA_000377	Metallopeptidase M24 family	-4.3	1.10747E-42	ChrII
VDA_001632	Oligopeptide ABC transporter OppA	-4.2	9.9638E-59	ChrI
VDA_001633	Oligopeptide ABC transporter OppB	-3.5	1.21575E-52	ChrI
VDA_001634	Oligopeptide ABC transporter OppC	-3.0	7.71823E-42	ChrI
VDA_001635	Oligopeptide ABC transporter OppD	-2.8	5.5165E-35	ChrI
VDA_001636	Oligopeptide ABC transporter OppF	-3.3	6.69827E-54	ChrI
VDA_001382	Methionine ABC transporter, substrate binding component	-3.3	4.52395E-44	ChrI
VDA_001251	Iron-molybdenum cluster-binding protein with NifB/NifX domain	-6.1	1.2384E-133	ChrI
VDA_001252	NADH:quinone oxidoreductase NqrM	-3.0	2.51284E-19	ChrI
VDA_002257	Alanine dehydrogenase	-4.3	7.76228E-76	ChrI
VDA_001997	Agmatinase	-3.9	9.89728E-95	ChrI
VDA_001605	Aspartate-semialdehyde dehydrogenase	-2.1	3.49578E-26	ChrI
VDA_002504	Citrate synthase	-3.5	5.08558E-45	ChrI
VDA_002724	Malate synthase	-3.1	8.95602E-40	ChrI
VDA_002723	Isocitrate lyase	-3.0	3.72706E-32	ChrI
VDA_000495	Putative hydrolase, alkyl/aryl sulfatase	-4.1	2.35121E-79	ChrII
VDA_002425	Acetoacetyl-CoA-reductase	-3.7	4.67185E-25	ChrI
VDA_002349	Alpha acetolactate decarboxylase	-3.3	3.9868E-50	ChrI
VDA_001166	Alpha-1,2-mannosidase	-3.2	1.30795E-38	ChrII
Stress response and host defence				
VDA_003169	Cold-shock protein	-4.6	8.21889E-58	ChrI
VDA_000629	RpoS	-3.5	5.16984E-57	ChrII
VDA_001327	Glutamate decarboxylase	-3.3	9.17337E-45	ChrI
VDA_001328	Glutaminase	-3.4	2.2451E-42	ChrI
VDA_001329	Glutamate/GABA antiporter	-3.0	7.67865E-16	ChrI
VDA_001116	Multidrug resistance efflux pump	-3.5	6.15611E-72	ChrII
VDA_000570	Methionine sulfoxide reductase MsrQ	-3.1	9.25296E-34	ChrII
VDA_000571	Methionine sulfoxide reductase MsrP	-4.6	1.28296E-51	ChrII
VDA_001099	Cu-Zn Superoxide dismutase	-3.1	1.9361E-122	ChrII
Virulence factors				
VDA_002242	Phospholipase PlpV	-1.8	2.02363E-17	ChrI
Hypothetical proteins of unknown function				
VDA_000632	Hypothetical protein	-6.2	8.1218E-133	ChrII
VDA_001326	Hypothetical protein	-3.4	2.00678E-63	ChrI

(*Continued*)

Table 2. (Continued)

Gene ID	Product/Function	Fold Change	p-value	Location
VDA_000814	Hypothetical protein	-3.8	7.28892E-46	ChrII
VDA_000647	Hypothetical protein	-3.6	2.93637E-36	ChrII
VDA_001949	Hypothetical protein	-3.5	1.82412E-53	ChrI
VDA_001746	Hypothetical protein	-3.4	1.43464E-49	ChrI
VDA_002346	Conserved hypothetical membrane protein	-3.3	1.30302E-29	ChrI
VDA_001892	Putative transporter	-3.1	4.50201E-55	ChrI
VDA_001660	Hypothetical protein	-3.0	2.70017E-34	ChrI
VDA_003210	Hypothetical protein	-3.0	4.74045E-89	ChrI
VDA_000496	Hypothetical protein	-3.0	7.01092E-40	ChrII
VDA_003208	Hypothetical protein	-3.0	8.59014E-50	ChrI
VDA_000405	Hypothetical protein	-3.0	9.44929E-53	ChrII
VDA_002379	Hypothetical protein	-3.0	4.8787E-117	ChrI
VDA_000439	Hypothetical protein	-3.0	1.36579E-08	ChrII

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system *oppABCDF* whose main function is predicted to be nutritional [78]. Interestingly, the expression of *oppABCDF* in *Vibrio alginolyticus* was found to be sensitive to temperature [79]. The role of the *opp* operon in *Pdd* physiology has not been studied so far and further evaluation of these genes is necessary to assess their possible contribution to bacterial fitness at low temperatures.

VDA_001251 contains a NifB/NifX domain for synthesis of iron-molybdenum cofactors. These cofactors bind the active site of dinitrogenase enzyme which participates in nitrogen fixation [80]. Although nitrogen fixation has not been studied in *Pdd*, some members of the *Vibrionaceae* family have this ability [81]. Enzymes of amino acid metabolism were also downregulated at 25˚C. The upregulation of specific amino acid biosynthetic pathways at both temperatures might be in part attributable to the need to provide optimal amino acid ratios for the different types of proteins produced at each temperature.

Genes of the Krebs cycle were downregulated at 25˚C: citrate synthase, malate synthase and isocitrate lyase. In addition to its role in the cell bioenergetics, the Krebs cycle is connected to iron acquisition in *Pdd* where endogenous citrate is used as iron scavenger [45, 82]. Thus, the use of ferric citrate as an iron source might be favoured in *Pdd* at low temperatures.

Biosynthesis and uptake of betaine glycine constitutes an adaptation for growth at low temperatures and is part of the cold-stress response of the marine fish pathogen *Vibrio anguillarum* [83]. Of note, a glycine betaine transporter showed a strong downregulation at 25˚C in our study (Table 2). Only one gene among the main 50 downregulated genes at 25˚C encoded a cold shock protein (VDA_003169). In contrast, some mesophilic bacteria such as *V*. *cholerae* [84] and *V*. *parahaemolyticus* [85] overexpress cold shock proteins following shifts to low temperatures close to 15˚C. The alternative sigma factor RpoS (VDA_000629) is downregulated at 25˚C. RpoS is a major regulator of the general stress response pathway in bacteria [86]. Important traits regulated by RpoS include virulence and colonization in *V*. *cholerae* and *V*. *parahaemolyticus* [87, 88]. A three-gene operon encoding a glutamate decarboxylase, a glutaminase and a glutamate/GABA antiporter is potentially involved in acid resistance [89]. The biological roles of this system in *Pdd* are unknown, but amino acid decarboxylation has been reported in other species of the *Vibrionaceae* family as a strategy for acid tolerance [90]. An operon which encode the methionine sulfoxide reductase system MsrPQ was downregulated in *Pdd* at 25˚C. The MsrPQ systems participate in the repair of oxidative damage [91].

Growth at 25˚C does not upregulate expression of the major cytotoxins of *Pdd*: Dly cytotoxin is within the 10 most expressed genes at 15˚C

Considering their importance in the pathogenicity of *Pdd* for fish, expression of the cytotoxins Dly, PhlyP, PhlyC and PlpV would be expected to be upregulated at 25˚C. Unexpectedly, expression of damselysin (Dly) (VDA_000159), PhlyP (VDA_000160) and PhlyC (VDA_002420) did not experience significant expression changes in growth at 25˚C compared to 15˚C (S3 Table), and PlpV (VDA_002242) was slightly downregulated at 25˚C (Table 2). This clearly indicates that levels of cytotoxin expression at 15˚C do not represent a limiting step that would prevent disease outbreaks in fish from occurring at low temperatures. These observations support that, as suggested above, the major influence of increased seawater temperatures in the onset of *Pdd* outbreaks in fish farms may be connected to the upregulation of other cellular processes (higher division rate, motility, chemotaxis, other plasmid-encoded putative virulence factors, etc) and not to a differential production of the four major cytotoxins.

This observation prompted us to analyze the RNAseq data to identify which are the most expressed genes in the genome of *Pdd* at each temperature of the study. Transcript abundance was quantified as Fragments Per Kilobase of transcript per Million mapped reads (FPKM), a method that allows the comparison of transcripts abundance among samples and conditions (S6 Table). Notably, the *dly* gene was the ninth most expressed gene at 15˚C (Table 3) with transcript abundance levels similar to genes of ribosomal proteins, which are among the most actively transcribed genes in fast-growing prokaryotic cells [92]. Two cold shock proteins and the NAD-dependent glyceraldehyde-3-phosphate dehydrogenase were included within the 10 most expressed genes at 15˚C. The 10 most expressed genes at 25˚C all corresponded to ribosomal protein genes (Table 4). LSU ribosomal protein L24p (L26e) (VDA_003450) was the most expressed gene under both conditions.

To illustrate the dominance of damselysin toxin transcripts, the FPKM values of *dly* and other virulence-related genes, as well as a selection of genes related with secretion systems and housekeeping cellular functions were compared (Fig 5). Although far from the top 10 most expressed genes, the FPKM values of the mRNA levels for the two pore-forming toxins PhlyP and PhlyC were also higher than those of housekeeping genes as *gyrB*, *recA*, *mreB* and *ftsZ*. Dly was the most highly expressed virulence factor at the two temperatures of the study, being particularly the case at 15˚ C, and its transcript levels were almost 3 orders of magnitude higher than those of the PlpV phospholipase, which is considered to only have a minor contribution to virulence and cell toxicity [25]. The plasmid-encoded putative virulence factors OmpU,

Table 3. List of most expressed genes at 15˚C and their corresponding FPKM values. Damselysin (Dly) toxin is highlighted in bold. FPKM values shown correspond to mean values of the three biological replicates.

Locus tag	Protein	FPKM
VDA_003450	LSU ribosomal protein L24p (L26e)	14782
VDA_000346	Putative cold shock-like protein	14687
VDA_003244	LSU ribosomal protein L34p	14467
VDA_003169	Cold shock protein	12798
VDA_001583	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	12190
VDA_003449	LSU ribosomal protein L14p (L23e)	11923
VDA_003447	LSU ribosomal protein L29p (L35e)	11833
VDA_003446	LSU ribosomal protein L16p (L10e)	11690
VDA_000159	**Damselysin toxin (Dly)**	10938
VDA_003456	SSU ribosomal protein S5p (S2e)	10255

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Table 4. List of most expressed genes at 25˚C and their corresponding FPKM values. FPKM values shown correspond to mean values of the three biological replicates.

Locus tag	Protein	FPKM
VDA_003450	LSU ribosomal protein L24p (L26e)	16486
VDA_003447	LSU ribosomal protein L29p (L35e)	15622
VDA_003093	LSU ribosomal protein L7/L12 (L23e)	15513
VDA_003446	LSU ribosomal protein L16p (L10e)	14870
VDA_003461	SSU ribosomal protein S13p (S18e)	13609
VDA_003444	LSU ribosomal protein L22p (L17e)	13577
VDA_003449	LSU ribosomal protein L14p (L23e)	13557
VDA_003456	SSU ribosomal protein S5p (S2e)	13188
VDA_003244	LSU ribosomal protein L34p	12838
VDA_003463	SSU ribosomal protein S4p (S9e)	12813

https://doi.org/10.1371/journal.pone.0210118.t004

Vep07 and Vep20 showed transcript abundance levels largely inferior to Dly cytotoxin, again reinforcing the dominance of Dly as the top-expressed virulence factor in *Pdd*. This observation is in agreement with early studies which described highly virulent *Pdd* strains are producers of "large amounts of a cytolytic toxin in vitro" [93] and reinforces the major role of

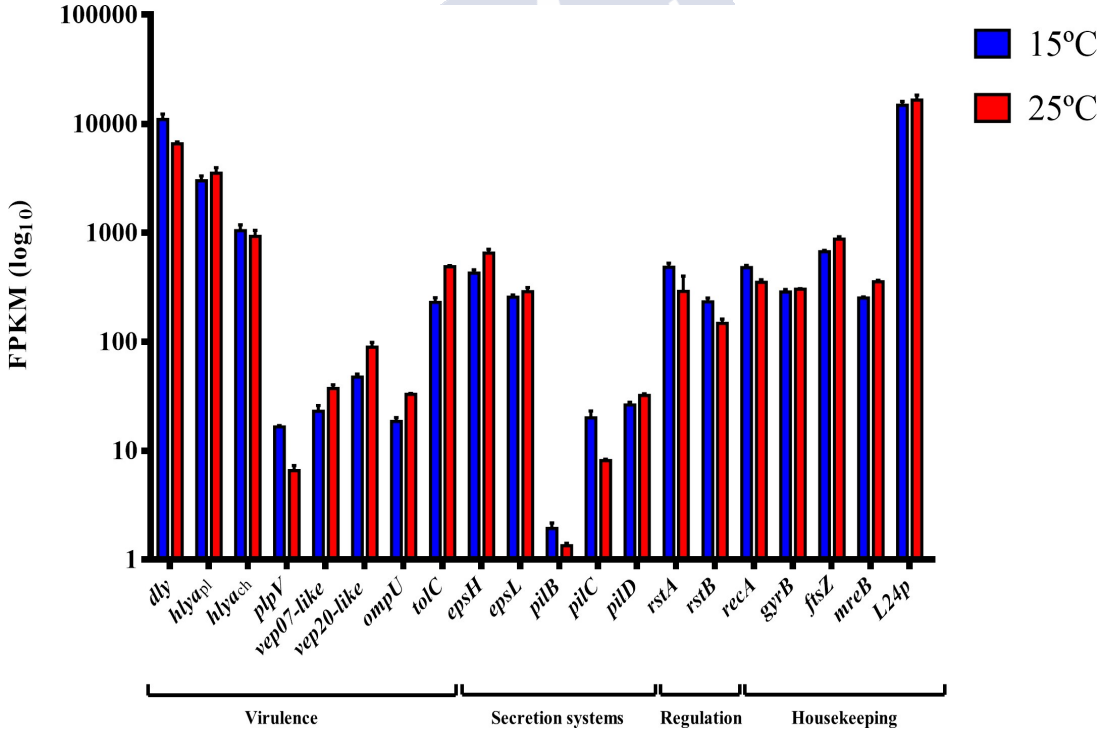

Fig 5. Damselysin toxin gene *dly* is one of the most highly expressed genes in *Pdd*. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values at the two assayed temperatures (S6 Table) were obtained for a selection of virulence and regulatory genes, secretion system genes and housekeeping genes including the top-expressed gene encoding ribosomal protein L24p, and compared using a logarithmic scale. Vertical error bars represent standard deviation of biological triplicates.

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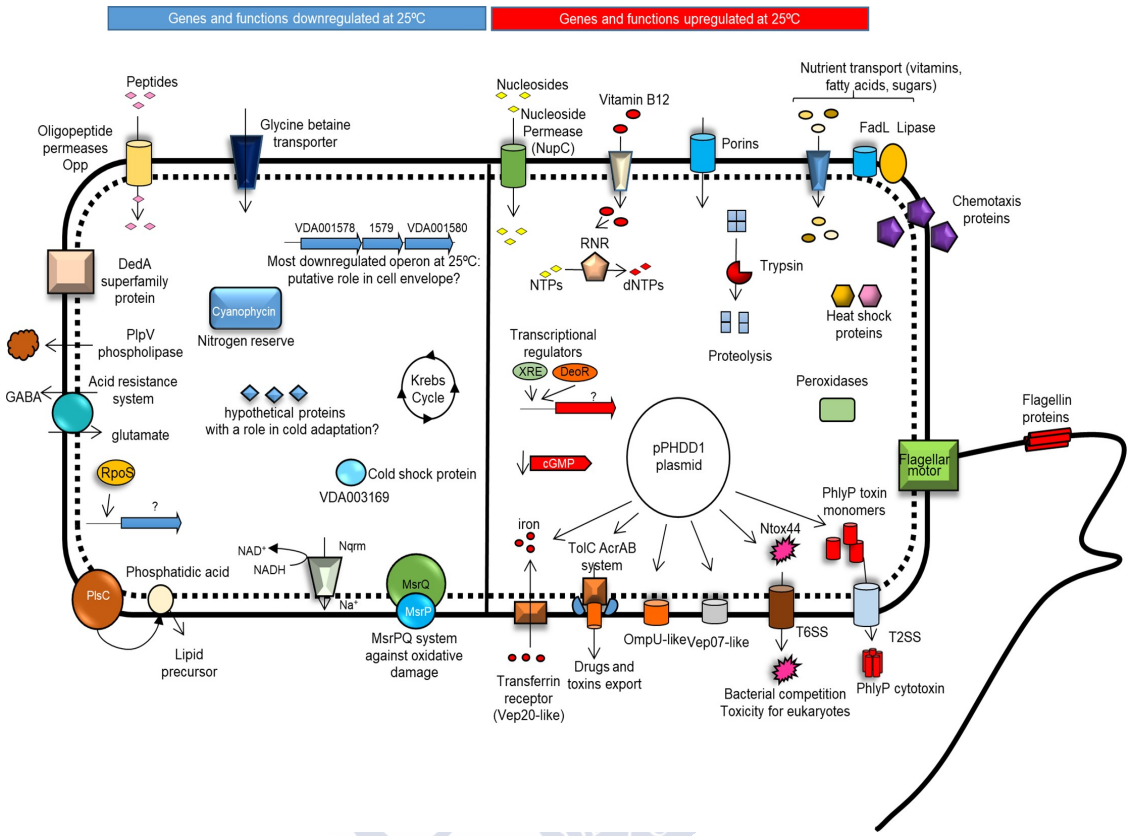

Fig 6. Diagrammatic summary of genes upregulated (right side) and downregulated (left side) at 25˚, relative to 15˚C, in *Pdd*. Growth at 25˚C upregulated motility and chemotaxis-related functions, as well as nutrient uptake and utilization genes. Potential virulence factors encoded within pPHDD1 plasmid were also upregulated at 25˚C. Genes with lower expression at 25˚C include a number of gene operons of yet unknown function, functions related to cell envelope, *rpoS*, and specific amino acid biosynthesis routes, among others. This diagram has been constructed based on transcriptomic data, and further studies are needed to establish the proposed model.

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pPHDD1 as a virulence plasmid that contributed to the evolution of highly hemolytic and highly virulent lineages of *Pdd*.

Conclusions

Regulation of expression of virulence factors in response to temperature has been widely studied in pathogenic bacteria infecting homeotherms [94–96]. Meanwhile, much less is known about the temperature-dependent regulation of virulence factors in fish bacterial pathogens [59, 97]. The aim of the current study was to investigate which genes are differentially regulated in *Pdd* in a heat stress condition (25˚C), relative to a colder condition (15˚C), considering that the majority of fish farm outbreaks occur during warm summer months at temperatures close to 25˚C. Fig 6 features a diagram of differentially expressed functions that are upregulated (right panel) or downregulated (left panel) at 25˚C.

Growth at 25˚C resulted in the upregulation of motility- and chemotaxis-related functions, as well as nutrient uptake and utilization genes. Notably, potential virulence factors encoded

within pPHDD1 related to iron acquisition (transferrin receptor), adhesion (OmpU), serum resistance (Vep07-like) and defence against competitors, were also upregulated at 25˚C (Fig 6). Overall, this study unveils a large set of previously overlooked genetic networks in *Pdd* and points at a number of cell functions related to virulence and acclimatization to changes in the environment.

Notably, damselysin toxin was one of the most highly expressed genes in the cell at the two assayed temperatures, with expression levels comparable to the most expressed genes encoding ribosomal proteins. This finding highlights the importance of this phospholipase D for the bacterium and suggests that this toxin might fulfil other biological roles in addition to virulence. To the best of our knowledge, this study represents the first transcriptome-based analysis of *Pdd*, and it has allowed us to identify a large set of gene functions that surely will constitute a foundation for future studies. Currently we are in the process of investigating the role of the top-differentially expressed genes identified in this study in the pathobiology of *Pdd*.

Supporting information

S1 Table. OD_{600} data for each biological replicate at 15˚C and 25˚C used to generate growth curves depicted in Fig 1.
(XLSX)

S2 Table. Reads mapping for each biological replicate.
(XLSX)

S3 Table. List of Differentially Expressed Genes (DEGs) at 25˚C vs 15˚C mapped to type strain genome (CIP102761).
(XLSX)

S4 Table. List of Differentially Expressed Genes (DEGs) at 25˚C vs 15˚C mapped to RM-71 genome.
(XLSX)

S5 Table. List of Differentially Expressed Genes (DEGs) within virulence plasmid pPHDD1.
(XLSX)

S6 Table. FPKM values for each biological replicate at 15˚C and 25˚C.
(XLSX)

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Exposure of the Opportunistic Marine Pathogen *Photobacterium damselae* subsp. *damselae* to Human Body Temperature Is a Stressful Condition That Shapes the Transcriptome, Viability, Cell Morphology, and Virulence

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Photobacterium damselae subsp. *damselae* (*Pdd*), an important pathogen for marine animals, is also an opportunistic human pathogen that can cause fatal necrotizing fasciitis. The regulatory changes triggered by the temperature shift experienced by this marine pathogen upon entering the human body, are completely unknown. Here we report an RNA-seq approach combined with phenotypical assays to study the response of *Pdd* to cultivation at 37°C in comparison to 25°C. We found that cultivation of a *Pdd* highly virulent strain for fish and mice, RM-71, at 37°C, initially enhanced bacterial growth in comparison to 25°C as evidenced by the increase in optical density. However, cells were found to undergo a progressive loss of viability after 6 h cultivation at 37°C, and no viable cells could be detected from 30 h cultures at 37°C. In contrast, at 25°C, viable cell counts achieved the highest values at 30 h cultivation. Cells grown at 25°C showed normal rod morphology by scanning electron microscopy analysis whereas cells grown at 37°C exhibited chain-like structures and aberrant long shapes suggesting a defect in daughter cell separation and in septum formation. Cells grown at 37°C also exhibited reduced tolerance to benzylpenicillin. Using a RNA-seq approach we discovered that growth at 37°C triggered a heat-shock response, whereas genes involved in motility and virulence were repressed including iron acquisition systems, the type two secretion system, and damselysin toxin, a major virulence factor of *Pdd*. Human isolates did not exhibit advantage growing at 37°C compared to fish isolates, and comparative genomics did not reveal gene markers specific of human isolates, suggesting that any *Pdd* genotype existing in the marine environment might potentially cause disease in humans. Altogether, these data indicate that the potential of *Pdd* to cause disease in humans is an accidental condition rather than a selected trait, and that

human body temperature constitutes a stressful condition for *Pdd.* This study provides the first transcriptome profile of *Pdd* exposed at human body temperature, and unveils a number of candidate molecular targets for prevention and control of human infections caused by this pathogen.

Keywords: *Photobacterium damselae*, zoonosis, temperature, transcriptome, hemolysin, heat-shock response

INTRODUCTION

Photobacterium damselae subsp. *damselae* (hereafter *Pdd*) is a bacterium of the family *Vibrionaceae* pathogenic for a broad range of hosts including marine animals and humans (Osorio et al., 2018). It represents a major threat for marine fish aquaculture worldwide, and disease outbreaks in fish farms are typically preceded by increases in sea water temperature during summer months (Fouz et al., 1992; Pedersen et al., 2009; Eissa et al., 2018; Yu et al., 2019). In humans, *Pdd* can cause severe wound infections and necrotizing fasciitis that may evolve into a fatal outcome despite prompt antibiotic administration (Morris et al., 1982; Clarridge and Zighelboim-Daum, 1985; Yuen et al., 1993; Asato and Kanaya, 2004; Yamane et al., 2004; Hundenborn et al., 2013; Akram et al., 2015; Alhemairi et al., 2015). Notably, some authors recommend to surgically debride and to amputate at an early stage of the infection to save lives of patients (Goodell et al., 2004). Underlying diseases (as diabetes, liver disorders and immunodeficiency among others) may accompany infection (Knight-Madden et al., 2005; Nakamura et al., 2008; Kim et al., 2009), but they are not a prerequisite for the development of disease since apparently healthy individuals are also susceptible (Morris et al., 1982; Barber and Swygert, 2000; Yamane et al., 2004). Human infections by *Pdd* originate through exposure of subcutaneous tissue to the marine environment or through wounds inflicted during fish handling. *Pdd* is considered one of the main zoonotic pathogens acquired topically from fish (Lehane and Rawlin, 2000; Austin, 2010), and the majority of human cases concentrate in coastal areas of the United States of America, Japan and Australia, during the warm season.

The major reported virulence factors of *Pdd* are cytotoxins with hemolytic activity (Osorio et al., 2018). The virulence plasmid pPHDD1 encodes the cytotoxins damselysin (Dly) and phobalysin P (PhlyP) (Rivas et al., 2011), and two additional cytotoxins, phobalysin C (PhlyC) and phospholipase PlpV are encoded within chromosome I (Rivas et al., 2013; Vences et al., 2017). These virulence factors are secreted via the type II secretion system (Rivas et al., 2015a; Vences et al., 2017). Recently, it was reported that the two-component regulatory system RstAB regulates transcription of the genes encoding Dly, PhlyP, and PhlyC toxins, and consequently its inactivation strongly impairs virulence (Terceti et al., 2017, 2019).

Despite the aggressive outcome of *Pdd* infections in humans, nothing is known about how this bacterium responds to the temperature shift that it encounters in its transition from warm sea waters to invading a human host. In order to shed light on this, in the current work we compared the transcriptomes of *Pdd* at 25 and 37°C, mimicking the environmental vs. the host temperature conditions. In addition, the genome sequences of two human isolates of *Pdd* are reported for the first time, and a comparative genomics analysis between human and fish strains is presented. Growth assays, viability tests and cell morphology studies, together with the transcriptomics data, all suggest that growth at 37°C represents a stressful condition and not an evolutionarily selected trait for *Pdd*. Overall, these data are expected to contribute to the investigation of novel approaches for prevention and for less invasive control of human infections caused by *Pdd* in a global warming scenario.

MATERIALS AND METHODS

Culture Conditions, Viability Assays, and Fatty Acid Methyl Ester (FAME) Analysis

Pdd strains were grown at 25 or 37°C on tryptic soy agar (TSA) or in tryptic soy broth (TSB) supplemented with NaCl up to 1% (TSA-1 and TSB-1, respectively). For growth curves, three replicates for each temperature of the assay (25 and 37°C) were grown in TSB-1 until obtaining exponentially growing precultures (OD_{600}: 0.3). Then, 1:100 dilutions of each preculture were grown in 100 µl of TSB-1 in 96 well plates and the optical density (OD_{600}) was measured using the spectrophotometer Epoch2 microplate reader (Biotek). For drop-plating viability assays, three independent cultures were grown at each temperature using the spectrophotometer Epoch2 microplate reader under the same conditions used for growth curve analysis, and 5 µl aliquots of 10-fold dilutions were drop-plated in TSA-1 plates after 6, 12, 24, 30, 36, and 48 h of incubation, and plates were incubated at 25°C for 24 h. FAME analysis was performed by extracting and preparing fatty acid methyl esters from 24 h *Pdd* RM-71 cultures on TSA-1 at 25 or 37°C and using the MIDI Sherlock® Microbial Identification System (MIDI, Inc., United States), following manufacturer's recommendations.

Genome Sequencing and Comparative Genomics

Draft genome sequences of human isolates CDC-2227-81 (Kreger, 1984) and 80077637 (Hundenborn et al., 2013) were obtained using an Illumina platform as previously described (Abushattal et al., 2019) and annotated using RAST tool (Aziz et al., 2008). Sequence similarity analysis by comparison of orthologous fragments between pairs of genomes was conducted with OrthoAni (Lee et al., 2016). Core genome and unique genes were calculated using RAST (Aziz et al., 2008). Genomic BLAST file of *Photobacterium damselae* subsp. *damselae* strains 80077637, CDC-2227-81, RM-71, CIP102761,

A-162, and LD-07 was downloaded from NCBI[1], and the dendrogram was visualized by Interactive Tree of Life (iTOL v5) (Letunic and Bork, 2019). The draft genome sequences obtained in this study have been deposited at GenBank/EMBL/DDBJ under accession number VZUQ00000000 (CDC-2227-81) and WAEO00000000 (80077637).

Scanning Electron Microscopy

Exponentially growing cultures of *Pdd* RM-71 in TSB-1 at each temperature were stopped when they reached an OD_{600} of 0.55, cells were carefully pelleted down by centrifugation (4,000 *g*) and fixed for 3 h at 4°C in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and postfixed for 1.5 h in 1% osmium tetroxide in the same buffer. Samples were washed three times in dH_2O, dehydrated using a series of graded ethyl alcohols, chemically dried using HMDS (hexamethyldisilazane) (Sigma), sputter-coated with iridium, and viewed and photographed in an Ultra Plus ZEISS scanning electron microscope.

E-test Assay

To determine the susceptibility to benzylpenicillin at the two assayed temperatures, *Pdd* strain RM-71 was grown at a temperature of 25°C in 10 ml TSB-1 in 100 ml flasks, and collected when cultures reached a sharp optical density at 600 nm (OD_{600}) of 0.5. Aliquots of 100 µl were spread onto TSA-1 plates in the presence of *E*-test gradient antibiotic strips (bioMérieux), and incubated for 24 h at 25°C and 37°C.

RNA-Seq

For RNA-seq, three independent precultures of RM-71 strain for each temperature condition were started and grown until an OD_{600} of 0.3. Then, 1:100 dilutions of each preculture were grown in 10 ml of TSB-1 in 100 ml flasks until they reached a sharp OD_{600} of 0.55. Cells were immediately treated with RNAprotect Bacteria Reagent (Qiagen) for stabilization of RNA following manufacturer's instructions. Pelleted cells were then carefully resuspended in 100 µl TE buffer (30 mM Tris·Cl, 1 mM EDTA, pH 8.0), adding 10 µl lysozyme (15 mg/ml) (Sigma Aldrich) and 15 µl Proteinase K (20 mg/ml) (Qiagen). RNA was extracted with RNeasy Mini Kit (Qiagen), and DNase I treatment was performed using the on-column kit RNase-free DNase (Qiagen). The quality and the quantity of the total RNA was determined using a Bioanalyzer 2100 (RNA 6000 Nano chip assay) and a Qubit 3.0 (Quant-It dsRNA BR Assay).

Total RNA was rRNA-depleted using the Ribo-Zero rRNA Removal Kit (Gram Negative Bacteria) (Illumina) and cDNA libraries were obtained using the TruSeq RNA kit following Illumina's recommendations. Briefly, rRNA-depleted RNA was chemically fragmented prior to reverse transcription and cDNA generation. The cDNA fragments then went through an end repair process, the addition of a single "A" base to the 3′ end and then ligation of the adapters. Finally, the products were purified and enriched by PCR to create the indexed final double stranded cDNA library. The pool of libraries was sequenced on an Illumina HiSeq 2500 sequencer. The quality control of the raw reads was performed using FastQC[2] program as previously described (Matanza and Osorio, 2018). The raw pair-end reads were mapped against the reference genome of the *Pdd* type strain CIP102761 (GenBank accession number NZ_ADBS00000000.1), using Bowtie2 (Langmead and Salzberg, 2012) v2.2.6 algorithm. Several quality control steps were performed. Reads displaying a very low quality were removed by using Samtools (Li et al., 2009) and Picard Tools software[3]. Furthermore, one of the key factors that can condition the sequencing process is the GC content of samples which was checked as normal (distribution between 40 and 60%) in our experiment. Likewise, distribution of duplicates was evaluated to confirm the normal small proportion. The process of genetic quantification was carried out by the HTSeq (Anders et al., 2015) software (0.6.1 version).

Concordance between samples of the same condition (replicates of each of the two assayed temperatures) was carried out by a study of correlation and distance considering the whole transcriptome normalized by the size of the library. This process was made using the statistics program R. Differential expression analysis was assessed using DESeq2 (Love et al., 2014) method (1.18.1 version). The analysis of Differentially Expressed Genes (DEG) was done by using statistical packages designed by Python and R, using the DESseq2 (Love et al., 2014) algorithm applying a differential negative binomial distribution for the statistics significance. Comparison between the two different conditions (37°C vs. 25°C) was set as fixed effect in DEseq2. A Python script developed at Sistemas Genómicos (Valencia, Spain) was employed to generate a data matrix for each group condition with the counts obtained from HTSeq count for each sample (each of the three replicates at each of the two temperatures). Genes with Fold Change (FC) value lower than -1.5 or higher than 1.5 and a *P*-value adjusted by False Discovery Rate (FDR) ≤ 0.05 were considered as differentially expressed. FPKM (Fragments per kilobase per million fragments mapped) values calculated with Cufflinks v2.2.1 (Langmead and Salzberg, 2012) were used to calculate the expression of each individual gene. FPKM is used for normalization of the data since it indicates the number of lectures of a given gene per kilobase (independently of the length of the gene), and per million reads (independently of the size of the library).

Construction of Insertional Mutants in Heat-Shock Related Genes

For insertional mutation of *clpB*, *groEl,* and *htpG* genes encoding heat-shock proteins, an internal fragment of each gene was PCR amplified and cloned into the suicide vector pNidKan (Mouriño et al., 2004). The mutant allele constructions were mobilized from *Escherichia coli* S17-1-λpir into the *Pdd* parental strain RM-71. Insertions of the suicide vectors into the chromosome by a single crossover would result in a Km^r phenotype and disruption of the coding sequence. The insertional mutants were selected

[1]https://www.ncbi.nlm.nih.gov/genome/675

[2]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[3]http://broadinstitute.github.io/picard/

on thiosulfate-citrate-bile-sucrose (TCBS) agar plates containing kanamycin (50 mg L^{-1}), and the presence of the plasmid inserted into the target gene was confirmed by PCR. Mutants were grown in parallel with the parental strain RM-71 at 25 and 37°C to assess differences in growth, using the spectrophotometer Epoch2 microplate reader (Biotek).

Screening of a Mini-Tn*10* Transposon Insertional Library

A mini-Tn*10* transposon mutant library of approximately 2,000 clones was generated in *Pdd* parental strain RM-71 in a previous study (Terceti et al., 2017), and clones were always grown at a temperature of 25°C. The library, which consisted of clones maintained frozen at -80°C in 96-well plates, was used in the present study for the screening of putative mutant clones exhibiting impaired growth at 37°C. To this aim, each 96-well plate was carefully thawed, and clones were replicated into 96-well plates containing fresh TSB-1 medium. Two replicas per plate were incubated at 25 and 37°C respectively, and bacterial growth was monitored for 24 h using the spectrophotometer Epoch2 microplate reader (Biotek).

β-Galactosidase Assays

RM-71 strain derivatives carrying the *dly*, $hlyA_{pl}$, and $hlyA_{ch}$ hemolysin gene promoters fused to a promotorless *lacZ* gene in reporter plasmid pHRP309 were grown in TSB-1 at 25 and 37°C, and β-galactosidase activities measured as previously described (Rivas et al., 2013). Three independent experiments with 3 replicates each were conducted. The statistical analysis of the expression data was carried out with the Student's *t*-test (adjusted $P < 0.05$). Mann–Whitney test was used for non-parametric comparison of the mean values.

RESULTS

Growth at 37°C Causes Impairment in Viability, Changes in Cell Morphology, Reduced Tolerance to Benzylpenicillin, and Modification of Membrane Lipid Composition

In order to cause disease in a human host, a marine bacterium has to be able to replicate at temperatures near 37°C. The ability to grow at temperatures > 30°C is indeed a differential phenotypic trait of *P. damselae* subsp. *damselae* in comparison to its sibling subspecies, the subsp. *piscicida* (*Pdp*). This difference is supposed to contribute to the ability of *Pdd* to colonize and establish an infection in homeothermic animals, whereas *Pdp* is pathogenic only for fish (Romalde, 2002).

To assess the effect of temperature in *Pdd* growth, the highly virulent strain RM-71, isolated from a disease outbreak in a turbot farm (Fouz et al., 1992), was cultured at 25 and 37°C for 48 h. Cultivation at 37°C triggered an earlier entry into exponential phase than at 25°C, but OD_{600} values dropped after 24 h suggesting a cell death scenario (**Figure 1A**). In order to test this hypothesis, viable cells were quantified by drop-counting on agar plates. As a result, there was a > 3.5-fold reduction in the number of colony-forming units (CFU) at 12 h incubation in cultures at 37°C in comparison to 25°C. Notably, CFU were no longer detected from cultures at 37°C after 30 h (**Figures 1B,C**). The observation of cell viability reduction at 37°C prompted us to study cell morphology by scanning electron microscopy, in exponentially-growing (OD_{600} of 0.55) cultures at 25 and 37°C. Remarkably, cells grown at 25°C showed normal rod morphology by scanning electron microscopy analysis whereas cells grown at 37°C exhibited chain-like structures and aberrant long shapes suggesting a defect in daughter cell separation and in septum formation (**Figure 2A**). Quantitatively, mean cell length values at 37°C were significantly higher than at 25°C (**Figure 2B**). In addition, growth at 37°C caused a reduction in the intrinsic tolerance of *Pdd* RM-71 to benzylpenicillin in comparison to 25°C (**Figure 2C**).

Bacteria can adapt to variations in environmental temperature by altering the ratio between saturated (SFA) and unsaturated (UFA) fatty acids in membranes (Marr and Ingraham, 1962). However, the effects of temperature in membrane lipids composition in *Pdd* have not been studied so far. We here carried out an analysis of fatty acid methyl esters (FAME) from *Pdd* RM-71 cultures at 25 and 37°C. We observed that the percentage of saturated fatty acids in membranes increased at 37°C with respect to 25°C, and the three saturated fatty acids 16:0 N alcohol, 16:0 iso and 19:0 cyclo w8c were exclusively detected at 37°C (**Supplementary Table S1**).

Human Isolates Are Not Better Adapted for Growth at 37°C Than Fish Isolates, and Comparative Genomics Does Not Reveal Human-Specific Gene Markers

We selected two strains from human clinical cases (CDC 2227-81 and 80077637), and three strains from diseased fish (RM-71, LD-07, and A-162), and growth of all the isolates was monitored at 25 and 37°C. The ability to grow at 37°C was not a differential trait of human isolates since all the strains showed similar dynamics: early exponential growth and a drop in the OD_{600} after approximately 24 h (**Figures 3A,B**). Of note, human strain 80077637 achieved the least OD_{600} values at the two temperatures. These data, together with the above described survival assays, suggest that 37°C, rather than being a normal condition, represents a stress condition for *Pdd*.

To assess the existence of gene markers characteristic of human isolates, we here obtained for the first time the draft genome sequences of two *Pdd* strains isolated from humans, CDC-2227-81 (Kreger, 1984) and 80077637 (Hundenborn et al., 2013), and a comparative genomics analysis was conducted including the genomes of four fish isolated strains, RM-71, LD-07, A-162, (Vences et al., 2017) and CIP102761. Ortho Average nucleotide identity (OrthoANI) values, which are derived from the pairwise comparison of strains taking into consideration the core genes shared by all the strains, ranged from 97.19 to 98.97 between strains (**Figure 3C**). Two genomes are considered the same species when the

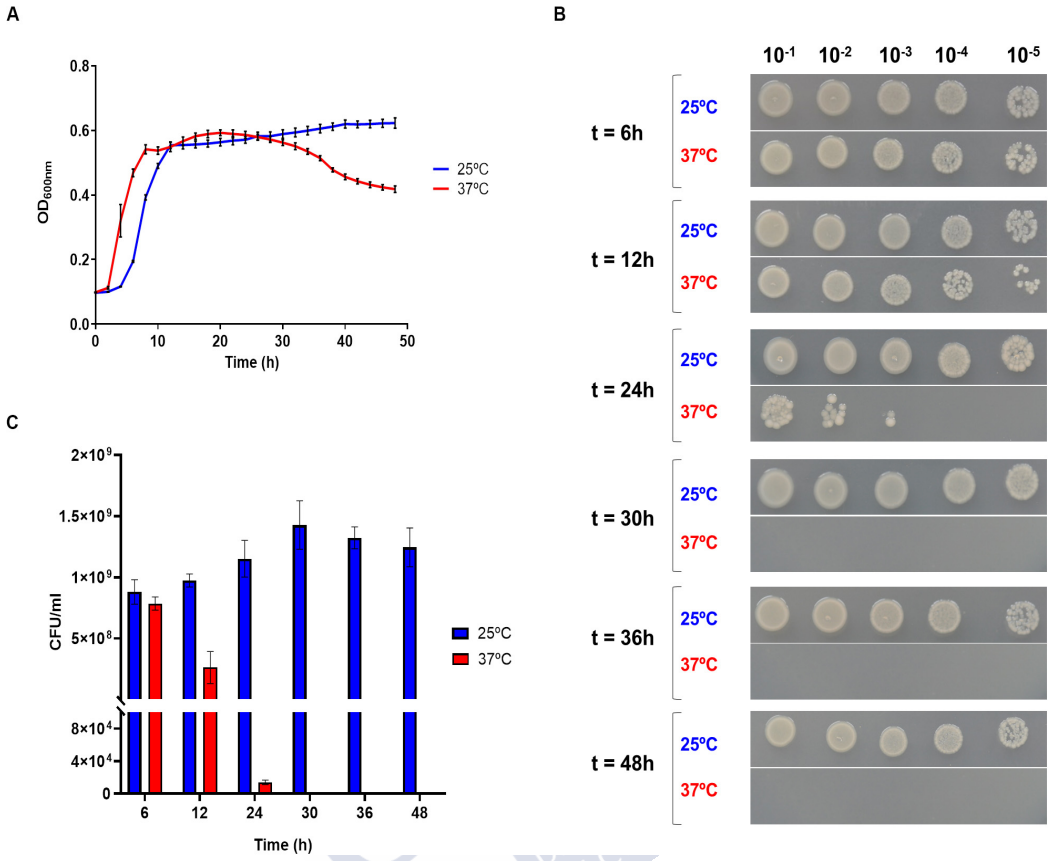

FIGURE 1 | Growth at 37°C impairs cell survival in *Pdd*. **(A)** Growth curves of *Pdd* RM-71 at 25 and 37°C in TSB-1, showing the brief stationary phase and the start of the death phase at 37°C, contrasting with the prolonged stationary phase at 25°C. Mean data of three independent experiments are shown. **(B)** Growth at 37°C causes a drastic reduction in CFU numbers as detected by drop-plate assays. Serial decimal dilutions of cultures at 25 and 37°C were prepared from samples taken at 6, 12, 24, 30, 36, and 48 h incubation, and 5 μl aliquots were applied as drops onto TSA-1 plates and incubated at 25°C for 24 h. **(C)** Quantitative representation of the drop-plate assay data. Viable counts are zero after 30 h cultivation at 37°C whereas at 25°C the number of CFUs is still increasing.

ANI value is higher than 95–96% (Lee et al., 2016). The 4 strains harboring the virulence plasmid pPHDD1 clustered together independently of their source of isolation, and human isolates were not more similar between them than to the other genomes, when core genes where compared. In fact, the highest similarity value was found between RM-71 (fish isolate) and 80077637 (human isolate). Notably, genomics analysis unveiled a large number of strain-specific genes, with 265 genes unique to strain CDC-2227-81 and 132 genes unique to strain 80077637 (**Figure 3D**). Four genes were common to the human isolates and absent in the fish isolates. These genes encoded a racemase (locus F6450_06750), an HD domain-containing protein (F6450_11305), a MFS transporter (F6450_16290), and a multidrug transporter (F6450_07685) (GenBank loci tags correspond to CDC 2227-81). However, BLAST searches in GenBank database unveiled that three of these genes were also present in other *Pdd* strains isolated from fish, and the multidrug transporter F6450_07685 was found in a *Pdd* isolate from the porpoise *Neophocaena asiaeorientalis* and in non-pathogenic *Photobacterium* species (data not shown). A phylogenetic tree constructed based on the genome alignments (including core and accessory genome) of *Pdd* strains revealed that human-isolated strains do not represent an independent evolutionary line with respect to the fish isolates, and human clinical strain 80077637 fell into the same clade with fish-isolated RM-71 (**Figure 3E**).

RNA Sequencing Reveals Major Changes in the *Pdd* Transcriptome in Response to Growth at 37°C

The RNAseq analysis of RM-71 resulted in 1607 differentially expressed genes (DEGs): 804 upregulated (Foldchange (FC) higher than 1.5) and 803 downregulated (FC lower than −1.5)

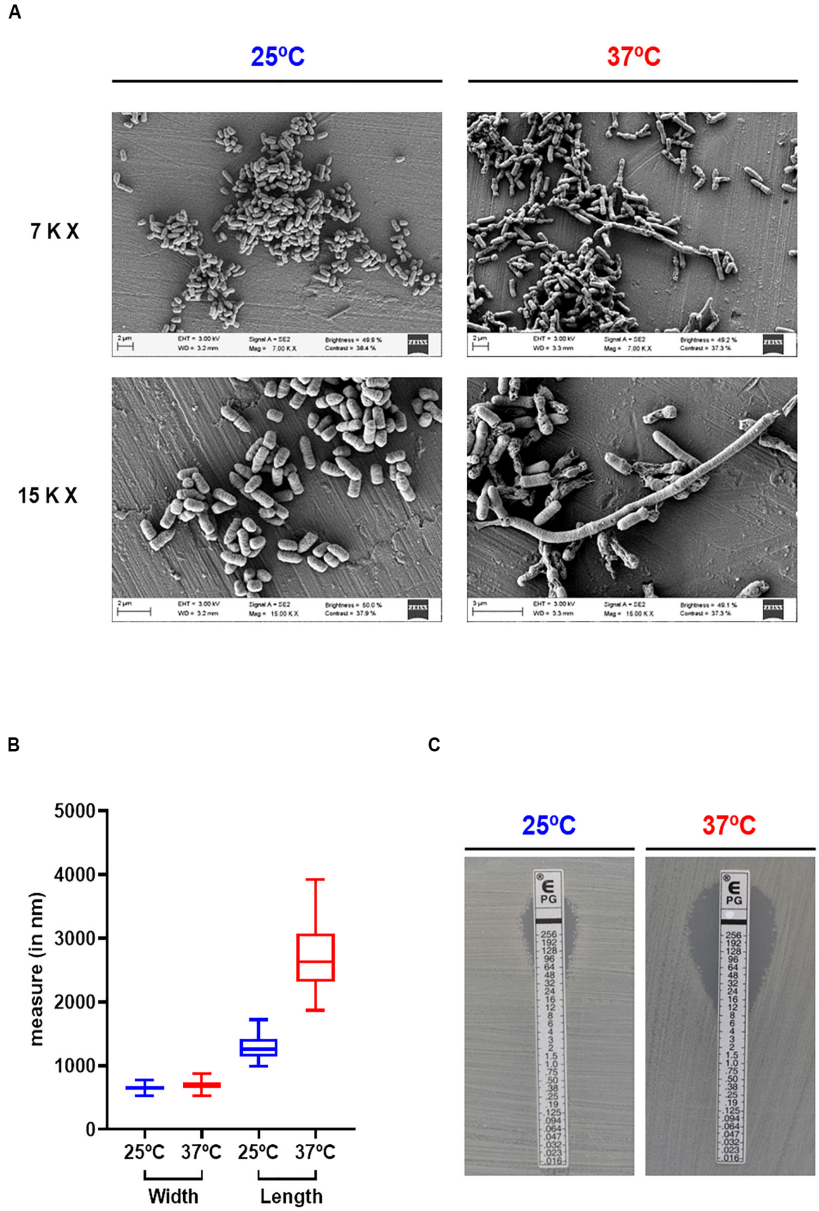

FIGURE 2 | Growth at 37°C impairs cell morphology and benzylpenicillin tolerance in *Pdd*. **(A)** Scanning electron microscopy images showing the increase in cell length and the impairment in daughter cell separation in *Pdd* cells grown at 37°C with respect to the normal cell size at 25°C. Pictures from two magnifications, 7,000× (7 K X) and 15,000× (15 K X) are shown. **(B)** Box plot graphs showing cell width and cell length values at 25 and at 37°C. Whiskers indicate min and max values. Data correspond to the measurements of 50 independent cells per temperature assayed. **(C)** *E*-tests for benzylpenicillin of *Pdd* RM-71 cultivated at 25 and 37°C, showing the decreased tolerance to this antimicrobial compound at 37°C.

at 37°C with respect to 25°C (**Supplementary Table S2**). The principal component analysis (PCA) showed that samples grown at each different temperature were distinguishable and replicates clustered together (**Figure 4A**). A heat map with gene expression plotted as z-score normalized FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value for differentially expressed genes (DEGs) was performed. Hierarchical clustering showed the different expression profiles of

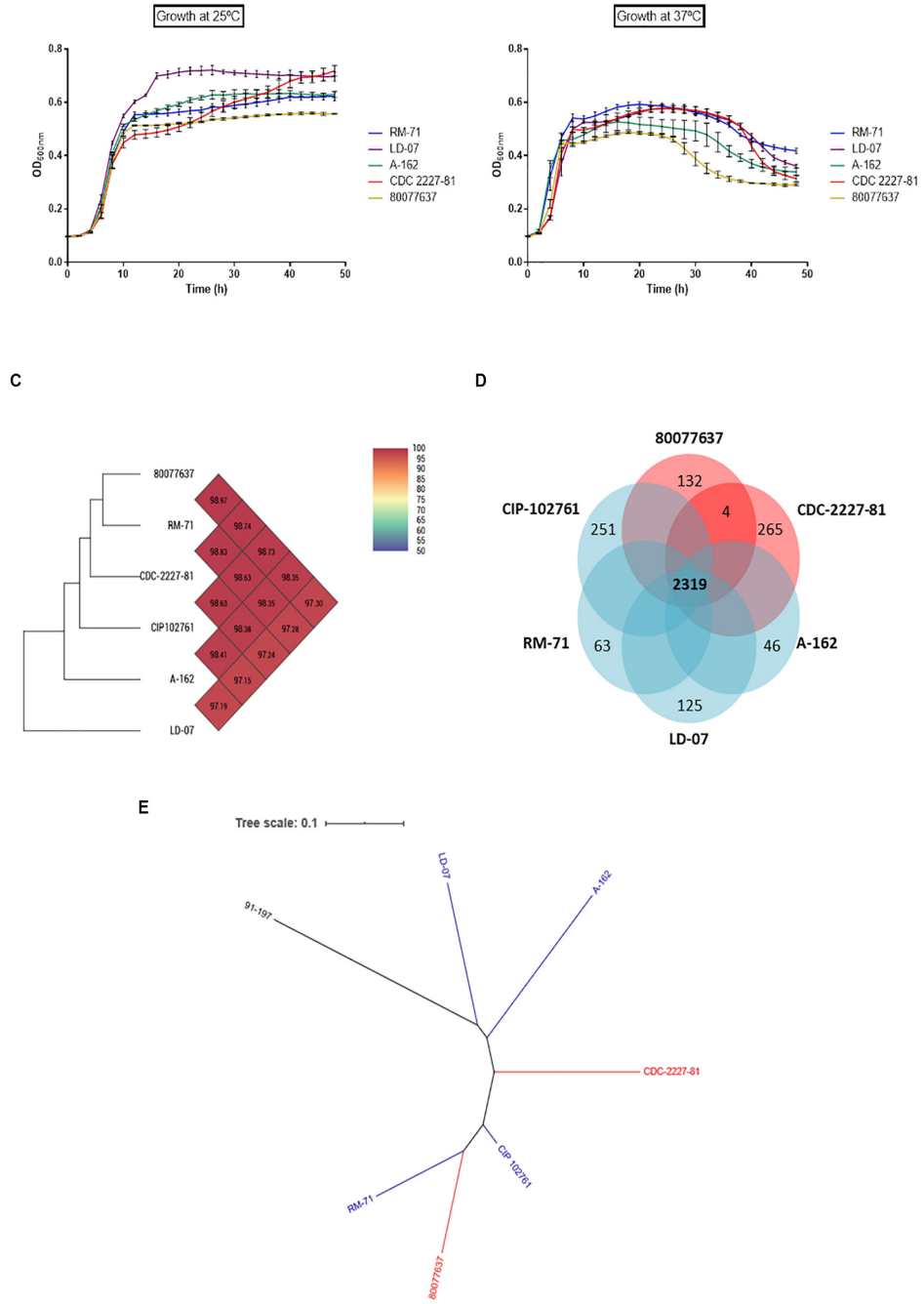

FIGURE 3 | Continued

FIGURE 3 | *Pdd* isolates from human origin do not exhibit an advantage for growth at 37°C compared to fish isolates, and do not contain specific gene markers unique to human isolates. Growth curves of *Pdd* strains from human (CDC 2227-81; 80077637) and fish (RM-71; LD-07; A-162) origin, cultivated at 25°C **(A)** and at 37°C **(B)**. Note that human clinical strain 80077637 achieves the least OD_{600} values of all strains at the two temperatures. **(C)** Heatmap generated by comparison of core genes with OrthoANI values calculated from the OAT software shows the absence of close phylogenetic association of human isolates vs. fish isolates. **(D)** Venn diagram depicting the comparative genomics of six *Pdd* strains. The core genome was estimated in 2319 genes, and each isolate has a varying number of strain-unique genes, ranging from 46 unique genes to strain A-162 to the 265 unique genes to strain CDC-2227-81. Of note, the two human-isolated strains only share 4 specific genes which are absent from the fish strains. **(E)** Dendrogram of *Pdd* strains based on genomic BLAST, showing that human clinical strains do not form an independent clade with respect to fish isolates. The genome of the sibling subspecies *P. damselae* subsp. *piscicida* strain 91–197 was included for comparative purposes. The genomic BLAST file was downloaded from the NCBI database and the tree was visualized by Interactive Tree Of Life (iTOL v5). The genome of the *Pdd* type strain CIP 102761 was included in the genomic analyses depicted in **(C–E)**.

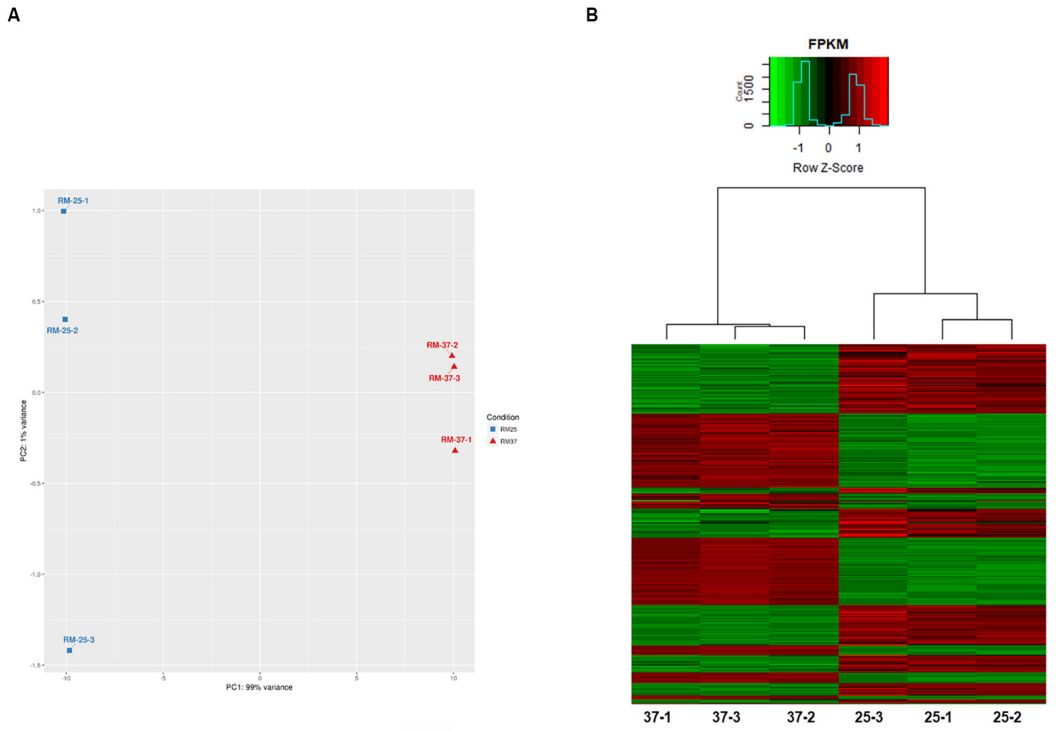

FIGURE 4 | RNA sequencing reveals changes in the *Pdd* transcriptome in response to growth at 37°C vs. 25°C. **(A)** Hierarchical clustering of all genes by Principal Component Analysis (PCA) demonstrates clear separation of the 2 sample groups (25°C vs. 37°C). **(B)** Heat map with gene expression plotted as z-score normalized FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value for differentially expressed genes (DEGs). The hierarchical clustering shows the different expression profiles of RM-71 at the two temperatures and provides strong evidence of the grouping of the biological replicates.

RM-71 at the two temperatures and the grouping of the biological replicates (**Figure 4B**).

Growth at 37°C Triggers a Strong Stress Response: Heat Shock Proteins and Defense-Related Mechanisms Are Upregulated at 37°C

To better illustrate the impact of human body temperature on gene expression, a heat map was generated with 32 selected DEGs that showed remarkable FC values (**Figure 5**). Notably, heat-shock proteins and molecular chaperones were among the most upregulated genes at 37°C (**Table 1** and **Figure 5**). The genes encoding ClpB, GroEl and HtpG heat-shock proteins were selected for construction of insertional mutants, using the suicide plasmid pNidKan (Mouriño et al., 2004) containing an internal fragment of the target gene. A *htpG* insertional mutant was successfully generated although it did not show any impairment in growth at 37°C compared to 25°C (data not shown). Notably, we were unable to obtain insertional mutants for *clpB* and *groEL* despite numerous attempts, suggesting that these genes are essential, even for growth at 25°C (the temperature at which strains were incubated during the mutant construction process). We thus aimed to gain a further insight into the existence of gene markers essential for growth at 37°C but dispensable at 25°C. Identification of such markers would be of interest for the design

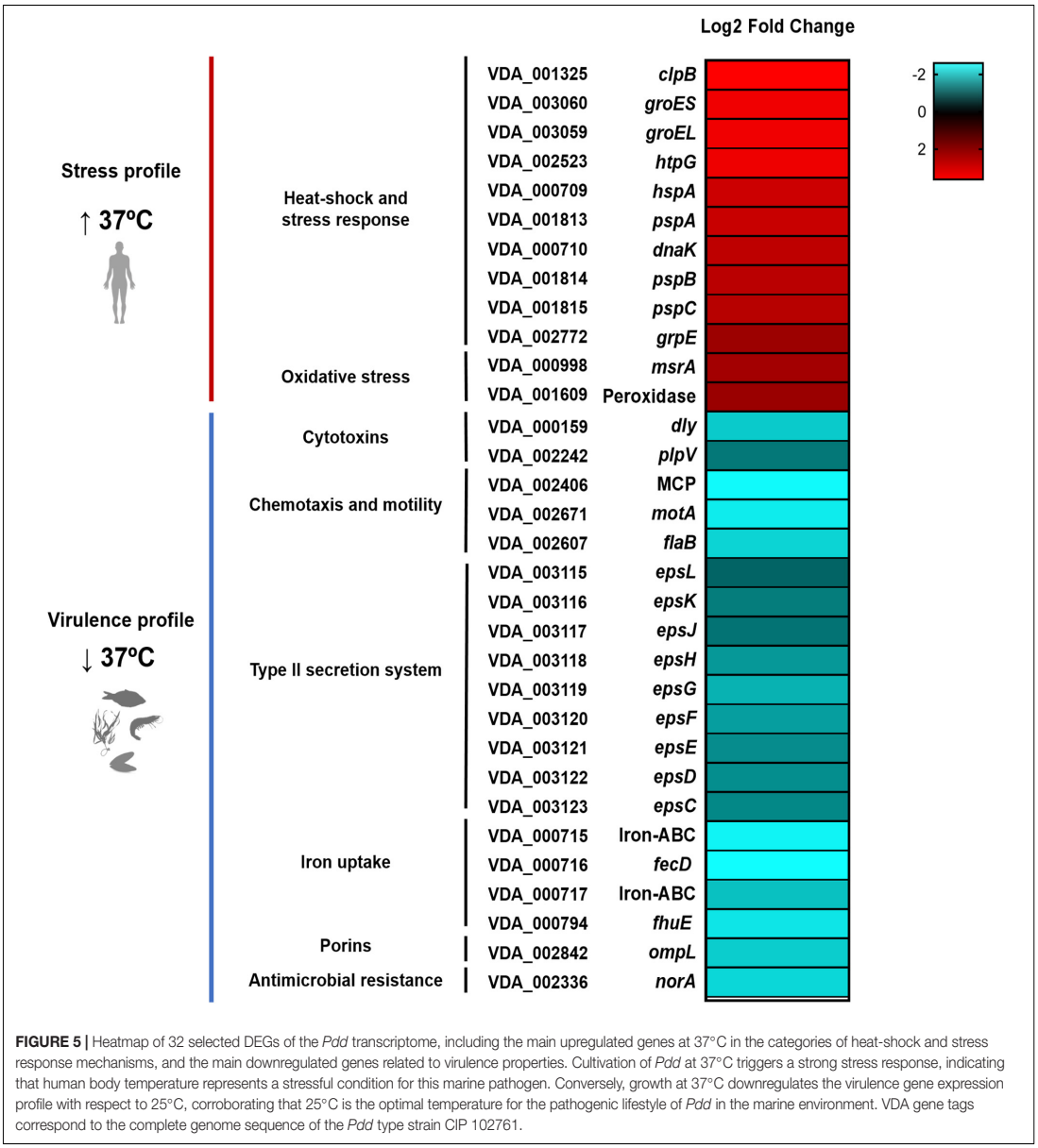

FIGURE 5 | Heatmap of 32 selected DEGs of the *Pdd* transcriptome, including the main upregulated genes at 37°C in the categories of heat-shock and stress response mechanisms, and the main downregulated genes related to virulence properties. Cultivation of *Pdd* at 37°C triggers a strong stress response, indicating that human body temperature represents a stressful condition for this marine pathogen. Conversely, growth at 37°C downregulates the virulence gene expression profile with respect to 25°C, corroborating that 25°C is the optimal temperature for the pathogenic lifestyle of *Pdd* in the marine environment. VDA gene tags correspond to the complete genome sequence of the *Pdd* type strain CIP 102761.

of control strategies of human infections caused by *Pdd*. For this, we screened a > 2000-clone Tn*10* transposon mutant library of *Pdd* RM-71 constructed in a previous study (Terceti et al., 2017), and each clone was grown in TSB-1 in 96-well plates per duplicate at 25 and at 37°C. However, all the mutant clones were able to grow at 37°C without any evident signs of impairment (data not shown), which suggests that any potential transposon-insertional mutation that would prevent growth at 37°C, would also be deleterious for growth at 25°C (the temperature at which the transposon library was originally obtained).

Genes involved in prevention of oxidative damage were found among the most up-regulated at 37°C (**Table 1** and **Figure 5**), including the peptide methionine sulfoxide reductase MsrA, alkyl hydroperoxide reductase subunit C-like protein and the thioredoxin peroxidase, among others. These enzymes are produced by bacteria as defensive strategies against host defenses. Together with the viability assays, this association between high temperatures and upregulation of stress and defense mechanisms supports the idea that 37°C constitutes a stressful condition for *Pdd*.

TABLE 1 | List of selected top DEGs whose expression is upregulated at 37°C.

Gene ID	Product/Function	Fold change	*p*-value	Location
Stress response and defense mechanisms				
VDA_001325	ClpB protein	12.1	0	ChrI
VDA_003060	Heat shock protein 60 family co-chaperone GroES	10.4	0	ChrI
VDA_003059	Heat shock protein 60 family chaperone GroEL	10.4	0	ChrI
VDA_002523	Chaperone protein HtpG	10.1	0	ChrI
VDA_000709	Heat shock protein A	7.3	0	ChrII
VDA_001813	Phage shock protein A	7.1	8.97261E−76	ChrI
VDA_000710	Chaperone protein DnaK	6.4	0	ChrII
VDA_001814	Phage shock protein B	6.1	1.624E−197	ChrI
VDA_001815	Hypothetical phage shock protein C	5.9	1.7736E−117	ChrI
VDA_002771	Chaperone protein DnaK	5.9	0	ChrI
VDA_003125	Heat-shock chaperonin	5.5	0	ChrI
VDA_000998	Peptide methionine sulfoxide reductase MsrA	4.9	0	ChrII
VDA_001609	Alkyl hydroperoxide reductase subunit C-like protein	4.4	0	ChrI
VDA_002772	Heat shock protein GrpE	4.6	0	ChrI
VDA_000104	Lipoprotein precursor OmpA family	5.4	5.73249E−76	pPHDD1
Metabolism				
VDA_002723	Glyoxylate cycle isocitrate lyase	4.0	1.1169E−114	ChrI
VDA_001102	ATP synthase gamma chain	4.1	8.8225E−67	ChrII
VDA_001103	ATP synthase alpha chain	3.5	1.55568E−69	ChrII
VDA_002810	Phosphopentomutase	3.2	1.7648E−261	ChrI
VDA_002812	Deoxyribose-phosphate aldolase	4.8	0	ChrI
VDA_002811	Thymidine phosphorylase	3.6	7.7733E−277	ChrI
VDA_003271	Thiamine biosynthesis ThiC	4.5	1.325E−176	ChrI
VDA_002967	Thiamine ABC transporter	4.4	1.3878E−296	ChrI
VDA_002261	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	13.5	0	ChrI
VDA_003183	Oligopeptidase A	5.5	0	ChrI
VDA_002812	Deoxyribose-phosphate aldolase	4.8	0	ChrI
VDA_000814	Hypothetical protein (DUF 4832)	12.5	1.2613E−200	ChrII
VDA_002964	3-isopropylmalate dehydratase large subunit	7.1	0	ChrI
VDA_002965	3-isopropylmalate dehydratase small subunit	7.1	0	ChrI
VDA_002963	3-isopropylmalate dehydrogenase	6.6	0	ChrI
VDA_001041	Adenylosuccinate synthetase	6.5	1.6314E−128	ChrII
VDA_002962	2-isopropylmalate synthase	6.3	0	ChrI
VDA_002881	Homoserine kinase	4.7	0	ChrI
VDA_002880	Threonine synthase	4.7	0	ChrI
Transporters, adaptation, and colonization				
VDA_002633	Sulfate transporter CysZ	3.1	3.0492E−158	ChrI
VDA_003428	Gluthatione-regulated potassium-efflux system protein KefB	3.4	4.4457E−183	ChrI
VDA_000372	Putative choline-glycine betaine transporter	7.7	0	ChrII
VDA_002854	$Na^{(+)}/H^{(+)}$ antiporter NhaA type	4.7	6.0472E−303	ChrI
VDA_000302	Urease accessory protein UreG	3.2	2.30662E−44	ChrII
VDA_000303	Urease accessory protein UreF	4.8	2.62317E−51	ChrII
VDA_000304	Urease accessory protein UreE	4.8	2.62317E−51	ChrII
VDA_000305	Urease subunit alpha UreC	3.5	4.26325E−17	ChrII
VDA_000306	Urease gamma subunit UreA	3.3	1.4581E−58	ChrII
Lipases				
VDA_000412	Hypothetical protein (patatin similar to Yjju protein)	10.6	0	ChrII
VDA_002140	Putative lipase/esterase protein	3.5	2.11351E−81	ChrI
Pilus assembly				
VDA_000103	Protein TadG associated with Flp pilus assembly	5.4	1.55701E−93	pPHDD1
VDA_000134	IncF plasmid conjugative transfer pilus assembly protein TraF	5.0	3.42729E−12	pPHDD1
VDA_000102	Flp pilus assembly protein TadD	4.9	1.09531E−19	pPHDD1
VDA_000142	Mfp1 (mar binding filament-like protein 1)	4.9	0	pPHDD1

(Continued)

TABLE 1 | Continued

Gene ID	Product/Function	Fold change	*p*-value	Location
Transcriptional regulators and DNA-binding proteins				
VDA_001014	Hypothetical transcriptional regulator	5.7	6.7055E−294	ChrII
VDA_000143	DNA-binding protein H-NS	5.0	0	pPHDD1
VDA_001042	Transcriptional regulator LysR family	4.6	3.1515E−180	ChrII
VDA_000105	Site-specific recombinase resolvase family	8.3	1.925E−296	pPHDD1
VDA_000152	Chromosome segregation ATPase	5.0	9.68836E−64	pPHDD1

Enhanced expression at 37°C is denoted by positive Fold change (FC) values. Genomic location of each DEG in either ChrI (chromosome I), ChrII (chromosome II) or in virulence plasmid pPHDD1 is detailed.

Metabolic Pathways, Transporters, ATP Generation, and Nutrient Acquisition Are Upregulated at 37°C

Cultivation at 37°C causes a rapid initial growth of *Pdd* as shown above. In the RNA seq analysis, the expression of genes encoding enzymes of catabolic and anabolic pathways were strongly up-regulated at 37°C (**Table 1**), being the glycolytic enzyme NAD-dependent glyceraldehyde-3-phosphate dehydrogenase one of the most strongly upregulated genes at 37°C. Leucine, isoleucine, threonine and thiamine biosynthetic genes, enzymes of the phosphate pentose pathway and the purine/pyrimidine metabolism were upregulated at 37°C, likely to supply precursors for proteins, nucleotides and coenzymes to sustain the rapid initial growth of *Pdd* observed at 37°C. Accordingly, genes encoding the ATP-synthase subunits were upregulated. A number of pH-dependent and independent transporters were among the most upregulated genes at 37°C, including a Na^+/H^+ antiporter of NhaA-type. In *V. cholerae* this gene contributes to resistance to Na^+ at alkaline pH leading to improved fitness in the environment (Herz et al., 2003). Also upregulated were several sulfate transporters, and the potassium-efflux system protein KefB. Of relevance, *kefB* belongs to the locus of heat resistance in *E. coli* (Mercer et al., 2017) and it has been studied for its ability to acidify intracellular pH in response to electrophilic stress (Ferguson et al., 1995).

Pdd is one of the few urease-producer species within the *Vibrionaceae* family, and genes encoding urease subunits were upregulated at human body temperature. Urease is a well-studied enzyme in gastric pathogens for its role in maintaining periplasmic pH near neutrality in highly acidic environments (Sachs et al., 2003) and it also constitutes a key virulence factor of numerous urinary pathogens (Nielubowicz and Mobley, 2010; de Paiva-Santos et al., 2018). To date no functional studies of *ure* genes have been carried out in *Pdd*. This up-regulation of *ure* genes might play a role in some *Pdd* infections as this pathogen has been isolated as causative agent of a urinary tract infection in a pregnant woman in warm coastal areas of the Caribbean Sea (Alvarez et al., 2006).

Genes encoding degradative functions were upregulated, including several putative lipases (VDA_000412 and VDA_002140) and an operon for utilization and transport of galactosamine and glycosaminoglycans (VDA_001072-VDA_001083). Glycosaminoglycans are major components in extracellular matrix of animals (Gandhi and Mancera, 2008). *Pdd* is considered a generalist pathogen of a wide range of animals (Osorio et al., 2018) so genetic networks involved in the utilization of different carbon sources allows colonization of different niches and hosts. At 37°C, *Pdd* also upregulated genes of two pili structures encoded within the virulence plasmid pPHDD1, including the *tra* genes for conjugative pilus biogenesis, and the genes of the Flp/Tad pilus, with a potential role in adhesion/colonization.

A Virulence Plasmid-Encoded H-NS Repressor Is Upregulated at 37°C

How *Pdd* modulates genetic expression in response to external conditions remains unknown. Several LysR family transcriptional regulators were upregulated at 37°C (VDA_001014, VDA_001042, VDA_000547, and VDA_001035). Of special relevance is the observation that the DNA-binding protein H-NS (VDA_000143), encoded within the virulence plasmid pPHDD1 showed a > fivefold upregulation at 37°C. In *Vibrio cholerae*, this protein functions as a virulence repressor controlling motility and biofilm formation (Wang et al., 2015). As detailed below, growth at 37°C was found to downregulate virulence and motility functions in *Pdd*.

Growth at 37°C Causes Downregulation of Virulence Factors

In a previous work, we demonstrated that many *Pdd* virulence factors were up-regulated at 25°C in comparison with 15°C, thus contributing to partially explain the onset of disease outbreaks in marine fish farms when seawater temperature is close to 25°C (Matanza and Osorio, 2018). We here investigated how human body temperature affects virulence gene expression in *Pdd*. Of note, well-known virulence factors of *Pdd* were downregulated at 37°C, suggesting that infection of a human host is not a condition that has been favored during evolution of this marine pathogen (**Table 2** and **Figure 5**). Damselysin (Dly) toxin, a major virulence factor of *Pdd*, showed a fourfold down-regulation at 37°C. The two pore-forming toxins PhlyP and PhlyC did not show a differential expression at 37°C, and the phospholipase PlpV showed downregulation at 37°C. β-galactosidase assays of transcriptional fusions of the hemolysin gene promoters to *lacZ* gene validated the RNA-seq results (**Figure 6**). Despite the absence of upregulation at 37°C, the three major cytotoxins Dly, PhlyP, and PhlyC exhibited high FPKM values (**Supplementary Table S3**), in accordance with previous

TABLE 2 | List of selected top DEGs whose expression is downregulated at 37°C.

Gene ID	Product/Function	Fold change	*p*-value	Location
Defense and virulence				
VDA_000717	Iron ABC-transporter	−3.9	2.7907E−177	ChrII
VDA_000716	Iron(III) dicitrate transport system permease protein FecD	−6.1	6.4554E−244	ChrII
VDA_000715	ABC type periplasmic iron siderophore/cobalamin binding protein	−5.6	0	ChrII
VDA_000794	Ferrichrome-iron receptor	−5.1	0	ChrII
VDA_000159	Damselysin	−4.2	8.6351E−269	pPHDD1
VDA_002336	Multidrug efflux protein NorA	−4.6	1.7265E−164	ChrI
VDA_002842	OmpL porin-like protein L precursor	−4.3	4.4961E−185	ChrI
VDA_000341	ABC transporter periplasmic spermidine putrescine-binding protein PotD	−7.9	0	ChrII
VDA_001897	Thiol-disulfide isomerase	−4.6	6.0694E−145	ChrI
VDA_002117	ABC transporter periplasmic spermidine putrescine-binding protein PotD	−4.2	7.62485E−75	ChrI
VDA_003028	Flavohemoprotein	−4.2	0	ChrI
VDA_000342	Oxidoreductase	−3.2	4.73193E−72	ChrII
Cold response				
VDA_003169	Cold shock protein	−16.3	0	ChrI
VDA_000346	Putative Cold shock-like protein	−8.4	3.1036E−252	ChrII
VDA_000863	Cold-shock DEAD-box protein A	−5.3	9.3703E−299	ChrII
Hypothetical proteins of unknown function				
VDA_002460	Lipoprotein putative	−15.9	0	ChrI
VDA_002316	Hypothetical protein (Helix-turn-helix domains)	−14.2	0	ChrI
VDA_001898	Putative heat shock protein YegD	−7.3	7.2519E−209	ChrI
Histone acetylation				
VDA_000822	Histone acetyltransferase HPA2	−10.8	4.0491E−204	ChrII
Flagellar motility and chemotaxis				
VDA_002406	N-acetylglucosamine regulated methyl-accepting chemotaxis protein	−6.0	5.4912E−139	ChrI
VDA_002671	Flagellar motor rotation protein MotA	−5.4	1.0403E−179	ChrI
VDA_001613	Sodium-type flagellar protein MotY precursor	−4.9	1.5077E−180	ChrI
VDA_003029	Sodium-type polar flagellar protein MotX	−4.8	3.9208E−150	ChrI
VDA_001198	Putative methyl-accepting chemotaxis protein	−4.1	1.6095E−186	ChrI
VDA_003044	Methyl-accepting chemotaxis protein	−4.0	3.2241E−137	ChrI
VDA_002619	Chemotaxis protein CheV	−3.4	6.3682E−259	ChrI
VDA_002670	Flagellar motor rotation protein MotB	−4.1	9.0774E−129	ChrI
VDA_002607	Flagellin protein FlaB	−4.5	2.4193E−132	ChrI
Porins, permeases, and transporters				
VDA_001789	Nucleoside permease NupC	−10.7	0	ChrI
VDA_001677	Sulfate permease	−6.5	0	ChrI
VDA_002944	Probable low-affinity inorganic phosphate transporter	−6.8	0	ChrI
VDA_002943	Phosphate transport regulator	−7.4	0	ChrI
Transcriptional regulators				
VDA_003227	Putative transcriptional regulator DeoR family protein	−3.4	1.2526E−136	ChrI
VDA_001543	Hypothetical response regulator	−3.7	2.486E−250	ChrI
VDA_002957	Putative LuxZ	−4.6	0	ChrI
Metabolism				
VDA_002716	Myo-inositol-1(or 4)-monophosphatase	−7.4	1.66767E−53	ChrI
VDA_001806	Orotidine 5′-phosphate decarboxylase	−5.1	2.4264E−246	ChrI
VDA_003379	Fructose-1.6-bisphosphatase GlpX type	−4.5	4.51805E−64	ChrI
VDA_001717	Cytochrome c-type protein TorC	−4.3	3.762E−125	ChrI
VDA_000979	HNH nuclease	−10.9	3.2236E−205	ChrII
VDA_002963	Inosine-5′-monophosphate dehydrogenase	−6.6	0	ChrI
VDA_003340	Orotate phosphoribosyltransferase	−4.1	1.4272E−176	ChrI
Adjustment of membrane composition				
VDA_002169	Phosphatidylglycerophosphatase B	−3.9	1.8426E−114	ChrI
VDA_002932	1-acyl-sn-glycerol-3-phosphate acyltransferase	−3.5	3.0484E−200	ChrI
VDA_002087	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase	−4.2	0	ChrI
VDA_002463	UDP-2.3-diacylglucosamine hydrolase	−4.1	6.7473E−233	ChrI

Note that downregulated expression at 37°C is denoted by negative fold change values.

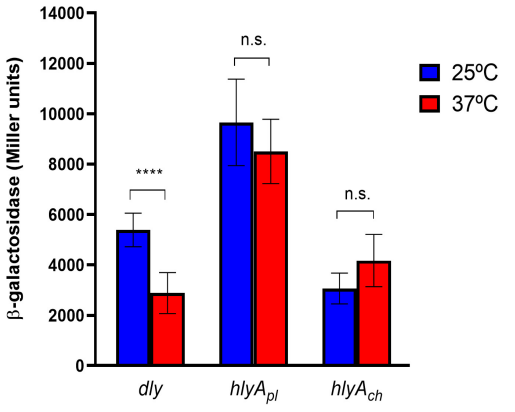

FIGURE 6 | Transcription activities of the three major cytotoxin genes of *Pdd*, damselysin (*dly* gene), phobalysin P ($hlyA_{pl}$ gene), and phobalysin C ($hlyA_{ch}$ gene) under the temperature conditions of a human host (37°C) in comparison to the temperature in warm seawaters (25°C), as determined by transcriptional fusions of *dly*, $hlyA_{pl}$, and $hlyA_{ch}$ promoters to *lacZ* reporter gene. The β-galactosidase values (expressed in Miller units) show that *dly* is significantly downregulated at 37°C whereas expression of $hlyA_{pl}$ and $hlyA_{ch}$ is not significantly affected at 37°C vs. 25°C, thus validating the results of the transcriptomics analysis. ****denotes the degree of significance in the Mann-Whitney test (*p* value was <0.0001).

studies that demonstrated that the three cytotoxin genes are highly expressed and the toxins represent a major fraction of the *Pdd* secretome (Matanza and Osorio, 2018; Terceti et al., 2019). Genes of the type II secretion system (*eps* genes, VDA_003115-VDA_003123) that play a major role in the secretion of the cytotoxins and therefore in virulence (Rivas et al., 2015a; Vences et al., 2017), were downregulated at 37°C (**Figure 5**). The two-component RstAB system, a major positive regulator of virulence in *Pdd* (Terceti et al., 2017, 2019), was not significantly affected by temperature.

Other virulence-related functions were downregulated at 37°C (**Table 2** and **Figure 5**): an iron ABC-transporter (VDA_00715-VDA_000717), a TonB-dependent siderophore receptor (VDA_000794), and the *potABCD* operon (VDA_002114-VDA_002117, VDA_000341) encoding a polyamine (spermidine and putrescine) transport system. Polyamines are essential for normal growth and proliferation in bacteria (Shah and Swiatlo, 2008). In *Vibrio vulnificus* expression of *pot* genes is up-regulated in presence of human serum (Williams et al., 2014) and in *V. cholerae* they regulate biofilm formation (McGinnis et al., 2009).

Porins are outer membrane proteins that modulate pathogenicity of many *Vibrio* species. VDA_002842 is downregulated at 37°C and shows similarity to *Vibrionaceae* porins, including OmpU. OmpU has been pointed for its role in adhesion, host recognition and pathogenesis in several *Vibrio* species (Goo et al., 2006; Duperthuy et al., 2011; Sakharwade and Mukhopadhaya, 2015). Multidrug efflux protein VDA_002336 showed a down-regulation at 37°C. It belongs to the multidrug and toxic-compound extrusion (MATE) proteins group, involved in protecting cells against antibiotics and other substances in human pathogenic *Vibrios* as *Vibrio parahaemolyticus* and *Vibrio cholerae* (Begum et al., 2005; Otsuka et al., 2005).

Motility and adhesion to hosts are considered of special relevance in *Pdd* pathogenicity (Fouz et al., 2000; Rivas et al., 2015b). A recent study demonstrated that disruption of chemotaxis regulator *cheA* reduced motility and production of PhlyP, and subsequently impaired bacterial adhesion to human cell lines (von Hoven et al., 2018). The current study revealed a large set of genes with a role in chemotaxis and flagellar motility which were downregulated at 37°C (**Table 2** and **Figure 5**). All these data indicate that exposure of *Pdd* at human body temperature triggers a transcriptomic pattern of defense against stress rather than a high virulence profile.

Genes Encoding Cold Shock Response, Membrane Transporters, Metabolic Functions, and Adjustment of Membrane Composition Are Downregulated at 37°C

There was a downregulation of cold shock functions at 37°C (**Table 2**), including two proteins that might play a role in cold tolerance in *Pdd*: VDA_002460 and VDA_002316. Phosphate, nucleoside and vitamin transporters, and permeases for inorganic ions were downregulated at 37°C (**Table 2**). DNA can be utilized as a source of phosphate, carbon and nitrogen. Phosphate limitation is encountered by marine pathogens in either the environment or inside the host. We have found that a phosphate transport regulator (VDA_002943) and a low-affinity inorganic phosphate transporter (VDA_002944), which shares homology with *E. coli pitA* (identity 33%), are down-regulated at 37°C. As well, nucleoside permeases NupC (VDA_001789 and VDA_002313) were also down-regulated at 37°C. A previous study (Matanza and Osorio, 2018) showed that NupC VDA_001789 presented a high up-regulation at 25°C compared to 15°C. These results suggest that *Pdd* takes advantage of the utilization of DNA as a source of biomolecules especially at 25°C (temperature at which outbreaks in fish farms are favored). An operon of type ECF (energy coupling factor) ABC transporters (VDA_002275-VDA_002277) was down-regulated at 37°C, as well as the putative vitamin transporter VDA_003148. The myo-inositol-1 (or 4) – monophosphatase (VDA_002716), which participates in myo-inositol biosynthesis, showed a remarkable down-regulation at 37°C. This function has been associated with a role in correct rRNA biosynthesis (Singh et al., 2016).

During anaerobic respiration, bacteria use alternative electron acceptors such as trimethylamine N-oxide (TMAO) to oxidize organic compounds. The cytochrome c-type protein TorC (VDA_001717) that takes part in this electronic transfer (Gon et al., 2001) shows a down-regulation at 37°C. The association between anaerobic growth and temperature in *Pdd* has not been explored to date. Functions related to nucleotide and pyrimidine biosynthesis, and of carbohydrate metabolism were also downregulated. Functions for the maintenance of lipid

membranes, phospholipid biosynthesis, fatty acid biosynthesis and lipid IVA biosynthesis were markedly downregulated at 37°C. These observations might correlate with the observed changes in membrane lipid composition in *Pdd* cultured at 37°C vs. 25°C as reported above (**Supplementary Table S1**).

DISCUSSION

Different to other human pathogenic Vibrios (as *V. cholerae* and *V. parahaemolyticus*) that cause diarrhea in humans and therefore gain exit from the host, infection of a human by *Pdd* can be seen as an end-point for the bacterium. Most clinical cases evolve as wound infections that may or may not complicate into fatal necrotizing fasciitis, and there is no evident exit strategy for the successful *Pdd* genotypes to go back to the marine environment. The lines of evidence presented in this study clearly suggest that the ability of *Pdd* to infect humans is more an anecdotic circumstance than an evolutionarily selected trait. *Pdd* cells growing at human body temperature undergo stressful conditions that impair viability, control of cell shape, and size. This evidence is in agreement with earlier studies that reported the difficulties to isolate *Pdd* from bullae fluid and blood in a human clinical case, whereas streaking the specimens on blood agar plates caused hemolysis in absence of growth (Clarridge and Zighelboim-Daum, 1985). In a rapidly fatal infection case, wound specimens yielded *Pdd* in pure culture but blood cultures were negative, and it was suggested that a systemic toxin released by *Pdd* contributed to the rapid fatal outcome and that septicemia did not (Fraser et al., 1997). Other studies were unable to detect *Pdd* cells upon Gram staining of specimens from tissues in infected individuals (Coffey et al., 1986; Goodell et al., 2004). All these observations can be explained, on the one hand, by the low viability of *Pdd* at 37°C as demonstrated in the present study and, on the other hand, by the high amounts of hemolysins produced, that would accumulate in host fluids during the initial phases of infection. Accumulated hemolysins might cause further tissue damage in absence of bacterial replication. Recent studies have demonstrated that under laboratory growth conditions at 25°C, the hemolysin genes are among the most highly expressed genes in *Pdd* (Matanza and Osorio, 2018; Terceti et al., 2019), and the FPKM values obtained in the present study (**Supplementary Table S3**) also confirmed that the three major hemolysins, Dly, PhlyP, and PhlyC exhibit high transcription levels at 37°C. Our findings on affected cell morphology and size in *Pdd* upon growth at 37°C are in agreement with previous observations of bacilli with large dimensions in Gram-stained sections from *Pdd*-infected tissues (Coffey et al., 1986) and with aberrant cell morphologies (Clarridge and Zighelboim-Daum, 1985; Goodell et al., 2004).

Similar to other species of the genera *Vibrio* and *Photobacterium*, *Pdd* exhibits high levels of intrinsic tolerance to bactericidal inhibitors of cell wall biosynthesis, as benzylpenicillin. Recent studies reported that *Pdd* RM-71 regulatory mutants in the RstAB two-component system exhibited aberrant cell morphologies, increased cell size and length, and reduced tolerance to benzylpenicillin (Terceti et al., 2019). In the present study, growth at 37°C was also found to cause increased sensitivity to benzylpenicillin with respect to 25°C. Thus, conditions that impair cell size and shape also impair penicillin tolerance in *Pdd*. A recent study reported that tolerance to beta lactams in *Vibrio cholerae* has a pleiotropic nature, and genes involved in cell envelope biogenesis and modification also play a major role in tolerance to beta lactams (Weaver et al., 2018).

A conserved strategy used by bacteria to face variations in environmental temperature is the modulation of membrane fluidity by adjustment of lipid composition (Sinensky, 1974; Rock and Cronan, 1996; Mansilla et al., 2004). The predominant strategy consists of altering the ratio between saturated (SFA) and unsaturated (UFA) fatty acids in membranes (Marr and Ingraham, 1962; Russell, 1983; Zhang and Rock, 2008). The analysis of FAME in *Pdd* RM-71 at 25 and 37°C in the present study has demonstrated that the percentage of saturated fatty acids in membranes increases at 37°C with respect to 25°C, and three saturated fatty acids are exclusively detected at 37°C. It is conceivable that the ability of this marine pathogen to inhabit different environments may rely on its skills to reversibly modify its membranes, assuring the survival outside and inside the diversity of hosts it can successfully colonize.

On the light of the current study, it is not possible to propose gene markers specific for human-adapted strains vs. fish-adapted strains in *Pdd*. The pPHDD1 virulence plasmid shared by the two human isolates analyzed here, also occurs in fish isolates (Rivas et al., 2011). A close look at the hemolytic activities of human isolates reported in an early study (Kreger, 1984) suggests that some human isolates fall within the category of low hemolytic activity (isolates that lack pPHDD1). These lines of evidence suggest that any *Pdd* genotype thriving in the marine environment might potentially cause an opportunistic infection in humans.

Recent transcriptomic studies have revealed the connection between temperature and virulence in marine pathogenic bacteria (Williams et al., 2014; Kong et al., 2017; Huang et al., 2018). In the current work, we demonstrate that *Pdd* undergoes a stress response at 37°C, with heat-shock proteins accounting for the most upregulated functions at this temperature. On the contrary, *Vibrio* species pathogenic for humans and marine animals as *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholerae* are routinely cultured at 37°C because such temperature does not represent a limiting factor. Indeed, *V. parahaemolyticus* shows the highest percentage of genes with stable expression at 37°C (Urmersbach et al., 2015).

Growth at 37°C caused the downregulation of most of the virulence-related functions known in *Pdd*. This not only pertains to the *Pdd* cytotoxins, but also to iron acquisition systems, motility and chemotaxis. Previous studies have reported the importance of iron acquisition in *Pdd* virulence for fish and mammals (Fouz et al., 1994, 1997). In the present study, it was demonstrated that growth at 37°C caused downregulation of iron ABC transporters and siderophore receptors. Downregulated genes included VDA_000794 encoding a TonB-dependent siderophore receptor. Of note, this gene was

found to be induced when *Pdd* is cultured under iron-limitation conditions (Puentes et al., 2017).

A previous study that compared the *Pdd* transcriptomes at 15 and 25°C, revealed that warm seawater temperatures (25°C) upregulated genes for chemotaxis, flagellar motility and virulence with respect to 15°C (Matanza and Osorio, 2018). This clearly suggests that the optimal temperature for *Pdd* to trigger a virulence expression profile is close to 25°C. Proteins for prevention of oxidative damage, found among the most up-regulated genes at 37°C, are produced by bacteria as defensive strategies against host defenses, and constitute interesting targets for the design of antimicrobial compounds (Ezraty et al., 2017). In this line, another gene upregulated at 37°C was an outer membrane protein (VDA_000104) whose homologs in *V. cholerae* (VC1835) and *V. parahaemolyticus* (VP1390) are related to virulence and antimicrobial activities (Bina et al., 2003; Ronholm et al., 2016). Some of the most DEGs genes unveiled in this study still await further investigation. It is the case of VDA_000412, a protein of the patatin-like phospholipase superfamily that exhibits a 10-fold upregulation at 37°C. The patatin-like ExoU plays an important role in pathogenesis in *Pseudomonas aeruginosa* (Sato and Frank, 2014). Overall, the transcriptomics data reported here are expected to contribute to the investigation of novel approaches for prevention and for less invasive control of human infections caused by *Pdd*. On balance, these results integrate transcriptomics, genomics and phenotypic data providing strong evidence that human infections caused by *Pdd* are opportunistic rather than originated by specific clones adapted to the human host. Transcriptomics analysis reveals that, under human host temperature conditions, *Pdd* triggers a defensive strategy by upregulating heat-shock proteins and defense mechanisms, while downregulating a virulence expression profile.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank: VZUQ00000000 and WAEO00000000.

AUTHOR CONTRIBUTIONS

XM and CO conceived the study, designed the experiments, analyzed the data, and wrote the manuscript. XM performed the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01771/full#supplementary-material

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