CENTRO INTERNACIONAL DE ESTUDOS
DE DOUTORAMENTO E AVANZADOS
DA USC (CIEDUS)

TESIS DE DOCTORADO

# *PHOTOBACTERIUM DAMSELAE* SUBSP. *DAMSELAE*: ANALYSIS OF THE GENETIC DIVERSITY AND CHARACTERIZATION OF A CONSERVED TWO-COMPONENT SYSTEM REGULATING VIRULENCE

De Souza Terceti Mateus

**ESCUELA DE DOCTORADO INTERNACIONAL**

**PROGRAMA DE DOCTORADO EN AVANCES EN BIOLOGÍA MICROBIANA Y PARASITARIA**

SANTIAGO DE COMPOSTELA

2018

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

## DECLARACIÓN DEL AUTOR DE LA TESIS

**_Photobacterium damselae_ subsp. _damselae_: analysis of the genetic diversity and characterization of a conserved two-component system regulating virulence.**

D. Mateus de Souza Terceti,

*Presento mi tesis, siguiendo el procedimiento adecuado al Reglamento, y declaro que:*

1) *La tesis abarca los resultados de la elaboración de mi trabajo.*

2) *En su caso, en la tesis se hace referencia a las colaboraciones que tuvo este trabajo.*

3) *La tesis es la versión definitiva presentada para su defensa y coincide con la versión enviada en formato electrónico.*

4) *Confirmo que la tesis no incurre en ningún tipo de plagio de otros autores ni de trabajos presentados por mí para la obtención de otros títulos.*

*En Santiago de Compostela, 19 de Octubre de 2018*

Fdo: Mateus de Souza Terceti

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

## *AUTORIZACIÓN DEL DIRECTOR / TUTOR DE LA TESIS*

**_Photobacterium damselae_ subsp. _damselae_: analysis of the genetic diversity and characterization of a conserved two-component system regulating virulence.**

D. Carlos Rodríguez Osorio

INFORMA:

*Que la presente tesis, corresponde con el trabajo realizado por D. Mateus de Souza Terceti, bajo mi dirección, y autorizo su presentación, considerando que reúne los requisitos exigidos en el Reglamento de estudios de Doctorado de la USC, y que como director de ésta no incurre en las causas de abstención establecidas en Ley 40/2015. De acuerdo con el artículo 41 del Reglamento de estudios de Doctorado declara también que la presente tesis doctoral es idónea para ser defendida en base a la modalidad de COMPENDIO DE PUBLICACIONES, en los que la participación del doctorando fue decisiva para su elaboración. La utilización de estos artículos en esta memoria, está en conocimiento de los coautores, tanto doctores como no doctores. Además, estos últimos tienen conocimiento de que ninguno de los trabajos aquí reunidos podrá ser presentado en ninguna otra tesis doctoral.*

*En Santiago de Compostela, 19 de Octubre de 2018*

Fdo: Carlos Rodríguez Osorio

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

D. Mateus de Souza Terceti

**Declaro:**

1) No tener ningún conflicto de intereses relevante en lo que concierne al contenido presentado en esta tesis doctoral.

2) El protocolo de experimentación con animales utilizados en este estudio (Código de procedimiento 15004/14/003) ha sido revisado y aprobado por el Comité de Ética de Experimentación Animal (CEEA) de la Universidad de Santiago de Compostela

En Santiago de Compostela, 19 de Octubre de 2018

Fdo: Mateus de Souza Terceti

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

El trabajo descrito en la presente memoria ha sido financiado por los siguientes **proyectos competitivos**:

-EM2012/043 (Xunta de Galicia).

-AGL2013-48353-R (Ministerio de Economía y Competitividad).

-AGL2016-79738-R (Ministerio de Economía y Competitividad).

Todos los proyectos arriba mencionados han estado cofinanciados por el programa FEDER de la Unión Europea.

Durante la realización de esta tesis se ha disfrutado de una beca CAPES, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil, do programa Ciências Sem Fronteiras.

Una parte de este trabajo de tesis se realizó durante una estancia de **4 meses** (Septiembre-Diciembre de 2017 y mayo de 2018) en la Universidade do Porto (Portugal), en el Instituto de Investigação e Inovação em Saúde - I3s, Departamento de Biologia Celular e Molecular, bajo la supervisión del Dr. Nuno dos Santos y de la Dr$^{a}$ Ana do Vale.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

*"Bom mesmo é ir à luta com determinação,*
*abraçar a vida com paixão,*
*perder com classe*
*e vencer com ousadia,*
*porque o mundo pertence a quem se atreve*
*e a vida é muito para ser insignificante"*

(Augusto Branco)

*"There is a driving force more powerful than steam,*
*electricity and nuclear power:*
*the will."*

(Albert Einstein, 1879-1955)

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Aos meus pais **Antônio e Elizabeth,** que dignamente me apresentaram à importância da família e ao caminho da honestidade e persistência.

Sem vocês nenhuma conquista valeria a pena.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

## Acknowledgments

Ao meu director de tese Dr. Carlos Rodríguez Osorio, por ser un exemplo de científico e por todas as leccións, orientacións, oportunidades, paciencia e incentivos. Moitas grazas pola confianza. Síntome privilexiado de ter formado parte do seu grupo de investigación.

Aos meus compañeiros de laboratorio Ana Vences, Xosé Matanza, Alba Vázquez e Lucía Vázquez, vós transformastes os días tristes en alegres e as tarefas difíciles en fáciles. A amizade, aprendizaxe e historias graciosas (engrasadas) que tiven con vós xamáis as esquencerei. Que saibades que sempre teredes un irmán brasileiro cos brazos abertos para o que necesitedes. NOSSA! Moitas grazas.

Aos pesquisadores do Instituto de Investigação e Inovação em Saúde (I3s) da Universidade do Porto (Portugal) Dr[a] Ana Vale, Dr. Nuno Santos, Dr[a]. Cassilda, Dr[a]. Ines e Dr. Johnny por terem me recebido tão bem nesta instituição e que por 4 meses me proporcionaram ampliar meus conhecimentos e obter resultados valiosos para a tese.

To the researchers Dr. Hamdi Ogut of Bursa Technical University (Turkey), Dr. Karl Pedersen and Dr. Inger Dalsgaard of Technical University of Denmark (Denmark), Dr. Felipe Cava of Umea University (Sweden) and Dr. Manuel Noia of University of Santiago of Compostela (Spain) for your valuable scientific collaboration.

Con mucho cariño agradezco a mis compañeros del Instituto de Acuicultura, Saqr, Laura, Fátima, Marta, Gabriel, Diego, Miguel, Sol, Fabián, Pamela, Sandra e Placeres por el incentivo y momentos de relajación, vosotros hicieron la diferencia.

Sem dúvida alguma minha tia Conceição (*in memory*) e a minha prima Juliana têm um papel muito importante nesta conquista. Há 18 anos compartilhávamos um sonho e mesmo quando tudo parecia impossível a vitamina de abacate, o pão de cebola e as palavras de carinho e força da eterna tia Con funcionavam como uma porção mágica incrível que me impulsionou a seguir tentando, tentando e tentando. Minha querida prima Jurema, não tenho palavras para agradecer todo seu apoio, amizade e por me fazer acreditar que eu era capaz. Minha gratidão.

Minha família é certamente a maior responsável por esta tese de doutorado existir. Sem o apoio, amor e a educação dos meus pais Antônio e Elizabeth, eu nunca chegaria tão longe. São exemplos de força, bondade, honestidade, dedicação, perseverança e de trabalhadores. Essas experiências me ensinavam o valor do trabalho e que não há dia nem hora para o fazer acontecer. Minha gratidão aos meus irmãos/amigos Vinícius e Nayara pelo companherismo, amor e por cuidarem do nosso pai e nossa minha mãe nestes quatro anos e meio que não estive presente fisicamente e que por vezes tiveram que resolver problemas sem minha ajuda. Ao meu cunhado Éder por estar sempre presente na família, nos momentos alegres e complicados. Por fim, agradeço uma pessoinha que ainda não conheço, meu afilhado Heitor, por renovar minhas energias e esperanças para seguir sonhando. Amo vocês incondicionalmente.

To you all mi moito obrigado!

## PUBLICATIONS

**Publications derived from the present doctoral thesis:**

**Article 1.**
Terceti MS, Ogut H, Osorio CR. (2016). *Photobacterium damselae* subsp. *damselae*, an Emerging Fish Pathogen in the Black Sea: Evidence of a Multiclonal Origin**.** ***Applied and Environmental Microbiology***, 82: 3736-3745. Journal impact factor 2016: **3.807**.

**Article 2.**
Terceti MS, Vences A, Matanza XM, Dalsgaard I, Pedersen K, Osorio CR. (2018) Molecular epidemiology of *Photobacterium damselae* subsp. *damselae* outbreaks in marine rainbow trout farms reveals extensive horizontal gene transfer and high genetic diversity. ***Frontiers in Microbiology***, 19;9:2155. Journal impact factor 2016: **4.076**.

**Article 3.**
Terceti MS, Rivas AJ, Alvarez L, Noia M, Cava F, Osorio CR. (2017). *rstB* Regulates Expression of the *Photobacterium damselae* subsp. *damselae* Major Virulence Factors Damselysin, Phobalysin P and Phobalysin C. ***Frontiers in Microbiology***, 8:1-11. Journal impact factor 2016: **4.076**.

**Papers that will be submitted for publication:**

**Article 4.**
Terceti MS, Vences A, Matanza X.M, Barca A.V, Noia M., Lisboa J, Santos NMS, Vale A, and Osorio CR. Mutation of the RstAB two-component regulatory system impairs virulence, motility, cell morphology, antimicrobial resistance and production of type II secretion system-dependent factors in the fish and human pathogen *Photobacterium damselae* subsp. *damselae*.

**Article 5.**
Terceti MS & Osorio CR. Evidence of Interaction Between VarAS and RstAB Systems in the Regulation of the Hemolytic Activity of *Photobacterium damselae* subsp. *damselae.*

**Other publication related whith the present doctoral thesis:**

**Article 6.**
Osorio CR, Vences A, Matanza XM, Terceti MS. (2018). *Photobacterium damselae* subsp. *damselae*, a generalist pathogen with unique virulence factors and high genetic diversity. ***Jounal of Bacteriology***, 1: 1-12. Journal impact factor 2016: **3.143**.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

## ABBREVIATIONS & ACRONYMS

**°C:** Celsius Degree

**CDS:** Predicted Coding Sequences

**ColP**: *Pdd* Collagenase

**Dly:** Damselysin

**OD:** Optical Density

**Kan:** Kanamycin

**Kb:** Kilobase

**KDa:** KiloDaltons

**LB:** Luria Bertani medium

**ORF:** Open Reading Frame

**bp:** base pairs

**nm:** nanometre

**PCR:** Polimerase chain reaction

***Pdd:*** *Photobacterium damselae* subsp. *damselae*

**PhlyC:** Phobalysin C

**PhlyP:** Phobalysin P

**PlpV:** *Pdd* Phospholipase

**T2SS:** Type II Secretion System

**TCBS:** Thiosulfate Citrate Bile Sucrose medium

**TCS:**.Two-Component System

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

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

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

## SUMMARY

*Photobacterium damselae* subsp. *damselae* is considered an emerging pathogen of importance in aquaculture. Outbreaks of diseases caused by this pathogen in marine fish cultures have shown a marked increase in their geographical distribution in recent years, appearing in countries and fish species where it has never been reported before. In humans, *P. damselae* subsp. *damselae* can cause an extreme variant of severe necrotizing fasciitis, resulting fatal in some cases.

Its pathogenicity is attributed to the production of up to four different toxins and two major categories of strains can be distinguished. On the one hand, strains harboring the virulence plasmid pPHDD1 produce two toxins encoded within this plasmid, Damselysin (Dly) and Phobalysin P (PhlyP), in addition to the toxins encoded within chromosome I, Phobalysin C (PhlyC) and phospholipase PlpV. On the other hand, strains lacking pPHDD1 produce only PhlyC and PlpV.

The first part of this thesis is devoted to the study of the genetic diversity of this fish pathogen to better understand its epidemiology and also to better elucidate if the outbreaks caused by this pathogen are consequence of infection and proliferation of a single adapted clone or of several clones. For this, it was essential to have collections of strains of *P. damselae* subsp. *damselae* isolated from disease outbreaks in the same geographical region. In this sense, we demonstrate using phylogenetic data, together with analyses of phenotypic and genotypic diversity, that strains of *P. damselae* subsp. *damselae* isolated from sea bass farms in the Black Sea (Turkey) and from marine rainbow trout farms in Denmark, clearly originated not from the clonal spread of a single strain, but probably from the simultaneous occurrence of several clones.

Regarding the main virulence factors of *P. damselae* subsp. *damselae*, as no previous work has addressed the study of how the expression of hemolysins Dly, PhlyP and PhlyC is regulated, we proposed as the second branch of investigation of this thesis to begin

this research topic, searching for regulatory genes that may be ubiquitous in this bacterium.

The two-component system is a dominant form of bacterial genetic control that responds to changes in its environment. Using transposon insertion mutagenesis we identified a mutant with a disrupted *rstB* gene that showed impaired hemolytic activity. Here, we demonstrate that the two-component RstAB system positively regulates the expression of the cytotoxins Dly, PhlyP and PhlyC in *P. damselae* subsp. *damselae*. We analysed the effect of *rstA* and *rstB* mutations in cell fitness and in diverse virulence-related features. Mutants in the *rstA* and *rstB* genes were impaired in hemolysis and Dly-dependent phospholipase activity, and expression of dly, $hlyA_{pl}$ and $hlyA_{ch}$ genes was greatly impaired in a *rstB* mutant as demonstrated by transcriptional fusions to a *lacZ* reporter gene. However, mutations of the *rstAB* genes did not cause a detectable effect on PlpV-dependent phospholipase and ColP-dependent gelatinase activities. We did not find deffects in the growth of *rstA* and *rstB* mutants under varying conditions of temperature and concentration of NaCl, with respect to the parental strain. However, *rstA* and *rstB* mutants grown at 0.5% NaCl exhibited reduced motility, increased cell size, and impaired ability to separate daughter cells after cell division. Mutation of either gene also caused an increased sensitivity to benzylpenicillin.

Notably, the *rstA* and *rstB* mutants showed impaired secretion of various proteins dependent on the type II secretion system, which included the major cytotoxins Dly, PhlyP and PhlyC, as well as four hitherto unreported proteins secreted in this bacterial species and which may constitute new virulence factors. Both *rstA* and *rstB* mutants were severely impaired on virulence in a sea bass model.

This study establishes the role of the two-component RstAB system as the main regulator of virulence and several cellular functions in *P. damselae* subsp. *damselae*. These regulators might constitute useful targets to control infections caused by this pathogen.

## RESUMO

*Photobacterium damselae* subsp. *damselae* é considerada un patóxeno emerxente de importancia na acuicultura. Brotes de enfermidade causados por este patóxeno en cultivos de peixes mariños mostraron un aumento acentuado na súa distribución xeográfica nos últimos anos, aparecendo en países e especies de peixes onde nunca foran antes descritos. En humanos, *P. damselae* subsp. *damselae* pode causar unha variante extrema de fascite necrotizante grave, resultando mortal nalgúns casos.

A súa patoxenicidade atribúese á produción de ata catro toxinas diferentes e pódense distinguir dúas grandes categorías de cepas. Por unha banda, as cepas que albergan o plásmido de virulencia pPHDD1 que codifica para dúas toxinas, a damselisina (Dly) e a fobalisina P (PhlyP), ademais das toxinas codificadas no cromosoma I, a fobalisina C (PhlyC) e fosfolipasa PlpV. Por outra banda, as cepas que carecen de pPHDD1 producen só PhlyC e PlpV.

A primeira parte desta tese está dedicada ao estudo da diversidade xenética de cepas de *P. damselae* subsp. *damselae* coa fin de facilitar a comprensión da súa epidemioloxía e elucidar se os brotes causados por este patóxeno son consecuencia da infección e proliferación dun único clon adaptado ou de varios clons. Para iso, foi esencial contar con coleccións de cepas deste patóxeno, illadas a partir de brotes de enfermidades da mesma rexión xeográfica. Neste sentido, utilizando datos filoxenéticos xunto con análises de diversidade fenotípica e xenotípica, demostramos que cepas de *P. damselae* subsp. *damselae* illadas de robaliza en granxas no Mar Negro (Turquía) e de troitas arco da vella mariñas procedentes de granxas de cultivo de Dinamarca, son claramente orixinadas non da propagación clonal dunha única cepa, pero probablemente da aparición simultánea de varios clons.

En canto aos principais factores de virulencia de *P. damselae* subsp. *damselae*, ningún traballo previo abordara o estudo da expresión e regulación das hemolisinas Dly, PhlyP e PhlyC, polo que propuxemos considerar este tema como a segunda rama de investigación desta tese, enfocándonos na busca de xenes reguladores

que poden ser omnipresentes nesta bacteria. O sistema de regulación de dous compoñentes é unha forma dominante de control xenético bacteriano que responde a cambios ambientais. Usando a mutaxénese por inserción de transposón identificamos un mutante co xene *rstB* interrumpido que mostraba actividade hemolítica alterada. Aquí, demostramos que o sistema de dous compoñentes RstAB regula positivamente a expresión das citotoxinas Dly, PhlyP e PhlyC en *P. damselae* subsp. *damselae*. Analizamos o efecto das mutacións *rstA* e *rstB* na fisioloxía celular e en varias características relacionadas coa virulencia. Os mutantes nos xenes *rstA* e *rstB* resultaron estar afectados na hemólise e na actividade fosfolipasa dependente de Dly, e a expresión dos xenes *dly*, $hlyA_{pl}$ e $hlyA_{ch}$ viuse prexudicada de maneira significativa no mutante *rstB*, como se demostrou mediante fusións transcricionais co xene *lacZ*. Con todo, as mutacións dos xenes *rstAB* non causaron un efecto detectábel nas actividades fosfolipase dependente de PlpV e xelatinase dependente de ColP. Non atopamos defectos no crecemento dos mutantes *rstA* e *rstB* baixo condicións de temperatura e concentración de NaCl diferentes, en relación coa cepa salvaxe. Ademais, os mutantes *rstA* e *rstB* crecidos a 0,5% de NaCl exhibiron motilidade reducida, aumento do tamaño das células e dificultade para separarse as células fillas logo da división celular. A mutación do xene *rstA* tamén causou unha maior sensibilidade á bencilpenicilina.

Notablemente, os mutantes *rstA* e *rstB* mostraron prexudicada a secreción de varias proteínas dependentes do sistema de secreción tipo II, que inclúe as principais citotoxinas Dly, PhlyP e PhlyC, así como catro proteínas secretadas, non descritas nesta especie bacteriana ata entón, e que poden constituír novos factores de virulencia. Tanto o mutante *rstA* como o *rstB* amosaron ter gravemente alterada a virulencia, como se demostrou nun modelo de lubina.

Este estudo establece o papel do sistema de dous compoñentes RstAB como o principal regulador da virulencia e de varias funcións celulares en *P. damselae* subsp. *damselae*. Estes reguladores poden ser considerados puntos clave co obxectivo de controlar as infeccións causadas por este patóxeno.

## RESUMEN

*Photobacterium damselae* subsp. *damselae* es un patógeno emergente de importancia en la acuicultura. Los brotes de enfermedades causadas por este patógeno en cultivos de peces marinos han mostrado un aumento acentuado en su distribución geográfica en los últimos años, apareciendo en países y especies de peces, donde nunca se había descrito antes. En humanos, *P. damselae* subsp. *damselae* puede causar una variante extrema de fascitis necrotizante grave, resultando fatal en algunos casos.

Su patogenicidad se atribuye a la producción de hasta cuatro toxinas diferentes, y se distinguen dos categorías principales de cepas. Por una parte, las cepas que albergan el plásmido de virulencia pPHDD1 y, por lo tanto, producen dos toxinas codificadas en dicho plásmido, la damselisina (Dly) y la fobalisina P (PhlyP), además de las toxinas codificadas en el cromosoma I, la fobalisina C (PhlyC) y la fosfolipasa PlpV. Por otro lado, existen cepas carentes de pPHDD1 productoras sólo de PhlyC y PlpV.

La primera parte de esta tesis está dedicada al estudio de la diversidad genética de cepas de *P. damselae* subsp. *damselae* a fin de entender mejor su epidemiología y también para aclarar si los brotes causados por este patógeno son consecuencia de la infección y proliferación de un único clon adaptado o de varios clones. Para ello, fue esencial tener colecciones de este patógeno aisladas de brotes de enfermedades de una misma región geográfica. En este sentido demostramos, utilizando datos filogenéticos junto con análisis de diversidad fenotípica y genotípica, que cepas de *P. damselae* subsp. *damselae* aisladas de lubina de granjas en el Mar Negro (Turquía) y de truchas arco iris de granjas marinas de cría en Dinamarca, están claramente originadas no de la propagación clonal de una sola cepa, sino probablemente de la ocurrencia simultánea de varios clones.

En relación a los principales factores de virulencia de *P. damselae* subsp. *damselae*, dado que ningún trabajo anterior abordó el estudio de cómo está regulada la expresión de las hemolisinas Dly, PhlyP y PhlyC, propusimos iniciar este estudio en la segunda parte de esta tesis, buscando genes reguladores que puedan ser comunes a todas las

cepas de esta bacteria. El sistema de regulación de dos componentes es una forma dominante de control genético bacteriano que responde a cambios ambientales. Utilizando mutagénesis por inserción del transposón miniTn10, identificamos un mutante con el gen *rstB* interrumpido, que mostró una actividad hemolítica muy reducida. En este trabajo demostramos que el sistema de dos componentes RstAB regula positivamente la expresión de las citotoxinas Dly, PhlyP y PhlyC en *P. damselae* subsp. *damselae*. Analizamos el efecto de las mutaciones *rstA* y *rstB* en la fisiología celular y en diversas características relacionadas con la virulencia. Los mutantes en los genes *rstA* y *rstB* están muy afectados en la hemólisis y en la actividad fosfolipasa dependiente de Dly, y la expresión de los genes *dly*, $hlyA_{pl}$ y $hlyA_{ch}$ mostró una notable disminución en un mutante *rstB*, como se demostró por fusiones transcripcionales con el gen *lacZ*. Sin embargo, las mutaciones de los genes *rstAB* no causaron un efecto detectable en las actividades fosfolipasa dependiente de PlpV y gelatinasa dependiente de ColP. No encontramos defectos en el crecimiento de los mutantes *rstA* y *rstB* bajo condiciones variables de temperatura y concentración de NaCl, en relación a la cepa parental. Sin embargo, los mutantes *rstA* y *rstB* crecidos a 0,5% de NaCl mostraron motilidad reducida, aumento del tamaño de las células y dificultades para separar las células hijas después de la división celular. La mutación de *rstA* también causó un aumento de la sensibilidad a la benzilpenicilina.

Los mutantes *rstA* y *rstB* mostraron una fuerte afectación en la secreción de varias proteínas dependientes del sistema de secreción tipo II, que incluyó las principales citotoxinas Dly, PhlyP y PhlyC, así como cuatro proteínas secretadas, no caracterizadas hasta el momento en esta especie bacteriana, y que podrían constituir nuevos factores de virulencia. Ambos mutantes *rstA* y *rstB* mostraron una severa disminución en la virulencia para lubinas.

Este estudio demuestra el papel del sistema de dos componentes RstAB como un principal regulador de la virulencia y de varias funciones celulares en *P. damselae* subsp. *damselae*. Estos reguladores pueden constituir dianas útiles para controlar las infecciones causadas por este patógeno.

## RESUMO

*Photobacterium damselae* subsp. *damselae* é considerada um patógeno emergente de importância na aquicultura. Surtos de doenças causadas por este patógeno em cultivos de peixes marinhos têm mostrado um aumento acentuado em sua distribuição geográfica nos últimos anos, aparecendo em países e espécies de peixes, onde nunca foram relatados antes. Em humanos, *P. damselae* subsp. *damselae* pode causar uma variante extrema de fascite necrotizante grave, resultando fatal em alguns casos.

Sua patogenicidade é atribuída à produção de até quatro toxinas diferentes e duas categorias principais de cepas podem ser distinguidas. Por um lado, as cepas que abrigam o plasmídeo de virulência pPHDD1 e portanto, produzem duas toxinas codificadas nesse plasmídeo, a damselisina (Dly) e a fobalisina P (PhlyP), além das toxinas codificadas no cromossomo I, a fobalisina C (PhlyC) e fosfolipase PlpV. Por outro lado, cepas carentes de pPHDD1 e portanto, produtoras apenas de PhlyC e PlpV.

A primeira parte desta tese é dedicada ao estudo da diversidade genética de cepas de *P. damselae* subsp. *damselae* a fim compreender sua epidemiologia e também melhor elucidar se os surtos causados por este patógeno é conseqüência da infecção e proliferação de um único clone adaptado ou de vários clones. Para isso, foi essencial ter coleções deste patógeno isolados de surtos de doenças de uma mesma zona geográfica. Neste sentido, demonstramos utilizando dados filogenéticos, juntamente com análises de diversidade fenotípica e genotípica, que cepas de *P. damselae* subsp. *damselae* isoladas de robalo cultivados em viveiros no Mar Negro (Turquia) e de trutas arco-íris marinhas de viveiros na Dinamarca, são claramente originadas não da propagação clonal de uma única cepa, mas da ocorrência simultânea de vários clones.

Em relação aos principais fatores de virulência de *P. damselae* subsp. *damselae*, como nenhum trabalho anterior abordou o estudo de como a expressão das hemolisinas Dly, PhlyP e PhlyC são reguladas, propusemos iniciar este tópico como o segundo ramo de investigação desta tese, buscando genes reguladores que possam ser onipresentes

em esta bacteria marinha. O sistema de regulação de dois componentes é uma forma dominante de controle genético bacteriano que responde a mudanças ambientais. Utilizando a mutagênese por inserção de transposon identificamos um mutante com o gene *rstB* rompido que mostrou atividade hemolítica comprometida. Aqui, demonstramos que o sistema de dois componentes RstAB regula positivamente a expressão das citotoxinas Dly, PhlyP e PhlyC em *P. damselae* subsp. *damselae*. Analisamos o efeito das mutações *rstA* e *rstB* na fisiologia celular e em diversas características relacionadas à virulência. Os mutantes nos genes *rstA* e *rstB* foram prejudicados na hemólise e na atividade fosfolipase dependente de Dly, e a expressão dos genes *dly*, $hlyA_{pl}$ e $hlyA_{ch}$ foi grandemente prejudicada em um mutante *rstB*, como demonstrado por fusões transcricionais com o gene repórter *lacZ*. No entanto, as mutações dos genes *rstAB* não causaram um efeito detectável nas atividades fosfolipase dependente de PlpV e gelatinase dependente de ColP. Não foi encontrado alterações no crescimento dos mutantes *rstA* e *rstB* sob variadas condições de temperatura e concentração de NaCl, em relação à estirpe parental. No entanto, os mutantes *rstA* e *rstB* cultivados a 0.5% de NaCl exibiram motilidade reduzida, aumento do tamanho das células e capacidade prejudicada para separar células filhas após a divisão celular. A mutação de um dos genes também permitiu aumentar a sensibilidade à benzilpenicilina.

Notavelmente, os mutantes *rstA* e *rstB* mostraram a secreção prejudicada de várias proteínas dependentes do sistema de secreção tipo II, que incluiu as principais citotoxinas Dly, PhlyP e PhlyC, bem como quatro proteínas secretadas até então não relatadas nesta espécie bacteriana e que podem constituir novos fatores de virulência. Ambos os mutantes *rstA* e *rstB* foram severamente prejudicados na virulência em um modelo de robalo.

Este estudo estabelece o papel do sistema de dois componentes RstAB como o principal regulador da virulência e de várias funções celulares em *P. damselae* subsp. *damselae*. Esses reguladores podem constituir alvos úteis para controlar infecções causadas por esse patógeno.

# INTRODUCTION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

# 1. INTRODUCTION

## 1.1. THE IMPORTANCE OF AQUACULTURE

Aquaculture has a history of 4000 years, however, it has been in the last 50 years that it has become a socioeconomic activity of world importance. According to the United Nations Food and Agriculture Organization (FAO), aquaculture is the production in water of animals and plants both in coastal and inland areas using specialized techniques to make production income more efficient. One of the characteristics that differentiates aquaculture from fishing is that throughout all or at least part of its life cycle the species produced are owned by some person (Apromar, 2018).

Aquaculture is a great prospective practice as the resources required to produce one kilo Gram of food for consumption are smaller in water than on land. In addition, it has in its favor the fact that 70% of the surface of the planet is water (Apromar, 2018). Without a doubt, the biggest challenge facing humanity in the coming decades, the part of obtaining energy, will be to feed the 9,600 million people who will inhabit the planet Earth in the year 2050 (Apromar, 2018).

Never before in mankind has consumed the amount of aquatic products like today. Fish are extraordinarily nutritious as a source of vitamins (D, A and B), mineral micronutrients (calcium, phosphorus, iodine, zinc, iron and selenium), proteins with bioavailability of 5 to 15% higher than proteins derived from vegetables as well as containing amino acids essential for human health, lipids composed of

long chain polyunsaturated fatty acids that offer benefits for adult health and for child development (Apromar, 2018).

Global aquaculture production in 2016 was 110.2 million tons (worth € 194,778 mill.), 4.5% more than in the previous year, and surpassing fishing production by 18.2 million tons (Apromar, 2018).

The top 10 producing countries in world aquaculture in thousands of tonnes were: China (63,721), Indonesia (16,616), India (5,703); Vietnam (3,334), Bangladesh (2,203), Philippines (2,200), Republic of Korea (1,859), Egypt (1,370), Norway (1.326) and Japan (1,067) (FAO, 2016, Apromar, 2018).

The aquaculture production of the European Union (EU) in 2016 was 1,292 thousands of tonnes (€ 3,729 mill.).In the UE, the main aquaculture products are fish and molluscs. The aquaculture of crustaceans, algae or other invertebrates is very small. The main species produced in the UE are mussels, with 476,388t. in 2016, followed by rainbow trout with 185,400 t. and atlantic salmon with 181,030t.

Spain is the Member State of the EU with the largest aquaculture production, with 283,831t. in 2016, followed by the UK with 194,492t. (15.0%) and France with 166,640t. (12,9%) (Apromar, 2018). However, when considering the value of production, the UK is the main producer Member State with € 905.3 million (24.3% of the total value), followed by France with € 550.9 million (14,8%) and Greece with €465.0 million (12,5%) (Apromar, 2018). Spain ranks fourth with 449.4 million euros (12,1%), followed by Italy. The main production of aquatic products in Spain in 2016 (aquaculture and fisheries) was reduced by 5.3% compared to 2015 (Apromar, 2018).

In order to address the major challenges facing aquaculture, research and innovation initiatives should be encouraged and targeted to optimize their efficiency and productivity, both on small and large scale systems. These investigations should improve the optimization of feed and raw materials, improve farm management, as well as the domestication of new species and provide improvement and maintenance of the health of the animals reared.

## 1.2. PATHOGENS IN AQUACULTURE

Fish are susceptible to diseases that are more incidental in aquaculture than in the natural environment due to several factors such as inadequate feeding, stress caused by exposure to an extreme environmental factor, high population densities, water temperature, lack of dissolved oxygen, and the attack of pathogenic organisms (FAO, 2016).

Every year, Western Europe loses $ 60 million in aquaculture due to viral haemorrhagic septicemia (Alday, 2010). In 1993 China lost more than $ 250 million due to the pathogen white spot virus, which was also responsible in 1997 in Thailand for the economic loss of 600 million dollars (Alday, 2010).

The bacterium *Photobacterium damselae* subsp. *damselae* is one of the causal agents of economic losses in the world marine aquaculture industry because it is responsible for one type of vibriosis (Fouz *et al*., 1992; Labella *et al*., 2010a). This marine pathogen was isolated from several important fish species in Spanish aquaculture such as turbot in Galicia (*Scophthalmus maximus*; Fouz *et al.*, 1991; Fouz *et al.*, 1992),sea bass on the Mediterranean coast near Valencia (*Dicentrarchus labrax*; Botella *et al*., 2002; Labella *et al.*, 2010a), the seabream in the Mediterranean (*Sparus aurata*, Vera *et al.*, 1991; Botella*et al*, 2002; Pujalte *et al*, 2003; Labella *et al.*, 2010a) and several species recently introduced in farms in southern Spain as snapper (*Pagrus Auriga*; Labella *et al*., 2006; García-Rosado*et al*., 2007), common dentex (*Dentex dentex*; Company *et al*., 1999) and sea bream in the mediterranean (*Diplodus sargus*; García-Rosado *et al*., 2007).

*P. damselae* subsp. *damselae* has also been isolated from fish farmed in various parts of the world, such as rainbow trout in Denmark (*Oncorhynchus mykiss*; Pedersen *et al.*, 1997; Pedersen *et al*., 2009), eels in Australia (*Anguilla reinhardtii*, Ketterer and Eaves, 1992),barramundi in Thailand and Tahiti (*Lates calcarifer*, Renault *et al*., 1994; Kanchanopas-Barnette *et al.*, 2009), a kind of sole (*Cynoglossus semilaevis*; Zhang *et al.*, 2011) and White pompano (*Trachinotus ovatus*; Zhao *et al.*, 2009) in China. It was also isolated

from sea bass and sea bream cultures of the Egyptian shores (Abdel-Aziz *et al*., 2013) and in Tunizia (Khouadja *et al*., 2014) as well as from sea bass from the Turkish Black Sea (Uzun and Ogut, 2015; Hassanzadeh *et al*., 2015) and more recently from cobia grown in India (Sharma *et al*., 2017).

## 1.3. *PHOTOBACTERIUM DAMSELAE* SUBSP. *DAMSELAE*

### 1.3.1. Taxonomy

The marine bacterium *Photobacterium damselae* is Gram-negative and is included in the family *Vibrionaceae*, belonging to the phylum Proteobacteria (Gauthier *et al*., 1995). It is divided into two subspecies, *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* and in the past these subspecies were denominated *Vibrio damsela* (family *Vibrionaceae*) and *Pasteurella piscicida* (family *Pasteurellaceae*), respectively. The subsp. *damselae* was first isolated in 1971 from ulcerative lesions on the skin of an angel wild fish (*Chromis punctipinnis*), being attributed to the genus *Vibrio* (family *Vibrionaceae*) under the name of *Vibrio damsela* (Love *et al*., 1981). A phylogenetic study comparing the ribosomal 5S gene from 31 strains of the *Vibrionaceae* family proposed the creation of the genus *Listonella*, in which *V. damsela* and *V.anguillarum* would be renamed *Listonella damsela* and *L.anguillarum*, respectively (Mac Donell and Colwell 1984). A comparative study of the phenotypic characteristics of several species of the genus *Photobacterium* has replaced *Listonella damsela* with the genus *Photobacterium*, becoming known as *Photobacterium damselae* (Smith *et al*., 1991). *Photobacterium damsela* and *Pasteurella piscicida* were considered as two subspecies belonging to the same species (*Photobacterium damselae*) due to the high level of similarity observed in DNA-DNA hybridization. In this way, they became known thereafter as *Photobacterium damselae* subsp. *damselae* and *Photobacterium damselae* subsp. *piscicida*, respectively (Gauthier *et al*., 1995).

### 1.3.2. Morphological and Biochemical Characteristics

*P. damselae* subsp.*damselae* is motile due to its polar flagellum (Fig. I.1) and exhibits a bacillary form (Fig. I.2). Biochemical characteristics of this pathogen include: oxidase positive, facultative anaerobic, sensitive to the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropyl phosphate depteridine), grows in the differential medium for vibrios TCBS (Thiosulfate Citrate Bile Sucrose), and is positive for the methyl red and Voges-Proskauer tests. In addition, it is positive for the decarboxylation of arginine and negative for the decarboxylation of lysine and ornithine. It is able to ferment maltose, mannose and D-glucose, being associated with the latter the production of gas. It shows strict NaCl requirement, reaching tolerance of up to 6, and even 8% (Fouz *et al.*, 1992; Abdel-Aziz *et al.*, 2013; Wu *et al.*, 2006). Strains have a relatively wide range of growth temperature, ranging from 12°C to 37°C. However, some studies reported that there are strains of *P. damselae* subsp. *damselae* that can grow at 4 and 40°C (Botella *et al*., 2002).

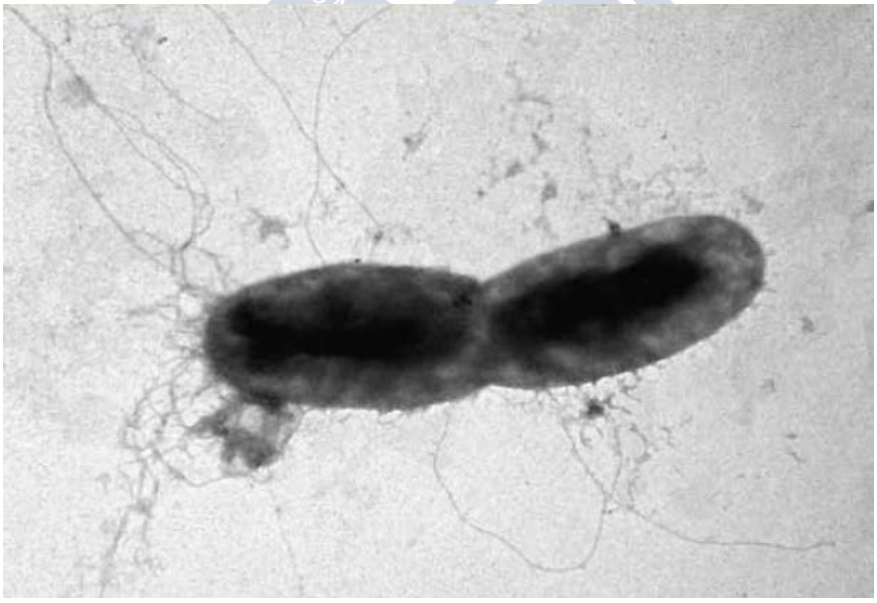


**Fig.I.1**: Transmission electron microscopy of *P.damselae* subsp. *damselae*.

This marine bacterium has been shown to have high serological diversity, being reported several different serogroups as well as different membrane protein patterns (Smith *et al*., 1991; Fouz *et al*., 1992; Fouz *et al*., 1997).

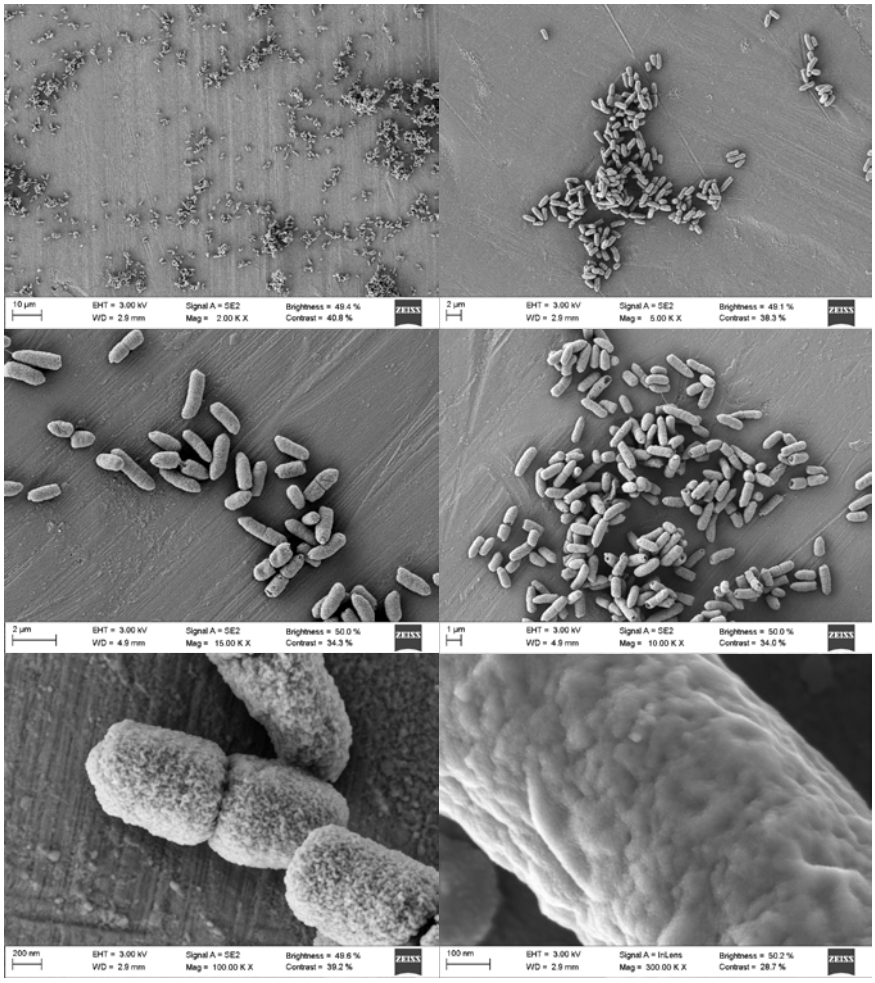



**Fig.I.2:** Scanning electron microscopy of *P. damselae* subsp. *damselae.*

*P. damselae* subsp. *damselae* can synthesize various degradative enzymes such as gelatinase (Labella *et al*., 2010a; Zhang *et al*., 2011), lipase (Pedersen *et al*., 1997) and amylase (Labella *et al.*, 2010a), urease (Thyssen *et al.*, 1998; Botella *et al.*, 2002), phospholipase (Vences *et al*., 2017), acetylcholinesterase (Pérez *et al*., 1998), caseinase (Khouadja *et al*., 2014) and hemolysins (Osorio *et al*., 2000b; Rivas *et al*., 2011; Rivas *et al.*, 2013b; Rivas *et al*., 2014; Rivas *et al*., 2015a). It is important to note that the values of these activities are not the same in all the isolates.

The main phenotypic differences between *P. damselae* subsp. *damselae* and the *piscicida* subspecies are flagellar motility, urease activity, hemolysis of lamb erythrocytes, and the ability to grow at 37°C, all of which are considered positive only in the *damselae* subspecies (Fig. I.3). In addition, the *piscicida* subspecies, has as host only fish, while the subspecies *damselae* can infect not only fish, but also mollusks, crustaceans, reptiles, marine mammals and even humans.

| | Motility | Nitrate reductase | Bipolar stain | Gas from glucose | Urease | Lipase | Amylase | Production of acid from maltose | Hemolysis of lamb erythrocytes | Hemolysis of trout erythrocytes | Serological diversity | Growth in TCBS | Growth in 5% NaCl | Growth at 37ºC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. damselae* subsp. *damselae* | + | + | - | + | + | + | + | + | + | + | + | + | + | + |
| *P. damselae* subsp. *piscicida* | - | - | + | - | - | - | - | - | - | + | - | - | - | - |

**Fig.I.3:** Main differential phenotypic characters between *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida.*

### 1.3.3. Virulence

Pathogenicity is usually a multifactorial feature that involves a complex interaction between the host and the pathogen and is defined as the ability of a microbe to cause damage to a host (Casadevall and Pirofski, 2001). The host-pathogen interaction depends on several host factors such as age, sex and immune system status (Bouzid *et al*., 2013), as well as genotypic and phenotypic characteristics of the pathogen (Méthot and Alizon 2014).

*P. damselae* subsp. *damselae* can cause infection in a healthy host and therefore is considered a primary pathogen. This species has been isolated from several environments such as estuaries, sea water, sediments and healthy aquatic animals (Buck, 1990; Ghinsberg *et al*., 1995) and can survive in sea water and sediments for a long time, maintaining infectivity potential (Fouz *et al*., 2000). Infectious outbreaks caused by this pathogen generally match with periods of higher sea-water temperature, and with less resistance of the host's immune system (Alvarez *et al*., 2006; Austin, 2010).

It is suggested that cutaneous lesions precede the infections of this bacterium, being skin a poposed route of entry in the host (Fouz *et al*., 2000). By experimental studies of infection, it is known that virulent strains by the intraperitoneal route, are also infective through water (Fouz *et al*., 2000). The most notable external signs caused in fish are: presence of hemorrhages around the mouth, anus, eyes and at the base of the fins, abdominal distension, and in some cases presence of cutaneous ulcers (Fig. I.4). Usual internal signs are accumulation of ascitic fluid in the peritoneal cavity and, in some cases, a liver with a pale appearance and abundant hemorrhagic petechiae (Fig. I.4) (Fouz *et al*., 1991; Fouz *et al*., 1992).

*P. damselae* subsp. *damselae* poses the ability to affect species of homeotherms, such as whales (*Balaenopteraedeni*; Buck *et al.*, 1991), dolphins (*Tursiops truncatus*, *Delphinus delphis* and *Stenella coeruleoalba*; Fujioka *et al.*, 1988; Casalone *et al.*,2014) and humans (Morris *et al.*, 1982). The majority of infections by this pathogen in humans, have their primary origin in wounds exposed to brackish seawater, caused by handling fish or fishing tools (Table I.1). There are also cases of infection after the intake of raw seafood (Kim *et al*., 2009), by exposure of the urinary tract to seawater (Alvarez *et al.*, 2006), or by injuries caused by the keel of a boat (Hundenborn *et al*., 2013). In humans, this bacterium can cause an extreme variant of severe necrotizing fasciitis, being fatal in some cases when administration of antibiotics is a treatment incapable of controlling the progression of the infection. Although cases have been detected in completely healthy patients, a large part of the clinical symptoms

caused by this pathogen occur in patients with some type of underlying disease.

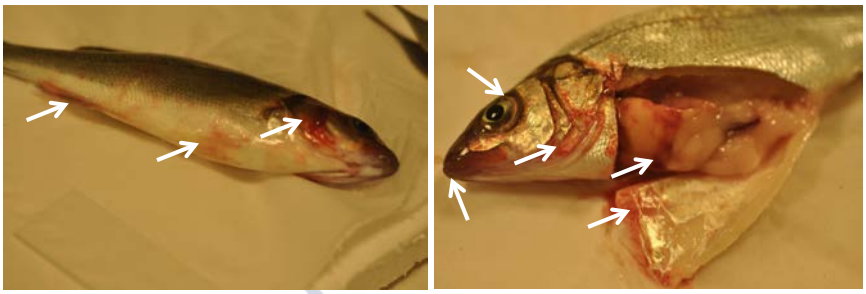


**Fig.I.4:** External signs caused in sea bass by infection of *P. damselae* subsp. *damselae.* Bleeding is reported around the mouth, eye, operculum, fins, liver and internal musculature.

## 1.4. VIRULENCE FACTORS OF *P. DAMSELAE* SUBSP. *DAMSELAE*

Casadevall and Pirofski, 2001, defines virulence as the degree or measure of pathogenicity that microorganisms have and is determined, in part, by the possession of different virulence factors that are genetic, biochemical or structural, that allow an organism to produce disease in a host. Virulence factors are involved in the adhesion, colonization, invasion and evasion of the host immune system. Virulence can be considered a quantitative characteristic, due to the expression to a greater or lesser extent of the different virulence factors.Virulence factors can be as diverse as exotoxins (cytotoxins, hemolysins, proteases, lipases), components of the capsule and cell surface, flagellum, iron uptake systems, factors of adherence, colonization and invasion, among others (Casadevall and Pirofski, 2001). Bacterial virulence factors may be encoded in the chromosome, in bacteriophage DNA, in plasmids or in pathogenicity islands (Peterson, 1996). The virulence factors described to date in *P. damselae* subsp. *damselae* include: iron acquisition systems, phospholipases, collagenase and, of major importance, cytotoxins with hemolytic activity.

**Table I.1:** Clinical cases of infections caused by *P. damselae* subsp. *damselae.* (SD): no data available.

| Origin | Patient | Infection Mode | Medical History | Result | Reference |
|---|---|---|---|---|---|
| SD | 6 cases | Wounds exposed to seawater | Healthy individuals | SD | Morris *et al.*, (1982) |
| USA | 61 year old man | Wound handling catfish | Pancreatitis and diabetes | Death | Clarridge and ZighelboimDaum (1985) |
| USA | 38 year old man | Wound manipulating fish | Healthy man | Necrotizing infection | Coffey *et al.*, (1986) |
| Australia | SD | Infectious wound | SD | SD | Dryden *et al.*, (1989) |
| Asian | 62 year old man | Wound manipulating *Siganus vulpinus* | Healthy man | Death (fasciitis necrotizing) | Yuen *et al.*, (1993) |
| USA | 70 year old man | Wound manipulating fish | Healthy man | Death (septicemia) | Pérez-Tirse *et al.*, (1993) |
| SD | Man of 63 years | SD | Diabetes and cirrhosis | SD | Shin *et al.*, (1996) |
| USA | 64 year old man | Wound by hook | Heart disease | Death (multiple failure) | Fraser *et al.*, (1997) |
| USA | 43 year old man | Wounded manipulating stripe | SD | Necrotizing fasciitis | Barber and Swygert (2000) |
| UK | 69 year old man | SD | SD | Death (multiple failure) | Goodell *et al.*, (2004) |
| Japan | 58 year old man | Fishing | Diabetes | Death (fasciitis necrotizing) | Asato and Kanaya (2004) |
| Japan | 76 year old man | Fishing | Healthy man | Death (multiple failure) | Yamane *et al.*, (2004) |
| Jamaica | child | SD | Sickle-cell anemia | SD | Knight-Madden *et al.*, (2005) |
| USA | 22 year old woman | SD | Pregnant | Urinary infection | Alvarez *et al.*, (2006) |
| Japan | 68 year old man | Harpoon wound | Diabetes | Necrotizing fasciitis | Nakamura *et al.*, (2008) |
| USA | 14 year old man | Wound while surfing | SD | Septicemia | Aigbivbalu and Maraqa (2009) |
| USA | 46 year old man | Oral ingestion | Cirrhosis | Septicemia and later death due to liver failure | Kim *et al.*, (2009) |
| Australia | 64 year old man | Injury in the tibia by a keel | Healthy man | Infectious wound | Hundenborn *et al.*, (2013) |

### 1.4.1. Iron Acquisition Systems

Iron is an essential nutrient for most bacteria in different cellular processes (Saha *et al*., 2013). In marine habitats the bioavailability of iron is low because it is in its oxidized iron form insoluble. In vertebrates, the reduction of iron availability is one of the mechanisms of non-specific immune defense against microbial pathogens (Cassat and Skaar, 2013; Ganz and Nemeth, 2015).

The most widespread strategies for the acquisition of iron among pathogenic bacteria are the uptake of the heme group and the production of siderophores (Saha *et al*., 2013; Ellermann and Arthur, 2017). Siderophores are low molecular weight, iron-chelating compounds, which can either solubilize the iron or remove it from other chelators and transport it to the cell through the corresponding membrane receptor proteins. Some bacteria not only produce their own siderophores but also express receptors capable of carrying xenosiderophores produced by other organisms (Cornelis and Andrews, 2010). Fouz *et al*., 1994 have shown that all strains of *P. damselae* subsp. *damselae* were able to use hemoglobin and ferric ammonium citrate as sole sources of iron and that all isolates of this bacterium produced some type of siderophore when grown under iron deficient conditions (Fouz *et al*., 1994, Fouz *et al*., 1997). It was demonstrated in a human isolate of *P. damselae* subsp. *damselae* that a cluster of genes encode a heme group capture system, making it able to use hemoglobin and hemin as sources of iron (Rio *et al*., 2005). Virulence tests performed on animals that had previously been injected with an iron source had already shown an increase in the susceptibility of fish and mice to infection by this pathogen, indicating that iron is important for this marine pathogen to establish an infection in an organism (Fouz *et al*., 1994).

Under iron limiting conditions some strains of *P. damselae* subsp. *damselae* are able to synthesize vibrioferrin as siderophore, whose synthesis and transport genes are thought to be located within a genomic island acquired by horizontal transfer since they occur only in a fraction of strains (Puentes *et al.*, 2017; Balado *et al.*, 2017). Most of the strains of this microorganism release endogenous citrate to the

extracellular medium in response to iron limitation, suggesting that besides being part of the molecule of some siderophores, the citrate moiety can itself be used for the absorption of iron by *P. damselae* subsp. *damselae* (Balado *et al*., 2017).

### 1.4.2. Phospholipases and Collagenases

The growth and proliferation of pathogenic bacteria in a host are the result of a multifactorial process that depends to a large extent on the action of proteolytic and lipolytic enzymes secreted by the microorganism (Travis *et al*., 1995; Roberts *et al*., 2013). In general, these extracellular enzymes, including those of the family *Vibrionaceae*, have been considered as important virulence factors, since they can directly interfere with the functions of the host cells, or be involved in the processing of other bacterial virulence factors (Matsushita and Okabe, 2001; Frees *et al*., 2013; Zhang *et al*., 2015; Stehr *et al*., 2003; Bender and Flieger, 2010; Hausmann and Jaeger, 2010). They play a key role in the colonization of the host, since they help the propagation of the pathogen in its tissues by degrading extracellular components, contribute to the evasion of the host immune system, to the availability of amino acids for survival and bacterial growth and facilitate the diffusion of toxins (Bond and Wart, 1984; Salyers and Witt, 1994; Harrington, 1996; Matsushita and Okabe, 2001; Eckhard *et al*., 2009; Duarte *et al*., 2014). Bacterial phospholipases are responsible for hydrolyzing phospholipids and participate in the acquisition of phosphate from carbon sources, and in some cases, act as virulence factors (Macfarlene *et al*., 1941; Kreger *et al*., 1987). Regarding the classification of the phospholipases, it takes into account the site of attack to the phospholipid, that is, the ester bond that they split. Grade letters A, B, C and D are used to differentiate phospholipase types, and indicate the specific binding directed to the phospholipid molecule (Mollby, 1978).

A cytolysin named damselysin (Dly), from *P. damselae* subsp. *damselae* is a hemolytically active toxin and has phospholipase D activity against sphingomyelin, phosphatidylcholine and phosphatidylethanolamine (Kreger *et al*., 1987; Daniel *et al*., 1988).

Vences and collaborators in 2017, combining analysis of genomes and mutants by gene deletion in the LD07 strain of *P. damselae* subsp. *damselae* lacking virulence plasmid pPHDD1, identified a chromosomal locus encoding a phospholipase (PlpV). PlpV was omnipresent in the *damselae* subspecies and exerted hemolytic activity against trout erythrocytes, which was improved in the presence of lecithin. *P. damselae* subsp. *damselae* mutants for *epsL* and *pilD* genes of the type II secretion system (T2SS) exhibited alterations in phospholipase activities (Vences *et al*., 2017). Sea bass virulence experiments and cell culture assays demonstrated important contributions of phospholipase PlpV to virulence and toxicity (Vences *et al*., 2017).

Collagen plays a central role as a primary extracellular component in the ultra structure of several animal tissues, contributing to the shape of organs and the integrity of vertebrate tissues. Collagen serves as an important source of nutrients and energy in the marine environment and its degradation by various microorganisms contributes to the global nitrogen and carbon cycle (Duarte *et al*., 2014). Enzymes capable of hydrolyzing specifically native collagen (tropocollagen) under physiological conditions of pH and temperature, as well as soluble denatured collagen (Harrington, 1996) are called collagenases. According to the discrimination of the active sites, the MEROPS database (Rawling *et al*., 2016) classified the collagenolytic enzymes into: metalloproteases, cysteine proteases, serine proteases and some proteases of the U32 family. Recently it was demonstrated in some strains of *P. damselae* subsp. *damselae* the production of a collagenase dubbed ColP (metalloprotease), which contributes to virulence (Vences *et al*., 2017). These authors revealed by genome analysis that ColP was restricted to a fraction of the isolates of *P. damselae* subsp. *damselae* and that is, as well as PlpV, dependent on the T2SS for its secretion.

### 1.4.3. Cytotoxins with Hemolytic Activity

*P. damselae* subsp. *damselae* produces large quantities of a cytolysin that has hemolytic activity against the blood of various animal species, being more active against erythrocytes of rat and mouse, and the amount of this toxin produced was variable between strains and those that presented significantly higher values of the cytolysin had lower lethal doses for mice (Kreger, 1984). It was determined from a purified fraction that this cytolysin has a molecular weight of 69 KDa (Kothary and Kreger, 1985), this fraction being also more active against mouse erythrocytes. They also revealed that less purified fractions were active against the erythrocytes of four animal species, suggesting the presence of other enzymes with hemolytic activity (Kothary and Kreger, 1985). This hypothesis was supported by the results obtained by thin layer of isoelectric focusing, which revealed the presence of three bands, one major and two minor, that subsequently proved to be hemolytically active (Kothary and Kreger, 1985). This cytolysin of *P. damselae* subsp. *damselae* was named damselisin (Dly), a hemolytically active toxin with phospholipase D activity (Kreger *et al*., 1987; Daniel *et al*., 1988). Like other sphingomyelinases, Dly acted synergistically with the delta-toxin of *Staphylococcus aureus* against sheep erythrocytes, increasing the hemolytic effect of the delta-toxin due to the elimination of polar sphingomyelin choline heads (Kreger *et al*., 1987). This was the first evidence that Dly can act synergistically with hemolysins produced by other cells.

Cutter and Kreger (1990) hypothesized that the *dly* gene was part of a prophage, since highly hemolytic *P. damselae* subsp. *damselae* strains lost this gene spontaneously, significantly reducing hemolysis. These authors also demonstrated that the presence of the *dly* gene was revealed in the strains that presented higher values of hemolytic activity.

Fouz *et al.* (1993) reported the great variability of the hemolytic capacity of several strains of this microorganism. Osorio *et al.* (2000a) revealed the existence of hemolytic strains not harboring the *dly* gene and the ability of these strains to trigger a pathology in fish and mice.

This analysis suggested that the hemolytic activity of this pathogen did not reside only in damselysin (Osorio *et al*., 2000a). Negative strains for *dly* gene were toxic to homeotherm and poikilotherm cell lines (Labella *et al*., 2010a). For decades, the genomic context of the *dly* gene remained uninvestigated.

Rivas *et al*., 2011 identified a 150 Kb plasmid, called pPHDD1 that was present in strains isolated from fish and humans. The characterization of pPHDD1 would reveal the prediction of 171 ORFs (Open Reading Frame) and the distinction of five gene modules: a replication module (*rep* genes), a partition module (*par* genes), a conjugation module (*tra* genes), an adhesion modulus (*tad* genes, tight adhesion), and finally, a hemolysis module consisting not only of the *dly* gene, but also of the gene $hlyA_{pl}$ encodinga potential pore-forming hemolysin (Rivas *et al*., 2011). Interestingly, the genes of the Dly and $HlyA_{pl}$ toxins are contiguous in the sequence of pPHDD1, but are transcribed from divergent chains (Rivas *et al*., 2011). In order to uncover the contribution of Dly and $HlyA_{pl}$ to hemolysis, these authors analyzed single *dly*, single $hlyA_{pl}$ and double *dly*-$hlyA_{pl}$ knockout mutants and evaluated their hemolytic phenotype. They found that mutation of the *dly* gene did not completely abolish hemolysis. Interestingly, the mutation of the $hlyA_{pl}$ gene caused only a slight reduction in the radius of the hemolysis halo. Based on these results, they proposed that hemolysis in *P. damselae* subsp. *damselae* is mainly due to the sum of the contributions of Dly and $HlyA_{pl}$. As expected, the $hlyA_{pl}$-*dly* double mutant showed a > 80% reduction in the hemolytic halo. These results suggested that Dly was a major contributor to hemolysis and that there would be another hemolytic factor since hemolysis in the *hlyA*-*dly* double mutant was not eliminated at 100%.

The pore-forming toxin $HlyA_{pl}$ has been renamed as phobalysin P (PhlyP), which means "plasmid-encoded *Photobacterium* lysin" and is a β-toxin with hemolytic activity (Rivas *et al*., 2015a). It has been shown that all isolates of *P. damselae* subsp. *damselae*, regardless of whether they contain pPHDD1, also harbour a gene $hlyA_{ch}$ encoding a novel pore-forming hemolytic toxin (Rivas *et al*., 2013b) in its chromosome I. This gene encodes Phobalysin C (PhlyC), a toxin that

shares 92% identity in the amino acid sequence with PhlyP (Rivas *et al*., 2014). Thus, based on these studies, strains harboring pPHDD1 produce three different hemolysins (Dly, PhlyP and PhlyC), whereas strains that do not carry pPHDD1 synthesize only the hemolysin PhlyC.

To better understand the contribution of Dly, PhlyP and PhlyC to hemolysis, Rivas *et al*., (2013b) generated mutants for these hemolysins with different genetic contexts. Damselysin alone is unable to produce hemolysis in sheep blood but is required to produce synergistic effects with either of the two phobalysins (PhlyP and PhlyC), which demonstrates that at least one of the two pore-forming toxins is required and sufficient to produce detectable hemolysis (Rivas *et al*., 2013b). On the other hand, Dly alone is able to lyse mouse and turbot erythrocytes (Rivas *et al*., 2013b). The two pore-forming toxins, PhlyP and PhlyC, have the ability to lyse the erythrocytes of sheep, mice and turbot by themselves.

In hemolytic assays in sheep erythrocytes performed with extracellular bacterial products, it was disclosed that the hemolytic phenotype produced by the combined action of PhlyP and PhlyC constituted a phenomenon of addition, but not of synergy (Rivas *et al*., 2013b). These authors also demonstrated that the synergy process between Dly and HlyA can be seen by CAMP effect, so that the Dly producing strains generate CAMP reactions with a non-Dly producing strain.

With respect to the pathogenic potential, Rivas *et al*. (2013b) demonstrated that the virulence for mice of pPHDD1 carrier strains resides mainly in Dly and PhlyP. On the contrary, as regards virulence in fish (turbot), the same authors would conclude that the two pore-forming toxins PhlyP and PhlyC alone do not cause death in turbot, but that the presence of Dly (synergistic effect) is necessary or of the other HlyA hemolysin (additive effect) to cause the death.

Thus, it was demonstrated that the phenotype of the large haemolytic halo of pPHDD1-bearing strains would be the result of secretion of the three hemolysins and the synergistic effect between Dly and PhlyP/PhlyC and that the small hemolytic halo of strains lacking pPHDD1 results from the secretion of PhlyC. In addition, the

intermediate hemolytic halos observed in strains containing the plasmid pPHDD1 are possibly the result of point mutations that affect the activity of any of the three hemolysins (Rivas *et al*., 2011; Rivas *et al*., 2013b; Rivas *et al*., 2014).

It is also known that the three hemolysins are secreted by the type II secretion system (T2SS) (Rivas, *et al*., 2015a). Mutation of the *epsL* and/or *pilD* gene in a strain bearing pPHDD1 caused an almost complete abolition of hemolytic activity against sheep erythrocytes, indicating that *epsL* and *pilD* play an important role in plasmid-encoded PhlyP and Dly secretion. This was further demonstrated by analysis of different combinations of mutated genes from hemolysins and by strain complementation assays (Rivas *et al*., 2015a). Analysis of promoter expression has suggested that the impairment of hemolysin secretion in *epsL* and *pilD* mutants may constitute a signal that affects the gene expression of hemolysins and T2SS at the transcriptional level (Rivas *et al*., 2015a). It was also demonstrated that hemolysins promote bacterial adhesion. When analyzing mammalian (HaCaT) cells infected with *P. damselae* subsp. *damselae* by differential interference contrast microscopy, it was revealed that a parental strain of *P. damselae* subsp. *damselae* decorated the surfaces of the HaCaT cells, while comparatively few bacteria from the mutants for hemolysin genes adhered to these cells (Rivas *et al*., 2015b).

Although the molecular basis of virulence in this pathogen has been extensively studied in recent years, regulatory mechanisms by which expression of the PhlyC, PhlyP and Dly hemolysins and PlpV phospholipase are unknown. Pathogens should detect environmental changes such as osmolarity, temperature, nutrients, metal ion concentration, among others and produce virulence factors only when necessary and thus regulate the virulence genes according to their environment. It is expected that *P. damselae* subsp. *damselae*, a pathogen that also has a free lifestyle, has such detection systems to control hemolysin production. Although several regulatory systems are predicted to be encoded in the genome of *P. damselae* subsp. *damselae*, to date none of these systems have been characterized, and

their possible role in the regulation of virulence factors is hitherto unknown.

## 1.5. GENETIC DIVERSITY IN *P. DAMSELAE* SUBSP. *DAMSELAE*

Early studies had pointed at the observation that not all the isolates exhibited the same hemolytic activity (Kreger *et al*., 1984). In addition, other studies demonstrated that *P. damselae* subsp. *damselae* isolates from outbreaks in rainbow trout farms in Denmark exhibited a high diversity of Ribotype profiles and of PFGE (Pulsed-Field-Gel-Electrophoresis) patterns (Pedersen *et al*., 1997; Pedersen *et al*., 2009).

The discovery of pPHDD1 plasmid in 2011, (Rivas *et al*., 2011) and of the chromosome I-encoded PhlyC hemolysin (Rivas *et al*., 2013a), fuelled studies that analyzed the presence of these three hemolysin genes in larger collections of isolates. Such studies demonstrated that only a fraction of the strains harbour pPHDD1 plasmid, whereas PhlyC (encoded by $hlyA_{ch}$ gene) is virtually ubiquitous in the subspecies with the exception of a few negative isolates (Rivas *et al*., 2014). Similarly, it was shown that all the *P. damselae* subsp. *damselae* strains harbour *plpV* gene, but only a fraction of strains harbour *colP* gene (Vences *et al*., 2017).

Before the completion of the present thesis, only a few genomes of *P. damselae* subsp. *damselae* had been sequenced and subjected to comparative genomics analysis. A recent study obtained the draft sequence of the three strains RM-71, LD-07 and A-162, and after a comparative analysis with the genome of the type strain CIP 102761 it was demonstrated that each strain harboured an important number of strain-specific genes, ranging from 262 genes unique to RM-71, to 34 genes unique to strain A-162 (Vences *et al*., 2017). These four strains were isolated from different parts of the world and from different host species.

## 1.6. TWO COMPONENT REGULATORY SYSTEMS

The two-component system (TCS) is a family of signal transduction proteins present in eukaryotes and prokaryotes (Stock *et al*., 2000). Two-component bacterial system are a dominant form of genetic control that responds to changes in their environment. They are related to the response to environmental stress (Freeman *et al*., 2013, Raivio 2014), in pathogenesis (Herrera *et al*., 2014; Kim *et al.* 2015; Mey *et al*., 2015; Goudenège *et al*., 2015), symbiotic relationships (Raghavan and Groisman, 2010; Norsworhy and Visick, 2015) and interactions with eukaryotic hosts (Quon *et al.*, 1996; Dubrac *et al.*, 2008). Recently, TCS have been increasingly used as microbial biosensors, designed for bioremediation and biorefinery (Ravikumar *et al*., 2017).

The classical TCS captures an environmental signal and transfers this information to the intracellular medium through a cascade of phosphorylations that culminates in altered gene expression and/or biochemical activities of target proteins. That is, a sensor protein responds to an environmental signal by modifying the phosphorylated state of a cognate regulatory protein. (Fig. I.5A). Regulatory proteins are generally repressors and / or activators of gene transcription. Thus, the induction of TCS causes a change in the transcription profile of the bacterium. The response regulator protein (histidine kinase), in its phosphorylated condition, has greater affinity for the promoter of a gene (Zwir *et al*., 2012; Gao *et al*., 2015). Some regulators do not have DNA binding domains and exert their effects establishing direct interactions with protein targets (Hengge, 2008) or RNA (Shu and Zlulin, 2002). The sensors use ATP to autophosphorylate a conserved histidine residue that serves as a phosphodonor for its regulatory partner, which is phosphorylated in a conserved aspartic acid residue. The recognition surface that confers specificity between sensor and regulator as well as the biochemical properties of these proteins are widely conserved between systems and species (Laub and Goulin, 2007). In contrast, signals acting on regulated sensors and genes generally differ between TCS (Mascher *et al.*, 2006). When a TCS receives inducer conditions there is an increase in the levels of

phosphorylated regulator. A regulator can be phosphorylated not only from a sensor but also from a low molecular weight phosphodonor such as acetyl phosphate (McCleary and Stock, 1994; Kosono *et al*., 2015; Schilling *et al*., 2015). In addition, a signal can alter the synthesis and/or activity of a protein which in turn can modify the ability of a sensor to act as autokinase or phosphatase (Ishii *et al*., 2013), or a phosphorylated regulator is dephosphorylated by cognate sensor (Kato and Groisman, 2004). These observations show how complex the mechanism of bacterial gene regulation may be. Hybrid TCS constitute a family of proteins that harbor sensing and regulatory domains of a classical TCS fused to a single polypeptide (Xu *et al*., 2003; Xu *et al*., 2004; Raghavan and Groisman, 2010) (Fig. I.5B). Phosphorelay TCS is a version of the two-component system that involves four phosphoryl transfer events (Perego and Brannigan, 2001) (Fig. I.5C).

Normally, the sensor only affects the phosphorylated state of its cognate regulator (Fig. I.5A) (Verhamme *et al*., 2002; Laub and Goulian, 2007). However, certain regulators may be phosphorylated from cognate and non-cognate sensors (Fig. I.5D), allowing the regulator's output to reflect different inputs from cognate and non-cognate sensors (Botella *et al*., 2014). Consequently, different signals acting on different sensors can converge to a given regulator (Fig. I.5D). Beside, a particular sensor may serve as a phosphodonor for multiple regulators (Fig. I.5E), thus expanding the spectrum of genes, proteins, and activities affected by the stimulus in a given sensor (Mika and Hengge, 2005).

Genetic control systems often use feedback mechanisms (Fig. I.6) to adjust responses according to an environmental signal (Mitrophanov and Groisman, 2008). Feedback mechanisms play critical roles in responding to stress conditions (Hsieh and Wanner 2010) and developmental pathways (Biondi *et al*., 2006). In addition, they are essential for pathogens to cause disease (Shin *et al*., 2006) for symbiotic organisms to reside in their eukaryotic hosts (Tu *et al*., 2010) and for the synthesis of antibacterial agents (Sherwood and Bibb, 2013), resistance to antibacterial agents (Chen and Groisman, 2013) and persistence in the presence of antibacterial agents (Balaban,

2011). In general, the proteins of the two-component system control their production by altering the amount of active regulator. In figure I.6 we can observe the extrinsic control of a two-component system that is self-regulating, besides regulating the effector gene and transcriptional control of the regulator. The effector can affect the input access to the sensor (Park and Groisman, 2014; Raghavan *et al*., 2014), by binding to the sensor modifying its enzymatic activities (Raivio *et al.*, 1999; Lippa and Goulian, 2009) or modifying the phosphorylation state of the regulator directly or indirectly (Wang *et al.*, 2014). For certain operons of the TCS, a single promoter is used to provide baseline and self-regulated data (Martin *et al.*, 2010). In some cases, the genes of theTCS can be transcribed in different ways even if they belong to the same operon (Pescaretti *et al*., 2010). This is due to the existence of a separate promoter located within the coding region of the adjacent operon gene (Pescaretti *et al*., 2009).

High levels of sensor and/or regulator may result in cross-talk through two or more TCS because of the competition between cognate and non-cognate proteins, thus altering the output of a system (Yamamoto *et al.*, 2005). It appears that during evolution, cross-protein cross-linking of TCShas been avoided in two ways: by maintaining basal levels of proteinexpression from the two-component low-level system, and by using feedback to increase levels of cognate sensor and regulator proteins only when the stimulus environment is present (Groisman, 2016).

The number of sensor and regulator molecules for a given TCS is not constant under inducing and non-inducing conditions, even when the corresponding genes are part of an operon. In the vast majority of cases examined, the number of regulatory molecules is larger than the number of molecules in the sensor (Schrecke *et al*., 2013). Increasing the phosphorylated regulator results in a corresponding increase in promoter occupancy by the regulator and in raising mRNA levels of target genes (virulence genes for example) (Shin *et al*., 2006).

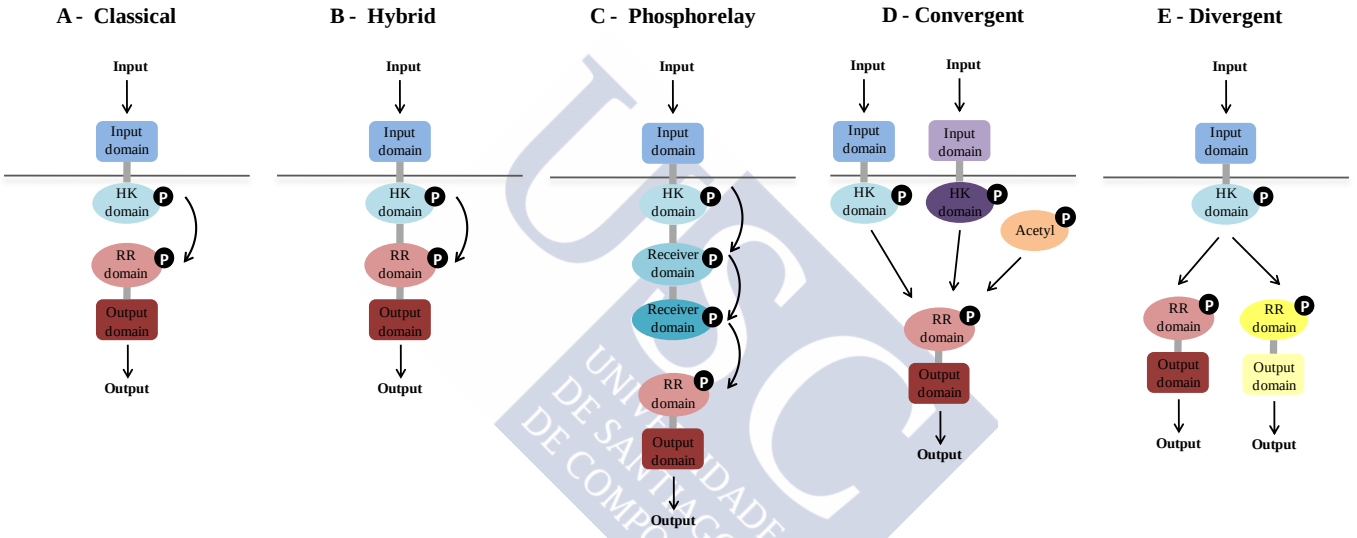



**Fig.I.5:** Architectural classes of two-component signal transduction systems, and of signaling pathways using two-component system proteins. **A**-Classical, **B**-Hybrid, **C**-Phosphorelay, **D**-Convergent, **E**-Divergent. Graph based on information available in (Groisman, 2016).

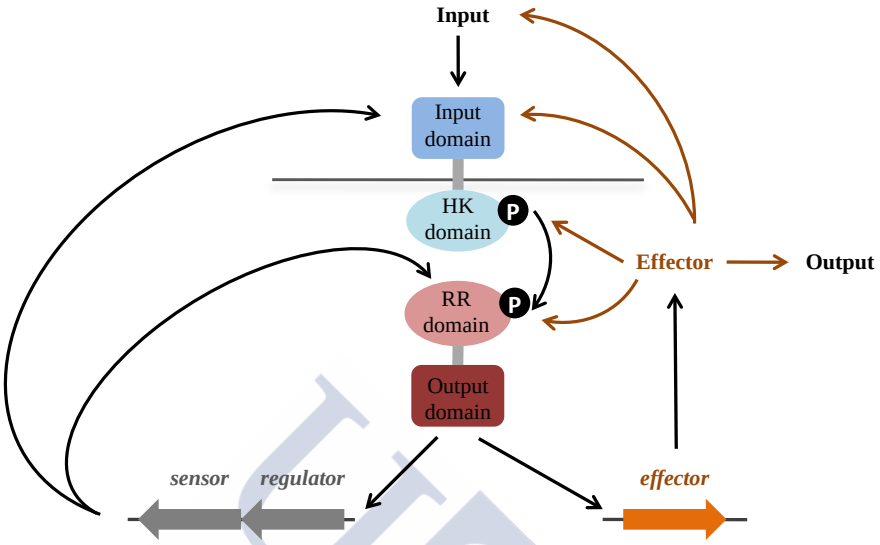



**Fig.I.6:** Feedback control of two-component system. Graph based on information available in (Groisman, 2016).

## 1.7. JUSTIFICATION OF THE UNITY AND COHERENCE OF THIS THESIS

As mentioned above, there is increasing evidence that *P. damselae* subsp. *damselae* is a genetically diverse microorganism. One of the questions that is awaiting for a definite response is whether the outbreaks caused by *P. damselae* subsp. *damselae* in fish farms are caused by specialized clonal lineages or by multiclonal populations. This question is of major importance, since, as described above, not all the strains produce the same virulence factors. It has to be said that several lines of evidence point at a multiclonal origin of the outbreaks. In this sense, previous studies have demonstrated that *P. damselae* subsp. *damselae* isolates from outbreaks in rainbow trout farms in Denmark exhibited a high diversity of Ribotype profiles and of PFGE (Pulsed-Field-Gel-Electrophoresis) patterns (Pedersen *et al*., 1997; Pedersen *et al*., 2009). However, when these studies were conducted, there was not information on the genetic basis of most of the *P. damselae* subsp. *damselae* virulence factors, and therefore such

studies could only provide information about the genetic diversity in a broad sense.

In order to provide an answer to the query of the monoclonal or multiclonal nature of the *P. damselae* subsp. *damselae* outbreaks, it would be necessary to analyze collections of isolates from a same geographical area, and when posible, from a same outbreak, under the light of the recent discoveries on the genetic basis of hemolysis and virulence in this pathogen. For this purpose, in the present thesis we will analyze two important collections of strains of this pathogen, isolated from outbreaks in Turkey (sea bass farms), and in Denmark (marine rainbow trout farms), respectively.

It is notable that from 1971, when it was isolated as a causative agent of human infection, to this day, much progress has been made on the knowledge of the characteristics of the marine bacterium *P. damselae* subsp. *damselae*, and a number of virulence factors have been identified and characterized (Fig. I.7). However, how expression of these virulence factors is regulated, remains almost uninvestigated. Therefore, as one of the main objectives of the present work, we will attempt to elucidate the mechanisms by which the expression of hemolysins, damselysin (Dly), Phobalysin P (PhlyP) and Phobalysin C (PhlyC) of *P. damsela*e subsp. *damselae* is regulated.

Considering the evidence that *P. damselae* subsp. *damselae* is a genetically diverse pathogen, and that different strains contain different virulence gene repertoires, we also expect to identify regulatory mechanisms of virulence that might be common to all the strains, regardless of their content of virulence genes. The completion of this objective would surely provide novel molecular targets for the development of novel treatments for the vibriosis caused by this pathogen in fish.

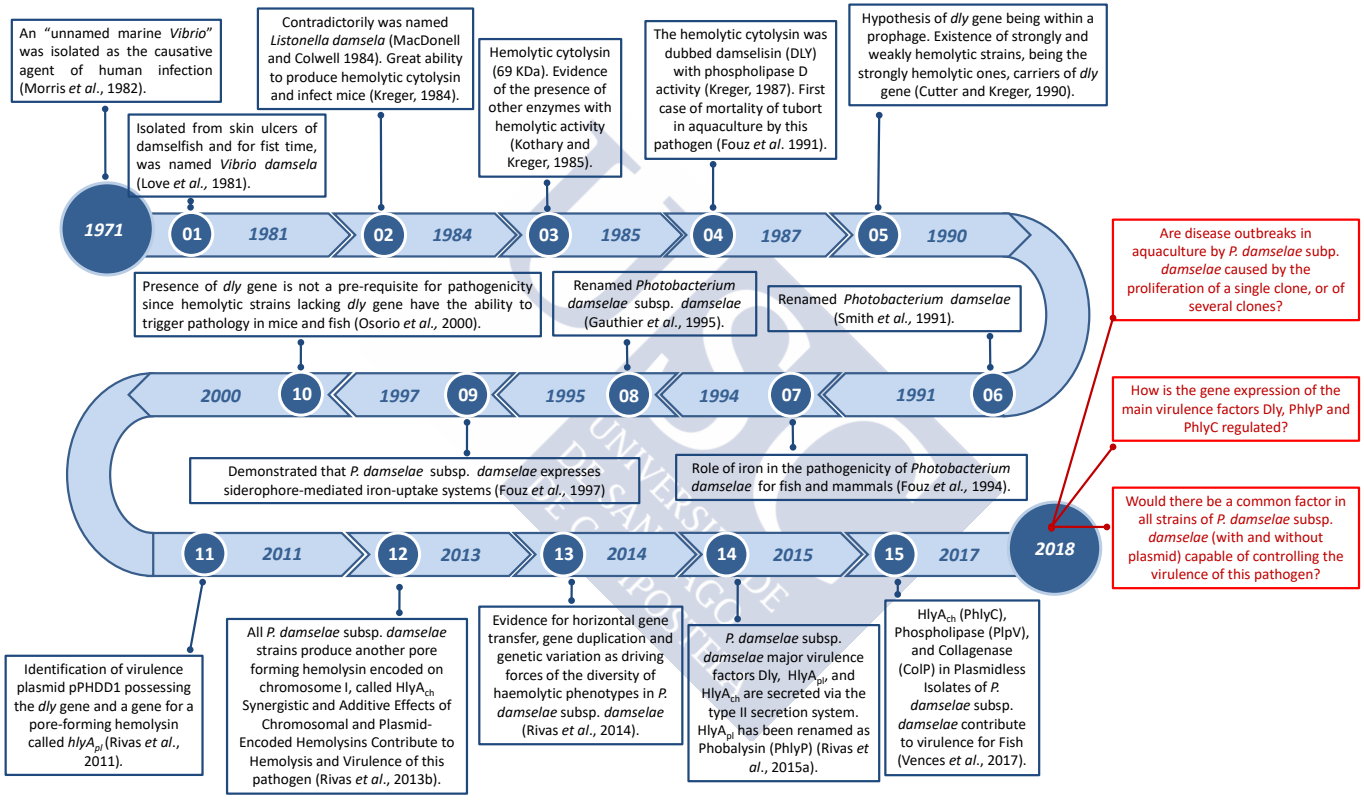



**Fig.I.7:** Some examples of relevant advances in the knowledge of *P. damselae* subsp. *damselae* and future perspectives of this work.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

# OBJECTIVES

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

# 2. OBJECTIVES

The first part of this thesis is devoted to the study of the genetic diversity of the fish and human pathogen *P. damselae* subsp. *damselae* in order to better understand its epidemiology and also whether the outbreaks caused by this pathogen are a consequence of infection and proliferation of a single adapted clone or of several clones. For this, it was essential to have strains of *P. damselae* subsp. *damselae* isolated from outbreaks in a same geographical region. We thus then began studying the collections provided by our collaborator Prof. Dr. Hamdi Ogut of Bursa Technical University (Turkey) and by Prof. Dr. Karl Pedersen and Prof. Dr. Inger Dalsgaard of the Denmark Technical University (DTU), who had collections of *P. damselae* subsp. *damselae* with the requirements we were looking for.

It has been mentioned in the introduction of this work that there are pPHDD1 virulence plasmid-bearing strains which, therefore, produce the hemolysins Dly, PhlyP and PhlyC, and also strains that do not harbor pPHDD1, and then produce only PhlyC. Since no previous works have tackled the study of how expression of these hemolysins is regulated, we here propose to initiate this investigation, looking for regulatory genes that could be ubiquitous in *P. damselae*. subsp. *damselae*. Thus, these regulators could be used in the future as a weak point to control infections caused by both plasmid-containing and by plasmidless strains.

Considering what has been presented so far in the above description, and in the Introduction section of this thesis, we propose the following Objectives:

1. Genetically characterize isolates of *P. damselae* subsp. *damselae* from the same outbreak or from geographically-related outbreaks, in order to understand the epidemiology and clonality of the populations of this pathogen.

2. Identify and characterize genes involved in the regulation of gene expression of Dly, PhlyP and PhlyC hemolysins (and other virulence factors) of *P. damselae* subsp. *damselae*, and verify if such regulatory genes are ubiquitous in this subspecies.

# RESULTS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

# 3. RESULTS

## 3.1. Article 1

*Photobacterium damselae* subsp. *damselae*, an Emerging Fish Pathogen in the Black Sea: Evidence of a Multiclonal Origin.

https://aem.asm.org/content/82/13/3736

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

### 3.2. Article 2

Molecular Epidemiology of *Photobacterium damselae* subsp. *damselae* Outbreaks in Marine Rainbow Trout Farms Reveals Extensive Horizontal Gene Transfer and High Genetic Diversity.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6156455/

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

## 3.3. Article 3

*rstB* Regulates Expression of the *Photobacterium damselae* subsp. *damselae* Major Virulence Factors Damselysin, Phobalysin P and Phobalysin C.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5385354/

### 3.4. Article 4 (*to submit*)

Mutation of the RstAB Two-Component Regulatory System Impairs Virulence, Motility, Cell Morphology, Antimicrobial Resistance and Production of Type II Secretion System-Dependent Factors in the Fish and Human Pathogen *Photobacterium damselae* subsp. *damselae.*

# Mutation of the RstAB two-component regulatory system impairs virulence, motility, cell morphology, antimicrobial resistance and production of type II secretion system-dependent factors in the fish and human pathogen *Photobacterium damselae* subsp. *damselae*

Mateus S. Terceti [1], Ana Vences [1], Xosé M. Matanza [1], Alba V. Barca [1], Manuel Noia [2], Johnny Lisboa [3,4], Nuno M. S. dos Santos [3,4], Ana do Vale [3,4] and Carlos R. Osorio[1*]

[1]*Departamento de Microbioloxía e Parasitoloxía, Instituto de Acuicultura, Universidade de Santiago de Compostela, Santiago de Compostela, Spain*
[2] *Departamento de Bioloxía Funcional, Facultade de Bioloxía-CIBUS, Universidade de Santiago de Compostela, Santiago de Compostela, Spain*
[3] *Fish Immunology and Vaccinology Group, IBMC-Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal*
[4] *i3S-Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal*

**Running title:** RstAB regulates virulence and physiology in *P. damselae*

**Keywords:** RstAB, *Photobacterium damselae*, damselysin, phobalysin, vibriosis, CarSR

**Correspondence:**
Carlos R. Osorio
Departamento de Microbioloxía e Parasitoloxía, Instituto de Acuicultura, Universidade de Santiago de Compostela, Santiago de Compostela 15782, Galicia, Spain
Email: cr.osorio@usc.es
Tel. (+34) 881 816050
Fax. (+34) 881 816047

**Abstract**

The RstB histidine kinase of the two component system RstAB positively regulates the expression of Dly, PhlyP and PhlyC cytotoxins in the fish and human pathogen *Photobacterium damselae* subsp. *damselae*. However, the function of the predicted cognate response regulator RstA has not been studied so far, and the effects of *rstA* and *rstB* mutations in other cell functions and phenotypes remain uninvestigated. Here, we analysed the effect of *rstA* and *rstB* mutations in cell fitness and in diverse virulence-related features. Mutants in *rstA* and *rstB* genes were impaired in hemolysis and in Dly-dependent phospholipase activity. However, *rstAB* mutations did not cause a detectable effect on PlpV-dependent phospholipase and ColP-dependent gelatinase activities. We found no defects in growth of *rstA* and *rstB* mutants under varied conditions of temperature and NaCl concentration. However, *rstA* and *rstB* mutants grown at 0.5% NaCl exhibited impaired swimming motility, enlarged cell size and impaired ability to separate after cell division. Mutation of any of the two genes also caused an increased sensitivity to benzylpenicillin. Notably, *rstA* and *rstB* mutants showed impaired secretion of a number of type II secretion system-dependent proteins, which included the major cytotoxins Dly, PhlyP and PhlyC, as well as four hitherto unreported secreted proteins in this bacterial species which might constitute novel virulence factors. Both *rstA* and *rstB* mutants were severely impaired in virulence in a seabass model. This study establishes the role of RstAB as a major regulator of virulence and diverse cellular functions in *P. damselae* subsp. *damselae*.

**Introduction**

Two-component signal transduction systems enable bacteria to sense environmental stimuli and transfer this information across the cytoplasmic membrane to the cytoplasm. Such systems consist of a membrane-embedded protein kinase which acts as a sensory

component, and its cognate response regulator, a cytoplasmic transcriptional factor. When the sensory component of the pair is stimulated by a specific signal, it autophosphorylates a histidine residue and then transfers the phosphate group to a conserved aspartate residue of the response regulator (Stock et al., 2000).

*Photobacterium damselae* subsp. *damselae* is a marine bacterium pathogenic for a variety of marine animals as well as for humans, and represents an emerging threat for fish species of financial importance in marine aquaculture (Rivas et al., 2013a; Terceti et al; 2016; Osorio et al., 2018). pPHDD1 plasmid encodes two major virulence factors, the phospholipase-D damselysin (Dly) and the pore-forming toxin phobalysin P (PhlyP) (Rivas et al., 2011; Rivas et al 2015a). Additional virulence factors are encoded within the chromosomes and include the pore-forming toxin phobalysin C (PhlyC), the phospholipase PlpV and the collagenase ColP (Rivas et al., 2013b; Vences et al., 2017). While production of PhlyC and PlpV are almost ubiquitous traits in this subespecies, only a fraction of the isolates produce the collagenase ColP (Vences et al., 2017). Mutants in the gene encoding EpsL protein, an inner membrane component of the type II secretion system (T2SS) exhibit impaired hemolysis, phospholipase, and gelatinase activities, providing evidence that the T2SS secretes Dly, PhlyP, PhlyC, PlpV and ColP enzymes (Rivas et al., 2015b; Vences et al., 2017). Howewer, besides these cytotoxins, the secretome of *P. damselae* subsp. *damselae* remains largerly uncharacterized.

Recently, it was reported for the first time the functionality of a two-component regulatory system in this pathogen which, based on its similarity to the RstAB system originally described in *Escherichia coli*, was dubbed RstAB (Terceti et al., 2017). The *P. damselae* subsp. *damselae* RstAB system is thus predicted to consist of the histidine kinase RstB (locus VDA_000600) and its cognate cytoplasmic response regulator RstA (locus VDA_000601). Single *rstB* mutants

exhibited a strong impairment in the expression of the three hemolysins Dly, PhlyP and PhlyC as well as in virulence in a seabass fish model. However, the role of the putative cognate response regulator RstA has not been studied to date, and nothing is known about the role of RstAB system in the regulation of other virulence traits and of its effects on cell fitness.

In the present study, we have constructed and assayed for the first time single *rstA* mutants in the pPHDD1-harbouring strain RM-71, as well as *rstA* and *rstB* mutants in the plasmidless strain LD-07 of *P. damselae* subsp. *damselae*. Notably, we found that *rstA* mutation compromises virulence for fish and hemolytic activity at levels comparable to the *rstB* mutant. Most importantly, the RstAB system is essential for maintenance of cell shape and size and for full flagelar motility under conditions of low osmolarity. In addition, resistance to benzylpenicillin was impaired in *rstA* and *rstB* mutants. Mutation of either *rstA* or *rstB* strongly compromised the secretion of Dly, PhyP and PhlyC as well as of a number of type II secretion system-dependent proteins, some of which constitute potential novel virulence factors in *P. damselae*. Genes of the RstAB regulon comprised plasmid, chromosome I and chromosome II-encoded genes, and showed a notable differential distribution among isolates of this subspecies. These results demonstrate a major regulatory role of the RstAB system in the physiology and in virulence of this important marine pathogen, and open new paradigms in the study of the RstAB regulon in marine bacteria.

## Materials and Methods

**Bacterial Plasmids, Strains, and Culture Conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. In addition, 83 strains of *P. damselae* subsp. *damselae* from diverse isolation sources used in this study in the genetic screening of genes belonging to the RstAB regulon are included in Fig. 8.

*P. damselae* subsp. *damselae* cells were routinely grown at 25°C on tryptic soy agar (TSA) and broth (TSB) supplemented with NaCl up to 1% (TSA-1 and TSB-1, respectively). For experiments with other NaCl and temperature conditions, these variables were set as needed. *E. coli* was grown at 37°C in Luria-Bertani (LB) broth or LB agar. When necessary, antibiotics were used at the following final concentrations: kanamycin (Km) at 50 μg mL-1, chloramphenicol (Cm) at 20 μg mL-1. For growth curves, at least three replicates per strain were grown in independent experiments in 200 μl medium in a 96 well plate inoculated 1:100 from exponentially growing precultures (OD600=0.02) and analyzed using a Biotek plate reader by measuring OD600 at 10 min intervals.

**Assays for hemolysis, phospholipase and gelatinase activities, and swimming motility.** Motility assays were carried out using motility agar, which consisted of TSB (0.5 or 1% NaCl) supplemented with 0.25% bacteriological agar. For this assay, a single colony isolated from an 18-h culture agar plate for each strain was picked with a sterile plastic tip, and the tip was stabbed into the motility agar. They were incubated at 15 °C, 25°C and 37°C depending on the experiment. Hemolysis assays on agar plates were conducted by picking a colony of each strain previously grown on TSA-1, and inoculating it on sheep blood agar plates (Oxoid) and grown at 25°C. For the phospholipase/lecithinase activity assay, 10 μl of TSB-1 overnight cultures for each *P. damselae* subsp. *damselae* strain were spotted onto TSA-1 plates supplemented with 3% egg yolk extract (Oxoid), and results were evaluated after 24 h of culture at 25ºC. Hydrolysis of lecithin by the phospholipase yields water-insoluble diglycerides that cause the appearance of an opaque precipitate. The gelatinase activity assay was carried out by spotting 10 μl of a TSB-1 overnight culture onto TSA-1 plates supplemented with 1% gelatin (Oxoid), and results were developed after 48 h of incubation at 25 °C by covering the agar plate surface with a 12.5% (wt/vol) $HgCl_2$ solution. Hydrolysis of

gelatin by the gelatinase enzyme causes the appearance of a translucent halo around the bacterial colony upon addition of $HgCl_2$. All the assays were repeated three times to ensure that the hemolytic and enzymatic haloes and motility radius of the strains were reproducible.

**PCR assays.** Relevant PCR primers used in this study are listed in Table 2. PCR reactions were routinely performed with Kapa Taq DNA polymerase (Kapa) using a T-gradient termocycler (Biometra). Routinely, the following thermal cycling conditions were used: 95 ºC for 5 min, followed by 30 cycles of 95 ºC for 30 s, 52.5ºC for 30 s and an elongation step of 1 min at 72 ºC per kb.

Allelic-exchange deletion mutant construction and gene complementation. Nonpolar deletions of *rstA* and *rstB genes* were constructed using PCR amplification of the amino- and carboxy-terminal fragments of each gene, which, when fused together, would result in an in-frame deletion of more than 90% of the coding sequence. The primers used are described in Table 2. Amplification was carried out using Hi-Fidelity Kapa *Taq* (Kapa). Allelic exchange was performed using the $Km^R$ suicide vector pNidKan containing the *sacB* gene, which confers sucrose sensitivity, and R6K *ori*, which requires the *pir* gene product for replication. The plasmid constructs containing the deleted alleles were transferred from *E. coli* S17-1-λ*pir* into RM-71 strain. After conjugation for 48 h on TSA plates prepared with seawater, cells were scraped off the plate and resuspended in TSB-1. Next, 100-µl aliquots of serial decimal dilutions were spread on Thiosulfate citrate bile salts sucrose (TCBS) agar and subsequently selecting for sucrose resistance (15% [wt/vol]) for a second recombination event. This led to the *P. damselae* subsp. *damselae* mutant strains described in Table 1. The absence of the deleted alleles was confirmed in each case by PCR, and the genome region involved in the deletion was sequenced to verify that the deletion was nonpolar. For complementation of the

mutants, *rstAB* ORFs sequence together with the respective promoter sequence was amplified by PCR using Hi-Fidelity Kapa Taq, cloned into the $Cm^R$ mobilizable vector pMRB24 and mobilized from *E. coli* S17-1-λ*pir* into mutant strains MT319 (RM-71 Δ*rstA*), MT341 (LD-07 Δ*rstA*) and MT340 (LD-07 Δ*rstB*).

**Fish virulence assays.** In order to test the effect of *rstA and rstB* deletions in virulence of *P. damselae* subsp. *damselae* for fish, we carried out virulence assays using sea bass (*Dicentrarchus labrax*) as a model. Fish were obtained from IGAFA (Illa de Arousa, Galicia, Spain). Groups of 10 fish (6 ± 1.2 g) per strain tested and per dose were acclimated in 100 l aquaria at 24ºC for one week before performing the assays. Fish were inoculated intraperitoneally with 0.1 ml of bacterial suspensions of each strain in 0.85% NaCl solution at two different doses of $10^4$ and $10^3$ CFU/fish. A control group of 10 fish was inoculated with 0.1 ml of sterile 0.85% NaCl solution. Fish mortality was recorded daily for 10 days after inoculation. Re-isolation and identification of the bacteria from the kidney of dead fish were performed. 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.

**MIC assay**. To determine the susceptibility to benzylpenicillin, vancomycin, polymyxin, bacitracin, fosfomycin and teicoplanin, exponentially grown cultures of *P. damselae* subsp. *damselae* strains were adjusted to an optical density at 600 nm ($OD_{600}$) of 0.5 and seeded onto TSA-1 plates in the presence of E-test gradient antibiotic strips (bioMérieux),

**Scanning Electron Microscopy.** Exponentially growing cultures of *P. damselae* subsp. *damselae* strains in TSB with either 0.5% or 1% NaCl were used for scanning electron microscopy observation of cell shape and size. Bacteria were fixed for 3 h at 4 $^oC$ 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 then washed three times in $dH_2O$, dehydrated using a series of graded ethyl alcohols, chemically dried using HMDS (hexamethyldisilazane) (Sigma), sputter-coated with iridium, before finally being viewed and photographed in an Ultra Plus ZEISS scanning electron microscope.

**Molecular phylogenetic analysis.** Phylogenetic relationships of the sensor and regulator proteins of the two component systems RstAB of *P. damselae* subsp. *damselae* with homologous proteins of other bacteria were evaluated using MEGA6 (Tamura et al., 2013). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987), and evolutionary distances (number of residue substitutions per site) were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Numbers at the tree nodes represent bootstrap values, expressed as a percentage of 1,000 replications. Accession numbers are listed in parentheses to the right of each organism name.

**SDS-PAGE.** In order to identify the proteins secreted by the type II secretion system (T2SS) including those which were under the control of the two component regulatory system RstAB, we collected extracellular products (ECPs) from several replicates of liquid cultures of *P. damselae* subsp. *damselae* of the following strains: RM-71$^{wt,}$ Δ*rstA*, Δ*rstB*, a deletion mutant of *epsL* gene (Δ*epsL*) encoding a component of the type II secretion system (T2SS), and different mutant combinations for the genes encoding hemolysins Dly (*dly* gene), PhlyP ($hlyA_{pl}$ gene) and PhlyC ($hlyA_{ch}$ gene). The ECPs were obtained from cultures grown in TSB-1 at 25ºC to an optical density at 600 nm ($OD_{600}$) of 1.7, corresponding to the stationary phase of growth. Bacterial suspensions were centrifuged (6000× *g*, 5 min, 4 ºC), bacterial cell pellets discarded and the cultured supernatants collected and filtered through 0.22 µm-pore size filters (Schleicher & Shuell, Dassel, Germany). Proteins from 1.5 mL cell-free culture supernatants were precipitated with 10% (W/v) trichloroacetic acid

(TCA) for 30 min on ice and recovered by centrifugation. Protein pellets were washed in 10% (w/v) TCA followed by a washing in acetone, and air-dried. Precipitated ECPs were solubilized in SDS-sample buffer (50 mM Tris-HCl (pH 8.8), 2% SDS, 0.05% bromophenol blue, 10% glycerol, 2 mM EDTA, and 100 mM DTT) and subjected to SDS-PAGE in 10 or 12% polyacrylamide gels using the Laemmli discontinuous buffer system (Laemmli, 1970). Proteins in the gels were stained with Coomassie Brilliant Blue. For the identification of T2SS-dependent proteins, protein bands were cut from several independent polyacrylamide gels. Thus, we also made sure that the secreted protein profiles generated were always reproducible.

**Proteomic analysis.** The selected protein bands were excised from the gel, reduced with DTT, and alkylated with IAA as previously described (Gomes et al., 2013), following digestion with trypsin. The resulting peptides were desalted and concentrated using reverse phase C18 tips (ZipTips, Millipore) following the manufacturer's instructions, eluted in 60% ACN / 0.1% TFA, and allowed to dry (SpeedVac, Thermo Scientific). The ressolubilized peptides were analysed by liquid chromatography (LC) coupled to an Orbitrap Q-Exactive mass spectrometer (Thermo Scientific) using a nanospray ionization source (Easy-Spray, Thermo Scientific). Reverse phase peptide separation was performed with an Ultimate 3000 system (Thermo Scientific) fitted with a trapping cartridge (Acclaim PepMap C18 100Å, 5mm x 300 µm i.d., 160454, Thermo Scientific) in a mobile phase of 2% ACN, 0.1% FA at 10 µL/min. After 3 min of loading, the trap column was switched in-line to a PepMap RSLC C18, 3µm, 75 µmi.d. x 15 cm EASY-Spray analytical column (ES800, Thermo Scientific). Separation was generated by mixing A: 0.1% FA, and B: 80% ACN, 0.1% FA at 300 nL/min, with the following gradient: 30 min (2.5% B to 35% B), 5 min (35% B to 95% B), 5 min (hold 95% B). Data acquisition was controlled by Xcalibur 4.0 and

Tune 2.8 software (Thermo Scientific). The mass spectrometer was operated in data-dependent (dd)positive acquisition mode alternating between a full scan (m/z300-2000) and subsequent HCD MS/MS of the 10 most intense peaks from full scan (normalized collision energy of 27%). ESI spray voltage was 1.9 kV. Global settings: lock masses best (m/z 445.12003), lock mass injection Full MS, chrom. peak width (FWHM) 15s. Full scan settings: 70k resolution (m/z200), AGC target 3e6, maximum injection time 100 ms.dd settings: minimum AGC target 1e3, intensity threshold 1e4, charge exclusión (+) unassigned, 1, 5-8, >8, peptide match preferred, exclude isotopes on, dynamic exclusion 20s. MS2 settings: microscans 1, resolution 17.5k (m/z 200), AGC target 1e5, maximum injection time 100 ms, isolation window 2.0 m/z, isolation offset 0.0 m/z, spectrum data type profile. The raw data was processed using Proteome Discoverer 2.2.0.388 software (Thermo Scientific) and searched against the UniProt database for the taxonomic selection *Photobacterium* (September 2017 release) and Rapid Annotations using Subsystems Technology (RAST) server (Aziz et al., 2008). The Sequest HT search engine was used to identify tryptic peptides. The ion mass tolerance was 10 ppm for precursor ions and 0.02 Da for fragment ions. Maximum allowed missing cleavage sites was set 2. Cysteine carbamidomethylation was set as a constant modification. Methionine oxidation and N-terminal protein acetylation were defined as variable modifications. Gene nomenclature was used following that of *P. damselae* subsp. *damselae* type strain CIP102761 (GenBank accession number ADBS00000000.1). For those proteins absent in CIP102761, the gene nomenclature of strain RM-71 (GenBank accession number NZ_LYBT00000000.1) was used instead.

## RESULTS

### *rstA* mutants are impaired in hemolytic activity and in virulence for fish.

In a previous study, we generated a mini-Tn*10* transposon insertional library of *Pdd* RM-71 strain, and identified a mutant that exhibited impaired hemolytic activity. Such mutant contained a disrupted VDA_000600 locus, which was predicted to encode the histidine kinase partner RstB of a putative two-component regulatory system RstAB (Terceti et al., 2017). The upstream locus VDA_000601 encodes a putative response regulator RstA. Albeit this protein was considered the cognate pair of RstB by *in silico* analysis, to date no mutants of *rstA* gene have been constructed and assayed in *P. damselae* subsp. *damselae*.

We here constructed a *rstA* deletion mutant in RM-71 and found that its hemolytic activity for sheep blood erythrocytes was impaired at levels comparable to those of the Δ*rstB* strain (Fig. 1A). Hemolysis of sheep erythrocytes by RM-71 is known to be due to a synergistic effect of Dly phospholipase with the two pore-forming toxins PhlyP and PhlyC (Rivas et al., 2013). Of these three cytotoxins, only Dly has the ability to degrade phospholipids on agar plates supplemented with lecithin, which enables the study of impairment in Dly secretion. The effect of *rstA* or *rstB* mutations in phospholipase activity in *Pdd* has not been tested to date. Here, we demonstrate that single deletions of *rstA* and *rstB* in RM-71caused a strong impairment in the ability to degrade phospholipids (Fig. 1B), which demonstrates that Dly production is nearly abolished in *rstAB* mutants.

Previously, it was shown that *rstB* mutation drastically diminished virulence of RM-71 in a sea bass fish model (Terceti et al., 2017). Here we wanted to tested whether *rstA* mutation would compromise virulence of *Pdd* for fish. Virulence tests conducted with sea bass as a model fish crearly demonstrated that the single *rstA* and *rstB* mutants are strongly impaired in their virulence for sea bass (Fig. 2). All these

lines of evidence suggest that RstA, the protein encoded by VDA_000601 in *Pdd*, is the cognate response regulator of the histidine kinase RstB encoded by VDA_000600 and is necessary for hemolytic activity of *Pdd* and for full virulence for sea bass.

***Mutations in rstA and rstB* do not impair PlpV-dependent phospholipase and ColP-dependent collagenase activities.**

*P. damselae* subsp. *damselae* strains encode a phospholipase dubbed PlpV, whose contribution to lecithin degradation and to virulence for fish is inferior than that of Dly toxin. Plasmidless strains do not produce Dly and yield a thin phospholipase halo in lecithin-supplemented plates caused by the minor contribution of PlpV (Vences et al., 2017). Therefore, since RM-71 strain produces Dly and PlpV (Vences et al., 2017), the residual precipitation halo observed in the *rstA* and *rstB* mutants in the phospholipase assay might be attributable either to small amounts of Dly being produced, or to the contribution of PlpV, or to both. The role of the TCS RstAB on regulation of PlpV phospholipase has not been studied so far. In order to clarify this, we selected LD-07, a naturally plasmidless strain of *Pdd* isolated from sea bream (Table 1) that does not produce Dly and PhlyP toxins. The phospholipase phenotype of LD-07 is thus due exclusively to the production of PlpV, and its hemolytic activity againts sheep erythrocytes is exerted by the chromosome-encoded hemolysin PhlyC (encoded by $hlyA_{ch}$ gene) (Vences et al., 2017). Deletion mutants of the RstAB system were thus made in strain LD-07, generating the single mutants LD-07*ΔrstA* and LD-07*ΔrstB* that were seeded together with the parental strain LD-07 on sheep blood agar plates (to evaluate hemolytic activity) and on agar plates supplemented with egg yolk emulsion (to evaluate phospholipase activity). We observed that deletion of *rstA* and *rstB* did not cause any impairment in the lecithin degradation halo, confirming that *rstAB* system is not a regulator of the *plpV* gene (Fig. 1D). However, as

expected, mutation of either *rstA* or *rstB* abolished the hemolytic activity of LD-07 against sheep eryhrocytes, demonstrating that RstAB system is a positive regulator of $hlyA_{ch}$ gene and is essential for hemolytic activity in plasmidless isolates (Fig. 1C).

Recently, it was demonstrated that ColP collagenase has a minor contribution to virulence in LD-07 strain, and is the only gene responsible for gelatin degradation on agar plate tests (Vences *et al*., 2017). Since RM-71 strain lacks *colP* gene, we assayed the collagenase activity of *rstA* and *rstB* deletion mutants of strain LD-07 (*colP*-positive) on TSA-1 plates supplemented with 1% gelatin. We observed that the RstAB system is not involved in the regulation of *colP* at detectable levels (Fig. 1E). Complementation of the RM-71 and LD07 *rstAB* mutants with plasmid prstAB (Table 1) restored the hemolytic activity against sheep erythrocytes at levels of the parental strains, as well as the phospholipase activity in RM-71 derived mutants (data not shown).

**_rstAB_ mutants are not impaired in growth at different temperatures and NaCl concentrations in comparison to the parental strain**

In a previous study it was reported that mutation of *rstB* did not impair growth of *Pdd* RM-71 under standard laboratory conditions in TSB-1 medium at 25ºC (Terceti et al., 2017). Since *P. damselae* subsp. *damselae* isolates are known to grow through a wide range of temperatures and sodium concentrations, we here analyzed the growth of RM-$71^{wt}$, RM-71Δ*rstA* and RM-71Δ*rstB* strains using a Biotek plate reader at 15°C, 25°C and 37°C, and under NaCl concentrations of 0.5% and 1% NaCl.

No relevant differences were observed in the growth of these three strains when exposed to the same conditions of temperature and NaCl concentration (Fig. 3A). This result indicates that RstAB system is not essential for optimal growth under the assayed culture conditions.

However, the growth behaviour differed depending on the conditions. Parental and mutant strains reached the exponential growth stage more rapidly at 37°C with 1% NaCl, entering the stationary phase after 7 h post inoculation ($OD_{600}$max = 0.4) (Figure 3A). However, growth at 25°C with 1% NaCl was the optimal growth condition, as this allowed parental and mutant strains to reach an $OD_{600}$max= 0.5, even though they entered the exponential growth phase about 4 h later compared to growth at 37°C with 1% NaCl. At 15°C with 1% NaCl exponential growth was only observed after 20 h post inoculation (Fig. 3A).
When NaCl concentration was set at 0.5%, the growth of all the strains was impaired with respect to growth at 1% NaCl, independently of the temperature of the assay, and parental and mutant strains exhibited similar growth paterns (Fig. 2A). Unexpectedly, a sharp drop in growth was observed at 37°C and 0.5% NaCl, as no growth was detected for any of the three strains. This suggests that *P. damselae* subsp. *damselae* is not able to multiply under the combined effect of high temperature and low salinity, which might act as stressful conditions for this bacterium.

**_rstAB_ mutants show impaired motility and aberrant cell shape and size at 0.5 % NaCl, but not at 1% NaCl**

Swimming motility is believed to constitute an important factor in the pathogenicity of *Pdd* for fish and it has been demonstrated that seawater transmits the disease (Fouz et al., 2000). Since the effect of *rstAB* genes in motility has not been assayed so far, we here investigated the behaviour in motility agar of RM-71$^{wt}$, RM-71Δ*rstA* and RM-71Δ*rstB*. We found that mutant strains were not affected in swimming motility with respect to parental strain at any temperature at 1% NaCl, albeit the three strains showed different patterns of growth/motility in a temperature-dependent fashion, reflecting the differences in growth at each temperature mentioned above (Fig. 3B). Of note, growth at 37ºC generated a diffuse pattern of motility,

suggesting that growth at such temperature causes a subpopulation of cells to became less motile. However, when strains were assayed for motility at 0.5 NaCl, both the *rstA* and the *rstB* mutants exhibited impaired swimming motility haloes at 15ºC and at 25°C NaCl (Fig. 3B and 3C). In concordance with the data of growth curve analyses, cultivation at 37ºC in presence of 0.5% NaCl abolished bacterial growth. The impaired motility haloes of the *rstA* and *rstB* mutants are not due to differences in growth between parental strains and mutants, as demonstrated in the analysis of the growth curves (Fig. 3A). Altogether, these results suggest that the two-component RstAB system of *P. damselae* subsp. *damselae* contributes to regulation of swimming motility in this bacterium.

Previously, it had been observed that single mutation of *rstB* did not impair cell morphology of RM-71 grown in TSB and 1% NaCl (Terceti et al., 2017). Since motility is impaired in *rstAB* mutants only under growth at low salinity (0.5 % NaCl), we judged interesting to study the cell morphology of the three strains grown in TSB with 0.5 % and with 1% NaCl, respectively, by scanning electron microscopy. We observed that, at 1% NaCl, cells of the RM-71Δ*rstA* and RM-71Δ*rstB* mutants exhibited cell shapes and sizes similar to parental strain. However, when cells were cultured at 0.5% NaCl, differences in cell arrangement, morphology and size became evident (Fig. 4). Both the *rstA* and *rstB* mutants exhibited longer and swollen cells, and most often connected to each other forming chain-like structures, suggesting an impairment in daughter cell separation upon cell division. Mobilization of the complementing plasmid prstAB into the *rstA* and *rstB* mutants caused the reversion to normal cell shapes and size (data not shown). These results demonstrate that the RstAB system is essential for optimal cell division and control of cell shape and size under conditions of low NaCl concentrations.

**Mutations in *rstAB* increases benzylpenicillin and vancomycin sensitivity.**

Previous studies reported that mutations in RstAB-like systems cause an increased sensitivity to antimicrobial substances in some bacterial species. Such is the case in *Vibrio cholerae,* where mutations in *vprAB*/*carSR* genes (homologues of *Pdd rstAB*) increased sensitivity to polymyxin B (Herrera et al, 2014; Bilecen et al., 2015). However, *rstB* mutation in *Pdd* RM-71 was not found to cause an increase in polymyxin B in a previous study (Terceti et al., 2017). We here assessed whether *rstAB* mutation had any effect in *Pdd* sensitivity to a collection of antimicrobials targeted to the cell envelope. The antibiotic sensitivity of RM-71$^{wt}$, RM-71Δ*rstA* and RM-71Δ*rstB* strains was analyzed by E-test gradient strips. The two mutant strains showed an increased sensitivity for benzylpenicillin when cultured with 1% NaCl (Fig. 5). We also observed that mutant strains for the RstAB system became slightly sensitive to vancomycin, whereas the RM-71$^{wt}$ strain is completely resistant to it (Fig. 5). These results suggest that the two-component RstAB system may be directly or indirectly related to regulation of cell wall synthesis of *P. damsela*e subsp. *damselae*. However, the RM-71$^{wt}$, RM-71Δ*rstA* and RM-71Δ*rstB* strains were resistant to bacitracin and teicoplanin and sensitive to fosfomycin and polymyxin. Thus, RstAB appears to not interfere with the mechanism of sensitivity/resistance to bacitracin, fosfomycin, teicoplanin and polymyxin (Supplementary Figure S1).

***rstAB* mutants are impaired in production of type II secretion system-dependent proteins**

The four major toxins Dly, PhlyP, PhlyC and PlpV are predicted to be secreted via the type II secretion system (T2SS) since *epsL* mutants showed to be impaired in hemolytic and phospholipase activities (Rivas et al., 2015b; Vences et al., 2017). However, the T2SS-dependent secretome of RM-71 has not been further characterized. We

here wanted to gain an insight into the proteins secreted by RM-71, and into their dependence on the RstAB regulatory system. To this end, we conducted SDS-PAGE analysis of the extracellular products of the parental strain and the *rstA* and *rstB* mutants. In addition, in order to evaluate the dependence of the secreted proteins on a functional T2SS, we assayed the secreted proteins of an *epsL* mutant. The *epsL* gene encodes an inner-membrane spanning protein that constitutes an essential element in the T2SS of *P. damselae* subsp. *damselae* (Rivas et al., 2015b).

This analysis revealed 12 protein bands whose secretion was impaired in the *epsL* mutant (hence, T2SS-dependent proteins). These bands were extracted from the gels and subjected to protein identification by mass spectrometry, and it was found that they account for 13 distinct proteins (Fig. 6A and C). The genetic context of the genes encoding the identified proteins was analyzed and their location within the genome of *P. damselae* subsp. *damselae* RM-71$^{wt}$ was elucidated (Fig. 7A). In addition, the proteins identified in this study were analyzed for blastP homology in other bacterial species (Table 3) and for their domains by Pfam 31.0 (Table 4).

The proteins related to bands 1, 2, 3, 8 and 11 are only T2SS dependent and are not under control of the RstA/RstB system, whereas the remaining bands are T2SS- and RstAB-dependent. This observation indicates that mutation of the RstAB system does not prevent the correct function of the T2SS since bands 1, 2, 3, 8 and 11 are equally present in the secretome of the parental strain and in Δ*rstA* and Δ*rstB* mutants. In further support of this idea, as described above (Fig.1), *rstA* and *rstB* mutants were not affected in the lecithinase activity attributable to PlpV (mutants in the plasmidless strain LD-07), an enzyme known to be secreted via the T2SS (Vences et al., 2017).

Notably, secretion of seven T2SS-dependent proteins (included within bands 4, 5, 6, 7, 9, 10, 12) was strongly impaired in the *rstA* and *rstB* mutants, and for some of them secretion was practically abolished

(Fig. 6A). One of the most outstanding features in the secretome pattern of RM-71 was the presence of two major bands in the range of 60-80 kDa, that were absent in the *rstA*, *rstB* and *epsL* mutants. The molecular masses of these bands suggest that they correspond to the three hemolysins Dly, PhlyP and PhlyC, and we observed that these two major bands were absent in the protein profile of a RM-71 triple mutant for genes *dly*, $hlyA_{pl}$ and $hlyA_{ch}$ (Fig. 6A). To further determine which hemolysin(s) was contained in each band, we carried out SDS-PAGE analysis of all the combinations of mutants for these three hemolysins. As a result, we corroborated that the upper intense band of ca. 75 kDa corresponded to Dly cytotoxin and the lower band of ca. 65 kDa corresponded to PhlyP plus PhlyC, with a major contribution of PhyP over PhlyC (Fig. 6B). Thus, single mutation of each of the RstAB system genes, practically abolishes Dly, PhlyP and PhlyC production. In addition, and as previous studies had indirectly suggested (Rivas et al., 2015b), we demonstrate that mutation of *epsL* causes a strong impairment in Dly, PhlyP and PhlyC secretion.

Among the additional secreted proteins whose production was impaired in the *rstA* and *rstB* mutants (Fig. 6C) we found putative novel virulence factors of *Pdd*. Of note, the parental strain produced high amounts of a small protein of 11 kDa (identified as A0J47_07530) whose secretion was nearly abolished in the *rstAB* and *epsL* mutants. Although the gene encoding A0J47_07530 protein is present in the genome of the type strain CIP102761 it is not annotated as such in the GenBank database and therefore we here provide its gene locus in RM-71 genome. The analysis of the genetic context of A0J47_07530 unveiled that it is located upstream (and likely cotranscribed with) the gene encoding a putative deltaendotoxin (VDA_002799) (Fig. 7). Indeed, this deltaendotoxin is also *rstAB* and *epsL* dependent as it was identified as the major component of bands 6 and 7 (Fig. 6A and 6C). The delta-endotoxin corresponding to band 6 has coverage of the identified peptides ranging from residues 197 to

529 and the delta-endotoxin for band 7 has a coverage ranging from residue 197 to 474. This analysis suggests that this protein is processed in its C-terminal region being evident in gel in two forms: non-truncated (band 6) and truncated at C-terminus (band 7) (Fig. 6A).

Finally, two uncharacterized secreted proteins were also *rstAB* dependent and corresponded to the pPHDD1 plasmid-encoded VDA_000112 and the chromosome II-encoded VDA_000358. Similarity searches failed to unveil well-characterized homologues for these two proteins (Table 3), and no conserved domains could be identified in a sequence analysis (Table 4).

**The *P. damselae* subsp. *damselae* T2SS-dependent secretome includes additional hitherto uncharacterized proteins.**

Six additional proteins were identified as T2SS-dependent although their secretion was not affected in *rstA* and *rstB* mutants (Fig. 6A and 6C). Since the secretome of *P. damselae* subsp. *damselae* has been poorly characterized so far, we judged of high interest to further analyze these six proteins. VDA002460 corresponds to a T2SS-dependent putative lipoprotein (Table 3), although no Pfam-A matches to known sequences were found (Table 4). VDA_000694 corresponds to a putative sialidase and contains two Sial-lect-insert domains and a BNR_2 domain (Tables 3 and 4). It is known that pathogenic bacteria can utilize host sialic acid to form a protective coating that provides resistance to host immune response (Severi *et al*., 2007).

A0J47_15850 is a putative T2SS-dependent serine protease with peptidase S8 domain belonging to the family of subtilisin-like serine proteases (Bode *et al*., 1987). Subtilases are widespread, being found in eubacteria, archaebacteria, eukaryotes and viruses (Siezen and Leunissen, 1997). A0J47_09785 is also T2SS-dependent and corresponds to an uncharacterised *Pdd* protein with a trypsin-like

domain (Table 4) and with similarity (blastP) to proteases, metalloproteases and hemolysins (Table 3). It also appears as Hit (19% identity) the VesB protease from *Vibrio cholerae*, which has been described as a type II-secreted protease (Gadwal *et al*., 2014).
VDA_000966 is also T2SS dependent and is encoded within chromosome II of *Pdd*. It has homology to hypothetical proteins and porin family proteins (Table 3) and analysis by Pfam31.0 revealed an OMP_b-brl domain (Table 4). Another protein identified as T2SS dependent and encoded within chromosome II is VDA_000738 which has homology with ComEA helix-hairpin-helix (HHH) repeat competence proteins of various species (Table 3). By analysis by Pfam31.0 we have found that this protein has a HHH domain which is a short DNA-binding domain belonging to CL0198 clan superfamily (Table 4) (Witte *et al*., 2008).

**Genes of the RstAB regulon show differential presence in *P. damselae* subsp. *damselae* isolates**

As mentioned above, the RstAB regulon of RM-71 strain comprises at least 7 genes whose products are secreted by the T2SS. They include plasmid, chromosome I and chromosome II-encoded genes (Fig. 7). In order to assess the degree of conservation of the RstAB regulon in the subspecies, a total of 83 strains of *P. damselae* subsp. *damselae* isolated from a variety of geographical regions and host species were tested for the presence of the following target genes: $hlyA_{ch}$, $hlyA_{pl}$, *dly*, VDA_000112, VDA_002799, A0J47_07530 and VDA_000358 (Fig. 8). Presence of *rstAB* genes was also tested in all the strains. Some of these markers had already been tested in a fraction of these 83 isolates in previous studies (Rivas et al., 2011; Rivas et al., 2014; Terceti et al., 2016; Terceti et al., 2018) and such previous information is thus included within Fig. 8.
All the genes of the RstAB regulon showed differential distribution among the isolates. The three genes *dly*, $hlyA_{pl}$ and VDA_000112

were present in 33.73% (28/83) of the isolates, and occurred always together, an observation that is coherent with their being pPHDD1 plasmid-borne genes. The chromosome I-borne *hlyA*$_{ch}$ gene encoding PhlyC hemolysin is almost ubiquitous in the subspecies (77/83), with the exception of three Turkish and three Danish plasmidless isolates where this gene is truncated by an insertion sequence (Terceti *et al*., 2016; Terceti et al., 2018). In addition, 62.65% (52/83) of the isolates were carriers of the gene encoding VDA_000358 (Fig. 8). VDA_002799 (delta-endotoxin) and A0J47_07530 (the 11 kDa protein) co-occurred simultaneously in 48.2% (40/83) of the assayed strains. These two genes A0J47_07530 and VDA_002799 have been reported to be inserted into a highly variable genome region that is believed to constitute a host spot for the acquisition of horizontally-adquired DNA in *P. damselae* subsp. *damselae* (Terceti *et al*., 2018) (Fig. 7A).

Interestingly, *rstAB* genes were ubiquitous in the 83 isolates tested, demonstrating that this two-component regulatory system is highly conserved in the subspecies. Thus, the RstA/RstB system might be used as a weak point to control outbreaks caused by both plasmid-containing and plasmidless strains. Of note, we found that homologues of the *P. damselae* subsp. *damselae* RstAB proteins are widely distributed in *Vibrio* and *Photobacterium* species, including pathogenic and non-pathogenic species (Supplementary Fig. S2).

**Discussion**

Identification of the regulatory mechanisms in bacterial pathogens is of maximal interest in order to understand the environmental and host conditions that trigger the expression of virulence factors. *P. damselae* subsp. *damselae* is a generalist pathogen, capable of living as a free-swimming bacterium that causes outbreaks in cultured fish species when the conditions become favourable (Osorio et al., 2018). This pathogen produces a variety of toxins which are thought to be secreted

through the T2SS (Rivas et al., 2015b; Vences et al., 2018). So far, the only regulatory mechanism characterized in this subspecies is the putative histidine kinase RstB, which is genetically linked to a putative response regulator RstA (Terceti et al., 2017).

Here we demonstrated that single mutation of *rstA* impairs virulence in a seabass model, confirming the results previously obtained with single *rstB* mutants. Studies of the role of RstAB system in fish pathogens are very scarce. Notably, mutation of *rstB* in *Edwardsiella ictaluri* caused impaired colonization and virulence in a channel catfish model (Menanteau-Ledouble and Lawrence, 2013).

*P. damselae* subsp. *damselae* strains are able to grow through a range of temperatures and of NaCl concentrations (Kreger et al., 1984; Fouz et al., 1992; Fouz et al., 1998). Mutation of *rstA* and *rstB* genes did not compromise growth at 15, 25 and 37ºC at 1% NaCl. However, a drastic impairment in growth occurred at 37ºC and 0.5% NaCl, a combination of conditions that virtually abolished bacterial growth in the parental strain and in *rstA* and *rstB* mutants. To the best of our knowledge, this finding has not been previously reported, and suggests that such combination of temperature and salinity constitutes a stressful condition for this bacterium.

We found that mutations in *rstA* and *rstB* caused impairment in swimming motility at 0.5% NaCl. Although the connection between flagellum-based motility and the RstAB system in *Pdd* is so far unknown, recent studies have provided clues on the role of RstAB-lile systems in bacterial motility. Thus, silencing of the *rstA* or *rstB* genes resulted in impaired motility, hemolysis and virulence in *Vibrio alginolyticus* (Huang *et al.*, 2018). In *Salmonella typhimurium*, FlhA and MglB proteins involved in cell motility and chemotaxis are positively regulated by the RstAB system and a *rstB* mutant in this species showed a significant reduction in motility (Tran *et al*., 2016).

*rstA* and *rstB* mutants cultured at 0.5% NaCl exhibit impairment in cell separation upon cell division, as well as enlarged cell size. These

mutants also exhibited increased sensitivity to benzylpenicillin and, to a lesser extent, to vancomycin. In agreement with our results, overexpression of RstA in *E. coli* made this bacterium more resistant to β-lactam antibiotics such as ampicillin (Hirakawa *et al*., 2003a; Hirakawa *et al.*, 2003b). Similarly, *E. coli rstAB* mutants are hypersensitive to ketoprofen, pridinol, and troleandomycin, although the basis for these sensitivities has not been ascertained yet (Zhou et al., 2003). So far, the molecular mechanisms linking the RstAB system with antibiotic resistance and with maintenance of cell size and shape in *P. damselae* subsp. *damselae* remain unknown.

Among the T2SS-dependent proteins not regulated by the RstAB system we found predicted sialidases and proteases, among others. Identifying the function of these proteins constitutes a promising challenge and will require additional research. Fish cells secrete mucus glicoproteins containing sialic acid (Kimura et al., 1994), and pathogenic bacteria can use sialidases to remove sialic acid residues from host cells and coat themselves, thus gaining resistance to components of the host's innate immune response (Severi et al., 2007). In addition, pathogenic bacteria can bind to host sialic acid moiety to enhance adhesion and colonization. Thus, bacteria that produce sialidases might have a greater ability to colonize and stabilize themselves in the skin, gills, scales and intestines of animals that secrete mucosal glycoproteins containing sialic acids. Production of a trans-sialidase activity has been reported in *P. damselae* subsp. *damselae*, although its biological significance remains uninvestigated (Cheng *et al*., 2010). In addition, production of neuraminidase activity has been detected in strains potentially identified as *P. damselae* subsp. *damselae*, isolated from fish intestines in Japan and (Sugita *et al*., 2000).

Notably, our study has contributed to the identification of 4 hitherto unreported proteins belonging to the RstAB regulon, including plasmid-, chromosome I- and chromosome II-encoded proteins.

Therefore, the response regulator RstA is predicted to recognize target genes associated with the *P. damselae* subsp. *damselae* chromosomes as well as genes that have been acquired by horizontal gene transfer via conjugation. Notably, our study has brought to the forefront a number of hitherto uncharacterized, T2SS-dependent and RstAB-dependent proteins, and there are very few studies on these except for the three hemolysins Dly, PhlyP and PhlyC (Kreger, 1984; Kothary and Kreger 1985; Rivas *et al*., 2011; Rivas *et al*., 2013b; Rivas *et al*., 2015a; Vences *et al*., 2017). Of special interest are two genes which are likely cotranscribed as an operon, and encode the delta (δ)-endotoxin VDA_002799 and an associated 11 kDa small protein, respectively. These two genes are inserted within a potential hot spot for recombination within the genome of *Pdd* (Terceti *et al*., 2018). δ-endotoxins, also called Cry and Cyt toxins, are pore-forming toxins predicted to cross the cytoplasmic membrane via the twin-arginine (Tat) translocation pathway (Li *et al*., 1991; Cygler *et al*., 1995; Galitsky *et al.*, 2001). However, the role of this putative δ-endotoxin in *Pdd* is still unknown. Last, VDA_000112 and VDA_000358 are T2SS- and RstAB-dependent uncharacterized proteins encoded within plasmid pPHDD1 and chromosome II respectively (Fig. 7). Surely all these proteins will deserve an in-depth characterization in future studies.

*rstAB* genes are considered to be part of the PhoP/PhoQ regulon (a $Mg^{2+}$-sensing two-component system) (Oshima et al., 2002; Ogasawara et al., 2007). While some evidence suggests that the RstAB system might respond to acidic conditions (Ogasawara et al., 2007; Huang et al., 2017), the specific stimuli that trigger the activation of the sensor histidine kinase RstB remain unknown for all the RstAB-like systems studied to date (Ogasawara et al., 2007; Campbell et al., 2007; Flamez et al., 2008; Bilecen and Yildiz., 2009; Menanteau-Ledouble and Lawrence, 2013; Tran et al., 2016; Huang et al., 2018). In contrast, a number of genes and biological functions

under control of the RstAB system have been identified, demonstrating that the RstAB regulon is not predictive since it shows a high diversity among species. Functions regulated by homologous RstAB systems are as varied as pyrimidine metabolism, enterobactin biosynthesis, ferrous iron uptake and motility in *Salmonella enterica* (Tran et al., 2016), polysaccharide synthesis, biofilm formation, LPS modification in *Vibrio cholerae* (Bilecen and Yildiz, 2009; Herrera et al., 2014), or adhesion, biolfilm production, motility and hemolysis in *Vibrio alginolyticus* (Huang et al., 2017), among others.

This study has contributed to the knowledge of the bacterial RstAB regulon with a number of novel genes and functions. In addition, we here demonstrated that the RstAB regulon of *Pdd* RM-71 comprises genes of differential presence among strains, and no geographical or isolation source patterns could be identified. To summarize, the RstAB TCS plays a major role in regulation of virulence and of many aspects of cell physiology of *P. damselae* subsp. *damselae*. Ongoing studies are expected to unveil the roles of the novel RstAB-regulated genes reported in this study.

**Acknowledgements**

This work has been supported by grant AGL2016-79738-R (AEI/FEDER, EU) from the State Agency for Research (AEI) of Spain, and co-funded by the FEDER Programme from the European Union. The support of Xunta de Galicia (Spain) with grant GRC-2014/007 is also acknowledged. MT thanks the Brazilian Ministry of Education and CAPES (Coordenaçao de Aperfeiçoamento de Pessoal de Nível Superior) for a predoctoral fellowship. XM thanks Xunta de Galicia for a predoctoral fellowship. The mass spectrometry technique was performed at the Proteomics i3S Scientific Platform with the assistance of Hugo Osório. This work had support from the Portuguese Mass Spectrometry Network, integrated in the National

Roadmap of Research Infrastructures of Strategic Relevance (ROTEIRO/0028/2013; LISBOA-01-0145-FEDER-022125).

## FIGURE LEGENDS

**Figure 1.** Hemolysis of sheep blood agar plates, lecithin degradation (phospholipase activity) and gelatin degradation (collagenase activity) phenotypes of *P. damselae* subsp. *damselae* parental strains RM-71 and LD-07, and their mutant derivatives Δ*rstA* and Δ*rstB* mutant. Scale bar, 1 cm.

**Figure 2.** Mortality (%) of sea bass fish intraperitoneally challenged with two different doses ($10^4$ CFU/fish and $10^3$ CFU/fish) of the *P. damselae* subsp. *damselae* parental strain (RM-71) and the Δ*rstA* and Δ*rstB* mutants. For each dose assayed, a total of 10 fish were inoculated.

**Figure 3. (A)** Growth curves, and **(B, C)** swimming motility phenotypes of RM-71, *rstA* and *rstB* mutants at three different temperatures (15, 25 and 37ºC) in TSB medium with 0.5% or with 1% NaCl.

**Figure 4.** Scanning electron microscopy of *P. damselae* subsp. *damselae* parental strain RM-71, Δ*rstA* and Δ*rstB* mutants. Note the enlarged cell size of the mutants, which also form chain-like structures likely due to an impairment in daughter cell separation upon cell division.

**Figure 5.** E-tests for benzylpenicillin and vancomycin sensitivity, showing an increased sensitivity in both the *rstA* and the *rstB* mutants compared to parental strain RM-71.

**Figure 6. A:** Representative SDS-PAGE of culture supernatants from RM-71$^{WT}$, Δ*rstA*, Δ*rstB*, 3Δ (triple mutant for *dly*, *hlyA*$_{pl}$ and *hlyA*$_{ch}$ hemolysin genes) and Δ*epsL* (defective in T2SS) strains. The identified protein bands are denoted with numbers 1 to 12. Bands used for identification were excised from different gels. **B:** SDS-PAGE analysis of culture supernatants from RM-71$^{WT}$, Δ*hlyA*$_{ch}$, Δ*hlyA*$_{pl}$, Δ*dly*, Δ*hlyA*$_{ch}$Δ*hlyA*$_{pl}$, Δ*hlyA*$_{ch}$Δ*dly*, Δ*hlyA*$_{pl}$Δ*dly* and 3Δ (triple mutant). Comparative analysis of the protein profiles shows that band 4 corresponds to Dly and band 5 to PhlyP+PhlyC. **C:** Identification (gene barcodes) and characteristics of the T2SS-dependent proteins identified in this study, including those who also are RstAB-regulated.

**Figure 7:** Genetic context of the genes encoding the proteins identified in figure 6. Genomic location of the gene (i.e, chromosome I, II, or pPHDD1 plasmid is indicated).

**Figure 8:** Diagramm depicting the presence of *rstAB* genes, plus genes of the RstAB regulon, in 83 *P. damselae* subsp. *damselae* strains isolated from different geographical locations and from different hosts including marine animals and humans.

## REFERENCES

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., and Zagnitko, O. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* 9, 75.

Bilecen, K., and Yildiz, F. (2009). Identification of a calcium-controlled negative regulatory system affecting *Vibrio cholerae* biofilm formation. *Environ. Microbiol*. 11, 2015-2029.

Bilecen, K., Fong, J. C. N., Cheng, A., Jones, C. J., Zamorano-Sánchez, D., and Yildiz, F. (2015). Polymyxin B resistance and biofilm formation in *Vibrio cholerae* are controlled by the response regulator CarR. Infect. Immun. 83, 1199-1209.

Bode, W., Papamokos, E., and Musil, D. (1987). The high-resolution X-ray crystal structure of the complex formed between subtilisin Carlsberg and eglin c, an elastase inhibitor from the leech *Hirudo medicinalis*. Structural analysis, subtilisin structure and interface geometry. *Eur. J. Biochem.* 166, 673–692.

Campbell, T. L., Ederer, C. S., Allali-Hassani, A., and Brown, E. D. (2007). Isolation of the *rstA* gene as a multicopy suppressor of YjeE, an essential ATPase of unknown function in *Escherichia coli*. *J. Bacteriol*. 189, 3318-3321.

Cheng, J., Huang, S., Yu, H., Li, Y., Lau, K., and Chen, X. (2010). Trans-sialidase activity of *Photobacterium damsela* α2,6-sialyltransferase and its application in the synthesis of sialosides. *Glycobiology* 20, 260-268.

Cygler, M., Borisova, S., Grochulski, P., Masson, L., Pusztai-Carey, M., Schwartz, J.L., and Brousseau, R. (1995). *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. *J. Mol. Biol*. 254, 447–464.

Flamez, C., Ricard, I., Arafah, S., Simonet, M., and Marceau, M. (2008). Phenotypic analysis of *Yersinia pseudotuberculosis* 32777 response regulator mutants: new insights into two-component system regulon plasticity in bacteria. *Int. J. Med. Microbiol.* 298, 193-207.

Fouz, B., Larsen, J. L., Nielsen, B., Barja, J. L, and Toranzo, A. E. (1992). Characterization of *Vibrio damsela* strains isolated from turbot *Scophthalmus maximus* in Spain. *Dis. Aquat. Organ*. 12, 155-166.

Fouz, B., Toranzo, A. E, Marco-Noales, E., and Amaro, C. (1998). Survival of fish-virulent strains of *Photobacterium damselae* subsp.

*damselae* in seawater under starvation conditions. *FEMS Microbiol Lett.* 168, 181–186.

Gadwal, S., Korotkov, K. V., Delarosa, J. R., Hol, W. G., and Sandkvist, M. (2014). Functional and structural characterization of *Vibrio cholerae* extracellular serine protease B, VesB. *J. Biol. Chem.* 289, 8288-8298.

Galitsky, N., Cody, V., Wojtczak, A., Ghosh, D., Luft, J. R., Pangborn, W., and English, L. (2001). Structure of the insecticidal bacterial delta-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. *Acta Crystallogr.* D. 57, 1101–1109.

Gomes, C., Almeida, A., Ferreira, J. A., Silva, L., Santos-Sousa, H., Pinto-de-Sousa, J., et al. (2013). Glycoproteomic analysis of serum from patients with gastric precancerous lesions. *J. Proteome Res.* 12, 1454-1466.

Grimes, D. J., Stemmler, J., Hada, H., May, E. B., Maneval, D., Hetrick, et al. (1984). *Vibrio* species associated with mortality of sharks held in captivity. *Microb. Ecol.* 10, 271-282.

Herrera, C. M., Crofts, A. A., Henderson, J. C., Cassandra Pingali, S., Davies, B. W., and Trent, S. M. (2014). The *Vibrio cholerae* VprA-VprB two-component system controls virulence through endotoxin modification. *mBio.* 5, e02283-14.

Herrero, M., de Lorenzo, V., and Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* 172, 6557-6567.

Hirakawa, H., Nishino, K., Hirata, T., and Yamaguchi, A. (2003a). Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. *J. Bacteriol.* 185, 1851-1856.

Hirakawa, H., Nishino, K., Yamada, J., Hirata, T., and Yamaguchi, A. (2003b). Beta-lactam resistance modulated by the overexpression

of response regulators of two-component signal transduction systems in *Escherichia coli*. *J. Antimicrob. Chemother*. 52, 576-582.

Huang, L., Xu, W., Su, Y., Zhao, L., and Yan, Q. (2018). Regulatory role of the RstB-RstA system in adhesion, biofilm production, motility, and hemolysis. *Microbiologyopen*. 23, e00599.

Hundenborn, J., Thurig, S., Kommerell, M., Haag, H., and Nolte, O. (2013). Severe wound infection with *Photobacterium damselae* ssp. *damselae* and *Vibrio harveyi*, following a laceration injury in marine environment: a case report and review of the literature. *Case Rep. Med*. 610632.

Kimura, M., Hama, Y., Sumi, T., Asakawa, M., Rao, B. N. N., Horne, A. P., et al. (1994). Characterization of a deaminated neuraminic acid-containing glycoprotein from the skin mucus of the loach, *Misgurnus anguillicaudatus*. *J. Biol. Chem*. 269, 32138-32143.

Kothary, M. H., and Kreger, A. S. (1985). Purification and characterization of an extracellular cytolysin produced by *Vibrio damsela*. *Infect. Immun*. 49, 25–31.

Kreger, A. S. (1984). Cytolytic activity and virulence of *Vibrio damsela*. *Infect. Immun*. 44, 326–331.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Le Roux, F., Binesse, J., Saulnier, D., and Mazel, D. (2007). Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl. Environ. Microbiol*. 73, 777-784.

Le Roux, F., Davis, B. M., and Waldor, M. K. (2011). Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. *Nucleic Acids Res*. 39, 1004-1013.

Li, J. D., Carroll, J., and Ellar, D. J. (1991). Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. *Nature* 353, 815–821.

Love, M., Teebkenfisher, D., Hose, J.E., Farmer, J.J., Hickman, F.W., and Fanning, G.R. (1981). *Vibrio damsela*, a marine bacterium, causes skin ulcers on the damselfish *Chromis punctipinnis*. *Science*. 214, 1139-1140.

Menanteau-Ledouble, S., and Lawrence, M. L. (2013). Use of bioluminescence mutant screening for identification of *Edwardsiella ictaluri* genes involved in channel catfish (*Ictalurus punctatus*) skin colonization. *Vet. Microbiol*. 162, 724-730.

Mouriño, S., Osorio, C. R., and Lemos, M. L. (2004). Characterization of heme uptake cluster genes in the fish pathogen *Vibrio anguillarum*. *J. Bacteriol*. 186, 6159-6167.

Ogasawara, H., Hasegawa, A., Kanda, E., Miki, T., Yamamoto, K., and Ishihama, A. (2007). Genomic SELEX search for target promoters under the control of the PhoQP-RstBA signal relay cascade. *J. Bacteriol*. 189, 4791-4799.

Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B.L., et al. (2002). Transcriptome analysis of all two-component regulatory systems mutants of *Escherichia coli* K-12. *Mol. Microbiol*. 46, 281-291.

Osorio, C. R., Vences, A., Matanza, X. M., and Terceti, M. S. (2018). *Photobacterium damselae* subsp. *damselae*, a generalist pathogen with unique virulence factors and high genetic diversity. *J. Bacteriol*. 200, e00002-18.

Rivas, A. J., Balado, M., Lemos, M.L., and Osorio, C. R. (2011). The *Photobacterium damselae* subsp. *damselae* hemolysins damselysin and HlyA are encoded within a new virulence plasmid. Infect. Immun. 79, 4617-4627.

Rivas, A. J., Lemos, M.L., Osorio, C. R. (2013a). *Photobacterium damselae* subsp. *damselae*, a bacterium pathogenic for marine animals and humans. *Front. Microbiol*. 4, 283.

Rivas, A. J., Balado, M., Lemos, M. L., Osorio, C. R. (2013b). Synergistic and additive effects of chromosomal and plasmid-encoded hemolysins contribute to hemolysis and virulence in *Photobacterium damselae* subsp. *damselae*. *Infect. Immun*. 81, 3287-3299.

Rivas, A. J., Labella, A., Borrego, J. J., Lemos, M.L., Osorio, C. R. (2014). Evidences for horizontal gene transfer, gene duplication and genetic variation as driving forces of the diversity of haemolytic phenotypes in *Photobacterium damselae* subsp. *damselae*. *FEMS Microbiol. Lett*. 355, 152-162.

Rivas, A. J, Von Hoven, G., Neukirch, C., Meyenburg, M., Qin, Q., Füser, S., Boller, K., Lemos, M. L., Osorio, C.R., and Husmann, M. (2015a). Phobalysin, a small ß-pore-forming toxin of *Photobacterium damselae* subsp. *damselae*. *Infect. Immun.* 83, 4335-4348.

Rivas, A.J., Vences, A., Husmann, M., Lemos, M. L., and Osorio, C. R. (2015b). *Photobacterium damselae* subsp. *damselae* major virulence factors Dly, plasmid-encoded HlyA, and chromosome-encoded HlyA are secreted via the type II secretion system. *Infect. Immun*. 83, 1246-1256.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. 4, 406-425.

Severi, E., Hood, D. W., and Thomas, G. H. (2007). Sialic acid utilization by bacterial pathogens. *Microbiology* 153, 2817-2822.

Siezen, R. J., and Leunissen, J. A. (1997). Subtilases: the superfamily of subtilisin-like serine proteases. *Protein Sci.* 6, 501–523.

Song, Y.L., Cheng, W., and Wang, C.H. (1993). Isolation and characterization of *Vibrio damsela* infectious for cultured shrimp in Taiwan. *J. Invertebr. Pathol*. 61, 24-31.

Stock, A. M., Robinson, V. L., and Goudreau, P. N. (2000). Two-component signal transduction. *Annu. Rev. Biochem*. 69, 183-215.

Sugita, H., Shinagawa, Y., and Okano, R. (2000). Neuraminidase-producing ability of intestinal bacteria isolated from coastal fish. *Lett. Appl. Microbiol.* 31,10-13.

Tamura, K., Nei, M., and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA* 101, 11030-11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol*. 30, 2725-2729.

Terceti, M. S., Ogut, H., and Osorio, C. R. (2016). *Photobacterium damselae* subsp. *damselae*, an emerging fish pathogen in the Black Sea: evidences of a multiclonal origin. *Appl. Environ. Microbiol*. 82, 3736-3745.

Terceti, M. S., Rivas, A. J., Alvarez, L., Noia, M., Cava, F., and Osorio CR. (2017). *rstB* regulates expression of the *Photobacterium damselae* subsp. *damselae* major virulence factors Damselysin, Phobalysin P and Phobalysin C. *Front. Microbiol*. 8, 582.

Terceti, M.S., Vences, A., Matanza, X.M., Dalsgaard, I., Pedersen, K., and Osorio, C.R. (2018). Molecular epidemiology of *Photobacterium damselae* subsp. *damselae* outbreaks in marine rainbow trout farms reveals extensive horizontal gene transfer and high genetic diversity. *Front Microbiol*. 9:2155.

Tran, T. K., Han, Q. Q., Shi, Y., and Guo, L. (2016). A comparative proteomic analysis of *Salmonella typhimurium* under the regulation of the RstA/RstB and PhoP/PhoQ systems. *Biochim. Biophys. Acta.* 1864, 1686-1695.

Vences, A., Rivas, A. J., Lemos, M. L., Husmann, M., and Osorio, C. R. (2017). Chromosome-encoded hemolysin, phospholipase, and collagenase in plasmidless isolates of *Photobacterium damselae* subsp. *damselae* contribute to virulence for fish. *Appl. Environ. Microbiol*. 83, pii: e00401-17.

Vera, P., Navas, J.I., and Fouz, B. (1991). First isolation of *Vibrio damsela* from seabream (*Sparus aurata*). *Bull. Eur. Ass. Fish Pathol*. 11, 112-113.

Witte G., Hartung S., Buttner K., Hopfner K.P. (2008). Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. *Mol Cell*.30, 167-178.

**Table 1.** Bacterial strains and plasmids used and constructed in this study.

| Strain or plasmid | Description[a] | Reference/Source |
|---|---|---|
| **Strains** | | |
| ***P. damselae subsp. damselae*** | | |
| RM-71 | Isolated from turbot; pPHDD1-harboring strain | Fouz *et al.*, 1992 |
| MT151 | RM-71Δ*rstB* | Terceti *et al.*, 2017 |
| MT319 | RM-71Δ*rstA* | This study |
| MT157 | MT151 with prstAB (complemented mutant); $Cm^r$ | Terceti *et al.*, 2017 |
| MT245 | MT319 with prstAB (complemented mutant); $Cm^r$ | This study |
| LD-07 | Isolated from Gilthead seabream; not carrier of pPHDD1 | Vera *et al.*, 1991 |
| MT341 | LD-07Δ*rstA* | This study |
| MT340 | LD-07Δ*rstB* | This study |
| AR217 | RM-71Δ*epsL* | Rivas *et al.*, 2015b |
| AR129 | RM-71Δ$hlyA_{ch}$ | Rivas et *al.*, 2013b |
| AR133 | RM-71Δ$hlyA_{pl}$ | Rivas et *al.*, 2011 |
| AR64 | RM-71Δ*dly* | Rivas et *al.*, 2011 |
| AR158 | RM-71Δ$hlyA_{pl}$Δ$hlyA_{ch}$ | Rivas et *al.*, 2013b |
| AR119 | RM-71Δ*dly*Δ$hlyA_{ch}$ | Rivas et *al.*, 2013b |
| AR78 | RM-71Δ*dly*Δ$hlyA_{pl}$ | Rivas et *al.*, 2011 |
| AR89 (3Δ) | RM-71Δ*dly*Δ$hlyA_{pl}$Δ$hlyA_{ch}$ (also dubbed here 3Δ) | Rivas et *al.*, 2013b |
| ***E. coli*** | | |
| DH5α | Cloning strain | Laboratory stock |
| S17-1-λpir | RP4-2 (Km::Tn7,Tc::Mu-1) *pro-82 λpir recA1 endA1 thiE1 hsdR17 creC510* | Herrero *et al.*, 1990 |
| B-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** | | |
| pLOFKm | *Tn*10-based delivery plasmid; $Km^r$ | Herrero *et al.*, 1990 |
| pMRB24 | Cloning vector, mob, $Cm^r$ | Le Roux *et al.*, 2011 |
| prstAB | pMRB24 with *rstAB* genes; $Cm^r$ | Terceti *et al.*, 2017 |
| pNidkan | Suice vector derived from pCVD442: $Km^r$ | Mouriño *et al.*, 2004 |
| pWKS30 | Low-copy vector; $Amp^r$ | |

[a] $Cm^r$, chloramphenicol resistance; $Km^r$, kanamycin resistance: $Em^r$, erythromycin resistance; $Tc^r$, tetracycline resistance; $Amp^r$, ampicillin resistance; Δ, gene deletion

**Table 2.** Oligonucleotides used in this study.

| Target and oligonucleotides | Sequence | bp amplified | Reference |
|---|---|---|---|
| ***rstA* deletion** | | | |
| *1-2 fragment* | | | |
| RstA-mut1-xbaI-F | GCTCTAGAGGCTATAATCCAAACAATGG | 1968 | This study |
| RstA-mut2-smaI-R | GCCCCGGGTTAACTAAAGAATTACGTGG | | |
| *3-4 fragment* | | | |
| RstA-mut3-smaI-F | GCCCCGGGTTACCGTCAGAATGTGTTTG | 1957 | This study |
| RstA-mut4-apaI-R | GCGGGCCCCCTTTATCCATATCAATTCC | | |
| *internal* | | | |
| RstA-intF | CTTATTATCAATGGTTCTGT | variable | This study |
| RstA-intR | TTGGCTGTATAAGAACCATG | | |
| **PhlyC (*hlyA$_{ch}$*) screening** | | | |
| phlyC-5' | AATGTTTCTTTCCGTTGGGC | 353 | Terceti *et al.*, 2018 |
| phlyC-3' | CCGGAGTTCCACCAGTAAAT | | |
| **PhlyP (*hlyA$_{pl}$*) screening** | | | |
| PhlyP-5' | GCTATAAATGAATAAGAAAA | 767 | Terceti *et al.*, 2016 |
| PhlyP-3' | TTGAAGCTAACTCAAAAA | | |
| **Dly (*dly*) screening** | | | |
| Dly-5' | CTCCTATGGGACATGAATGG | 549 | Terceti *et al.*, 2016 |
| Dly-3' | TGCTCTAGGCTAAATGAATC | | |
| **VDA_000112 screening** | | | |
| VDA_000112 int -F | CATCAGATCTCAATGGTGCC | 350 | This study |
| VDA_000112 int -R | TCTTTAATAGGGTCTACGTA | | |
| **VDA_002799 screening** | | | |
| VDA_002799 int-F | TCCATTTCGTTCTTGTTCGA | 1500 | Terceti *et al.*, 2018 |
| VDA_002799 int-R | GATTTTTCAACGGACGATAT | | |
| **A0J47_07530 screening** | | | |
| A0J47_07530 int-F | CATGGAGCCCCTAATGAACC | 340 | This study |
| A0J47_07530 int-R | AGTAAAATCAGCCGTTTGTT | | |
| **VDA_000358 screening** | | | |
| VDA_000358 int -F | TGTCAAAGCATGGCTAGTTG | 410 | This study |
| VDA_000358 int -R | AGTCGAGAAAACAATCGCAT | | |
| **RstAB system (*rstAB*) screening** | | | |
| Kinasa int - F | TCAGCTTCATAATCTTCTAG | 236 | Terceti *et al.*, 2018 |
| Kinasa int - R | AATAAGATTGTGAGTCTACG | | |

**Table 3.** Homology and function of the *Photobacterium damselae* subsp. *damselae* proteins identified in this study using blastP.

| Protein ID | Predicted Function | Homologue Matches | | | |
|---|---|---|---|---|---|
| | | Accesion no. | Species | Identities | E-value |
| **VDA_002460** (T2SS dependent) | Hypothetical protein | WP_005300136.1 | *P.damselae* subsp.*damselae* | 100% | 0.0 |
| | Lipoprotein putative | EHN69669.1 | *Alitivibrio fischeri* | 26% | 6e-48 |
| | Lipoprotein putative | SGY87728.1 | *Moritella viscosa* | 29% | 2e-38 |
| | Lipoprotein putative | AUL94513.1 | *Vibrio vulnificus* | 27% | 3e-38 |
| **VDA_000694** (T2SS dependent) | Sialidase | WP_005305077.1 | *P.damselae* subsp.*damselae* | 100% | 0.0 |
| | Sialidase | WP_095465352.1 | *Vibrio cholerae* | 53% | 0.0 |
| | Sialidase | WP_067416827.1 | *Enterovibrio coralii* | 66% | 0.0 |
| | Sialidase | WP_078753142.1 | *Enterovibrio nigricans* | 64% | 0.0 |
| | Sialidase | WP_107273569.1 | *Photobacterium phosphoreum* | 65% | 0.0 |
| | Sialidase | WP_107210839.1 | *Photobacterium kishitanii* | 63% | 0.0 |
| | Sialidase | WP_080159127.1 | *Photobacterium piscicola* | 61% | 0.0 |
| **A0J47_15850** (T2SS dependent) | Hypothetical protein | WP_068946047.1 | *P.damselae* subsp.*damselae* | 100% | 0.0 |
| | Hypothetical protein | WP_086775603.1 | *Vibrio coralliirubri* | 61% | 6e-163 |
| | Serine protease | WP_063437776.1 | *Enterobacter cloacae* | 40% | 1e-107 |
| | Peptidase S8 | WP_012441023.1 | *Erwinia tasmaniensis* | 42% | 5e-107 |
| | Serine protease | WP_088205268.1 | *Enterobacter bugandensis* | 41% | 1e-105 |
| | Protease | CBJ46888.1 | *Erwinia amylovora* | 41% | 4e-102 |
| | Serine protease | WP_047054006.1 | *Enterobacter hormaechei* | 41% | 5e-97 |
| | Peptidase S8 | WP_012147543.1 | *Serratiaproteamaculans* | 39% | 6e-98 |
| **VDA_002799** (T2SS and RstAB dependent) | twin-arginine translocation pathway signal | WP_005301039.1 | *P.damselae* subsp.*damselae* | 100% | 0.0 |
| | twin-arginine translocation pathway signal | WP_044176547.1 | *P. damselae* subsp.*piscicida* | 99% | 0.0 |
| | twin-arginine translocation pathway signal | KJG31257.1 | *Photobacterium angustum* | 53% | 0.0 |
| | twin-arginine translocation pathway signal | WP_048246392.1 | *Laetiporus sulphureus* | 35% | 3e-84 |
| | twin-arginine translocation pathway signal | WP_059958603.1 | *Burkholderia cepacia* | 34% | 2e-82 |
| **A0J47_09785** (T2SS dependent) | Hypothetical protein | ODA21114.1 | *P.damselae* subsp.*damselae* | 100% | 0.0 |
| | trypsin family protein | WP_044175593.1 | *P. damselae* subsp.*piscicida* | 30% | 2e-23 |
| | peptidase S1 and S6, chymotrypsin/Hap | OAA35492.1 | *Cordyceps brongniartii* | 60% | 3e-103 |
| | Metalloprotease | PMB63986.1 | *Beauveria bassiana* | 58% | 1e-99 |
| | Hemolysin | WP_011517597.1 | *Cupriavidus metallidurans* | 53% | 6e-96 |
| | Hemolysin | WP_084624908.1 | *Xanthomonas cassavae* | 55% | 2e-94 |

**Table 3. (cont.)** Homology and function of the *Photobacterium damselae* subsp. *damselae* proteins identified in this study using blastP.

| Protein ID | Predicted Function | Homologue Matches | | | |
|---|---|---|---|---|---|
| | | Accesion no. | Species | Identities | E-value |
| **VDA_000112** (T2SS and RstAB dependent) | Hypothetical protein | WP_005306883.1 | *P.damselae* subsp.*damselae* | 100% | 0.0 |
| | Hypothetical protein | WP_023602899.1 | *Alitvibrio logei* | 40% | 3e-57 |
| | Hypothetical protein | WP_012552220.1 | *Alitvibrio salmonicida* | 39% | 3e-59 |
| | Hypothetical protein | WP_005431384.1 | *Vibrio campbelliti* | 39% | 4e-58 |
| | Hypothetical protein | WP_075478204.1 | *Moritella viscosa* | 41% | 2e-56 |
| | Lipase chaperone | WP_061066385.1 | *Vibrio harveyi* | 39% | 5e-56 |
| | sugar-binding protein | WP_110077592.1 | *Clostridium perfringens* | 35% | 1e-37 |
| **VDA_000358** (T2SS and RstAB dependent) | Hypothetical protein | SPY44430.1 | *P.damselae* subsp.*damselae* | 100% | 0.0 |
| | Hypothetical protein | WP_068968745.1 | *P. damselae* subsp.*piscicida* | 96% | 2e-168 |
| | Hypothetical protein | WP_107282639.1 | *Photobacterium lipolyticum* | 30% | 3e-20 |
| | Hypothetical protein | WP_107253499.1 | *Photobacterium indicum* | 32% | 4e-17 |
| | Hypothetical protein | WP_006231910.1 | *Photobacterium profundum* | 30% | 2e-15 |
| **VDA_000966** (T2SS dependent) | Hypothetical protein | WP_005305856.1 | *P.damselae* subsp.*damselae* | 100% | 3e-135 |
| | Porin family protein | WP_115060987.1 | *Photobacterium damselae* | 96% | 2e-133 |
| | Hypothetical protein | WP_044178211.1 | *P. damselae* subsp *piscicida* | 96% | 2e-126 |
| | Porin family protein | WP_001960583.1 | *Vibrio cholerae* | 40% | 4e-32 |
| | Porin family protein | WP_104970264.1 | *Vibrio diaboilicus* | 48% | 4e-48 |
| | Porin family protein | WP_025535933.1 | *Vibrio parahaemolyticus* | 46% | 1e-46 |
| **VDA_000738** (T2SS dependent) | competence ComEA helix-hairpin-helix repeat | WP_005305210.1 | *P.damselae* subsp.*damselae* | 100% | 1e-105 |
| | competence ComEA helix-hairpin-helix repeat | WP_044179754.1 | *P. damselae* subsp.*piscicida* | 99% | 9e-109 |
| | competence ComEA helix-hairpin-helix repeat | KFE32301.1 | *Vibrio cholerae* | 57% | 2e-15 |
| | competence ComEA helix-hairpin-helix repeat | WP_105062336.1 | *Photobacterium angustum* | 64% | 4e-58 |
| | competence ComEA helix-hairpin-helix repeat | WP_107234348.1 | *Photobacterium leiognathi* | 60% | 3e-54 |
| | competence ComEA helix-hairpin-helix repeat | WP_006229811.1 | *Photobacterium profundun* | 57% | 1e-50 |
| | competence ComEA helix-hairpin-helix repeat | WP_107252572.1 | *Photobacterium indicum* | 56% | 7e-50 |
| **AJ047_07530** (T2SS and RstAB dependent) | Hypothetical protein | WP_036764371.1 | *P. damselae* subsp.*damselae* | 100% | 0.0 |
| | Hypothetical protein | WP_086957603.1 | *P. damselae* subsp.*piscicida* | 96% | 7e-90 |
| | Hypothetical protein | WP_009601554.1 | *Vibriocaribbeanicus* | 32% | 1e-09 |
| | Hypothetical protein | ODU31474.1 | *Xanthomonadaceae bacterium* | 32% | 3e-06 |
| | Hypothetical protein | WP_071102108.1 | *Moorea producens* | 34% | 1e-04 |
| | Hypothetical protein | WP_036755189.1 | *Photobacterium galatheae* | 31% | 2e-05 |

**Table 4.** Analysis of the proteins identified in this study by Pfam 31.0 / * not find any Pfam-A matches to search sequence.

| Acession ID | Family/Domain | Description | Clan | Envelope | | Alignment | | Bit score | E-value |
|---|---|---|---|---|---|---|---|---|---|
| | | | | start | end | start | end | | |
| VDA_002460 * | - | - | - | - | - | - | - | - | - |
| VDA_000694 | Sial-lect-inser (PF09264.9) | *Vibrio cholerae* sialidase, lectin insertion | CL0004 | 67 | 233 | 82 | 214 | 36.7 | 8e-09 |
| | Sial-lect-inser (PF09264.9) | *Vibrio cholerae* sialidase, lectin insertion | CL0004 | 345 | 542 | 346 | 542 | 105.6 | 2e-30 |
| | BNR_2 (PF13088.5) | BNR repeat-like domain | CL0434 | 493 | 751 | 546 | 746 | 53.2 | 4e-14 |
| A0J47_15850 | Peptidase_S8 (PF00082.21) | Subtilase family | N/a | 71 | 458 | 207 | 442 | 53.2 | 4e-14 |
| VDA_002799 | Endotoxin_N (PF03945.13) | delta endotoxin, N-terminal domain | N/a | 65 | 273 | 68 | 273 | 73.5 | 8e-20 |
| A0J47_09785 | Trypsin (PF00089.25) | Trypsin | CL0124 | 50 | 273 | 63 | 273 | 31.5 | 4e-07 |
| VDA_000112* | - | - | - | - | - | - | - | - | - |
| VDA_000358* | - | - | - | - | - | - | - | - | - |
| VDA_000966 | OMP_b-brl (PF13505.5) | Outer membrane protein beta-barrel domain | CL0193 | 9 | 190 | 13 | 190 | 49.2 | 1e-13 |
| VDA_000738 | HHH_3 (PF12836.6) | Helix-hairpin-helix motif | CL0198 | 95 | 160 | 96 | 159 | 73.6 | 1e-20 |
| AJ047_07530* | - | - | - | - | - | - | - | - | - |

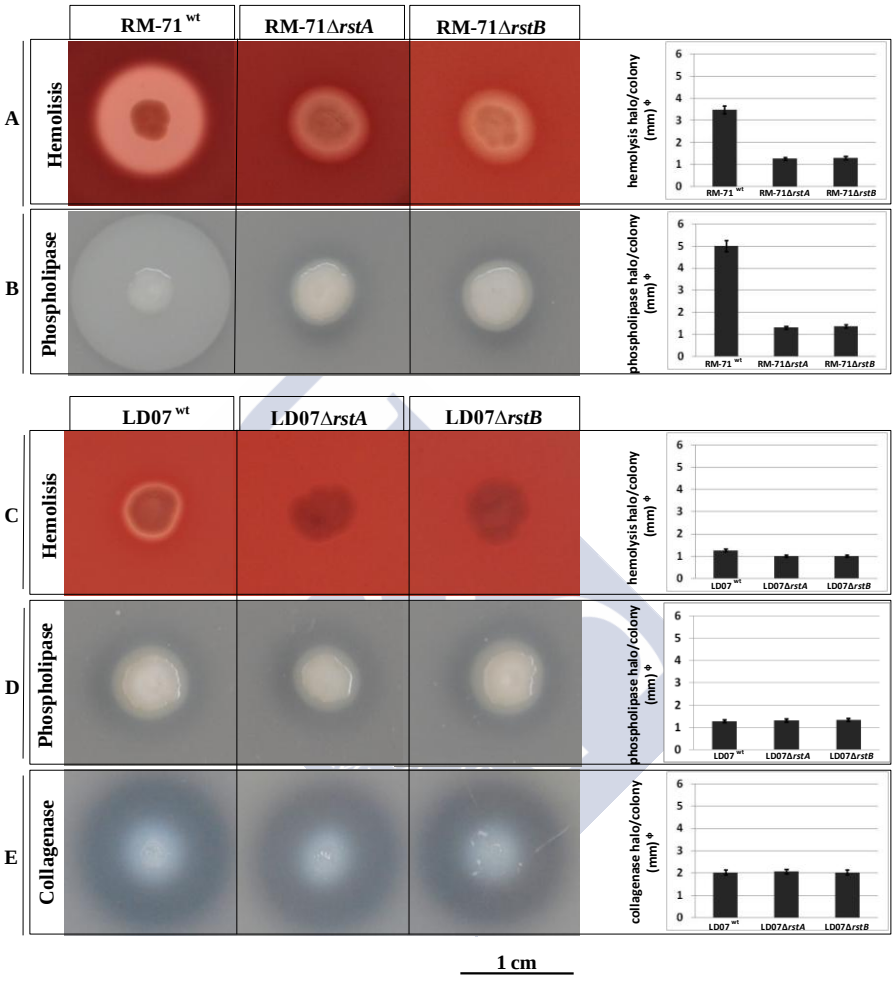

RM-71 wt
RM-71ΔrstA
RM-71ΔrstB
A
Hemolisis
hemolysis halo/colony (mm) Φ
B
Phospholipase
phospholipase halo/colony (mm) Φ
LD07 wt
LD07ΔrstA
LD07ΔrstB
C
Hemolisis
hemolysis halo/colony (mm) Φ
D
Phospholipase
phospholipase halo/colony (mm) Φ
E
Collagenase
collagenase halo/colony (mm) Φ
1 cm

Fig. 1

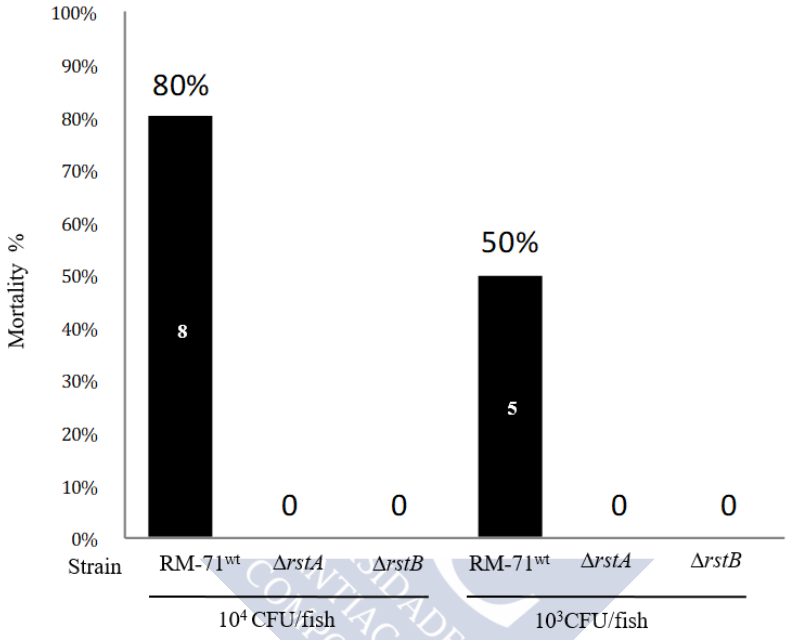

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Mortality %
80%
8
0
0
50%
5
0
0
Strain
RM-71wt
ΔrstA
ΔrstB
RM-71wt
ΔrstA
ΔrstB
10⁴ CFU/fish
10³CFU/fish

Fig. 2

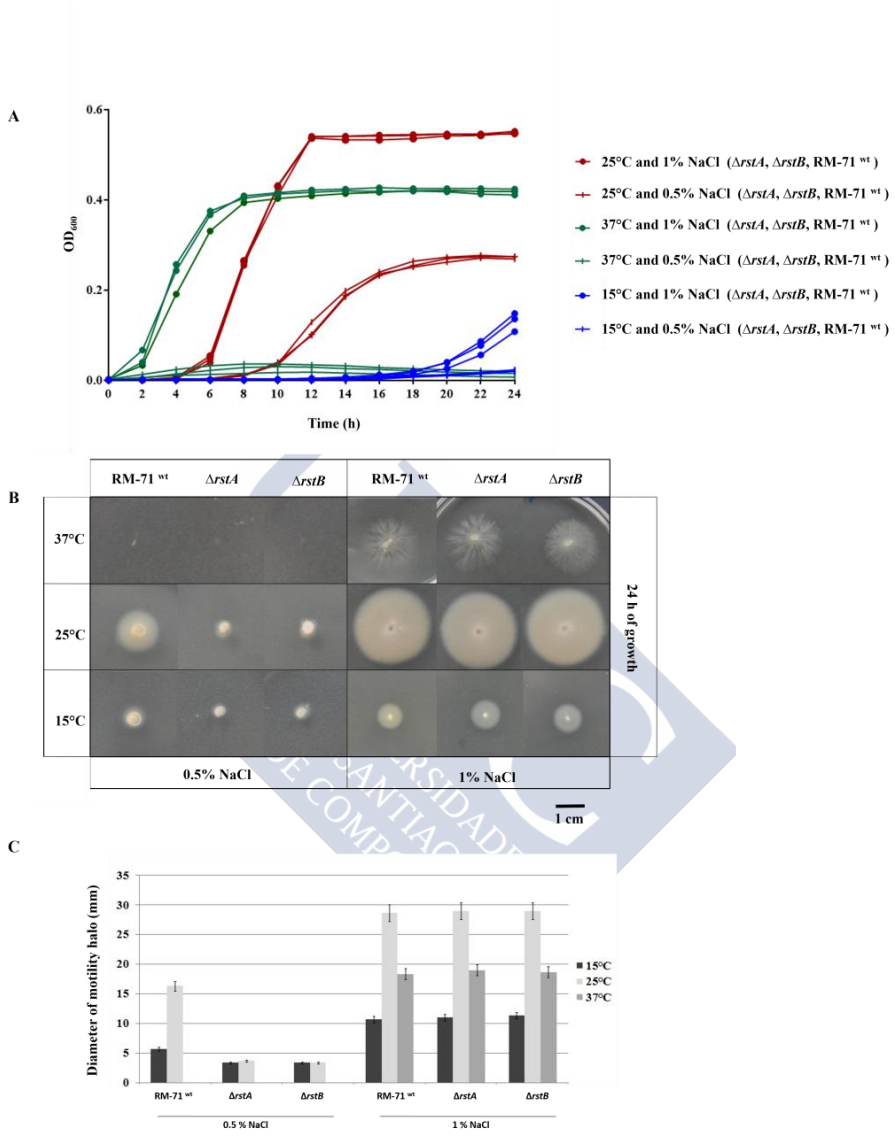

A
0.6
0.4
0.2
0.0
OD600
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
25°C and 1% NaCl (ΔrstA, ΔrstB, RM-71 wt)
25°C and 0.5% NaCl (ΔrstA, ΔrstB, RM-71 wt)
37°C and 1% NaCl (ΔrstA, ΔrstB, RM-71 wt)
37°C and 0.5% NaCl (ΔrstA, ΔrstB, RM-71 wt)
15°C and 1% NaCl (ΔrstA, ΔrstB, RM-71 wt)
15°C and 0.5% NaCl (ΔrstA, ΔrstB, RM-71 wt)
B
RM-71 wt
ΔrstA
ΔrstB
RM-71 wt
ΔrstA
ΔrstB
37°C
25°C
15°C
24 h of growth
0.5% NaCl
1% NaCl
1 cm
C
Diameter of motility halo (mm)
35
30
25
20
15
10
5
0
RM-71 wt
ΔrstA
ΔrstB
RM-71 wt
ΔrstA
ΔrstB
0.5 % NaCl
1 % NaCl
15°C
25°C
37°C

Fig. 3

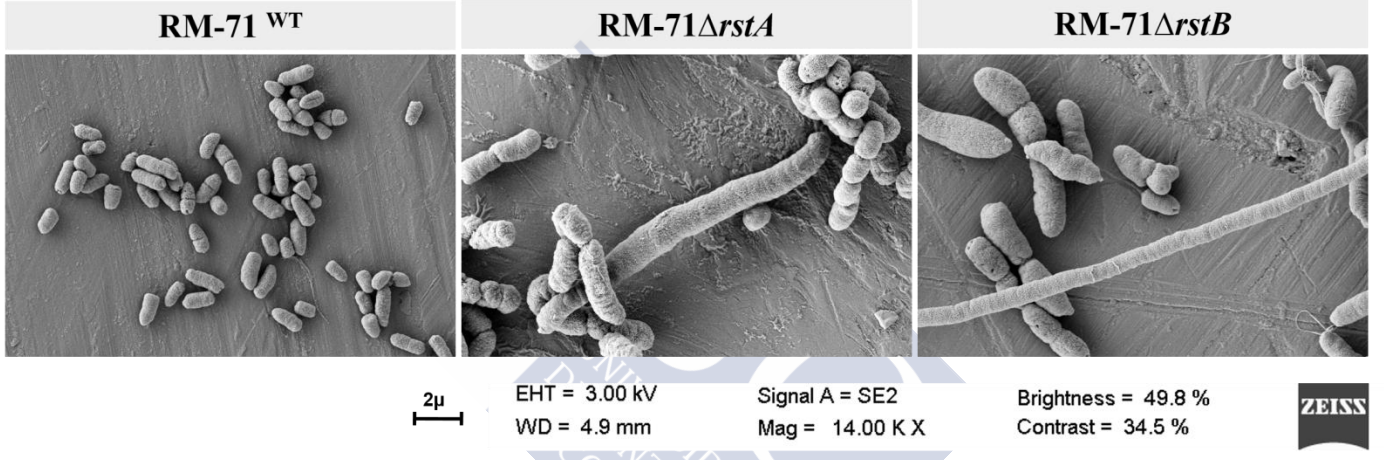

RM-71 WT
RM-71Δrst*A*
RM-71Δrst*B*
2µ
EHT = 3.00 kV
WD = 4.9 mm
Signal A = SE2
Mag = 14.00 K X
Brightness = 49.8 %
Contrast = 34.5 %
ZEISS

Fig. 4

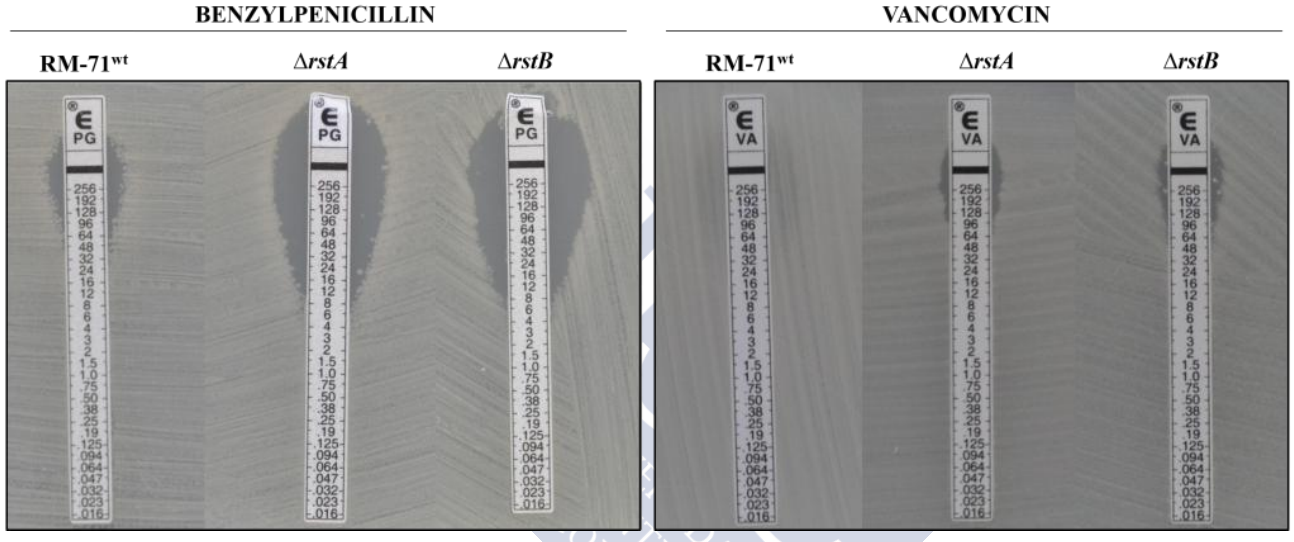
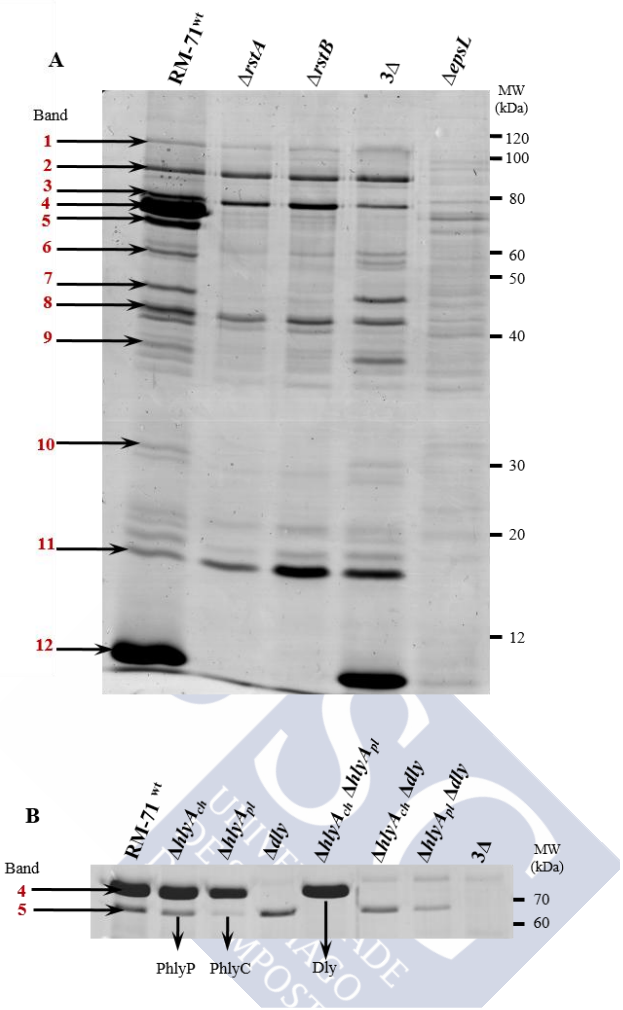

BENZYLPENICILLIN
VANCOMYCIN
RM-71wt
ΔrstA
ΔrstB
RM-71wt
ΔrstA
ΔrstB
PG
VA

Fig. 5




C

| Band | Acession ID | Function | Unique Peptides | Peptide Spectrum Matches | Predicted Signal Peptide | Mature protein MW | T2SS dependent | RstAB dependent |
|---|---|---|---|---|---|---|---|---|
| 1 | VDA_002460 | Lipoprotein (putative) | 26 | 48 | Sec1-25[AAA-TP] | 95.92 | yes | no |
| 2 | VDA_000694 | Sialidase (putative) | 36 | 271 | Sec1-25[AHV-YQ] | 83.05 | yes | no |
| 3 | A0J47_15850 | Serine protease (putative) | 62 | 447 | Sec1-23[TLA-NN] | 72.02 | yes | no |
| 4 | VDA_000159 | Damselisin (Dly) * | - | - | Sec1-19[AYA-FT] | 63.08 | yes | yes |
| 5 | VDA_000160 | Phobalisin P (PhlyP) * | - | - | Sec1-23[AIA-EV] | 65.62 | yes | yes |
| | VDA_002420 | Phobalisin C (PhlyC) * | - | - | Sec1-23[AIA-DV] | 65.80 | yes | yes |
| 6 | VDA_002799 | Delta-endotoxin (putative) | 22 | 89 | Tat1-30[SRA-NV] | 60.25 | yes | yes |
| 7 | VDA_002799 | Delta-endotoxin (putative) | 21 | 94 | Tat1-30[SRA-NV] | 60.25 | yes | yes |
| 8 | A0J47_09785 | Uncharacterized protein | 5 | 26 | Sec1-19[SNA-VV] | 43.94 | yes | no |
| 9 | VDA_000112 | Uncharacterized protein | 10 | 18 | Sec1-19[ANA-TQ] | 35.23 | yes | yes |
| 10 | VDA_000358 | Uncharacterized protein | 6 | 6 | Sec1-35[VSA-KV] | 24.83 | yes | yes |
| 11 | VDA_000966 | Uncharacterized protein | 9 | 17 | Sec1-22[TWA-AT] | 18.75 | yes | no |
| | VDA_000738 | Uncharacterized protein | 4 | 12 | Sec1-19[SQA-AT] | 15.05 | yes | no |
| 12 | A0J47_07530 | Uncharacterized protein | 5 | 9 | Sec1-21[SMA-NV] | 11.38 | yes | yes |

Fig. 6

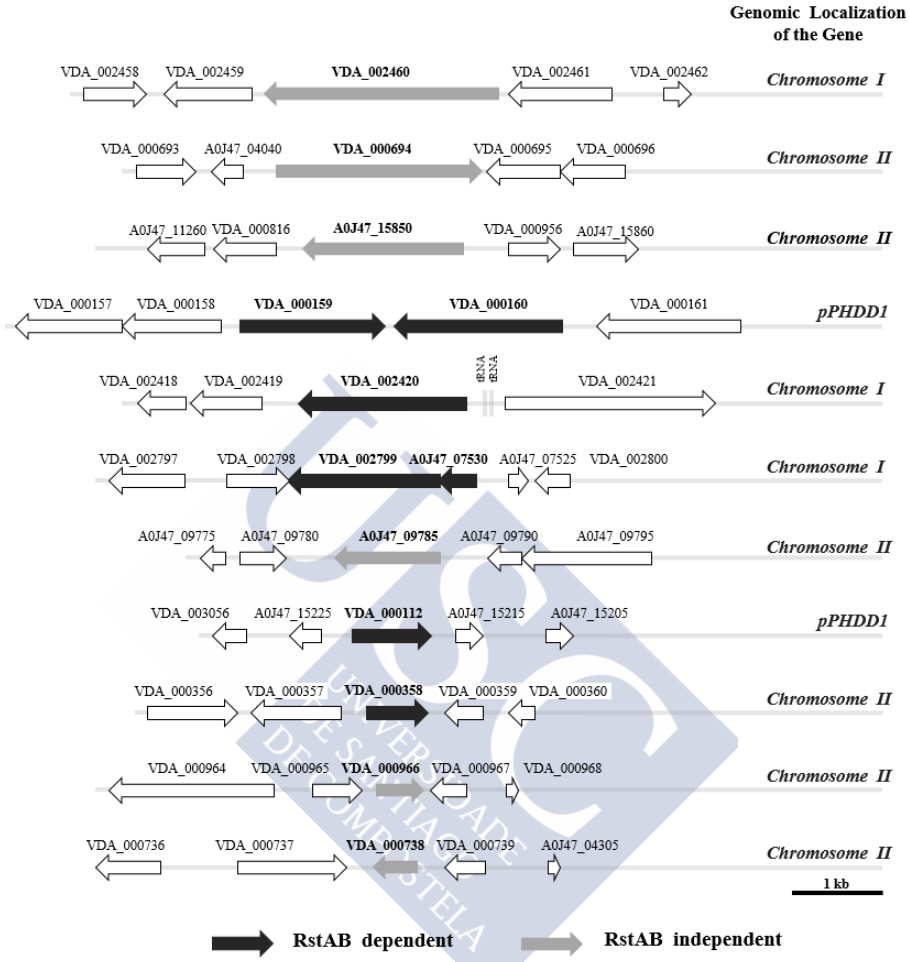

Genomic Localization of the Gene
VDA_002458 VDA_002459 VDA_002460 VDA_002461 VDA_002462 Chromosome I
VDA_000693 A0J47_04040 VDA_000694 VDA_000695 VDA_000696 Chromosome II
A0J47_11260 VDA_000816 A0J47_15850 VDA_000956 A0J47_15860 Chromosome II
VDA_000157 VDA_000158 VDA_000159 VDA_000160 VDA_000161 pPHDD1
VDA_002418 VDA_002419 VDA_002420 tRNA tRNA VDA_002421 Chromosome I
VDA_002797 VDA_002798 VDA_002799 A0J47_07530 A0J47_07525 VDA_002800 Chromosome I
A0J47_09775 A0J47_09780 A0J47_09785 A0J47_09790 A0J47_09795 Chromosome II
VDA_003056 A0J47_15225 VDA_000112 A0J47_15215 A0J47_15205 pPHDD1
VDA_000356 VDA_000357 VDA_000358 VDA_000359 VDA_000360 Chromosome II
VDA_000964 VDA_000965 VDA_000966 VDA_000967 VDA_000968 Chromosome II
VDA_000736 VDA_000737 VDA_000738 VDA_000739 A0J47_04305 Chromosome II
1 kb
RstAB dependent
RstAB independent

Fig.7

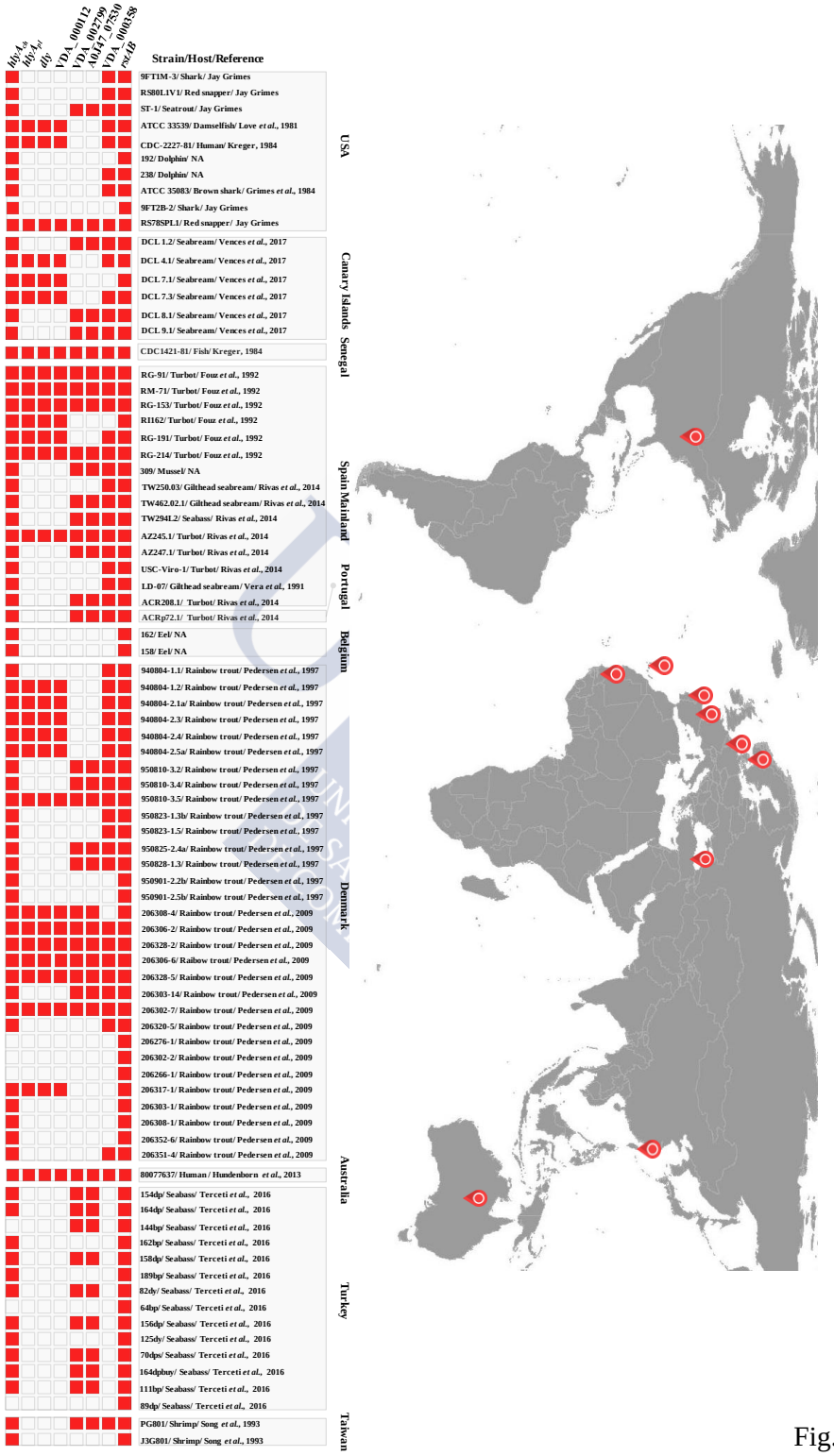

$hly^{+}A_{ch}$
$hly^{+}A_{fl}$
$hly^{-}$
VDA_0001112
VDA_0027599
A0A347_07530
VDA_0000358
pcAB
Strain/Host/Reference
9FT1M-3/ Shark/ Jay Grimes
RS80L1V1/ Red snapper/ Jay Grimes
ST-1/ Seatrout/ Jay Grimes
ATCC 33539/ Damselfish/ Love et al., 1981
CDC-2227-81/ Human/ Kreger, 1984
192/ Dolphin/ NA
238/ Dolphin/ NA
ATCC 35083/ Brown shark/ Grimes et al., 1984
9FT2B-2/ Shark/ Jay Grimes
RS78SPL1/ Red snapper/ Jay Grimes
DCL 1.2/ Seabream/ Vences et al., 2017
DCL 4.1/ Seabream/ Vences et al., 2017
DCL 7.1/ Seabream/ Vences et al., 2017
DCL 7.3/ Seabream/ Vences et al., 2017
DCL 8.1/ Seabream/ Vences et al., 2017
DCL 9.1/ Seabream/ Vences et al., 2017
CDC1421-81/ Fish/ Kreger, 1984
RG-91/ Turbot/ Fouz et al., 1992
RM-71/ Turbot/ Fouz et al., 1992
RG-153/ Turbot/ Fouz et al., 1992
RI162/ Turbot/ Fouz et al., 1992
RG-191/ Turbot/ Fouz et al., 1992
RG-214/ Turbot/ Fouz et al., 1992
309/ Mussel/ NA
TW250.03/ Gilthead seabream/ Rivas et al., 2014
TW462.02.1/ Gilthead seabream/ Rivas et al., 2014
TW294L2/ Seabass/ Rivas et al., 2014
AZ245.1/ Turbot/ Rivas et al., 2014
AZ247.1/ Turbot/ Rivas et al., 2014
USC-Viro-1/ Turbot/ Rivas et al., 2014
LD-07/ Gilthead seabream/ Vera et al., 1991
ACR208.1/ Turbot/ Rivas et al., 2014
ACRp72.1/ Turbot/ Rivas et al., 2014
162/ Eel/ NA
158/ Eel/ NA
940804-1.1/ Rainbow trout/ Pedersen et al., 1997
940804-1.2/ Rainbow trout/ Pedersen et al., 1997
940804-2.1a/ Rainbow trout/ Pedersen et al., 1997
940804-2.3/ Rainbow trout/ Pedersen et al., 1997
940804-2.4/ Rainbow trout/ Pedersen et al., 1997
940804-2.5a/ Rainbow trout/ Pedersen et al., 1997
950810-3.2/ Rainbow trout/ Pedersen et al., 1997
950810-3.4/ Rainbow trout/ Pedersen et al., 1997
950810-3.5/ Rainbow trout/ Pedersen et al., 1997
950823-1.3b/ Rainbow trout/ Pedersen et al., 1997
950823-1.5/ Rainbow trout/ Pedersen et al., 1997
950825-2.4a/ Rainbow trout/ Pedersen et al., 1997
950828-1.3/ Rainbow trout/ Pedersen et al., 1997
950901-2.2b/ Rainbow trout/ Pedersen et al., 1997
950901-2.5b/ Rainbow trout/ Pedersen et al., 1997
206308-4/ Rainbow trout/ Pedersen et al., 2009
206306-2/ Rainbow trout/ Pedersen et al., 2009
206328-2/ Rainbow trout/ Pedersen et al., 2009
206306-6/ Rainbow trout/ Pedersen et al., 2009
206328-5/ Rainbow trout/ Pedersen et al., 2009
206303-14/ Rainbow trout/ Pedersen et al., 2009
206302-7/ Rainbow trout/ Pedersen et al., 2009
206320-5/ Rainbow trout/ Pedersen et al., 2009
206276-1/ Rainbow trout/ Pedersen et al., 2009
206302-2/ Rainbow trout/ Pedersen et al., 2009
206266-1/ Rainbow trout/ Pedersen et al., 2009
206317-1/ Rainbow trout/ Pedersen et al., 2009
206303-1/ Rainbow trout/ Pedersen et al., 2009
206308-1/ Rainbow trout/ Pedersen et al., 2009
206352-6/ Rainbow trout/ Pedersen et al., 2009
206351-4/ Rainbow trout/ Pedersen et al., 2009
80077637/ Human / Hundenborn et al., 2013
154dp/ Seabass/ Terceti et al., 2016
164dp/ Seabass/ Terceti et al., 2016
144bp/ Seabass/ Terceti et al., 2016
162bp/ Seabass/ Terceti et al., 2016
158dp/ Seabass/ Terceti et al., 2016
189bp/ Seabass/ Terceti et al., 2016
82dy/ Seabass/ Terceti et al., 2016
64bp/ Seabass/ Terceti et al., 2016
156dp/ Seabass/ Terceti et al., 2016
125dy/ Seabass/ Terceti et al., 2016
70dps/ Seabass/ Terceti et al., 2016
164dpbuy/ Seabass/ Terceti et al., 2016
111bp/ Seabass/ Terceti et al., 2016
89dp/ Seabass/ Terceti et al., 2016
PG801/ Shrimp/ Song et al., 1993
J3G801/ Shrimp/ Song et al., 1993
USA
Canary Islands
Senegal
Spain Mainland
Portugal
Belgium
Denmark
Australia
Turkey
Taiwan

Fig. 8

## Supplementary Material

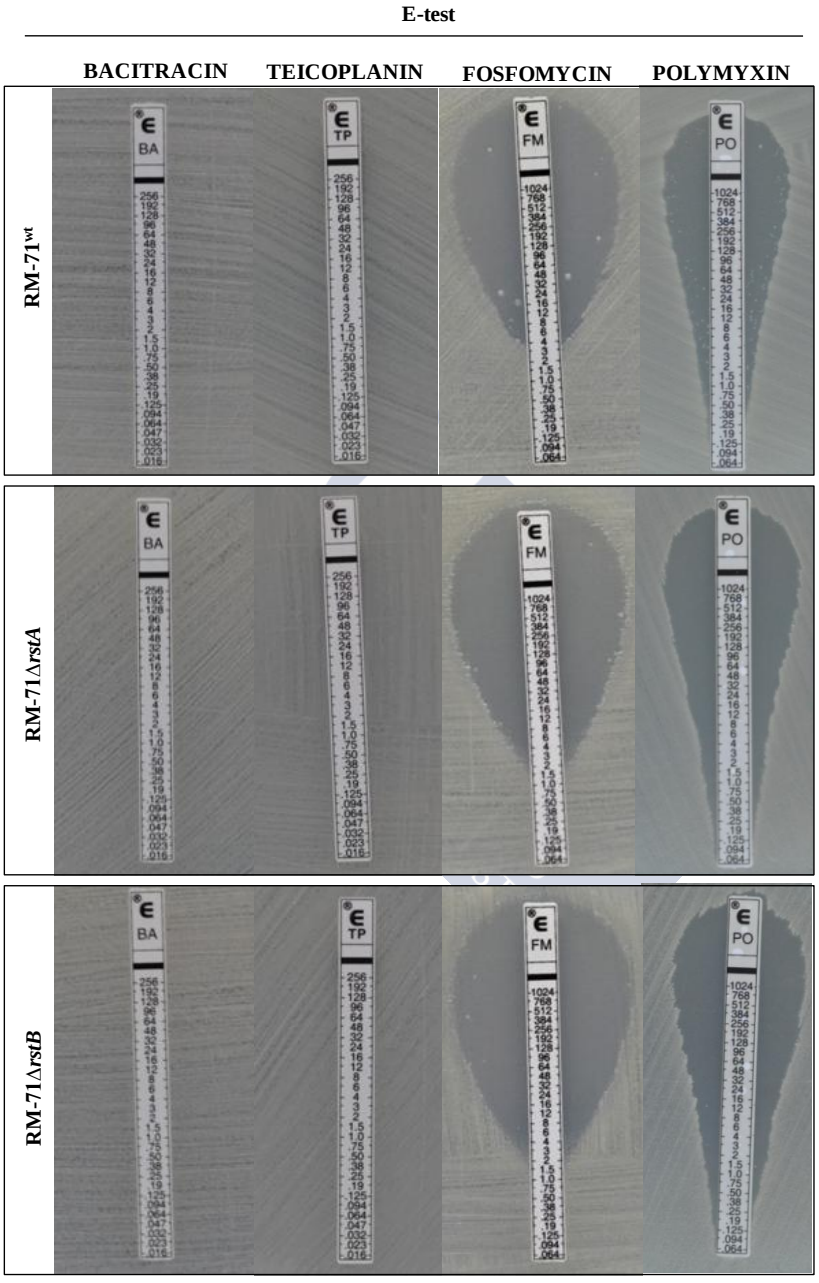

E-test
BACITRACIN
TEICOPLANIN
FOSFOMYCIN
POLYMYXIN
RM-71wt
RM-71ΔrstA
RM-71ΔrstB
BA
TP
FM
PO

Fig. S1

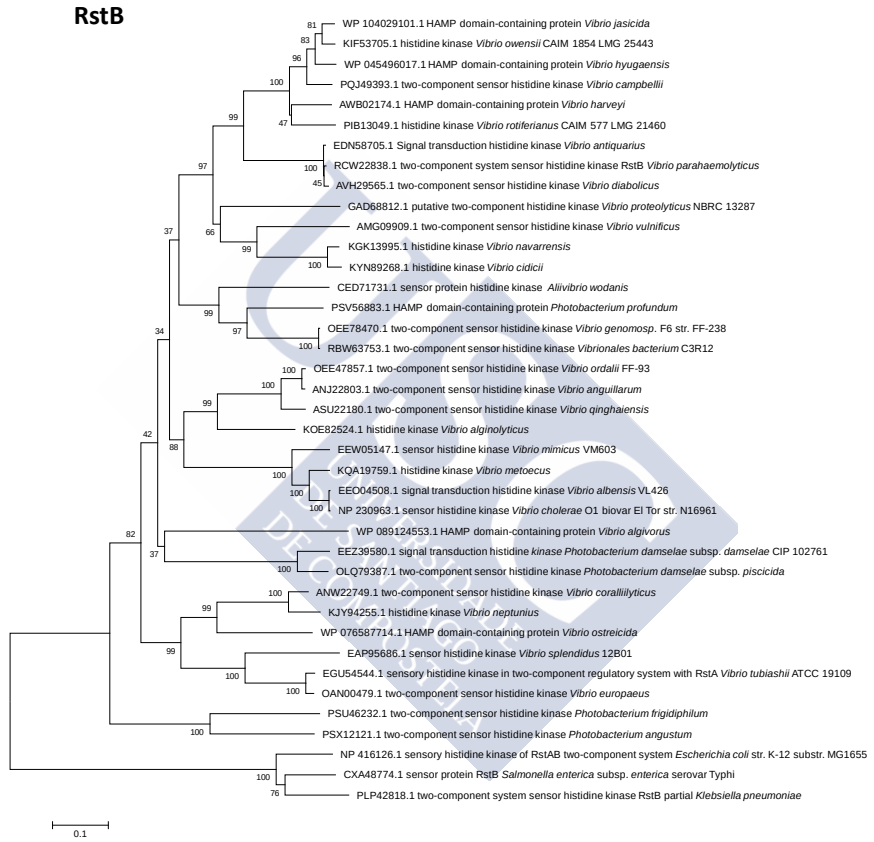

RstB
WP 104029101.1 HAMP domain-containing protein Vibrio jasicida
KIF53705.1 histidine kinase Vibrio owensii CAIM 1854 LMG 25443
WP 045496017.1 HAMP domain-containing protein Vibrio hyugaensis
PQJ49393.1 two-component sensor histidine kinase Vibrio campbellii
AWB02174.1 HAMP domain-containing protein Vibrio harveyi
PIB13049.1 histidine kinase Vibrio rotiferianus CAIM 577 LMG 21460
EDN58705.1 Signal transduction histidine kinase Vibrio antiquarius
RCW22838.1 two-component system sensor histidine kinase RstB Vibrio parahaemolyticus
AVH29565.1 two-component sensor histidine kinase Vibrio diabolicus
GAD68812.1 putative two-component histidine kinase Vibrio proteolyticus NBRC 13287
AMG09909.1 two-component sensor histidine kinase Vibrio vulnificus
KGK13995.1 histidine kinase Vibrio navarrensis
KYN89268.1 histidine kinase Vibrio cidicii
CED71731.1 sensor protein histidine kinase Alivibrio wodanis
PSV56883.1 HAMP domain-containing protein Photobacterium profundum
OEE78470.1 two-component sensor histidine kinase Vibrio genomosp. F6 str. FF-238
RBW63753.1 two-component sensor histidine kinase Vibrionales bacterium C3R12
OEE47857.1 two-component sensor histidine kinase Vibrio ordalii FF-93
ANJ22803.1 two-component sensor histidine kinase Vibrio anguillarum
ASU22180.1 two-component sensor histidine kinase Vibrio qinghaiensis
KOE82524.1 histidine kinase Vibrio alginolyticus
EEW05147.1 sensor histidine kinase Vibrio mimicus VM603
KQA19759.1 histidine kinase Vibrio metoecus
EEO04508.1 signal transduction histidine kinase Vibrio albensis VL426
NP 230963.1 sensor histidine kinase Vibrio cholerae O1 biovar El Tor str. N16961
WP 08912453.1 HAMP domain-containing protein Vibrio algivorus
EEZ39580.1 signal transduction histidine kinase Photobacterium damselae subsp. damselae CIP 102761
OLQ79387.1 two-component sensor histidine kinase Photobacterium damselae subsp. piscicida
ANW22749.1 two-component sensor histidine kinase Vibrio coralliilyticus
KJY94255.1 histidine kinase Vibrio neptunius
WP 076587714.1 HAMP domain-containing protein Vibrio ostreicida
EAP95686.1 sensor histidine kinase Vibrio splendidus 12B01
EGU54544.1 sensory histidine kinase in two-component regulatory system with RstA Vibrio tubiashii ATCC 19109
OAN00479.1 two-component sensor histidine kinase Vibrio europaeus
PSU46232.1 two-component sensor histidine kinase Photobacterium frigidiphilum
PSX12121.1 two-component sensor histidine kinase Photobacterium angustum
NP 416126.1 sensory histidine kinase of RstAB two-component system Escherichia coli str. K-12 substr. MG1655
CXA48774.1 sensor protein RstB Salmonella enterica subsp. enterica serovar Typhi
PLP42818.1 two-component system sensor histidine kinase RstB partial Klebsiella pneumoniae
0.1

Fig. S2

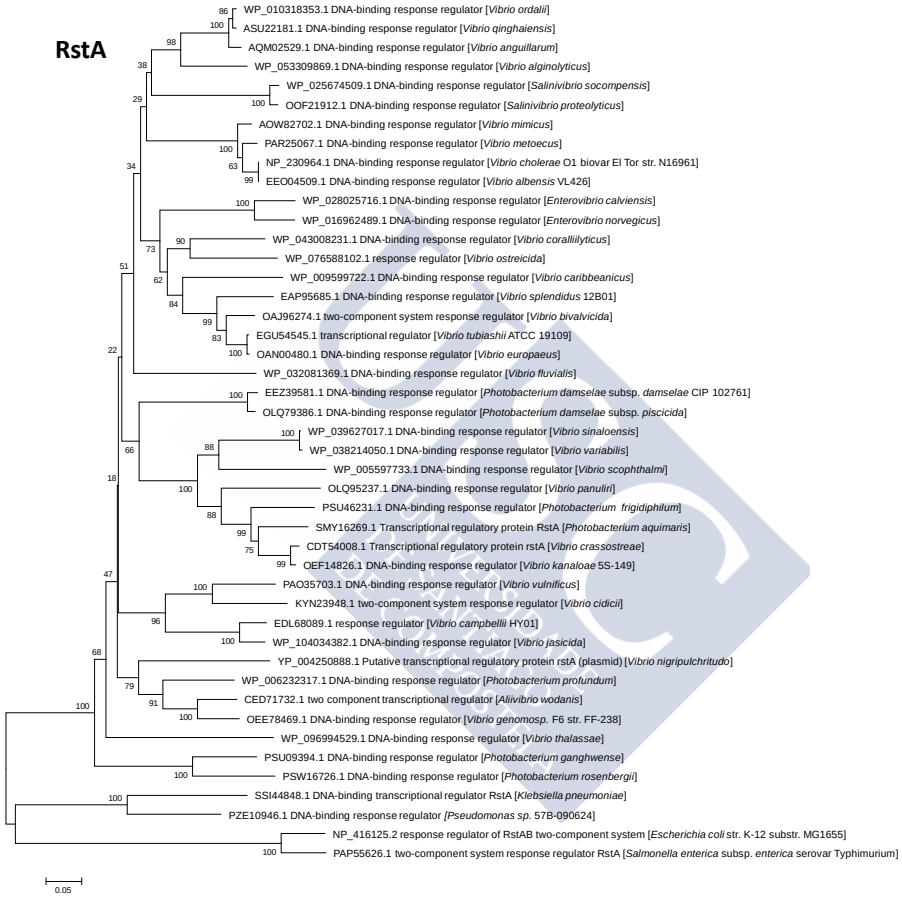

RstA
WP_010318353.1 DNA-binding response regulator [Vibrio ordalii]
ASU22181.1 DNA-binding response regulator [Vibrio qinghaiensis]
AQM02529.1 DNA-binding response regulator [Vibrio anguillarum]
WP_053309869.1 DNA-binding response regulator [Vibrio alginolyticus]
WP_025674509.1 DNA-binding response regulator [Salinivibrio socompensis]
OOF21912.1 DNA-binding response regulator [Salinivibrio proteolyticus]
AOW82702.1 DNA-binding response regulator [Vibrio mimicus]
PAR25067.1 DNA-binding response regulator [Vibrio metoecus]
NP_230964.1 DNA-binding response regulator [Vibrio cholerae O1 biovar El Tor str. N16961]
EEO04509.1 DNA-binding response regulator [Vibrio albensis VL426]
WP_028025716.1 DNA-binding response regulator [Enterovibrio calviensis]
WP_016962489.1 DNA-binding response regulator [Enterovibrio norvegicus]
WP_043008231.1 DNA-binding response regulator [Vibrio coralliilyticus]
WP_076588102.1 response regulator [Vibrio ostreicida]
WP_009599722.1 DNA-binding response regulator [Vibrio caribbeanicus]
EAP95685.1 DNA-binding response regulator [Vibrio splendidus 12B01]
OAJ96274.1 two-component system response regulator [Vibrio bivalvicida]
EGU54545.1 transcriptional regulator [Vibrio tubiashii ATCC 19109]
OAN00480.1 DNA-binding response regulator [Vibrio europaeus]
WP_032081369.1 DNA-binding response regulator [Vibrio fluvialis]
EEZ39581.1 DNA-binding response regulator [Photobacterium damselae subsp. damselae CIP 102761]
OLQ79386.1 DNA-binding response regulator [Photobacterium damselae subsp. piscicida]
WP_039627017.1 DNA-binding response regulator [Vibrio sinaloensis]
WP_038214050.1 DNA-binding response regulator [Vibrio variabilis]
WP_005597733.1 DNA-binding response regulator [Vibrio scophthalmi]
OLQ95237.1 DNA-binding response regulator [Vibrio panulirii]
PSU46231.1 DNA-binding response regulator [Photobacterium frigidiphilum]
SMY16269.1 Transcriptional regulatory protein RstA [Photobacterium aquimaris]
CDT54008.1 Transcriptional regulatory protein rstA [Vibrio crassostreae]
OEF14826.1 DNA-binding response regulator [Vibrio kanaloae 5S-149]
PAO35703.1 DNA-binding response regulator [Vibrio vulnificus]
KYN23948.1 two-component system response regulator [Vibrio cidicii]
EDL68089.1 response regulator [Vibrio campbellii HY01]
WP_104034382.1 DNA-binding response regulator [Vibrio jasicida]
YP_004250888.1 Putative transcriptional regulatory protein rstA (plasmid) [Vibrio nigripulchritudo]
WP_006232317.1 DNA-binding response regulator [Photobacterium profundum]
CED71732.1 two component transcriptional regulator [Aliivibrio wodanis]
OEE78469.1 DNA-binding response regulator [Vibrio genomosp. F6 str. FF-238]
WP_096994529.1 DNA-binding response regulator [Vibrio thalassae]
PSU09394.1 DNA-binding response regulator [Photobacterium ganghwense]
PSW16726.1 DNA-binding response regulator [Photobacterium rosenbergii]
SSI44848.1 DNA-binding transcriptional regulator RstA [Klebsiella pneumoniae]
PZE10946.1 DNA-binding response regulator [Pseudomonas sp. 57B-090624]
NP_416125.2 response regulator of RstAB two-component system [Escherichia coli str. K-12 substr. MG1655]
PAP55626.1 two-component system response regulator RstA [Salmonella enterica subsp. enterica serovar Typhimurium]
0.05

Fig. S2 (cont.)

### 3.5. Article 5 (*Results not yet published*)

Evidence of Interaction Between VarAS and RstAB Systems in the Regulation of the Hemolytic Activity of *Photobacterium damselae* subsp. *damselae.*

In this section we describe the partial results obtained in this doctoral thesis that have not yet been published because they require additional experimental studies in order to elucidate the various unknowns generated from these results. The hemolysis-regulating systems of .*P. damselae* subsp. *damselae* have been shown to constitute an extremely complex circuit due to the likely interactions among different regulatory systems.

# Evidence of Interaction Between VarAS and RstAB Systems in the Regulation of the Hemolytic Activity of *Photobacterium damselae* subsp. *damselae*

Mateus S. Terceti[1]& Carlos R. Osorio[1*]

[1]*Departamento de Microbioloxía e Parasitoloxía, Instituto de Acuicultura, Universidade de Santiago de Compostela, Santiago de Compostela, Spain*

**Keywords:** VarAS, RstAB, *Photobacterium damselae*, damselysin, phobalysin, vibriosis

**Correspondence:**
Carlos R. Osorio
Departamento de Microbioloxía e Parasitoloxía, Instituto de Acuicultura, Universidade de Santiago de Compostela, Santiago de Compostela 15782, Galicia, Spain
Email: cr.osorio@usc.es
Tel. (+34) 881 816050
Fax. (+34) 881 816047

**Abstract**

*Photobacterium damselae* subsp. *damselae* produces three cytotoxins, Damselysin, Phobalysin P and Phobalysin C, whose expression is positively regulated by the two-component system RstAB. Mutations in *rstA* or *rstB* genes impair hemolysin gene expression. Here we subjected single *rstA* and *rstB* mutants to transposon mutagenesis, and found that transposon insertions within a putative histidine kinase gene *varS* caused a partial recovery of hemolysis in the single *rstB* mutant. However, transposon mutagenesis of the *rstA* mutant did not yield revertants for hemolytic activity. Recovery of hemolysis was observed in *rstBvarS* double mutants constructed by allelic exchange, but not in *rstAvarS* mutants. A candidate response regulator gene *varA*, predicted to be the cognate pair of *varS*, was identified in the genome of strain RM-71. Deletion of *varA* also caused partial recovery of hemolytic activity in combination with deletion of *rstB*, but not in combination with *rstA*. The posible interactions between the RstAB and VarAS systems, and the effect of mutations in additional candidate regulatory genes is widely discussed.

## Introduction

The pathogenicity of *P. damselae* subsp. *damselae* is attributed to the production of up to four different toxins (Osorio *et al.*, 2018). Strains harboring the virulence plasmid pPHDD1 produce Damselysin (Dly) and Phobalysin P (PhlyP) (Rivas *et al*., 2011), in addition to the toxins encoded by chromosome I, Phobalysin C (PhlyC) and phospholipase (PlpV) (Vences *et al.*, 2017). Thus, strains lacking pPHDD1 produce only PhlyC and PlpV. Recently, we demonstrated that the two-component regulatory system (TCS) RstAB has a fundamental role in the regulation of hemolytic activity, phospholipase activity, swimming motility, ampicillin sensitivity, maintenance of cell morphology, and virulence of this pathogen (Terceti *et al*., 2017; Terceti *et al*., 2018b- *to submit).* It has also been shown that the RstAB system does not itself regulate the expression of PlpV and collagenase (ColP) in *P. damselae* subsp. *damselae* plasmidless strain LD-07 (Terceti *et al*., 2018b- *to submit*). In addition, we know that this system regulates the expression of several proteins dependent on the type II secretion system (T2SS) (Terceti *et al*., 2018b- *to submit*). The RstAB

regulatory system seems to be ubiquitous in all strains of *P. damselae* subsp. *damselae*, which makes this target system of interest since it could be a weak point to control infections caused both by plasmid as well as plasmidless strains. In this study, our objective was to search for new regulatory genes related to the hemolytic activity of *P. damselae* subsp *damselae* that could interact or not with the RstAB system.

## Materials and Methods

**Bacterial Plasmids, Strains, and Culture Conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *P. damselae* subsp. *damselae* cells were grown at 25°C on tryptic soy agar (TSA) and broth (TSB) supplemented with NaCl up to 1% (TSA-1 and TSB-1, respectively) and supplemented with antibiotics when appropriate. *E. coli* was grown at 37°C in Luria-Bertani (LB) broth or LB agar. When necessary, kanamycin (Km) were used at the following final concentration: at 50 μg mL-1.

**Hemolysis and motility assays.** Hemolysis assays on agar plates were conducted by picking a colony of each isolate previously grown on TSA-1, and inoculating it on sheep blood agar plates (Oxoid) and grown at 25°C. Motility assays were carried out using motility agar, which consisted of TSB (with either 0.5 or 1% NaCl) supplemented with 0.25% bacteriological agar and ampicillin, when appropriate. For this assay, a single colony isolated from an 18-h culture agar plate for each strain was picked with a sterile plastic tip, and the tip was stabbed into the motility agar. They were incubated at 15°C. For hemolysis and motility assays, pictures were taken at 24h post inoculation of the plates. Experiments were repeated three times to ensure that the hemolytic haloes and motility radius of the strains were reproducible.

**MIC assays**. To determine the susceptibility to benzylpenicillin, exponentially grown cultures of *P. damselae* subsp. *damselae* isolates were adjusted to an $OD_{600}$ of 0.5 and seeded onto TSA-1 plates in the presence of E-test gradient strips (bioMérieux).

**Assays for phospholipase.** The phospholipase activity was assayed using agar plates supplemented with egg yolk emulsion as a lecithin source. 10 µl of TSB-1 overnight cultures for each *P. damselae* subsp. *damsela*e strain were spotted onto TSA-1 plates supplemented with 3% egg yolk extract (Oxoid), and results were evaluated after 24 h of culture at 25ºC. Hydrolysis of lecithin by the phospholipase yields water-insoluble diglycerides that cause the appearance of an opaque precipitate.

**General PCR assays.** Genomic DNA was routinely extracted with the Easy-DNA kit (Invitrogen). Relevant PCR primers used in this study are listed in Table 2. PCR reactions were routinely performed with Kapa Taq DNA polymerase (Kapa) using a T-gradient termocycler (Biometra). Routinely, the following thermal cycling conditions were used: 95ºC for 5 min, followed by 30 cycles of 95ºC for 30 s, 52.5ºC for 30 s and an elongation step of 1 min at 72ºC per kb.

**Mini-Tn10 mutagenesis and identification of the disrupted gene.** With the objective of searching for direct or indirect repressor/activator genes of the hemolysis of *P. damselae* subsp.

*damselae* we decided to proceed with the random mutageneses by transposition against strain MT151 (Δ*rstB*), which producesa small halo of hemolysis. Thus, any transposon mutant derived from this strain and exhibiting a greater hemolysis halo than it, would constitute a major candidate for the analyses. Mini-*Tn*10 mutagenesis was performed using the suicide conjugative plasmid pLOFKm (Herrero *et al.*, 1990), with minor modifications as previously described (Rivas *et al*., 2015a). Genomic DNA from the clone with impaired hemolysis, Δ*rstB*, was purified with the genome DNA kit (Qbiogene), partially digested with BfuC1 and ligated to BamHI-digested plasmid pUC118. Ligation reactions were transformed into *E. coli* DH5α by electroporation (2.5 kV, 25 μF capacitance, and Pulse Controller Unit set to 200 Ω). Transformants were selected on LB agar plates supplemented with kanamycin, ampicillin and sheep blood (5%). Inserts containing the kanamycin resistance gene of mini-*Tn*10 plus flanking chromosomal DNA, were amplified by PCR and sequenced. DNA sequences were obtained using a capillary DNA Sequencer ABI 3730xl (Applied Biosystems).

**Table 1.** Bacterial strains and plasmids used and constructed in this study.

| Strain or plasmid | Description[a] | Reference/Source |
|---|---|---|
| **Strains** | | |
| ***P. damselae*** | **subsp. *damselae*** | |
| RM-71 | Isolated from turbot; pPHDD1-harboring | Fouz *et al*., 1992 |
| MT151 | RM-71Δ*rstB* | Terceti *et al.*, 2017 |
| MT319 | RM-71Δ*rstA* | Terceti *et al.*, 2018b |
| MT160 | MT151 broken gene not yet identified ::*Tn*10 ( revertant hemolysis) | This study |
| MT161 | MT151 broken gene not yet identified ::*Tn*10 ( revertant hemolysis) | This study |
| MT164 | MT151 broken gene not yet identified ::*Tn*10 ( revertant motility) | This study |
| MT165 | MT151 broken gene not yet identified ::*Tn*10 ( revertant motility) | This study |
| MT166 | MT151 broken gene not yet identified ::*Tn*10 ( revertant motility) | This study |
| MT162 | MT151 *varS* ::*Tn*10 ( revertant hemolysis) | This study |
| MT170 | RM-71Δ*varS* | This study |
| MT257 | RM-71Δ*varA* | This study |
| MT236 | RM-71Δ*varA*Δ*varS* | This study |
| MT358 | RM-71Δ*rstA*Δ*varA* | This study |
| MT255 | RM-71Δ*rstA*Δ*varS* | This study |
| MT350 | RM-71Δ*rstB*Δ*varA* | This study |
| MT351 | RM-71Δ*rstB*Δ*varS* | This study |
| MT235 | RM-71Δ*rstA*Δ*varA*Δ*varS* | This study |
| MT231 | RM-71Δ*rstB*Δ*varA*Δ*varS* | This study |
| MT209 | RM-71ΔluxO | This study |
| MT211 | RM-71Δ*luxO*Δ*rstB* | This study |
| MT203 | RM-71Δ*luxO* Δ*varS* | This study |
| MT317 | RM-71Δ*luxO*Δ*rstB*Δ*varA* | This study |
| MT212 | RM-71Δ*luxO*Δ*rstB*Δ*varS* | This study |
| MT107 | RM-71ΔtoxR | This study |
| MT172 | RM-71ΔtoxRΔ*varS* | This study |
| MT242 | RM-71Δ*phoP* | This study |
| MT261 | RM-71Δ*rstB* Δ*phoP* | This study |
| ***E. coli*** | | |
| DH5α | Cloning strain | Laboratory stock |
| S17λpir | RP4-2 (Km::*tn*7,Tc::Mu-1) pro-82 v | Herrero *et al.*, 1990 |
| B-3914 | F´RP-2TC::Mu; Δ*dapA*:: (erm-pir) gyA462 zei-298::*Tn*10 | Le Roux *et al.*, 2007 |
| **Plasmids** | | |
| pLOFKm | *Tn*10-based delivery plasmid; $Km^R$ | Herrero *et al.*, 1990 |
| pNidkan | Suice vector derived from pCVD442: $Km^R$ | Mouriño *et al.*, 2004 |

[a]$Km^r$, kanamycin resistance; Δ, gene deletion

**Table2.** Oligonucleotides used in this study.

| Target and oligonucleotides | Sequence | bp amplified | Reference |
|---|---|---|---|
| ***rstA* deletion** | | | |
| *1-2 fragment* | | | |
| rstA-mut1-xbal-F | GCTCTAGAGGCTATAATCCAAACAATGG | 1968 | Terceti *et al.*, 2018b |
| rstA-mut2-smaI-R | GCCCCGGGTTAACTAAAGAATTACGTGG | | |
| *3-4 fragment* | | | |
| rstA-mut3-smaI-F | GCCCCGGGTTACCGTCAGAATGTGTTTG | 1957 | Terceti *et al.*, 2018b |
| rstA-mut4-apaI-R | GCGGGCCCCCTTTATCCATATCAATTCC | | |
| *internal* | | | |
| rstA-intF | CTTATTATCAATGGTTCTGT | variable | Terceti *et al.*, 2018b |
| rstA-intR | TTGGCTGTATAAGAACCATG | | |
| ***varA* deletion** | | | |
| *1-2 fragment* | | | |
| varA-mut1-smaI-F | CGCCCGGGCTATGTTATCTATTTCATTT | 1982 | This study |
| varA-mut2-pstI-R | CGCTGCAGAACAGCTATCGTTACCGTCT | | |
| *3-4 fragment* | | | |
| varA-mut3-pstI-F | CGCTGCAGCGGCTTCCCCTACCACTTTA | 2019 | This study |
| varA-mut4-apaI-R | CGGGGCCCATCAACAGGGCGGTGTAGCA | | |
| *internal* | | | |
| varA-int-F | ACGTCTCTGTGTCTAACATA | variable | This study |
| varA-int-R | TATTCCTTGTAGATGATCAC | | |
| ***varS* deletion** | | | |
| *1-2 fragment* | | | |
| varS-mut1-xbal-F | CGTCTAGATATGGTATCGACTTTCTTCA | 1824 | This study |
| varS-mut2-smaI-R | CGCCCGGGTGAAGAGCACATTCTACAAC | | |
| *3-4 fragment* | | | |
| varS-mut3-smaI-F | CGCCCGGGATGGGGACCAAATTGCCCTT | 1986 | This study |
| varS-mut4-apaI-R | CGGGGCCCCATACAGCTAATTCAAACTT | | |
| *internal* | | | |
| varS-int-F | CATTATCACCACACATCACT | variable | This study |
| varS-int-R | GCCTTTAGTCAAGCAGATGC | | |
| ***luxO* deletion** | | | |
| *1-2 fragment* | | | |
| LuxO_mut1_xbal | GCTCTAGATTAGAATCTCGCCGTAAGCA | 1958 | This study |
| LuxO_mut2_smaI | GCCCCGGGCTCGGCATTTGATTTGTGTT | | |
| *3-4 fragment* | | | |
| luxO_mt3_smal | GCCCCGGGCGAATATTGCAGACACTGTT | 1995 | This study |
| luxO_mut4_ApaI | GCGGGCCCCATTTATGACAATTCACTCA | | |
| *internal* | | | |
| luxO_int_F | ACGTCTTCCTGATATGACCG | variable | This study |
| luxO_int_R | CAATCCACAGCGGCTCAATT | | |
| ***t oxR* deletion** | | | |
| *1-2 fragment* | | | |
| toxR_1_BamHI | GCGGATCCATTGCACTTCTGGCGCTTCT | 2057 | This study |
| toxR_2_PstI | GCCTGCAGTCGCTCCTGGTTCATCAATC | | |
| *3-4 fragment* | | | |
| toxR_3_PstI | GCCTGCAGATCCCTCTGGCAAGTTACTT | 2021 | This study |
| toxR_4_EcoRI | GCGAATTCGTCGATTGACAAGATTGAGT | | |
| *internal* | | | |
| toxR_int_F | TAGCTTAATTGACACAGTTG | variable | This study |
| toxR_int_R | CTCCTGCTGGTGGTTATCTA | | |
| ***phoP* deletion** | | | |
| *1-2 fragment* | | | |
| phoP_mut1_xbal | CGTCTAGAAGATGGACTTAGACCGCGCA | 1978 | This study |
| phoP_mut2_EcoR | CGGAATTCACGAAAGAAATTCACTAAGA | | |
| *3-4 fragment* | | | |
| phoP_mut3_EcoRI | CGGAATTCTGGTTTCCTAGCTCGCTAAG | 1988 | This study |
| phoP_mut4_SaLI | CGGTCGACGAAACTGTGTCGTGAACATT | | |
| *internal* | | | |
| phoP_mut_intF | CTGTGCTTGTAATTCAAAGA | variable | This study |
| phoP_mut_intR | ATGCGTATTCTTATCGTCGA | | |

**Allelic-exchange deletion mutant construction.** Nonpolar deletions of *varA, varS, phoP, luxo* and *toxR* genes were constructed using PCR amplification of the amino- and carboxy-terminal fragments of each gene, which, when fused together, would result in an in-frame deletion of more than 90% of the coding sequence. The primers used in are described in the table 2. Amplification was carried out using Hi-Fidelity Kapa *Taq* (Kapa). Allelic exchange was performed using the $Km^R$ suicide vector pNidKan containing the *sacB* gene, which confers sucrose sensitivity, and R6K *ori*, which requires the *pir* gene product for replication. The plasmid constructs containing the deleted alleles were transferred from *E. coli* S17-1-λ*pir* into RM-71 strain. After conjugation for 48 h on TSA plates prepared with seawater, cells were scraped off the plate and resuspended in TSB-1. Next, 100-µl aliquots of serial decimal dilutions were spread on Thiosulfate citrate bile salts sucrose (TCBS) agarand subsequently selecting for sucrose resistance (15% [wt/vol]) for a second recombination event. This led to the *P. damselae* subsp. *damselae* mutant strains describedin this study in Table 1. The absence of the deleted alleles was confirmed in each case

by PCR, and the genome region involved in the deletion was sequenced to verify that the deletion was nonpolar. To better understand the relationship between the two-component system VarAS and RstAB we proceeded to the construction of double and triple mutants combining genes from these two systems (Table 1).

## Results and Discussion

### Evidence of interaction between the two-components VarAS and RstAB in the regulation of the hemolytic activity of *Photobacterium damselae* subsp. *damselae*

In a previous study we demonstrated that deletion of the *rstB* gene in *P. damselae* subsp. *damselae* RM-71 to yield mutant strain MT151 (Δ*rstB*), considerably decreased the hemolytic halo on sheep blood agar when compared to parental strain and that it also strongly decreased virulence in a sea bass fish model (Terceti *et al.*, 2017). We also reported that the deletion of its cognate gene *rstA* of the two-component RstAB system, to yield mutant strain MT319 (RM-71Δ*rstA*), also caused a reduced hemolytic halo similar to that of

mutant strain MT151 (Terceti *et al*., 2018b- *to submit*). In addition, we have shown that the MT151 and MT319 strains show motility affected at 0.5% NaCl (Terceti *et al.*, 2017; Terceti *et al*., 2018b- *to submit*). In an attempt to find new direct or indirect regulatory genes for hemolysis in *P. damselae* subsp. *damselae*, we selected the deletion mutant for *rstB* gene (strain MT151) and subjected it to random transposon mutagenesis with mini-Tn10. We hypothesized that some mutants might exhibit a recovery of the hemolytic activity due to the mutagenesis of potential repressors. Among the thousands of colonies (MT151::*tn*10) analyzed in sheep blood agar, we observed that 3 colonies partially recovered the hemolytic activity (Fig. 1A) although they remained affected in swimming motility when grown at 0.5% NaCl (Fig. 1B). These three transposon mutants with partial recovery of hemolysis were named MT160, MT161 and MT162. The transposon mutants MT160 and MT161 have not yet been analyzed for the identification of the truncated gene.

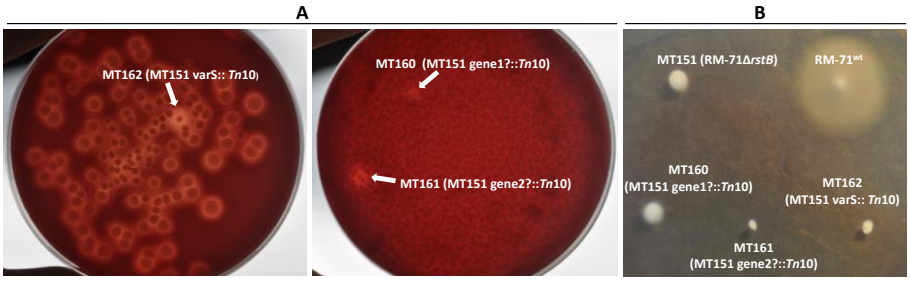



**Figure 1**. Transposon-mutagenized clones (MT151::*tn*10) that partially recovered hemolytic activity (A) but not motility (B).

We also searched for transposition mutants that recovered motility at 0.5% NaCl (MT164, MT165 and MT166, Table 1), but the truncated gene has not yet been identified, so they are available for future studies. Notably, motility revertants did not revert for hemolysis (data not shown). After sequencing the transposon-truncated gene in MT162 strain, we identified that mini-Tn10 had inserted within locus VDA_001267. This locus corresponds to *varS* gene, which was thus initially considered responsible for the revertance of hemolytic activity observed in clone MT162 (Δ*rstB*::*tn*10/Δ*varS*). The VarS protein is a membrane sensor of the VarAS two-component system and was first identified as a virulence factor in *Vibrio cholerae* by

Wong *et al*. (1998). We further searched the genome of the *P. damselae* subsp. *damselae* type strain CIP102761 to find the candidate gene encoding the homologue of VarA protein, the putative cognate response regulator of VarS. As a result, we found a candidate gene encoded by locus VDA_002274. This gene showed to be also present in the genome of RM-71 strain. So far there is no information in the scientific literature about the function of the VarAS system in the genus *Photobacterium*. There is indeed a wide variety of VarAS homologues in Gram-negative bacteria. For example, GacA/GacS in the genus *Pseudomonas* (Laville *et al*., 1992; Rich *et al.*, 1994; Rahme *et al.*, 1997)**,** BarA/UvrY in *Escherichia coli* (Moolenaar *et al.*, 1987), BarA/SirA in *Salmonella typhimurium* (Johnston *et al*., 1996) and LetA/LetS in *Legionella pneumophila* (Molofsky and Swanson, 2004, Bachman *et al*., 2004). In order to confirm that the reverted hemolytic phenotype of MT162 (Δ*rstB*::*tn*10/*varS*) was indeed due to a mutation in *varS* gene, we constructed by allelic exchange the double mutant MT351 (Δ*rstB*Δ*varS*) which also showed recovery of hemolysis (Fig 2A). This result suggests that the two-component VarAS system is

some what related to the RstAB system since the mutant strain MT151 (Δ*rstB*), which has low hemolysis, showed increased hemolysis when the transposon was inserted into the *varS* gene of MT151. In order to better understand the relationship between the RstAB and VarAS systems, we proceeded to construct several mutants combining deletion of genes from the two systems (Fig. 2) and further evaluated these mutants for hemolysis, phospholipase activity, motility at 0.5% and 1% NaCl and sensitivity to ampicillin (Fig. 2). Would mutant strains MT350 (Δ*rstB*Δ*varA*), MT255 (Δ*rstA*Δ*varS*) and MT358 (Δ*rstA*Δ*varA*) also be revertant for hemolysis? (Fig.2A). Would also recover phospholipase activity? (Fig.2B). And swimming motility at 0.5% NaCl? (Fig 2D). We demonstrated in a previous study that *P. damselae* subsp. *damselae* mutant strains with a deletion in the *rstA* or *rstB* gene became sensitive to ampicillin. Would MT351, MT350, MT255 and MT358 strains recover the ampicillin resistance levels of parental strain? (Fig. 2E). Interestingly, only the mutant strains MT351 (Δ*rstB*Δ*varS*), MT350 (Δ*rstB*Δ*varA*) were revertants for hemolytic activity and phospholipase activity (Fig. 2A, 2B and Fig. 3).

The MT257 (Δ*varA*), MT170 (Δ*varS*) and MT236 (Δ*varA*Δ*varS*) mutant strains, presented hemolytic haloes similar to the parental strain RM-71$^{wt}$ (Fig.2A). The hemolysis activity of the triple mutants MT235 (Δ*rstA*Δ*varA*Δ*varS*) and MT231 (Δ*rstB*Δ*varA*Δ*varS*) was low (Fig. 3A). In most of the evaluated mutants, there was a correlation between the presence of hemolytic halo and presence of opaque halo in egg yolk medium that is the result of phospholipase activity exerted by Dly phospholipase (Fig. 2). This relationship is not true for the triple mutant MT255 (Δ*rstB*Δ*varA*Δ*varS*) that shows no evident hemolytic activity in sheep blood, but has phospholipase activity (Fig. 2A, B and Fig. 3). None of the mutant combinations had their motility affected at 1% NaCl (Fig. 2C). However, at 0.5% NaCl, all mutants that had deletion of the *rstA* or *rstB* gene had their motility compromised, even in the mutant strains MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) that curiously had no motility revertence (Fig 2D).

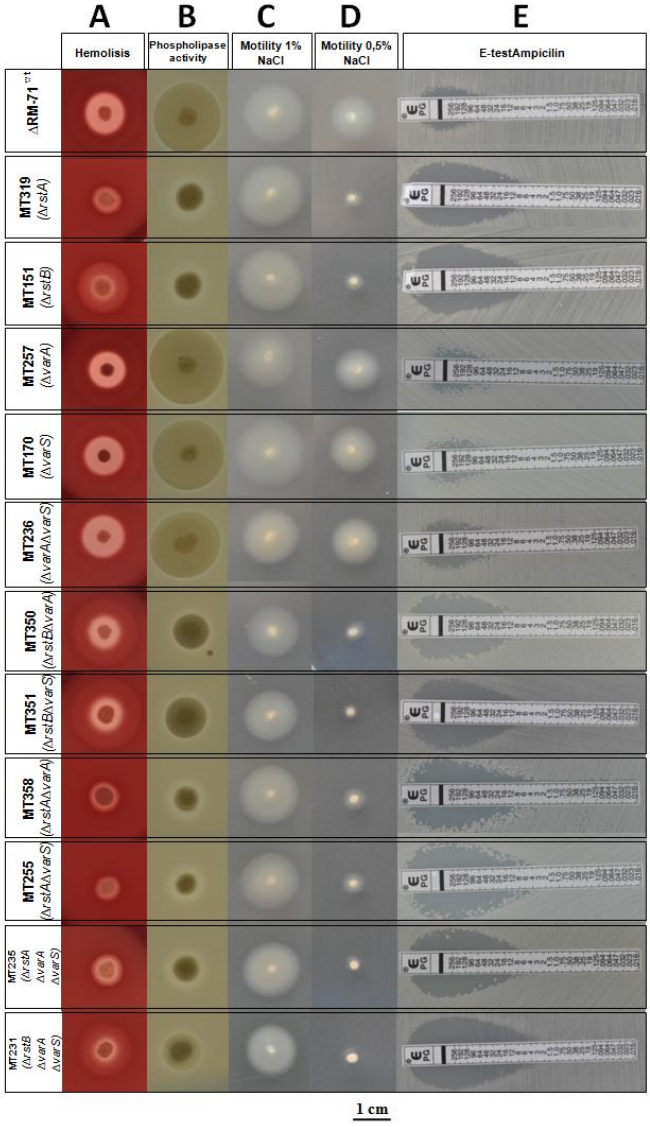



**Figure 2.** Phenotypes of mutant strains derived from combined deletions of genes from the two-component systems RstAB and VarAS.

Although RM-71$^{wt}$, MT257 (Δ*varA*), MT170 (Δ*varS*) and MT236 (Δ*varA*Δ*varS*) strains exhibited some degree of motility at 0.5% NaCl, this was done more efficiently at 1% NaCl (Fig. 2C). These results suggest that the two-component system RstAB of *P. damselae* subsp. *damselae* contributes to the osmotic balance of this bacterium and that the VarAS two-component system does not directly interfere with the motility of this pathogen since the mutants MT257 (Δ*varA*), MT170 (Δ*varS*) and MT236 (Δ*varA*Δ*varS*) have motility haloes similar to wild type strain RM-71$^{wt}$ under both culture conditions of 0.5% and 1% NaCl (Fig 2C; 2D). In a previous study we reported that the cell morphology of mutant strains MT151 (Δ*rstB*) and MT319 (Δ*rstA*) cultured 0.5% NaCl exhibited deformed cells, larger and swollen cells and often connected to each other, forming elongated structures, likely due to a difficulty indaughter cell separation during the cell division cycle (Terceti *et al*., 2018b- *to submit*). Here we demonstrate that all combinations of mutants that had deletion of the *rstA* or *rstB* gene also have impaired cell morphology (Fig.4). In addition, we demonstrated that mutant strains MT350 and MT351, which reverted in hemolysis

activity, do not recover their normal cell morphology (Fig.4). These results suggest once again that the two-component RstAB system contributes to the osmotic balance of *P. damselae* subsp. *damselae* and that probably the motility affected in 0.5% NaCl is also due to the deformed cells besides a possible defect in the flagellar motility system of this pathogen. It is interesting to note that mutant strains MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) of *P. damselae* subsp *damselae* had restoration of hemolysis and phospholipase activity, but not the restoration of motility and morphology at 0.5% NaCl, and did not recover the resistance to ampicillin. The mutants MT255 (Δ*rstA*Δ*varS*) and MT358 (Δ*rstA*Δ*varA*) continue to have hemolytic and phospholipase activity reduced, motility and morphology affected at 0.5% and aberrant morphology. This analysis suggests that the RstA response regulator protein may be essential for hemolysis since in strains where this gene is absent there is no recovery of hemolytic activity. The phenotypic results presented by the *P. damselae* subsp. *damselae* strains evaluated in this study are summarized in Figure 5.

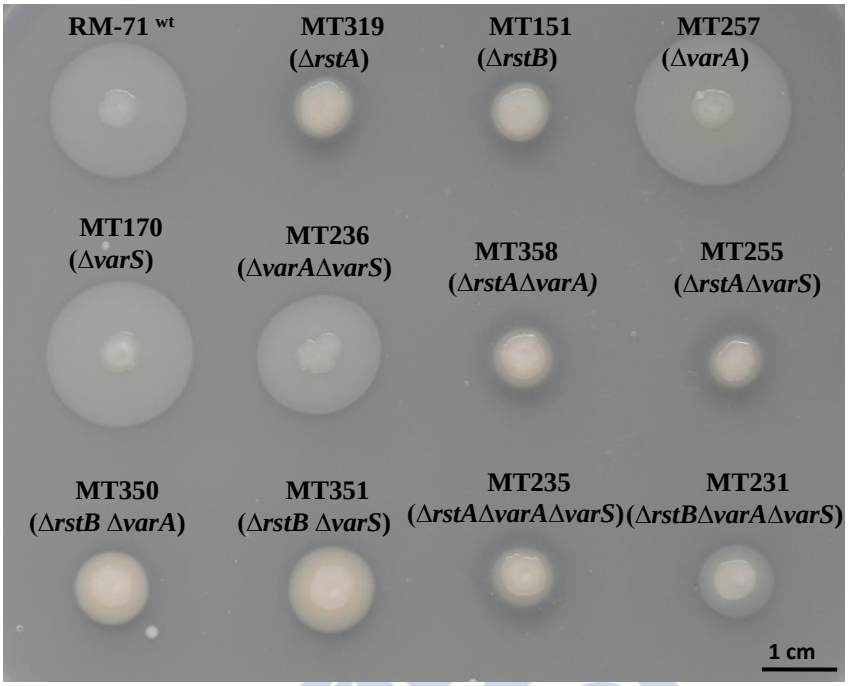



**Figure 3.** Phospholipase activity in egg yolk medium.

We know then that the two-component VarAS system is related in some way to the RstAB system. But what is the molecular basis of this relationship? Many bacterial pathogens utilize signal transduction proteins from the two-component regulatory system family to modulate the expression of virulence genes in response to environmental signals (Miller *et al*., 1989; Stock *et al*., 2000). The prototypical two-component system responds to a change in

environmental conditions by modifying gene expression and/or biochemical activities of the target proteins. Phosphorylation generally increases the affinity of a regulator for its target DNA (Gao and Stock, 2009; Zwir *et al*., 2012; Zwir *et al*., 2014). The sensors use ATP to autophosphorylate in a conserved histidine residue (H) (Fig.6).

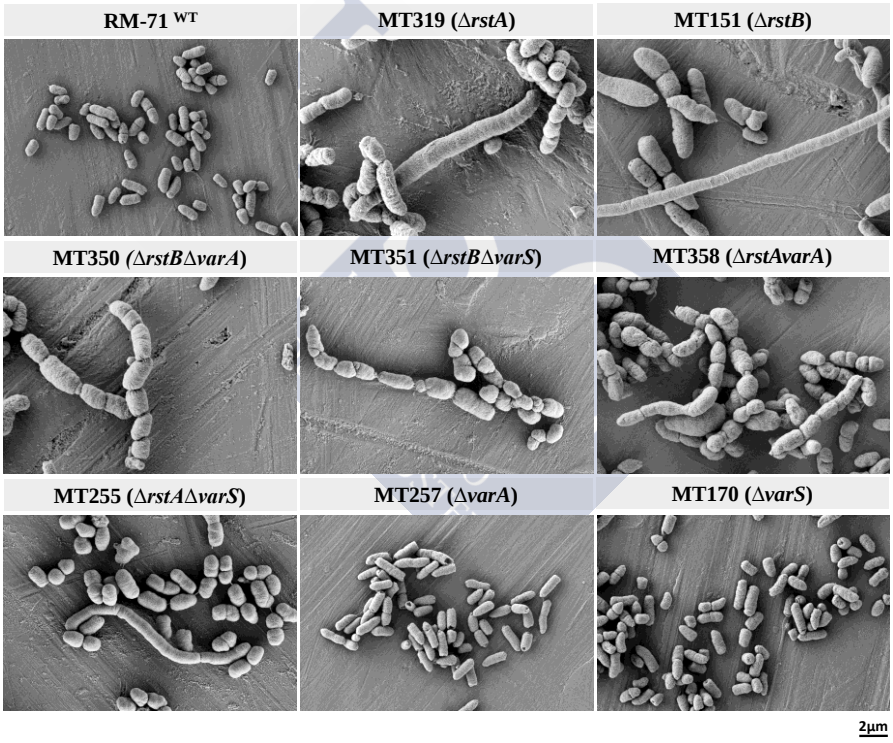



**Figure 4.** Scanning electron microscopy analysis of *P. damselae* subsp. *damselae* strains cultured in TSB medium with 0.5% NaCl.

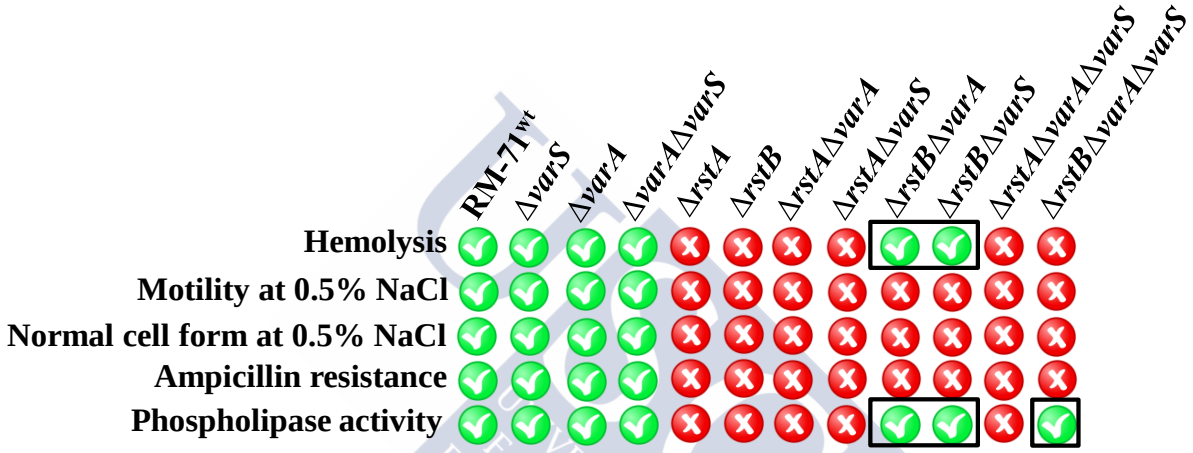



**Figure 5.** Graphical summary of the phenotypes exhibited by mutant strains of *P. damselae* subsp. *damselae* evaluated in this study. The highlight in the figure represents the phenotypes in revertant strains.

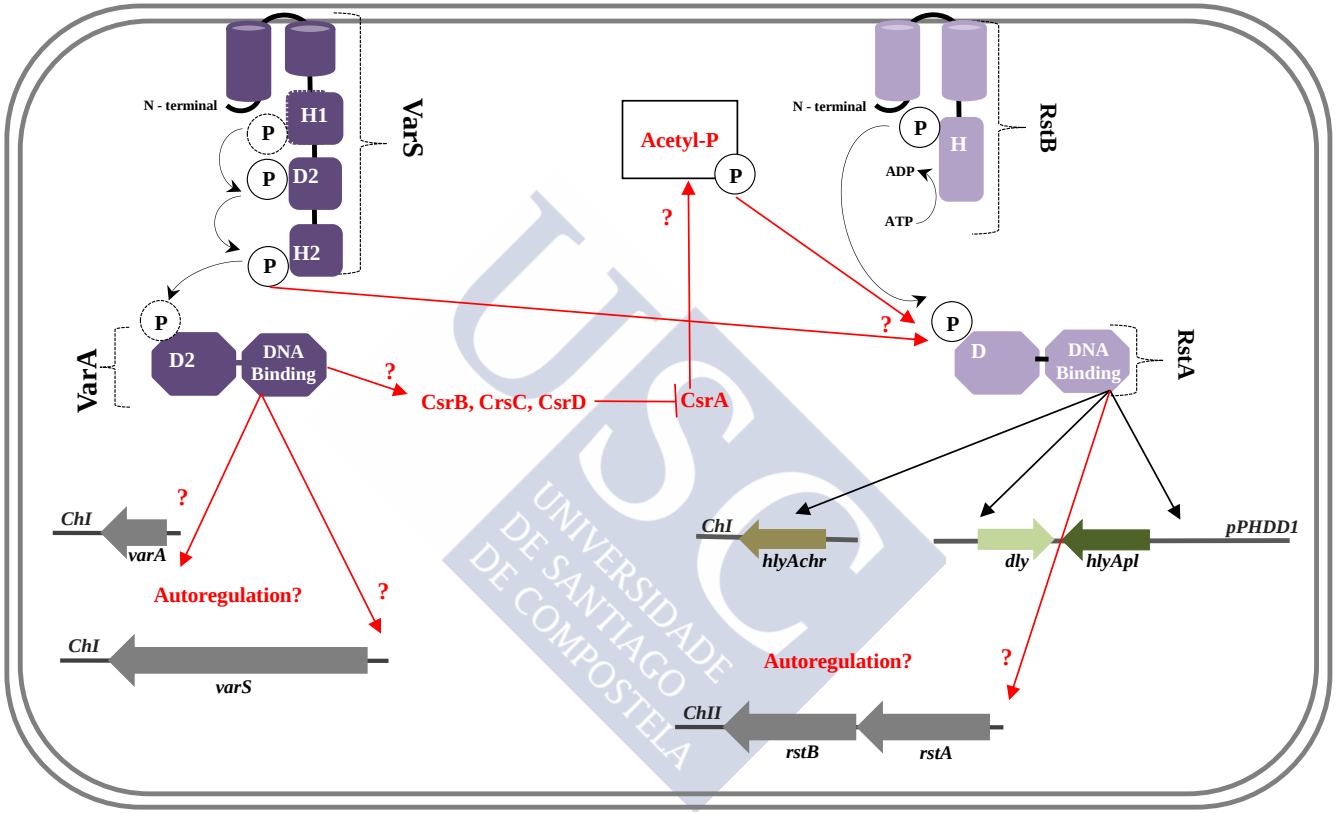



**Figure 6**. Some possible points of interaction between the VarAS, RstAB and acetyl phosphate systems.

The phosphorelay two-component system involves four phosphoryl transfer events. In phosphorelay TCS, an autophosphorylate sensor in a conserved histidine residue (H1) serves as a phosphoryl donor for a protein (or a domain of the same protein) that becomes phosphorylated in an aspartate residue (D1); in turn, donates the phosphoryl group to a third protein or domain, which is phosphorylated on a histidine residue (H2), and finally donates the phosphoryl group to a regulatory protein or domain whose activity changes when it is phosphorylated on a residue of conserved aspartate (D2) (Fig.6) (Perego and Brannigan, 2001). The existence of multiple phosphorylated intermediates provide ample opportunities for the phosphate transfer process to be aborted if there are additional repressor stimuli (Perego and Brannigan, 2001). According to literature data the two-component VarAS system in other bacterial species is of the phosphorelay type (Vakulskas *et al*., 2015). Analysis of VarS domains of *P. damselae* subsp. *damselae* suggests that the VarAS system of this pathogen, is also of the phosphorelay type (Fig. 6). In the vast majority of two-component systems, the sensor only

affects the phosphorylated state of its cognate regulator (Laub and Goulian, 2007; Verhamme *et al.*, 2002). However, certain regulators can be phosphorylated from cognate and non-cognate sensors, allowing the regulator's response (output) to reflect different stimuli (input) from cognate and non-cognate sensors (Botella *et al.*, 2014). Thus, different environmental stimuli acting on different sensors can converge to a given regulator. Is this the case of the two-component system RstAB and VarAS? Would the VarS sensor be able to phosphorylate the RstA regulator? (Fig.6). Taking into account the literature data and the experimental results obtained by us through mutant strains of *Photobacterium damselae* subsp. *damselae* from the gene deletion combination of the two-component systems VarAS and RstAB we can make the following reflections and queries:

A) **The presence of the *rstA* gene appears to be essential for full hemolytic activity.** Strains RM-71$^{wt}$, MT170 (Δ*varS*), MT257 (Δ*varA*), MT236 (Δ*varA*Δ*varS*) harbour functional *rstA* and *rstB* genes. Thus the RstB membrane sensing protein can phosphorylate RstA (RstA-P) which in turn could associate with

the promoters of the *hlyA*$_{ch}$, *hlyA*$_{pl}$ and *dly* genes by activating its expression and thus producing the hemolysins PhlyC, PhlyP and Dly respectively and consequently cause maximal hemolysis on sheep blood agar (Fig. 2A). Dly hemolysin exhibits phospholipase D activity, so phospholipase activity was observed in egg yolk medium in these mutants (Fig. 2B).

**B) The MT255 (Δ*rstA*Δ*varS*) MT358 (Δ*rstA*Δ*varA*) and MT235 (Δ*rstA*Δ*varA*Δ*varS*) mutants have low hemolytic activity and phospholipase since they do not possess the RstA protein due to the deletion of the *rstA* gene, therefore, there is low expression of the PhlyP, PhlyC and Dly (Fig. 2A, B and Fig.3).**

**C) There was a recovery of the hemolytic and phospholipase activity of mutant strains MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) (Fig. 2A, B) carrying the integral *rstA* gene.** But how could this be possible if the *rstB* gene, encoding RstB, is deleted? In what way could the RstA responsive regulatory protein become active (RstA-P) and bind to the hemolysin promoters if RstB protein is not present to transfer the phosphate group?. We

know that the phosphorylated sensor serves as a phosphodonor to its regulatory partner, which is phosphorylated in a conserved aspartic acid residue (D) (Fig. 6). A regulator can be phosphorylated not only from a sensor that continually autophosphorylates, but also from a low molecular weight phosphodonor, acetyl-P (McCleary and Stock, 1994, Trajtenberg *et al*., 2014; Christopher *et al*., 2015 Groisman, 2016). Therefore, the mutant strains MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) could have their RstA protein phosphorylated by a acetyl-P group. Supporting this hypothesis, the work of Kim *et al*., 2015, demonstrated that the Δ*varA* mutant strain of *Vibrio vulnificus* revealed that the *acsA* gene encoding acetyl-CoA synthetase was negatively regulated. If this were true for *P. damselae* subsp. *damselae* with deletions in the *varA* and *varS* genes, we would also expect the *acsA* gene to be downregulated, so that little Acetyl-CoA would be formed and an accumulation of Acetyl-P and Coenzyme A (CoA) groups would be observed. This supposed accumulation of Acetyl-P would explain the recovery of hemolysis

from the MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) strains that would have their RstA response regulator protein phosphorylated by these Acetyl-P groups. Interestingly, the *varA* and *varS* genes of *P. damselae* subsp. *damselae* exhibit 91% and 65% identity with the *varA* and *varS* genes of *Vibrio vulnificus* respectively (Table 3). It is also known that phosphorylation of the response regulator is more efficient when the sensors are used as phosphodonor than when they receive phosphoryl group of acetyl-P (Trajtenberg *et al*., 2014). This would explain why the hemolytic recovery observed in the mutant strains MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) was not 100%, ie, the diameter of the hemolytic halo of these strains was lower than that observed in the wild strain RM-71$^{wt}$ (Fig.2A).

**D) Why do mutant strains MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) recover hemolytic and phospholipase activity but do not regain normal cell motility and morphology when cultured at 0.5% NaCl? Why do they remain sensitive to ampicillin?** One would expect these mutants to regain normal

activity for all these phenotypes, since RstA is being phosphorylated by acetyl-P. Using the PhoPQ two-component system as an example, the PhoP regulator binds only to promoters with high affinity binding sites and/or those that are not subject to silencing by nucleotide-associated proteins, such as the structuring protein nucleoid similar to histone HNS (Zwir *et al*., 2012, Zwir *et al*., 2014). As the active PhoP-P concentration increases, promoters with sites of low binding affinity and/or subjected to H-NS mediated silencing will be occupied, and transcription of the corresponding genes will occur. Thus, there may be an expression hierarchy that reflects the number of molecules of the active regulator that may reflect on preferred physiological responses. (Beier and Gross, 2008; Fujita *et al*., 2005; Gao and Stock, 2009; Zwir *et al*., 2014). The fact that the mutant strains (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) recovered hemolytic activity and phospholipase but did not recover normal cell motility and morphology at 0.5% NaCl and remain sensitive to ampicillin could be due to RstA-P protein having affinity distinct from the

promoters of the genes they regulate. Thus, phospholylation of RstA (made less efficient by the acetyl-P groups when compared to sensor-phosphorylated) would generate active RstA-P with preferential affinity to the hemolysin promoters, which explains the recovery of hemolysis. Thus, the amount of RstA-P under these conditions, would not be sufficient to activate genes related to motility, cell morphology and ampicillin sensitivity.

**E) Considering the reflections made so far, why did the mutant MT151 (Δ*rstB*) carrying the *rstA* gene have reduced hemolytic activity? (Fig. 2A).** In *E. coli*, homologues of VarAS (BarA/UvrY) activate the transcription of genes encoding small sRNAs CsrB and CsrC which in turn inhibit the activity of the protein CsrA (Liu *et al*., 1997; Weilbacher *et al*., 2003; Lenz *et al*., 2005). CrsA is a post-transcriptional regulator of several processes including carbon metabolism, biofilm formation in *E. coli* (Romeo *et al*., 1993; Romeo, 1998). In *Pseudomonas* sp., the homologues of VarAS (GacA/GacS) activate the transcription of rsm genes encoding sRNAs that also inhibit a homolog of CsrA (Aarons *et*

*al*., 2000; Heeb *et al*., 2002; Valverde *et al*., 2003; Burrowes *et al*., 2005). Many defects conferred by mutations in homologues of the *varA* gene are due to their inability to alleviate repression of the CsrA protein, that key protein in central metabolism, motility and virulence (Blumer *et al*., 1999; Chancey *et al.*, 1999; Cui *et al*., 2001; Chatterjee *et al*., 2003;Vakulskas *et al*., 2015).The VarAS/CsrB, C, D/CsrA system was also demonstrated in *Vibrio vulnificus* (Jones *et al.*, 2008), *Vibrio fischeri* (Whisttler and Ruby, 2003; Nyholm and Mcfall-Ngai, 2004; Septer *et al*., 2015; Foxall *et al*., 2015) and *V. Cholera* (Jobling and Holmes, 1997; Zhu *et al*., 2002; Zhu and Mekalanos 2003; Hammer and Bassler, 2003; Lenz *et al*., 2005; Jang *et al*., 2010; Tsou *et al.*, 2011; Mey *et al*., 2015). It has recently been shown that the *Vibrio alginolyticus* VarAS two-component regulatory system regulates the expression of a collagenase A through a likely unidentified post-transcriptional regulator (Mima *et al.*, 2018). The degree of identity (%)/similarity of the two-component VarAS system of *P. damselae* subsp. *damselae* with the VarAS system of *E. coli*,

*Pseudomonas* sp., *Vibrio vulnificus, Vibrio fischeri, V. Cholera* and *Vibrio alginolyticus* is presented in table 3. Due to the high degree of identity (%)/similarity of the VarAS system of these species with the same system in *P. damselae* subsp. *damselae* we could assume that the two-component VarAS system of *P. damselae* subsp. *damselae* is also related to the activation of genes encoding small sRNAs which in turn would also inhibit the post transcriptional regulatory protein CsrA (identity of 91% with CsrA of*Vibrio fischeri, Vibrio vulnificus* and *V. cholerae,* table3). Thus, mutant strains MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) would not express the small sRNAs (CsrB, C, D) which would increase the levels of the CsrA protein which in turn would increase the levels of acetyl-P due to negative regulation of the *acsA* gene (less acetyl-coA synthase being produced), giving these mutants the recovery of hemolytic activity due to their ability to phosphorylate RstA. Using the same reasoning we can explain the low hemolytic activity of strain MT151 (Δ*rstB*). This mutant does not have the ability to phosphorylate the RstA protein because it

lacks the RstB membrane sensor. The MT151 (Δ*rstB*) strain also carries the VarA and VarS proteins, which in turn would activate the expression of small sRNAs which would consequently lower CsrA levels. Thus, acetyl-coa synthetase molecules would normally be produced which would decrease the acetyl-P number capable of phosphorylating RstA and therefore, reducing the hemolytic capacity of this mutant. Therefore, MT151 has reduced hemolytic activity because it would have neither RstB nor acetyl-P to phosphorylate RstA.

F) **Why does the MT231 (Δ*rstB*Δ*varA*Δ*varS*) mutant strain have low hemolytic activity (Fig. 2A) and recovers phospholipase activity (Fig. 3)?** Using the same interpretation to explain the recovery of the hemolytic activity of the mutant strains MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) for strain MT231 (Δ*rstB*Δ*varA*Δ*varS*), we expected that MT231 also recovered its hemolytic activity. What appears to be that for recovery of hemolytic activity, at least one of the VarAS system genes must be present. Would the *varA* and *varS* genes together be responsible

for the repression of some type of phospholipase? This could explain why the MT231 (*ΔrstBΔvarAΔvarS*) strain has some phospholipase activity (Fig. 3). All these interpretations are the results of hypotheses formulated from data presented in the literature from studies carried out with other bacterial species, therefore, they should be proven, refuted or reformulated from further experiments in the future. It is worth remembering that we were the pioneers to look for regulatory genes related to virulence factors in the species *Photobacterium damselae* and therefore, these partial results can act as an excellent starting point for later studies in order to elucidate this complex regulation network involving the RstAB and VarAS systems. However, we can say that there is interaction between these two regulation systems in a way still unknown. Thus, the Δ*rstB* mutant with reduced hemolytic activity, partially recovered the hemolytic activity after having the *varA* or *varS* gene deleted. This statement is true for strains derived from *Pdd* RM-71 that had their hemolytic activity evaluated in sheep blood.

**Table 3.** Identity of the *varA*, *varS* and *csrA* genes of *P. damselae* subsp. *damselae* with other bacterial species.

| locus_tag/ Feature ID (*P. damselae* subsp. *damselae*) | Homologue | locus_tag | Homologue Matches | | | |
|---|---|---|---|---|---|---|
| | | | Accesion nº. | Species | Ident. | E-value |
| **VarA ( Response Regulator)** | | | | | | |
| VDA_002274 (*varA*) | UvrY | b1914 | NP_416424.1 | *Escherichia coli* K-12 | 76% | 2e-123 |
| VDA_002274 (*varA*) | SirA | STY2155 | NP_456510.1 | *Salmonella enterica* Typhimurium | 75% | 1e-122 |
| VDA_002274 (*varA*) | LetA | PTVF89_03745 | KXB28168.1 | *Legionella pneumophila* | 51% | 1e-79 |
| VDA_002274 (*varA*) | GacA | DC048_17280 | PWB31412.1 | *Pseudomonas* sp. | 64% | 7e-99 |
| VDA_002274 (*varA*) | VarA | VC1213 | NP_230858.1 | *Vibrio cholerae* O1 El Tor N16961 | 88% | 1e-145 |
| VDA_002274 (*varA*) | VarA | K04M1_20050 | ARP03643.1 | *Vibrio alginolyticus* | 92% | 5e-150 |
| VDA_002274 (*varA*) | VarA | VF_1627 | YP_205010.1 | *Vibrio fischeri* | 89% | 6e-147 |
| VDA_002274 (*varA*) | VarA | VV1_3054 | AAO11378.1 | *Vibrio vulnificus* | 91% | 6e-148 |
| **VarS (sensory HK)** | | | | | | |
| VDA_001267 (*varS*) | BarA | b2786 | NP_417266.1 | *Escherichia coli* K-12 | 54% | 0.0 |
| VDA_001267 (*varS*) | BarA | STY3096 | NP_457354.1 | *Salmonella enterica* Typhimurium | 54% | 0.0 |
| VDA_001267 (*varS*) | LetS | PTVFX2014_11865 | KZX36238.1 | *Legionella pneumophila* | 30% | 1e-123 |
| VDA_001267 (*varS*) | GacS | CUR33_13515 | PJE40046.1 | *Pseudomonas* sp. | 38% | 0.0 |
| VDA_001267 (*varS*) | VarS | VC2453 | NP_232082.1 | *Vibrio cholerae* O1 El Tor N16961 | 64% | 0.0 |
| VDA_001267 (*varS*) | VarS | K04M1_26800 | ARP04300.1 | *Vibrio alginolyticus* | 66% | 0.0 |
| VDA_001267 (*varS*) | VarS | VF_2082 | YP_205465.1 | *Vibrio fischeri* | 69% | 0.0 |
| VDA_001267 (*varS*) | VarS | VV1_1573 | AAO09997.1 | *Vibrio vulnificus* | 65% | 0.0 |
| **CsrA (carbon storage regulator)** | | | | | | |
| VDA_001301 (*csrA*) | CsrA | CQ007_12255 | PRB80492.1 | *Pseudomonas* sp. | 86% | 7e-30 |
| VDA_001301 (*csrA*) | CsrA | VF_0538 | YP_203921.1 | *Vibrio fischeri* | 91% | 1e-36 |
| VDA_001301 (*csrA*) | CsrA | VV1_1595 | AAO10018.1 | *Vibrio vulnificus* | 91% | 3e-34 |
| VDA_001301 (*csrA*) | CsrA | VC0548 | NP_230199.1 | *Vibrio cholerae* O1 El Tor N16961 | 91% | 7e-38 |

**Deletion of regulatory genes *toxR, luxO* and *phoP* does not impair hemolytic activity in *Photobacterium damselae* subsp. *damselae***

In an attempt to find additional regulatory genes related to the hemolytic activity of *P. damselae* subsp *damselae* that may or may not interact with the RstAB system, we selected some candidate genes based on literature data and/or based on the results obtained in this work with the two-component VarAS system. The selected regulatory genes were *toxR, luxO* and *phoP* and are all present in chromosome I of *P. damselae* subsp. *damselae* (Fig. 7).

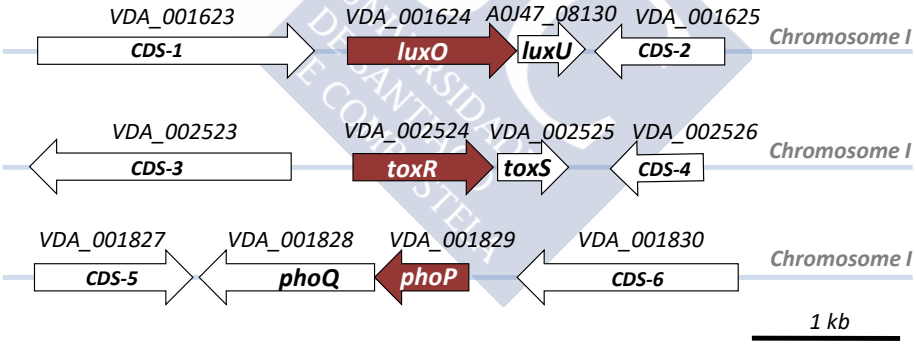



**Figure 7.** Genetic context of the *luxO*, *toxR* and *phoP* regulatory genes in *P. damselae* subsp. *damselae*.

These genes present in the wild type strain RM-71 have high homology with the same genes of type strain CIP 102761 (Table 4).

The *Vibrio cholerae* ToxR/S/T system has been reported as a key regulator of the expression of virulence proteins such as cholera toxin (CT) and toxin-co-regulated pili (TCP). (Miller and Mekalanos, 1987, Peterson and Mekalanos, 1988; Dirita *et al*., 1991; Childers and Klose, 2007; Bina *et al*., 2013, Peterson and Gellings, 2018). The *Vibrio cholerae* VarAS two-component system controls the expression of virulence proteins by regulating the expression of *toxT* (Jang *et al.*, 2011). The expression of ToxR in *Vibrio cholerae* depends on the global VarAS/CsrBCD/CrsA circuit and furthermore, it is known that the *ΔvarA* mutant overproduced the CsrA protein, exhibiting high levels of ToxR indicating that CsrA is a positive regulator of ToxR levels (Mey *et al.*, 2015).The homology of the *toxR* gene of *P. damselae* subsp. *damselae* is 47% with the *V. cholerae toxR* gene (Table 5). Given the key relationship of ToxR with the regulation of the virulence factors of *V. cholerae* and with the VarAS /CsrB, C, D/CrsA system we have found interesting to delete this *toxR* gene in *P. damselae* subsp. *damselae*, MT107 (Δ*toxR*), in order to evaluate if there were any consequences in the hemolysis of these pathogens.

**Table 4.** Homology between the *luxO*, *toxR*, *phoP* genes, adjacent genes of the RM-71 and CIP 10276 strains of *Pdd*.

| Feature ID | Length (bp) | Funcion of the predicted protein | Homologue Matches | | | |
|---|---|---|---|---|---|---|
| | | | Accesion nº. | Species | Ident. | E-value |
| VDA_001623/ CDS_1 | 2028 | excinuclease ABC subunit B | EEZ40596.1 | *P. damselae* subsp. *damselae* CIP 102761 | 100% | 0.0 |
| VDA_001624/ luxO | 1140 | putative repressor protein luxO | EEZ40597.1 | P. damselae subsp. damselae CIP 102761 | 100% | 0.0 |
| A0J47_08130/ *luxU* | 360 | phosphorelay protein LuxU (not annotated in CIP 102761) | ODA21700.1 | *P. damselae* subsp. *damselae* RM-71 | 100% | 0.0 |
| VDA_001625/ CDS_2 | 894 | adenylate kinase | EEZ41490.1 | *P. damselae* subsp. *damselae* CIP 102761 | 100% | 3e-151 |
| VDA_002523/CDS_3 | 1902 | chaperone protein HtpG | EEZ41491.1 | *P. damselae* subsp. *damselae* CIP 102761 | 100% | 0.0 |
| VDA_002524/ toxR | 900 | transcriptional activator ToxR | EEZ41492.1 | P. damselae subsp. damselae CIP 102761 | 100% | 0.0 |
| VDA_002525/ *toxS* | 564 | transmembrane regulatory protein ToxS | EEZ41493.1 | *P. damselae* subsp. *damselae* CIP 102761 | 100% | 6e-125 |
| VDA_2526/ CDS_4 | 603 | recombination protein RecR | EEZ41494.1 | *P. damselae* subsp. *damselae* CIP 102761 | 100% | 4e-126 |
| VDA_001827/ CDS_5 | 1203 | short-chain alcohol dehydrogenase family | EEZ40795.1 | *P. damselae* subsp. *damselae* CIP 102761 | 100% | 0.0 |
| VDA_001828/ *phoQ* | 1347 | sensor protein phoQ | EEZ40796.1 | *P. damselae* subsp. *damselae* CIP 102761 | 100% | 0.0 |
| VDA_001829/ phoP | 675 | putative transcriptional regulatory protein PhoP | EEZ40797.1 | P. damselae subsp. damselae CIP 102761 | 100% | 5e-167 |
| VDA_001830/CDS_6 | 1683 | TrkA Potassium channel-family protein | EEZ40798.1 | *P. damselae* subsp. *damselae* CIP 102761 | 99% | 0.0 |

**Table 5.** Homology between the *luxO*, *toxR* and *phoP* genes of *Pdd* and other bacterial species.

| locus_tag/Feature ID (*P. damselae* subsp. *damselae*) | Homologue | locus_tag | Homologue Matches | | | |
|---|---|---|---|---|---|---|
| | | | Accesion nº. | Species | Ident. | E-value |
| **LuxO (repressor protein)** | | | | | | |
| VDA_001624 (*luxo*) | LuxO | VC1021 | NP_230666.1 | *Vibrio cholerae* O1 El Tor N16961 | 70% | 0.0 |
| **PhoP ( Response Regulator)** | | | | | | |
| VDA_001829 (*phoP*) | PhoP | b1130 | NP_415648.1 | *Escherichia coli* K-12 | 51% | 6e-75 |
| VDA_001829 (*phoP*) | PhoP | STY1271 | NP_455723.1 | *Salmonella enterica* Typhimurium | 50% | 3e-74 |
| VDA_001829 (*phoP*) | PhoP | VC1638 | NP_231275.1 | *Vibrio cholerae* O1 El Tor N16961 | 39% | 6e-51 |
| **ToxR (transcriptional activator)** | | | | | | |
| VDA_002524 (*toxR*) | ToxR | VC0984 | NP_230630.1 | *Vibrio cholerae* O1 El Tor N16961 | 47% | 5e-76 |

We also constructed the double mutant MT172 (Δ*toxR*Δ*varS*) in order to give us clues about the operation of the VarAS/CsrB, C, D/CrsA system of *P. damselae* subsp. *damselae*. However, mutant strains MT107 and MT172 did not have their hemolytic activity altered in TSA-1 medium supplemented with sheep blood (data not shown). The *Vibrio cholerae* VarAS/CsrB,C,D/CrsA circuit, also acts on the quorum-sensing network of this pathogen by an alternative route, acting directly on the LuxO repressor protein. Then, the post-transcriptional protein, CsrA, promotes the activation of the LuxO protein which in turn activates the expression of sRNA genes that destabilize the HapR mRNA (key regulatory protein in the quorum-sensing of *V. cholerae*) thus repressing the production from HapR (Lenz *et al*., 2005; Lenz *et al*., 2007; Jang *et al*., 2010). It is known that the HapR regulatory protein activates or deactivates a variety of genes. For example, when HapR is produced, it activates the production proteases and represses the production of virulence factors and biofilm formation **(**Jobling and Holmes, 1997; Zhu *et al*., 2002; Hammer and Bassler, 2003; Zhu and Mekalanos, 2003; Jang *et al*.,

2010). A putative LuxO repressor protein from *P. damselae* subsp. *damselae* has 70% identity with the *V. cholerae* LuxO protein (Table 5). We constructed the mutants MT209 (Δ*luxO*), MT211 (Δ*luxO*Δ*rstB*), MT203 (Δ*luxO*Δ*varS*), MT212 (Δ*luxO*Δ*rstB*Δ*varS*) and MT317 (Δ*luxO*Δ*rstB*Δ*varA*) and evaluated the hemolytic activity in TSA-1 medium supplemented with sheep blood (data not shown). MT209, MT211 and MT203 did not have their hemolytic activity altered and MT212 and MT317 hemolytic activity similar to MT350 (Δ*rstB*Δ*varA*) and MT351 (Δ*rstB*Δ*varS*) mutants exhibiting partially recovered hemolytic activity (data not shown). We did not have evidence that LuxO regulates the hemolytic activity of *P. damselae* subsp. *damselae* under the conditions tested. The PhoPQ two-component system monitors availability of extracellular $Mg^{2+}$ and is more activated at low concentrations of $Mg^{2+}$. Increasing extracellular $Mg^{2+}$ concentrations (PhoPQ less active) led to decreased gene expression of the two-component RstAB system in *E. coli*, suggesting that the PhoPQ system activates the gene transcription of the two-component RstAB system in this pathogen (Minagawa *et al*., 2003;

Ogasawara *et al*., 2007). The interaction between the two-components PhoPQ and RstAB systems has also been reported in *Salmonella enterica* serovar Typhimurium (Nam *et al*., 2010; Tran *et al*., 2016). Data on the PhoPQ system in *V. cholerae* and *P. damselae* subsp. *damselae* is scarce. The PhoP response regulator protein of *P. damselae* subsp. *damselae* possesses 51%, 50% and 39% identity with *E. coli*, *Salmonella enterica* serovar Typhimurium and *V. cholerae* respectively (Table 5). We constructed the mutant strains MT242 (Δ*phoP*) and MT261 (Δ*rstB*Δ*phoP*) to evaluate the hemolytic activity. However, MT242 strain continued to have hemolytic activity similar to the wild-type strain RM-71$^{wt}$ (data not shown). The MT261 strain, however, exhibited reduced hemolytic activity due to deletion of the *rstB* gene (data not shown). No relationship was found between the *toxR, luxO* and *phoP* regulatory genes of *P. damselae* subsp. *damselae* strain RM-71$^{wt}$ with the hemolytic activity in sheep blood. The hemolysis regulating system of *Photobacterium damselae* subsp. *damselae* has been shown to be an extremely complex circuit because of the likely interaction of different regulatory systems.

## References


Aarons S., Abbas, A., Adams, C., Fenton, A., and O'Gara, F. (2000). A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113. *J Bacteriol* 182: 3913–3919.

Beier D., Gross R. (2008). The BvgS/BvgA phosphorelay system of pathogenic *Bordetellae*: structure, function and evolution. *Adv. Exp. Med. Biol.* 631:149–60.

Bina X.R., Taylor D.L., Vikram A.*et al*. (2013). *Vibrio cholerae* ToxR downregulates virulence factor production in response to Cyclo (PhePro). *mBio.* 4:e00366–13.

Botella E., Devine S.K., Hubner S., Salzberg L.I., Gale R.T., *et al*. (2014). PhoR autokinase activity is controlled by an intermediate in wall teichoic acid metabolism that is sensed by the intracellular PAS domain during the PhoPR-mediated phosphate limitation response of *Bacillus subtilis*. *Mol. Microbiol*. 94:1242–59.

Blumer C., Heeb, S., Pessi, G. and Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci U S A 96*. 14073–14078.

Burrowes E., Abbas, A., O'Neill, A., Adams, C., and O'Gara, F. (2005). Characterisation of the regulatory RNA RsmB from *Pseudomonas aeruginosa* PAO1. *Res Microbiol*. 156: 7–16.

Childers B.M., Klose K.E. (2007). Regulation of virulence in *Vibrio cholerae*; the ToxR regulon. *Future Microbiol*.2:335–44.

Chancey S.T, Wood D.W, Pierson 3rd L.S. (1999). Two-component transcriptional regulation of N-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol*. 65(6):2294–2299.

Cui Y, Chatterjee A., Chatterjee A.K. (2001). Effects of the two-component system comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the production of global regulatory rsmB RNA, extracellular enzymes, and harpinEcc. *Mol Plant Microbe Interact*. 14(4):516–26.

Chatterjee A., Cui Y., Yang H., Collmer A., Alfano J.R., Chatterjee A.K. (2003). GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. tomato DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol Plant Microbe Interact*. 16(12):1106–17.

DiRita V.J., Parsot C., Jander G.*et al*. (1991). Regulatory cascade controls virulence in *Vibrio cholerae*. *P Natl Acad Sci USA*.88:5403– 7.

Foxall R.L, Ballok A.E., Avitabile A., Whistler C.A. (2015). Spontaneous phenotypic suppression of GacA-defective *Vibrio fischeri* is achieved via mutation of *csrA* and *ihfA*. *BMC Microbiol*. 16;15:180.

Fujita M., Gonzalez-Pastor J.E., Losick R. (2005). High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. *J. Bacteriol.* 187:1357–68.

Gao R., and Stock, A. M. (2009). Biological insights from structures of two component proteins. *Annu. Rev. Microbiol*. 63, 133–154.

Groisman E.A. (2016). Feedback Control of Two-Component Regulatory Systems. *Annu Rev Microbiol.* 8;70:103-24.

Goodell K. H., Jordan M. R., Graham R., Cassidy C. and Nasraway S. A. (2004). Rapidly advancing necrotizing fasciitis caused by *Photobacterium (Vibrio) damsela*: a hyper aggressive variant. *Crit. CareMed*. 32(1):278- 281.

Hammer B.K., and Bassler, B.L. (2003). Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol*. 50: 101–104.

Heeb S., Blumer, C., and Haas, D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* 184: 1046–1056.

Herrero M., de Lorenzo, V., and Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol*. 172, 6557–6567.

Jang J., Jung K.T., Yoo C.K., Rhie G.E. (2010). Regulation of hemagglutinin/protease expression by the VarS/VarA-CsrA/B/C/D system in *Vibrio cholerae*. *Microb Pathog*. 48(6): 245-50.

Jang J., Jung, K. T., Park, J., Yoo, C. K., and Rhie, G. E. (2011). The *Vibrio cholerae* VarS/VarA two-component system controls the expression of virulence proteins through ToxT regulation. *Microbiology* 157, 1466–1473.

Jobling M.G., and Holmes, R.K. (1997). Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/ protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Mol Microbiol*. 26: 1023–1034.

Johnston C., Pegues D.A., Hueck C.J., Lee A., Miller S.I. (1996). Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. *Mol Microbiol*.22:715e27.

Jones M.K., Oliver J.D. (2009). *Vibrio vulnificus*: Disease and pathogenesis. *Infect Immun*. 77: 1723–33.

Kim M.J., Kim J., Lee H.Y., Noh H.J., Lee K.H., Park S.J. (2015). Role of AcsR in expression of the acetyl-CoA synthetase gene in *Vibrio vulnificus*. *BMC Microbiol.* 12;15:86.

Kreger A. S., Bernheimer, A. W., Etkin, L. A., and Daniel, L. W. (1987). Phospholipase D activity of *Vibrio damsela* cytolysin and its interaction with sheep erythrocytes. *Infect. Immun*. 55, 3209–3212.

Labella A., Berbel C., Manchado M., Castro D., Borrego J.J. (2011). *Photobacterium damselae* subsp. *damselae*, an emerging pathogen affecting new cultured marine fish species in southern Spain. Faruk A. and Zafer D. (Eds.), *Recent Advances in Fish Farns* (pp.135-152). New York: In Tech.

Laub M.T., Goulian M. (2007). Specificity in two-component signal transduction pathways. *Annu. Rev. Genet*. 41:121–45.

Laville J., Voisard, C., Keel, C., Maurhofer, M., Defago, G., and Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc Natl Acad Sci USA* 89: 1562–1566.

Lenz D.H., Bassler B.L. (2007). The small nucleoid protein Fis is involved in *Vibrio cholerae* quorum sensing. *Mol Microbiol*. 63:859–871.

Lenz D.H., Miller M.B., Zhu J., Kulkarni R.V., Bassler B.L. (2005). CsrA and three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. *Mol Microbiol*. 58(4):1186-202.

Liu M.Y., Gui G., Wei B., Preston J.F.*et al*. (1997). The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in *Escherichia coli*. *J. Biol. Chem.* 272:17502–17510.

McCleary W.R., Stock J.B. (1994). Acetyl phosphate and the activation of two-component response regulators. *J. Biol. Chem.* 269:31657–72.

Mey A.R., Butz H.A., Payne S.M. (2015). *Vibrio cholerae* CsrA Regulates ToxR Levels in Response to Amino Acids and Is Essential for Virulence. *MBio.* 4;6(4):e01064.

Miller V.L., Mekalanos J.J. (1987). Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. *Proc Natl Acad Sci USA* 81:3471–5.

Miller S.I., Kukral AM, Mekalanos J.J. (1989) A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proceedings of the National Academy of Sciences of the United States of America. 86(13):5054–8.

Minagawa S., Ogasawara H., Kato A., Yamamoto K., Eguchi Y., *et al*. (2003). Identification and molecular characterization of the $Mg^{2+}$ stimulon of *Escherichia coli*. *J. Bacteriol*. 185:3696–702.

Mima T., Gotoh K., Yamamoto Y., *et. al*. (2018). Expression of Collagenase is Regulated by the VarS/VarA Two-Component Regulatory System in *Vibrio alginolyticus*. *J Membr Biol*. 251(1):51-63.

Molofsky A.B., and Swanson, M.S. (2004) Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. *Mol Microbiol* 53: 29–40.

Moolenaar G.F., van Sluis C.A., Backendorf C., van de Putte P. (1987). Regulation of the *Escherichia coli* excision repair gene uvrC. Overlap between the uvrC structural gene and the region coding for a 24 kD protein. *Nucleic Acids Res.*15: 4273e89.

Nam D., Choi E, Kweon D.H., Shin D. (2010). The RstB sensor acts on the PhoQ sensor to control expression of PhoP-regulated genes. *Mol Cells*. 30(4):363-8.

Nyholm S.V., McFall-Ngai M.J. (2004). The winnowing: establishing the squid-vibrio symbiosis. *Nat Rev Microbiol.* 2(8):632–42.

Ogasawara H., Hasegawa, A., Kanda, E., Miki, T., Yamamoto, K., and Ishihama, A. (2007). Genomic SELEX search for target promoters under the control of the PhoQP-RstBA signal relay cascade. *J. Bacteriol*. 189, 4791–4799.

Osorio C. R., Vences, A., Matanza, X. M., and Terceti, M. S. (2018). *Photobacterium damselae* subsp. *damselae*, a generalist pathogen with unique virulence factors and high genetic diversity. *J. Bacteriol*. JB.00002-18.

Perego M., Brannigan J.A. (2001). Pentapeptide regulation of aspartyl-phosphate phosphatases. *Peptides* 22:1541–47.

Peterson K.M., Mekalanos J.J. (1988). Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. *Infect Immun*. 56:2822–9.

Peterson K. M. and Gellings P.S. (2018). Multiple intraintestinal signals coordinate the regulation of *Vibrio cholerae* virulence determinants. *Pathogens and Disease*. 1;76(1).

Rahme L.G., Tan M.W., Le L., Wong S.M., (1997). Tompkins R.G., Calderwood S.B., *et al*. Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. *Proc Natl Acad Sci USA*.94:13245e50.

Rich J.J., Kinscherf, T.G., Kitten, T., and Willis, D.K. (1994). Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. *J Bacteriol* 176: 7468–7475.

Rivas A. J., Balado, M., Lemos, M. L., and Osorio, C. R. (2011). The *Photobacterium damselae* subsp. *damselae* hemolysins

damselysin and HlyA are encoded within a new virulence plasmid. *Infect. Immun*. 79, 4617-4627.

Rivas A. J., Balado, M., Lemos, M. L., and Osorio, C. R. (2013a). Synergistic and additive effects of chromosomal and plasmid-encoded hemolysins contribute to hemolysis and virulence in *Photobacterium damselae* subsp. *damselae*. *Infect. Immun*. 81, 3287-3299.

Rivas A. J., Von Hoven, G., Neukirch, C., Meyenburg, M., Qin, Q., Füser, S., Boller, K., Lemos, M. L., Osorio, C. R., and Husmann, M. (2015a). Phobalysin, a small ß-pore-forming toxin of *Photobacterium damselae* subsp. *damselae*. *Infect. Immun*. 83, 4335-4348.

Rivas A. J., Vences, A., Husmann, M., Lemos, M. L., and Osorio, C. R. (2015b). *Photobacterium damselae* subsp. *damselae* major virulence factors Dly, plasmid encoded HlyA, and chromosome-encoded HlyA are secreted via the type II secretion system. *Infect. Immun*. 83, 1246-1256.

Romeo T. (1998). Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Mol Microbiol*. 29: 1321–1330.

Romeo T., Gong, M., Liu, M.Y., and Brun-Zinkernagel, A.M. (1993). Identification and molecular characterization of csrA, a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. *J Bacteriol*. 175: 4744– 4755.

Septer A.N., Bose J.L., Lipzen A., Martin J., Whistler C., Stabb E.V. (2015). Bright luminescence of *Vibrio fischeri* aconitase mutants reveals a connection between citrate and the Gac/Csr regulatory system. *Mol Microbiol.* 95(2):283–96.

Stock A.M., Robinson V.L., Goudreau P.N. (2000). Two-component signal transduction. *Annu. Rev. Biochem.* 69:183–215.

Terceti M. S., Rivas, A. J., Alvarez, L., Noia, M., Cava, F., and Osorio C.R. (2017). *rstB* regulates expression of the *Photobacterium damselae* subsp. *damselae* major virulence factors Damselysin, Phobalysin P and Phobalysin C. *Front. Microbiol*. 8, 582.

Terceti M. S., Vences A., Matanza X. M., Dalsgaard I., Perdersen K., Osorio C. R. (2018a). Molecular epidemiology of *Photobacterium damselae* subsp. *damseale* outbreaks in marine raibow trout farms reveals extensive horizontal gene transfer and high genetic diversity. *Front. Microbiol*. 9, 2155.

Terceti M. S., Vences A., Matanza X.M., Barca A.V., Noia M., Lisboa J., Santos N.M.S., Vale A., Osorio C. R. (2018b). Mutation of the RstAB two-component regulatory system impairs virulence, motility, cell morphology, antimicrobial resistance and production of type II secretion system-dependent factors in the fish and human pathogen *Photobacterium damselae* subsp. *damselae*. *(to submit).*

Tran T. K., Han Q. Q., Shi Y., and Guo, L. (2016). A comparative proteomic analysis of *Salmonella typhimurium* under the regulation of the RstAB and PhoPQ systems. *Biochim. Biophys*. Acta 1864, 1686–1695.

Trajtenberg F., Albanesi D., Ruetalo N., Botti H., Mechaly A.E., *et al.* (2014). Allosteric activation of bacterial response regulators: The

role of the cognate histidine kinase beyond phosphorylation. *mBio* 5:e02105.

Tsou A.M., Liu Z., Cai T., Zhu J. (2011) The VarS/VarA two-component system modulates the activity of the *Vibrio cholerae* quorum-sensing transcriptional regulator HapR. *Microbiology*. 157 (Pt 6):1620-8.

Vakulskas C.A., Potts A.H., Babitzke P., Ahmer B.M., Romeo T. (2015). Regulation of bacterial virulence by Csr (Rsm) systems. *Microbiol Mol Biol Rev*. 79:193–224.

Valverde C., Heeb, S., Keel, C., and Haas, D. (2003). RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. *Mol Microbiol 50*: 1361– 1379.

Verhamme D.T., Arents J.C., Postma P.W., Crielaard W, Hellingwerf K.J. (2002). Investigation of in vivo cross-talk between key two-component systems of *E. coli. Microbiology.* 148:69–78.

Vences A., Rivas A. J., Lemos M. L., Husmann M., and Osorio C. R. (2017). Chromosome-encoded hemolysin, phospholipase, and

collagenase in plasmidless isolates of *Photobacterium damselae* subsp. *damselae* contribute to virulence for fish. *Appl. Environ. Microbiol*. 83, pii: e00401-17.

Wong S.M., Carroll P.A., Rahme L.G., Ausubel F.M., Calderwood SB. (1998). Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two-component family of response regulators. *Infect Immun*. 66:5854-5861.

Weilbacher, T., Suzuki, K., Dubey, A.K., Wang, X., Gudapaty, S., Morozov, I., *et al*. (2003). A novel sRNA component of the carbon storage regulatory system of *Escherichia coli*. *Mol Microbiol*. 48: 657–670.

Whistler C.A. and Ruby E.G. (2003). GacA regulates symbiotic colonization traits of *Vibrio fischeri* and facilitates a beneficial association with an animal host. *J Bacteriol*. 185(24):7202–12.

Yuen K. Y., Ma L., Wong S. S. Y. and Ng W. F. (1993). Fatal necrotizing fasciitis due to *Vibrio damsela*. Scand. *J. Infect. Dis*. 25(5):659-661.

Zwir I., Latifi T., Perez J.C., Huang H., Groisman E.A. (2012). The promoter architectural landscape of the *Salmonella* PhoP regulon. *Mol. Microbiol*. 84:463–85.

Zwir I., Yeo W.S., Shin D., Latifi T., Huang H., Groisman E.A. (2014). Bacterial nucleoid-associated protein uncouples transcription levels from transcription timing. *mBio* 5:e01485–14.

Zhu J., and Mekalanos, J.J. (2003). Quorum sensingdependent biofilms enhance colonization in *Vibrio cholerae*. *Dev Cell*.5: 647–656.

Zhu J., Miller, M.B., Vance, R.E., Dziejman, M., Bassler, B.L., and Mekalanos, J.J. (2002). Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA*. 99: 3129–3134.

# DISCUSSION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

# 4. DISCUSSION

*Photobacterium damselae* subsp. *damselae* is recognized as an emerging pathogen in marine aquaculture (Labella *et al.,* 2011). Outbreaks of the disease caused by this bacterium in marine fish cultures have shown an evident increase in their geographical distribution in the last years, appearing in countries where it had never been reported before (Fig. IV.1). In addition, outbreaks in fish farms have been correlated with episodes of exceptionally high temperatures (Pedersen *et al.,* 1997, Pedersen *et al.,*2008; Pedersen *et al.,* 2009), following patterns similar to other infections caused by vibrios (Le Roux *et al.,* 2015). In humans, *P. damselae* subsp. *damselae* can cause an extreme variant of severe necrotizing fasciitis, resulting fatal in some cases (Clarridge and Zighelboim-Daum, 1985; Coffey *et al.,* 1986; Yuen *et al.,* 1993; Perez-Tirse *et al.,* 1993; Fraser *et al.,* 1997; Tang and Wong, 1999; Barber and Swygert, 2000; Goodell *et al.,* 2004; Asato and Kanaya, 2004; Alvarez *et al.,* 2006, Kim *et al.,* 2009a; Hundenborn *et al.,* 2013).

Despite the increasing number of reports on the isolation of this pathogen from new fish species and new geographical locations, studies at the genetic level to identify the presence of virulence markers have been scarce. Similarly, although the molecular basis of virulence in this pathogen has been extensively studied in recent years, little is known about how the expression of these toxins is regulated.

The proposal of our work was, on the one hand, to study the genetic diversity of *P. damselae* subsp. *damselae* in order to better understand its epidemiology and also whether the outbreaks caused by this pathogen are a consequence of infection and proliferation of a single adapted clone or several clones. For this, it was essential to analyze a collection of strains of *P. damselae* subsp. *damselae* from the same period and region of the outbreak. On the other hand, we here aimed at identifying some of the regulatory genes that modulate the production of virulence factors in *P. damselae* subsp. *damselae*.

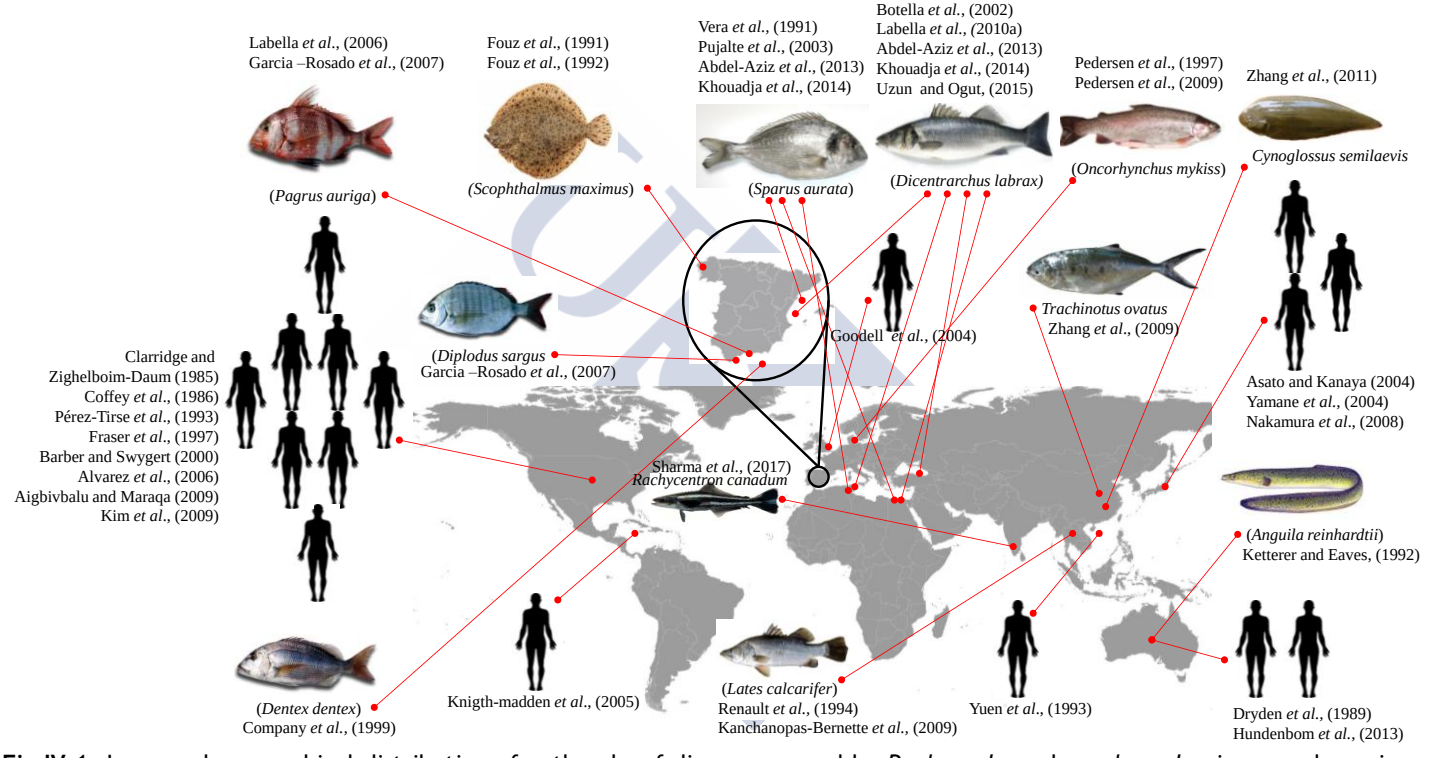



**Fig.IV.1**: Increased geographical distribution of outbreaks of diseases caused by *P. damselae* subsp. *damselae* in several species of fish and humans in the world.

So, we would then begin analyzing the valuable collection of isolates provided by Dr. Hamdi Ogut fromBursa Technical University (Turkey), which resulted in the publication of the paper **"*Photobacterium damselae* subsp. *damselae*, an Emerging Fish Pathogen in the Black Sea: Evidence of a Multiclonal Origin"(Terceti *et al.*, 2016).** Wesubsequently analysed the collection provided by Prof.Dr. Karl Pedersen and Prof. Dr. Inger Dalsgaard fromthe Technical University of Denmark, giving rise to paper "**Molecular Epidemiology of *Photobacterium damselae* subsp. *damselae* Outbreaks in Marine Rainbow Trout Farms Reveals Extensive Horizontal Gene Transfer and High Genetic Diversity" (Terceti *et al.*, 2018a)**. These two collections of *P. damselae* subsp. *damselae*metthe requirements we were looking for.These studies revealed high genetic diversity of the Turkish and Danish strains of *P. damselae s*ubsp. *damselae* causing outbreaks. (Both studies are discussed below in item 4.1).

It has been mentioned that there are strains bearing the virulence plasmid pPHDD1and therefore produce the hemolysins Dly, PhlyP and PhlyC, and other strains that do not harbor pPHDD1 and then produce only PhlyC. As we did not know anything about the regulation of the genetic expression of these hemolysins, we started the search for regulatory genes. We have shown that the two-component RstAB regulatory system plays an important role in controlling the hemolytic activity of *P. damselae* subsp. *damselae* and itcontrols the secretion of several other non-characterized proteins. In addition, this regulatory system proved to be ubiquitous inall strains of *P. damselae* subsp. *damselae* evaluated including both plasmid-bearing strains and strains lacking pPHDD1. From this research topic we published the paper entitled **"*rstB* Regulates Expression of the *Photobacterium damselae* subsp. *damselae* Major Virulence Factors Damselysin, Phobalysin P and Phobalysin"(Terceti *et al.*, 2017)** and the paper "**Mutation of the RstAB two-component regulatory system impairs virulence, motility, cell morphology, antimicrobial resistance and production of type II secretion system-dependent factors in the fish and human pathogen *Photobacterium damselae* subsp. *damselae* (Terceti *et al.*, 2018b** *to*

*submit***).** (Both discussed belowin item 4.2.). In section 4.2 we will also briefly discuss the unpublished results: "**Evidence of Interaction between VarAS and RstAB Systems in the Regulation of the Hemolytic Activity of *Photobacterium damselae* subsp. *damselae*".**

## 4.1. GENETIC DIVERSITY AND MULTICLONAL ORIGIN

We characterized a collection of 14 strains of *P. damselae* subsp. *damselae* isolated from sea bass during recent outbreaks in two farms in the Black Sea in Turkey, to carry out a study of their virulence gene background and to evaluate the pathogenicity potential forsea bass of strains producing PhlyC, and also of strains negative for the production of this hemolysin (Terceti *et al.*, 2016)**.** This study constitutes the first report, to our knowledge, on the molecular characterization of this pathogen isolated from disease outbreaks in the Black Sea, and our results indicated a multiclonal origin of the *P. damselae* subsp. *damselae* isolates in this area. Furthermore, to date, no study has been conducted to our knowledge to characterize the virulence of isolates of this emerging pathogen for sea bass, since most of the data on virulence were obtained using turbot (Fouz *et al.*, 1992), redbanded sea bream (Labella *et al.*, 2010b) and raibow trout (Pedersen *et al*. 2009) as models of infection. We also characterized a collection of 31 strains of this pathogen from outbreaks in rainbow trout farms in Denmark during three distinct years (Terceti *et al.*, 2018a). This collection of Danish strains had been previously characterized by DNA fingerprinting techniques (ribotyping and pulsed-field gel electrophoresis) in two studies (Pedersen *et al.*, 1997; Pedersen *et al*. 2009), revealing a lack of clonality between strains and suggesting that rainbow trout outbreaks were caused by genetically heterogeneous populations.

We used the gene encoding the transcriptional regulator ToxR as a phylogenetic marker to provide clues about the phylogeography of the Turkish and Danish collections of *P. damselae* subsp. *damselae*. The *toxR* sequence is increasingly used as a valuable molecular clock for finely tuned rate discrimination within *Vibrionaceae* because of its high variability and the existence of intrasubspecific variation in *P.*

*damselae* subsp. *damselae* (Osorio and Klose, 2000; Martins *et al.,* 2015)**.** Phylogeny based on the *toxR* gene revealed the existence of genetic heterogeneity among the Black Sea isolates (Terceti *et al.,* 2016) and Danish isolates (Terceti *et al.,* 2018a). Thus, we find interesting correlations between identity at the level of the *toxR* gene and the occurrence of the same phenotypic or genotypic characteristics. The hypothesis of multiclonality of the Turkish strains is reinforced by the observation that the *toxR* gene sequenced from the different groups of *P. damselae* subsp. *damselae* isolated from the Black Sea show a much greater degree of variability between them than with strains of *P. damselae* subsp. *piscicida* or with the group of *P. damselae* subsp. *damselae* isolated from an outbreak in turbot farms in Spain (Terceti *et al.,* 2016).

Regarding the Danish strains the isolation of DK2 and DK3 from two different fish individuals on farm A in 1994 indicated that the fish were colonized by at least two markedly different genotypes of *P. damselae* subsp *damselae*. Interestingly, the tree based on the *toxR* gene also revealed that some genotypes from 2006 were identical to the genotypes previously isolated in 1994 and 1995. This observation suggests that some genotypes of *P. damselae* subsp. *damselae* can thrive in the environment for long periods between outbreaks and cause fish outbreaks once environmental and/or host conditions are favorable (Terceti *et al.,* 2018a). Even so, the sampling procedure allowed the isolation of potentially clonal strains, exemplified by DK4 to DK7 from 1994 (Terceti *et al.,* 2018a).

We determined that the collection of Black Sea isolates of this pathogen exhibited variable phenotypes and genotypes. Although *P. damselae* subsp. *damselae* are recognized as urease-positive in most reports (Fouz *et al.,* 1992; Vera *et al.,* 1991; Takahashi *et al.,* 2008), previous studies have reported the existence of urease-negative isolates (Botella *et al.,* 2002). In our study, we found that among the Turkish strains the 70 dps isolate was the only isolate that tested negative for the *ureC* gene (Terceti *et al.,* 2016). The collection of Danish strains evaluated in this study were all urease positive (Pedersen *et al.,* 1997; Pedersen *et al.,* 2009).

Most *P. damselae* subsp. *damselae* strains do not ferment sucrose and therefore produce green colonies in TCBS medium (Fouz *et al.,* 1992; Botella *et al.,* 2002; Vaseeharan *et al.,* 2007; Abdel-Aziz *et al.,* 2013), but strains that form yellow colonies are isolated from time to time (Zhao *et al.,* 2009). We found that 6 of 14 Black Sea isolates and 1 of 31 Danish rainbow trout isolates produced yellow colonies on TCBS medium (Terceti *et al.,* 2016; Terceti *et al.,* 2018a).

We also revealed the great diversity in motility of Turkish and Danish strains, as demonstrated by motility agar assays and to our knowledge, this constitutes the first reported evidence of variability in this phenotypic trait in this pathogen to date (Terceti *et al.,* 2016; Terceti *et al.,* 2018a).

Virulence experiments using the highly hemolytic strain RM-71 indicate that pPHDD1 plasmid is an important virulence factor for sea bass, as this strain caused 100% fish death in the first 24 h after inoculation. The two hemolysins encoded by pPHDD1, Dly and PhlyP, are considered the main virulence factors for turbot, and in addition, PhlyC contributes to the virulence to athe lesser extent (Rivas *et al.,* 2013, Vences *et al.,* 2017). We have shown that plasmid pPHDD1 is absent in all Black Sea sea bass isolates (Terceti *et al.,* 2016). Recent studies have reported that strains of *P. damselae* subsp. *damselae* without pPHDD1 were virulent in redbanded sea bream model **(**Labella *et al.,* 2010b). In this respect, we demonstrated that negative isolates for pPHDD1 from the Black Sea environment have pathogenic potential for sea bass, and virulence was greatly diminished in a lineage with amutated $hlyA_{ch}$ gene version. Our finding that 3 non-hemolytic Black Sea isolates contained pseudogenes of the $hlyA_{ch}$ gene reinforces the hypothesis that the chromosomal region encoding PhlyC is a hot spot for recombination and insertion of IS elements into the genome of *P. damselae* subsp. *damselae* (Terceti *et al.,* 2016).

Interestingly, in Danish isolates, 3 non-hemolytic isolates without pPHDD1 (3/31) also harbored a $hlyA_{ch}$ pseudogene (Terceti *et al.,* 2018a). In addition, 13 isolates (13/31) were hemolytic and carrying plasmid pPHDD1 and 15 isolates (15/31) showed low hemolysis and negative for pPHDD1 (Terceti *et al.,* 2018a). This study confirmed

that the *dly* and *hlyA*$_{pl}$ genes, when present, always occur simultaneously, and their presence is linked to the detection of the origin of replication of the plasmid pPHDD1. Therefore, screening of virulence genes demonstrated that the rainbow trout outbreaks were probably caused by a mixture of pPHDD1 negative strains and pPHDD1 positive, or, in other words, strains with very different degrees of virulence. This observation may question whether strains without plasmids were actually disease-causing organisms or whether they behaved as secondary invaders with a minor role in infection.

The *plpV* gene encoding the PlpV phospholipase and its associated lecithinase activity proved to be ubiquitous in all Danish strains. These observations confirm that the phospholipase PlpV is the unique ubiquitous virulence gene marker described in *P. damselae* subsp. *damselae* so far, as previously suggested (Vences *et al.*, 2017).

In our study, we found that a pair of strains (DK2 and DK3) isolated from the same outbreak on the same fish farm contained a large number of strain-specific genes (Terceti *et al.,* 2018a). It is pertinent to say that the present study constitutes, as far as we know, the first report of comparative genomic analysis in the species *P. damselae*, in which strains of the same outbreak are compared. A recent study analyzed the genome sequence of three strains of this subspecies, but the strains came from different host species and from different geographic locations (Vences *et al.*, 2017).

Our study found the differential occurrence of several virulence-related genes in all 31 strains from rainbow trout from Denmark (Terceti *et al.,* 2018a). A putative cluster of genes for the synthesis and use of siderophore vibrioferrin has been shown to be unique to the DK2 lineage, corroborating previous observations that this genetic system is only characteristic of a fraction of *P. damselae* subsp. *damselae* strains (Balado *et al.*, 2017).

Genomic variation among *P. damselae* subsp. *damselae* genomes analyzed in some Danish strains appeared to be associated with the differential occurrence of putative mobile DNA (Terceti *et al.*, 2018a). This variable DNA fraction could have been gained by the contribution of each of the three main mechanisms of gene acquisition by horizontal gene transfer. Hence, the virulence plasmid pPHDD1

was likely acquired by conjugation, since a previous study demonstrated that this plasmid could be mobilized into recipient *P. damselae* cells (Rivas *et al.,* 2011). The major role of phages in DNA acquisition is exemplified by the number of putative phage-associated genes that were found in the genomes of the four *P. damselae* subsp. *damselae* strains analyzed (Terceti *et al.,* 2018a). The comparative analysis of these four genomes has also pointed at the existence of several hotspots for DNA acquisition (Terceti *et al.,* 2018a). Some hyper variable regions contain short repetitive sequences in tandem which overlap with the putative transcriptional terminators of genes (Terceti *et al.,* 2018a). These repeated sequences might therefore have played a role in the recombination of horizontally acquired DNA in some *P. damselae* subsp. *damselae* isolates. Additionally, a hyper variable region was characterized in the vicinity of the $hlyA_{ch}$ gene encoding PhlyC toxin (Terceti *et al.,* 2018a). This region, in addition to integrase genes and IS elements, also contained several tRNA genes. The role of tRNA genes as target loci for the insertion of horizontally acquired DNA sequences has been extensively documented (Schmidt and Hensel, 2004; Castillo *et al.,* 2017). The increasing evidence that *Vibrionaceae* species are indeed prone to gain foreign DNA by natural transformation (Sun *et al.,* 2013; Antonova and Hammer, 2015; Metzger and Blokesch, 2016), suggests that some of these variable DNA sequences might have been taken from the environment and inserted into the *P. damselae* subsp. *damselae* genomes via the aforementioned repeated sequences. This hypothesis will surely deserve an in-depth investigation in the near future.

Of particular significance is the high diversity within the gene clusters encoding cell envelope polysaccharides (LPS and capsular polysaccharides). It is known from early studies that this pathogen exhibits serological diversity (Fouz *et al.,* 1992). Our study with Danish strains provides supporting evidence that polysaccharide synthesis clusters of *P. damselae* subsp. *damselae* exhibit unique gene combinations in each analyzed strain (Terceti *et al.,* 2018a), as previously suggested (Osorio *et al.,* 2018). The reason for this high genetic diversity is currently unknown, and it might be related to the lifestyle of this generalist pathogen, that is capable of infecting a wide

range of animals and also of living as a free-swimming bacterium (Fouz *et al.,* 1998; Fouz *et al.,*2000).

Still on the analysis of the Danish collection, we revealed abundance and diversity of CRISPR-Cas systems occurring in *P. damselae* subsp. *damselae* genomes (Terceti *et al.,* 2018a). Genome sequencing of only four strains revealed as many as three distinct Cas gene clusters which, according to the recent classification of CRISPR-Cas (Makarova *et al.,* 2015) likely belong to the types I-F and I-E. The PCR screening revealed that most of the 31 analyzed strains from rainbow trout outbreaks harbor at least one of these systems (Terceti *et al.,* 2018a). As a pathogen that also thrives as a free-living bacterium in seawater, *P. damselae* subsp. *damselae* lives in close contact with bacteriophages (Novianty *et al.,* 2014; Yamaki *et al.,* 2015), hence the abundance of CRISPR-Cas systems. Not surprisingly, much of the variable DNA content that showed to be specific of each of the four genomes analyzed here contained typical features of prophage DNA. Recently, it has been reported that CRISPR-Cas systems may also play a regulatory role on endogenous genes. Thus, in addition to protecting from phages, these systems are increasingly being recognized as regulators of bacterial virulence (Louwen *et al.,* 2014; Ma *et al.,* 2018).

Phylogenetic data, together with phenotypic and genotypic diversity analyses, clearly indicate that *P. damselae* subsp. *damselae* isolates from sea bass in the Black Sea of Turkey and from rainbow trout from Denmark, originated not from the clonal spread of a single strain, but likely from the simultaneous occurrence of several clones.

### 4.2. TWO-COMPONENT REGULATORY SYSTEM RstAB

Identification of the regulatory mechanisms in bacterial pathogens is of maximal interest in order to understand the environmental and host conditions that trigger the expression of virulence factors. *P. damselae* subsp. *damselae* is a generalist pathogen, capable of living as a free-swimming bacterium that causes outbreaks in cultured fish species when the conditions become favourable (Osorio *et al.,* 2018).

This pathogen produces a variety of toxins which are thought to be secreted through the T2SS (Rivas *et al.,* 2015b; Vences *et al.,* 2018).

We began the search for regulatory genes that could control the expression of genes related to the hemolytic activity of *P. damselae* subsp. *damselae*. In this sense, we report in the paper (Terceti *et al.,* 2017) the isolation of a mini-Tn10 transposon mutant that showed a strong impairment in its hemolytic activity. The transposon disrupted a putative histidine kinase *rstB* gene sensor, which along with *rstA* is predicted to encode a putative two-component RstAB regulatory system. This system is homologous to CarSR/VprAB system in *Vibrio cholerae* (Bilecen and Yildiz, 2009; Herrera *et al.,* 2014). Reconstruction of the *rstB* mutant by allelic exchange showed equal involvement in hemolysis, and complementation with a plasmid expressing rstAB restored haemolysis to wild-type levels (Terceti *et al.,* 2017). We also demonstrated by analysis of promoter expression that reduced hemolysis in the *rstB* mutant was accompanied by a strong decrease in the transcription activities of the three hemolysin genes *dly* (damselysin), $hlyA_{pl}$ (Phobalysin P) and $hlyA_{ch}$ (Phobalysin C) (Terceti *et al*. 2017). We have also found that the reduced expression of the three virulence genes is correlated with a strong decrease in virulence in a bass model, demonstrating that RstB constitutes a master regulator of the three hemolysins of. *P. damselae* subsp *damselae* and plays a critical role in the pathogenicity of this bacterium (Terceti *et al.,* 2017). Previous studies reported that *V. cholerae vprAB* mutants (homologs of *P. damselae* subsp. *damselae* RstAB) exhibited sensitivity to polymyxin B (Herrera *et al.,* 2014; Bilecen *et al.,* 2015). We found that both the parental strain RM-71 and the *rstB* mutant were highly sensitive to polymyxin B (Terceti *et al.,* 2017). This study represented the first evidence of a direct role of a RstAB-like system in the regulation of bacterial toxins. Of the results generated from this work of 2017 some questions have arisen.

Would deletion of the *rstA* gene also compromise the hemolytic activity of this pathogen? And its virulence? Would there be other affected phenotypes resulting from the deletion of *rstA* and *rstB* genes? How would the motility of these mutants be? Would they become more or less sensitive to others antibiotics? Were there other

secreted proteins influenced by RstAB TCS? Is the RstAB TCS present only in a fraction of *P. damselae* subsp. *damselae* isolates, or is it ubiquitous in this subspecies? These inquiries have led us to continue studying and as a result we have produced an additional paper submitted for publication (Terceti *et al.,* 2018b-*to submit*). We demonstrate that single mutation of *rstA* impairs virulence in a sea bass model, confirming the results previously obtained with single *rstB* mutants. Studies of the role of RstAB system in fish pathogens are very scarce. Notably, mutation of *rstB* in *Edwardsiella ictaluri* caused impaired colonization and virulence in a channel catfish model (Menanteau-Ledouble and Lawrence, 2013).

*P. damselae* subsp. *damselae* strains are able to grow through a range of temperatures and of NaCl concentrations (Kreger *et al.,* 1984; Fouz *et al.,* 1992; Fouz *et al.,* 1998). We have previously reported that there are no differences in growth between the wild type strain RM-71 and the *rstB* mutant of *P. damselae* subsp. *damselae* in culture conditions with 1% NaCl at 25 °C (Terceti *et al.,* 2017). In the work submitted for publication we revealed that the mutation of *rstA* and *rstB* genes did not compromise growth at 15, 25 and 37ºC at 1% NaCl. However, a drastic impairment in growth occurred at 37ºC and 0.5% NaCl, a combination of conditions that virtually abolished bacterial growth in the parental strain and in *rstA* and *rstB* mutants. To the best of our knowledge, this finding has not been previously reported, and suggests that such combination of temperature and salinity constitutes a stressful condition for this bacterium.

We found that mutations in *rstA* and *rstB* caused impairment in swimming motility at 0.5% NaCl. Although the connection between flagellum-based motility and the RstAB system in *P. damselae* subsp. *damselae* is so far unknown, recent studies have provided clues on the role of RstAB-lile systems in bacterial motility (Terceti *et al.,* 2018b-*to submit*). Thus, silencing of the *rstA* or *rstB* genes resulted in impaired motility, hemolysis and virulence in *Vibrio alginolyticus* (Huang *et al.,* 2018). In *Salmonella typhimurium*, FlhA and MglB proteins involved in cell motility and chemotaxis are positively regulated by the RstAB system and a *rstB* mutant in this species showed a significant reduction in motility (Tran *et al.,* 2016). The cell

morphology of the *rstB* mutant under culture conditions with 1% NaCl at 25°C was not affected in comparison with the parental strain (Terceti *et al.*, 2017). However, in the paper in review, we provide strong evidence that *rstA* and *rstB* mutants cultured at 0.5% NaCl exhibit impairment in cell separation upon cell division, as well as enlarged cell size. These mutants also exhibited increased sensitivity to benzylpenicillin and, to a lesser extent, to vancomycin. In agreement with our results, overexpression of RstA in *E. coli* made this bacterium more resistant to β-lactam antibiotics such as ampicillin (Hirakawa *et al.*, 2003a; Hirakawa *et al.*, 2003b). Similarly, *E. coli rstAB* mutants are hypersensitive to ketoprofen, pridinol, and troleandomycin, although the basis for these sensitivities has not been ascertained yet (Zhou *et al.*, 2003). So far, the molecular mechanisms linking the RstAB system with antibiotic resistance and with maintenance of cell size and shape in *P. damselae* subsp. *damselae* remain unknown.

The work under review also contributed to the identification of 4 hitherto unreported proteins belonging to the RstAB regulon, including plasmid, chromosome I- and chromosome II-encoded proteins. Therefore, the response regulator RstA is predicted to recognize target genes associated with the *P. damselae* subsp. *damselae* chromosomes as well as genes that have been acquired by horizontal gene transfer via conjugation. Notably, our study has brought to the forefront a number of hitherto uncharacterized, T2SS-dependent and RstAB-dependent proteins, and there are very few studies on these except for the three hemolysins Dly, PhlyP and PhlyC (Kreger, 1984; Kothary and Kreger 1985; Rivas *et al.*, 2011; Rivas *et al.*, 2013b; Rivas *et al.*, 2015a; Vences *et al.*, 2017). Of special interest are two genes which are likely cotranscribed as an operon, and encode the delta (δ)-endotoxin VDA_002799 and an associated 11 kDa small protein, respectively. These two genes are inserted within a potential hot spot for recombination within the genome of *P. damselae* subsp. *damselae* (Terceti *et al.*, 2018a). δ-endotoxins, also called Cry and Cyt toxins, are pore-forming toxins predicted to cross the cytoplasmic membrane via the twin-arginine (Tat) translocation pathway (Li *et al.*, 1991; Cygler *et al.*, 1995; Galitsky *et al.*, 2001).

However, the role of this putative δ-endotoxin in *P. damselae* subsp. *damselae* is still unknown. Last, VDA_000112 and VDA_000358 are T2SS- and RstAB-dependent uncharacterized proteins encoded within plasmid pPHDD1 and chromosome II respectively. Surely all these proteins will deserve an in-depth characterization in future studies.

*rstAB* genes are considered to be part of the PhoP/PhoQ regulon (a $Mg^{2+}$-sensing two-component system) (Oshima *et al.*, 2002; Ogasawara *et al.*, 2007). While some evidence suggests that the RstAB system might respond to acidic conditions (Ogasawara *et al.*, 2007; Huang *et al.*, 2017), the specific stimuli that trigger the activation of the sensor histidine kinase RstB remain unknown for all the RstAB-like systems studied to date (Ogasawara *et al.*, 2007; Campbell *et al.*, 2007; Flamez *et al.*, 2008; Bilecen and Yildiz., 2009; Menanteau-Ledouble and Lawrence, 2013; Tran *et al.*, 2016; Huang *et al.*, 2018). In contrast, a number of genes and biological functions under control of the RstAB system have been identified, demonstrating that the RstAB regulon is not predictive since it shows a high diversity among species. Functions regulated by homologous RstAB systems are as varied as pyrimidine metabolism, enterobactin biosynthesis, ferrous iron uptake and motility in *Salmonella enterica* (Tran *et al.*, 2016), polysaccharide synthesis, biofilm formation, LPS modification in *Vibrio cholerae* (Bilecen and Yildiz, 2009; Herrera *et al.*, 2014), or adhesion, biolfilm production, motility and hemolysis in *Vibrio alginolyticus* (Huang *et al.*, 2017), among others.

We also demonstrate an evidence of interaction between VarAS and RstAB TCS in the regulation of the hemolytic activity of *P. damselae* subsp. *damselae* and that the deletion of the *toxR*, *luxO* and *phoP* regulatory genes in this pathogen does not alter its hemolytic activity. We found that the double mutants MT351 (Δ*rstB*Δ*varS*) and MT350 (Δ*rstB*Δ*varA*) partially recovered the hemolytic activity when compared to the hemolytic activity of the single mutant MT151 (Δ*rstB*) and wild type strain RM-71. This suggests that the response regulator RstA has a crucial function in the hemolysis of *P. damselae* subsp. *damsela*e since the mutants MT255 (Δ*rstA*Δ*varS*) and MT358 (Δ*rstA*Δ*varA*) do not recover the hemolytic activity. Figure IV.3 shows a hypothetical scheme of the possibilities of interaction

between VarAS and RstAB TCS. In addition Figure IV.3 shows the regulation exerted by the RstAB TCS on the hemolysin genes and other genes identified in this study, all T2SS-dependent. It is worth remembering that we were the pioneers to look for regulatory genes related to virulence factors in the species *Photobacterium damselae* and therefore, these results can act as an excellent starting point for further studies in order to elucidate this complex regulation network involving the RstAB and VarAS systems.

This study has contributed to the knowledge of the bacterial RstAB regulon with a number of novel genes and functions. We here demonstrated that the RstAB regulon of *P. damselae* subsp. *damselae* RM-71 comprises genes of differential presence among strains, and no geographical or isolation source patterns could be identified. To summarize, the RstAB TCS plays a major role in regulation of virulence and of many aspects of cell physiology of *P. damselae* subsp. *damselae*. Ongoing studies are expected to unveil the roles of the novel RstAB-regulated genes reported in this study. The RstAB TCS seems to be ubiquitous in all strains of *P. damselae* subsp. *damselae*, which makes this target system of interest since it could be a weak point to control infections caused both by plasmid as well as plasmidless strains.

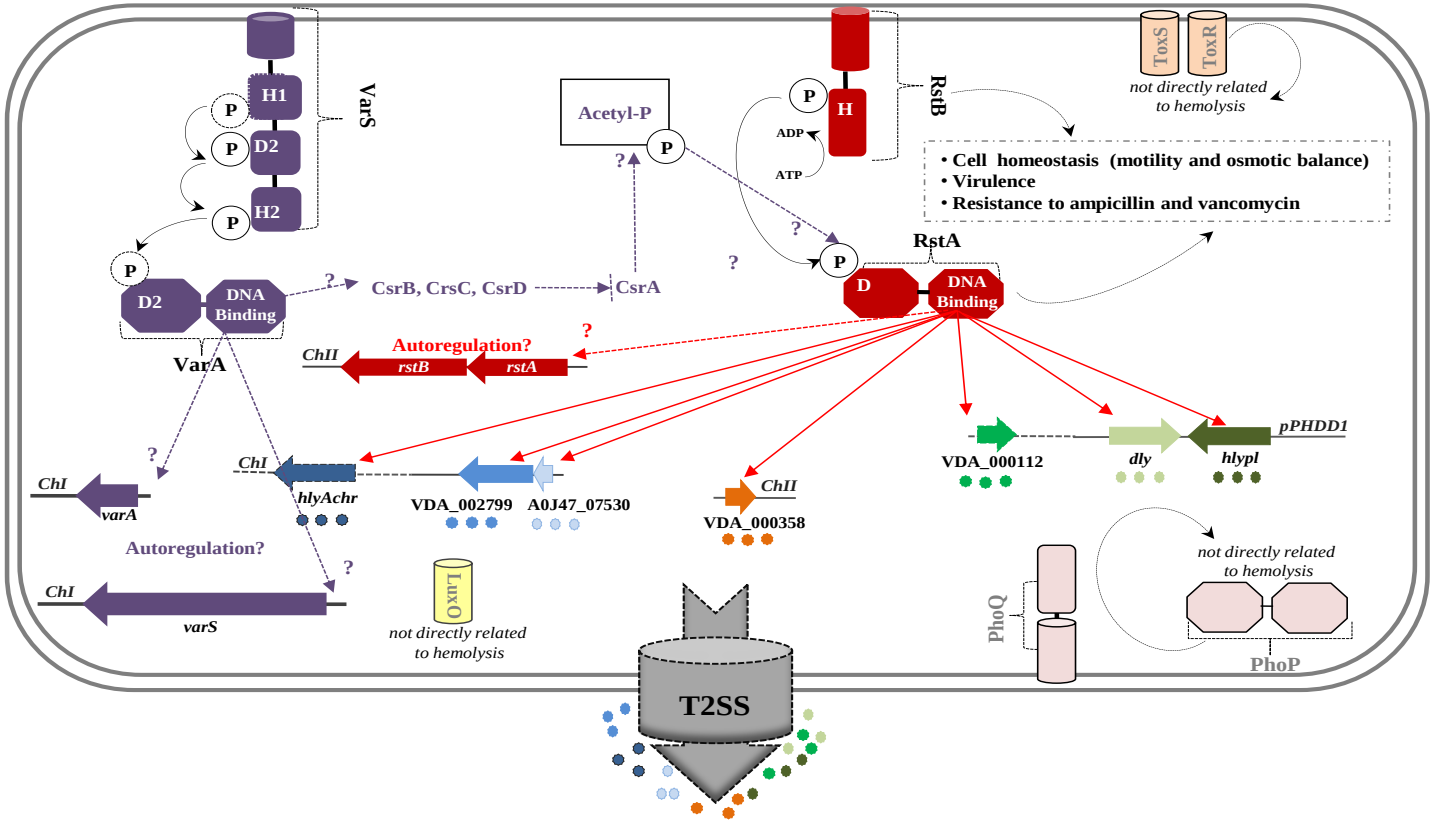



**Fig.IV.2:** Two-component RstAB system in *Pdd*. Possible interaction points between VarAS and RstAB TCS.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

# CONCLUSIONS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

# 5. CONCLUSIONS

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

1. Phylogenetic and genomic analyses of *P. damselae* subsp. *damselae* isolated from outbreaks in fish farms in Turkey and Denmark, suggest that the infectious agent was already present in the environment, not as a clonal pathogen but as multiclonal populations, waiting for stressful conditions (such as rising water temperature), to cause infection.

2. *P. damselae* subsp. *damselae* shows a high genetic diversity. Horizontal gene transfer has played an important role in the diversification of this subspecies, since a large part of the strain-specific DNA has characteristics related to plasmids, prophages and pathogenicity islands.

3. The two-component signal transduction system RstAB is a major positive regulator of the three cytotoxins Dly, PhlyP and PhlyC, and this regulation is exerted at the transcriptional level.

4. Mutations in the RstAB system impair diverse physiological functions under conditions of low NaCl concentration, including motility, cell shape and size, daughter cell separation following cell division, and antimicrobial resistance.

5. The T2SS-dependent secretome of *P. damselae* subsp. *damselae* includes RstAB-dependent and RstAB-independent proteins.

6. Mutation of RstAB system impairs the secretion of potentially novel virulence factors in *P. damselae* subsp. *damselae*.

7. Genes of the RstAB regulon show differential presence in *P. damselae* subsp. *damselae* strains. However, *rstAB* genes are ubiquitous, which makes this system a target of interest, a potential weak point to control infections caused by both plasmid-containing and by plasmidless strains.

8. There is evidence of interaction between the RstAB and the VarAS two-component signal transduction systems in regulation of the hemolytic activity of *Photobacterium damselae* subsp. *damselae*.

9. Deletion of regulatory genes *toxR, luxO* and *PhoP* does not impair hemolytic activity in *Photobacterium damselae* subsp. *damselae.*

# REFERENCES

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

# 6. REFERENCES

**A**


Aarons S., Abbas, A., Adams, C., Fenton, A., and O'Gara, F. (2000). A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113. *J Bacteriol* 182: 3913–3919.

Abdel-Aziz M., Eissa. A. E., Hanna. M., and Okada. M. A. (2013). Identifying some pathogenic *Vibrio*/*Photobacterium* species during mass mortalities of cultured gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) from some Egyptian coastal provinces. *Int. J. Vet. Sci. Med.* 1, 87-95.

Aigbivbalu L. And Maraqa N. (2009). *Photobacterium damsela* wound infection in a 14-year-old surfer. *South. Med. J.* 102(4):425-426.

Alba P., Caprioli, A., Cocumelli, C., Ianzano, A., Donati, V., Scholl, F., Sorbara, L., Terracciano, G., Fichi, G., Di Nocera, F., Franco, A., and Battisti, A. (2016). A new multilocus sequence typing scheme and its application for the characterization of *Photobacterium damselae* subsp. *damselae* associated with mortality in cetaceans. *Front Microbiol*. 7, 1656.

Alday V. (2010). El seguro de acuicultura. La necesidad de evaluación del riesgo de enfermedades para la sostenibilidad de una compañía. *Trébol* 53:4-13.

Alvarez J. R., Lamba S., Dyer K. Y. and Apuzzio J. J. (2006). An unusual case of urinary tract infection in a pregnant woman with *Photobacterium damsela*. *Infect. Dis. Obstet. Gynecol.* 2006:80682.

Ambur O. H., Frye, S. A., and Tønjum, T. (2007). New functional identity for the DNA uptake sequence in transformation and its presence in transcriptional terminators. *J. Bacteriol*. 189, 2077-2085.

Antonova E. S., and Hammer, B. K. (2015). Genetics of natural competence in *Vibrio cholerae* and other vibrios. *Microbiol. Spectr*. 3(3):VE-0010-2014.

Apromar (2007). La acuicultura en España (2018). Informe realizado por la Asociación Empresarial de Acuicultura de España (APROMAR): www.apromar.es

Asato J. and Kanaya F. (2004). Fatal infection of the hand due to *Photobacterium damsela*: A case report. *Clin. Infect. Dis*. 38(10):100-101.

Austin B. (2010). Vibrios as causal agents of zoonoses. *Vet. Microbiol.* 140(3-4):310-317. Ayers M., Howell P. L.

Aziz R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., *et al.* (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* 9, 75.

**B**

Balado M., Puentes, B., Couceiro, L., Fuentes-Monteverde, J. C., Rodríguez, J., Osorio, C. R., Jiménez, C., and Lemos, M.L. (2017). Secreted citrate serves as iron carrier for the marine pathogen *Photobacterium damselae* subsp *damselae*. *Front. Cell. Infect. Microbiol.* 7, 361.

Balaban N.Q. (2011). Persistence: mechanisms for triggering and enhancing phenotypic variability. *Curr. Opin. Genet. Dev.* 21:768–75.

Barber G. R. and Swygert J. S. (2000). Necrotizing fasciitis due to *Photobacterium damsela* in a man lashed by a stingray. *N. Engl. J. Med.* 342(11):824-824.

Beier D., Gross R. (2008). The BvgS/BvgA phosphorelay system of pathogenic *Bordetellae*: structure, function and evolution. *Adv. Exp. Med. Biol.* 631:149–60.

Bender J. and Flieger A. (2010). Lipases as pathogenicity factors of bacterial pathogens of humans. Timmis K. N. (Ed.), Microbiology of Hydrocarbons, Oils, Lipids and Derived Compounds (pp. 3241-3258). Berlín: Springer

Bernheimer A. W. (1988). Assay of hemolytic toxins. *Methods Enzymol*. 165, 213–217.

Bilecen K., and Yildiz, F. (2009). Identification of a calcium-controlled negative regulatory system affecting *Vibrio cholerae* biofilm formation. *Environ. Microbiol*. 11: 2015–2029.

Bilecen K., Fong, J. C. N., Cheng, A., Jones, C. J., Zamorano-Sánchez, D., and Yildiz, F. (2015). Polymyxin B resistance and biofilm formation in *Vibrio cholerae* are controlled by the response regulator CarR. *Infect. Immun.* 83, 1199–1209.

Bina X.R., Taylor D.L., Vikram A. *et al*. (2013). *Vibrio cholerae* ToxR down regulates virulence factor production in response to Cyclo (PhePro). *mBio.* 4:e00366–13.

Biondi E.G., Reisinger S.J., Skerker J.M., Arif M, Perchuk B.S., *et al*. (2006). Regulation of the bacterial cell cycle by an integrated genetic circuit. *Nature* 444:899–904.

Bode W., Papamokos, E., and Musil, D. (1987). The high-resolution X-ray crystal structure of the complex formed between subtilisin Carlsberg and eglin c, an elastase inhibitor from the leech *Hirudo medicinalis*. Structural analysis, subtilisin structure and interface geometry. *Eur. J. Biochem.* 166, 673–692.

Bond M. D. and Wart H. E. V. (1984). Purification and separation of individual collagenases from *Clostridium histolyticum* using red dye ligand chromatography. *Biochemistry (US).* 23(13):3077-3085.

Botella S., Pujalte M. J., Macian M. C., Ferrus M. A., Hernandez J., and Garay E. (2002). Amplified fragment length polymorphism (AFLP) and biochemical typing of *Photobacterium damselae* subsp. *damselae*. *J. Appl. Microbiol*. 93, 681-688.

Botella E., Devine S.K., Hubner S., Salzberg L.I., Gale R.T., *et al*. (2014). PhoR autokinase activity is controlled by an intermediate in wall teichoic acid metabolism that is sensed by the intracellular PAS domain during the PhoPR-mediated phosphate limitation response of *Bacillus subtilis*. *Mol. Microbiol*. 94:1242–59.

Bouzid M., Hunter P. R., Chalmers R. M. and Tyler M. (2013). *Cryptosporidium* pathogenicity and virulence. *Clin. Microbiol. Rev.* 26(1):115-134.

Buelow D.R., Raivio T.L. (2010). Three (and more) component regulatory systems—auxiliary regulators of bacterial histidine kinases. *Mol. Microbiol*. 75:547–66.

Buck J. D. (1990). Potentially pathogenic marine *Vibrio* species in seawater and marine animals in the Sarasota, Florida, area. *J. Coastal Res.* 6(4):943- 948.

Blumer C., Heeb, S., Pessi, G. and Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci U S A 96*. 14073–14078.

Burrowes E., Abbas, A., O'Neill, A., Adams, C., and O'Gara, F. (2005). Characterisation of the regulatory RNA RsmB from *Pseudomonas aeruginosa* PAO1. *Res Microbiol.* 156: 7–16.

Buck J. D., Overstrom N. A., Patton G. W., Anderson H. F. andGorzelany J. F. (1991). Bacteria associated with stranded cetaceans from the North east USA and South west Florida Gulf coasts. *Dis. Aquat. Org.* 10(2):147-152.

**C**

Cabeza M. L., Aguirre, A., Soncini, F. C., and García Véscovi, E. (2007). Induction of RpoS degradation by the two-component system regulator RstA in *Salmonella enterica. J. Bacteriol.* 189, 7335–7342.

Campbell T., L.,Ederer C.S., Allali-Hassani A., Brown E.D. (2007). Isolation of the *rstA* gene as a multicopy suppressor of YjeE, an essential ATPase of unknown function in *Escherichia coli. J Bacteriol.*189(8):3318-21.

Casadevall A. and Pirofski L. (2001). Host-pathogen interactions: the attributes of virulence. *J. Infect. Dis.* 184(3):337-344.

Casalone C., Mazzariol S., Pautasso A., Guardo G.D., Nocera F.D., Lucifora G., *et al*. (2014). Cetacean strandings in Italy: An unusual mortality evental on the Tyrrhenian Sea coast in 2013. *Dis. Aquat. Organ.* 109(1):81-86.

Cassat J. E. and Skaar E. P. (2013). Iron in infection and immunity. *Cell Host Microbe*. 13(5):509-519.

Castillo A., Tello, M., Ringwald, K., Acuña, L. G., Quatrini, R., and Orellana, O. (2017). A DNA segment encoding the anticodon stem/loop of tRNA determines the specific recombination of integrative-conjugative elements in *Acidithiobacillus* species. *RNA Biol* 20, 1-8.

Chancey S.T, Wood D.W, Pierson 3rd L.S. (1999) Two-component transcriptional regulation of N-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol*. 65(6):2294–2299.

Chatterjee A., Cui Y., Yang H., Collmer A., Alfano J.R., Chatterjee A.K. (2003). GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. tomato DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol Plant Microbe Interact*. 16(12):1106–17.

Chen H.D., Groisman E.A. (2013). The biology of the PmrA/PmrB two-component system: the major regulator of lipopolysaccharide modifications. *Annu. Rev. Microbiol*. 67:83–112.

Cheung R., and Diprio, J. (1986). Vancomycin: an update. Pharmacotherapy 6:153–169.

Childers B.M., Klose K.E. (2007). Regulation of virulence in *Vibrio cholerae*; the ToxR regulon. *Future Microbiol*.2:335–44.

Chiu T.H., Kao L.Y., Chen M.L. (2013). Antibiotic resistance and molecular typing of *Photobacterium damselae* subsp. *damselae*, isolated from seafood. *J Appl Microbiol* 114:1184–1192.

Clarridge J. E., and Zighelboim-Daum, S. (1985). Isolation and characterization of two hemolytic phenotypes of *Vibrio damsela* associated with a fatal wound infection. *J. Clin. Microbiol.* 21, 302–306.

Coffey J. A., Harris R. L., Rutledge M. L., Bradshaw M. W. and Williams T. W. (1986). *Vibrio damsela*: another potentially virulent marine vibrio. *J. Infect. Dis*. 153(4):800-802.

Cornelis P. and Andrews S. C. (eds) (2010). Iron uptake and homeostasis in microorganisms. Norfolk, UK: Caister Academic Press (HorizonPress).

Company R., Sitjà-Bobadilla, A., Pujalte, M. J., Garay, E., Alvarez-Pellitero, P., and Pérez-Sánchez, J. (1999). Bacterial and parasitic pathogens in cultured common dentex, *Dentex dentex* L. *J. Fish. Dis*. 22, 299-309.

Cui Y, Chatterjee A., Chatterjee A.K. (2001). Effects of the two-component system comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the production of global regulatory *rsmB* RNA, extracellular enzymes, and harpinEcc. *Mol Plant Microbe Interact*. 14(4):516–26.

Cutter D. L. and Kreger A. S. (1990). Cloning and expression of the damselysin gene from *Vibrio damsela. Infect. Immun*. 58(1):266-268.

Cygler M., Borisova S., Grochulski P., Masson L., Pusztai-Carey M., Schwartz J.L., and Brousseau R. (1995). *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. *J. Mol. Biol*. 254, 447–464.

**D**

Daniel L. W., King L. and Kennedy M. (1988). Phospholipase activity of bacterial toxins. *Methods Enzymol*. 165:298-301.

DiRita V.J., Parsot C., Jander G. *et al*. (1991). Regulatory cascade controls virulence in *Vibrio cholerae*. *P Natl Acad Sci USA* 88:5403– 7.

Dryden M., Legarde M., Gottlieb T., Brady L. and Ghosh H. K. (1989). *Vibrio damsela* wound infections in Australia. *Med. J. Aust.* 151(9):540-541.

Duarte A. S., Correia A. and Esteves A. C. (2014). Bacterial collagenases - A review. *Crit. Rev. Microbiol.* 42(1):106-126.

Dubrac S., Bisicchia P., Devine K.M., Msadek T. (2008). A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. *Mol. Microbiol*. 70:1307–22 .

Ducret A., Quardokus, E., and Brun, Y. V. (2016). MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. *Nat. Microbiol*. 1:16077.

**E**

Eckhard U., Schonauer E., Ducka P., Briza P., Nüss D. and Brandstetter H. (2009). Biochemical characterization of the catalytic domains of three different clostridial collagenases. *Biol. Chem.* 390(1):11-18.

Eissa I. A. M., Derwa, H. I., Ismail, M., El-Lamie, M., Dessouki, A. A., Elsheshtawy, H., and Bayoumy, E. M. (2018). Molecular and phenotypic characterization of *Photobacterium damselae* among

some marine fishes in Lake Temsah. *Microb. Pathog*. 114, 315–322.

Ellermann M. and Arthur J. C. (2017). Siderophore-mediated iron acquisition and modulation of host-bacterial interactions. *Free Radic. Biol. Med*. 105:68-78.

**F**

FAO (2016). The State of World Fisheries and Aquaculture (SOFIA) 2016. Departamento de Pesca. Roma. 2016.

Flamez C., Ricard, I., Arafah, S., Simonet, M., and Marceau, M. (2008). Phenotypic analysis of *Yersinia pseudotuberculosis* 32777 response regulator mutants: new insights into two-component system regulon plasticity in bacteria. Int. J. *Med. Microbiol*. 298, 193–207.

Fraser S. L., Purcell B. K., Delagdo B., Baker A. E. and Whelen A. C. (1997). Rapidly fatal infection due to *Photobacterium* (*Vibrio*) *damsela*. *Clin. Infect. Dis*. 25(4):935-936.

Freeman Z.N., Dorus S., Waterfield N.R. (2013). The KdpD/KdpE two-component system: integrating $K^+$ homeostasis and virulence. *PLOS Pathog*. 9:e1003201.

Frees D., Brondsted L. and Ingmer H. (2013). Bacterial proteases and virulence. *Subcell. Biochem*. 66:161-192

Fouz B., Larsen J. L. and Toranzo A. E. (1991). *Vibrio damsela* as a pathogenic agent causing mortalities in cultured turbot (*Scophthalmus maximus*). *Bull. Eur. Ass. Fish Pathol.* 11(2):80-81.

Fouz B., Larsen, J. L., Nielsen, B., Barja, J. L., and Toranzo, A.E. (1992). Characterization of *Vibrio damsela* strains isolated from

turbot *Scophthalmus maximus* in Spain. *Dis. Aquat. Organ*. 12, 155-166.

Fouz B., Barja J. L., Amaro C., Rivas C. and Toranzo A. E. (1993). Toxicity of the extracelular products of *Vibrio damsela* isolated from diseased fish. *Curr. Microbiol.* 27(6):341-347.

Fouz B., Toranzo A.E., Biosca E.G., Mazoy R. and Amaro C. (1994). Role of iron in the pathogenicity of *Vibrio damsela* for fish and mammals. *FEMS Microbiol. Lett.* 121(2):181-188.

Fouz B., Biosca E. G., and Amaro C. (1997). High affinity iron-uptake systems in: role in the acquisition of iron from transferrin. *J. Appl. Microbiol*. 82(2):157-167.

Fouz B., Toranzo, A. E., Marco-Noales, E., and Amaro, C. (1998). Survival of fish-virulent strains of *Photobacterium damselae* subsp. *damselae* in seawater under starvation conditions. *FEMS Microbiol. Lett*. 168, 181–186.

Fouz B., Toranzo, A. E., Milan, M., and Amaro, C. (2000). Evidence that water transmits the disease caused by the fish pathogen *Photobacterium damselae* subsp. *damselae*. *J. Appl. Microbiol*. 88, 531–535.

Foxall R..L, Ballok A.E., Avitabile A., Whistler C.A. (2015) Spontaneous phenotypic suppression of GacA-defective *Vibrio fischeri* is achieved via mutation of *csrA* and *ihfA*. *BMC Microbiol*. 16;15:180.

Fujioka R. S., Greco S. B., Cates M. B. and Schroeder J. P. (1988). *Vibrio damsela* from wounds in bottlenose dolphins *Tursiops truncatus. Dis. Aquat. Org*. 4:1-8.

Fujita M., Gonzalez-Pastor J.E., Losick R. (2005). High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. *J. Bacteriol.* 187:1357–68.

**G**

Gao R., Mack, T. R., and Stock, A. M. (2007). Bacterial response regulators: versatile regulatory strategies from common domains. *Trends Biochem. Sci*. 32, 225–234.

Gao R., and Stock, A. M. (2009). Biological insights from structures of two component proteins. *Annu. Rev. Microbiol*. 63, 133–154.

Gao Q., Ye, Z., Wang, X., Mu, X., Gao, S., and Liu, X. (2015). RstA is required for the virulence of an avian pathogenic *Escherichia coli* O2 strain E058. *Infect. Genet. Evol*. 29, 180–188.

Gadwal S., Korotkov, K.V., Delarosa, J.R., Hol, W.G., and Sandkvist, M. (2014). Functional and structural characterization of *Vibrio cholerae* extracellular serine protease B, VesB. *J. Biol. Chem*. 289, 8288-8298.

Galitsky N., Cody, V., Wojtczak, A., Ghosh, D., Luft, J.R., Pangborn, W., and English, L. (2001). Structure of the insecticidal bacterial delta-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. *Acta Crystallogr*. D. 57, 1101–1109.

Ganz T. and Nemeth E. (2015). Iron homeostasis in host defence and inflammation. *Nat. Rev. Immunol.* 15(8):500-510.

García-Rosado E., Cano I., Martín-Antonio B., Labella A., Manchado M., Alonso M. C., *et al*. (2007). Co-occurrence of viral and bacterial pathogens in disease outbreak saffecting newly cultured sparid fish. *Int. Microbiol*. 10(3):193-199.

Gauthier G., Lafay B., Ruimy R., Breittmayer V., Nicolas J. L., Gauthier M., *et al*. (1995). Small-subunit rRNA sequences and whole DNA related Ness concurfor there assignment of *Pasteurella piscicida* (Snieszko *et al*.) Janssen and Surgalla to the genus *Photobacterium* as *Photobacterium damselae* subsp. *piscicida* comb. nov. *Int. J. Syst. Bacteriol*. 45(1):139-144.

Ghinsberg R. C., Drasinover V., Sheinberg Y. and Nitzan Y. (1995). Seasonal distribution of *Aeromonas hydrophila* and *Vibrio* species in Mediterrane an coast al water and beaches: a posible health hazard. *Biomed. Lett*. 51:151-159.

Goodell K. H., Jordan M. R., Graham R., Cassidy C. and Nasraway S. A. (2004). Rapidly advancing necrotizing fasciitis caused by *Photobacterium (Vibrio) damsela*: a hyper aggressive variant. *Crit. CareMed*. 32(1):278- 281.

Gomes C., Almeida, A., Ferreira, J. A., Silva, L., Santos-Sousa, H., Pinto-de-Sousa, J., *et al.* (2013). Glycoproteomic analysis of serum from patients with gastric precancerous lesions. *J. Proteome Res*. 12, 1454-1466.

Goudenège D.,Travers M. A.,Lemire A., *et. al*. (2015). Single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogen icity in oysters. *Environ Microbiol*. 17(11):4189-99.

Grimes D. J., Colwell R. R., Stemmler J., Hada H., Maneval D., Hetrick F. M., *et al*. (1984a). *Vibrio species* as agents of elasmobranch disease. *Helgol. Meeresunters* 37(1-4):309-315.

Grimes D. J., Stemmler J., Hada H., May E. B., Maneval D., Hetrick F. M., *et al*. (1984b). *Vibrio species* associated with mortality of sharksheld in captivity. *Microb. Ecol.* 10(3):271-282.

Grimes D. J., Brayton P., Colwell R. R. and Gruber H. (1985). Vibrios as autochthonous flora of neritic sharks. *Syst. Appl. Microbiol.* 6(2):221-226.

Groisman E.A. (2001). The pleiotropic two-component regulatory system PhoP-PhoQ. *J. Bacteriol*. 183:1835–42.

Groisman E.A. (2016). Feedback Control of Two-Component Regulatory Systems. *Annu Rev Microbiol.* 8;70:103-24.

**H**

Hammer B.K., and Bassler, B.L. (2003). Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol*. 50: 101–104.

Hassanzadeh Y., Bahador N. and Baseri-Salehi M. (2015). First time isolation of *Photobacterium damselae* subsp. *damselae* from *Caranxsex fasciatus* in Persian Gulf, Iran. I*ran. J. Microbiol.* 7(3):178-184.

Harrington D. J. (1996). Bacterial collagenases and collagen-degrading enzymes and their potential role in human disease. *Infect. Immun.* 64(6):1885-1891.

Hausmann S. and Jaeger K. E. (2010). Lipolytic enzymes from bacteria. In: Timmis KN, editor. Handbook of hydrocarbon and lipid microbiology. *Berlin Heidelberg: Springer*; 2010. pp. 1099-1126.

Hengge R. (2008). The two-component network and the general stress sigma factor RpoS (σS) in *Escherichia coli.* Adv. *Exp. Med. Biol*. 631:40–53.

Heeb S., Blumer C., and Haas D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct

formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* 184: 1046–1056

Herrera C. M., Crofts, A. A., Henderson, J. C., Cassandra Pingali, S., Davies, B. W., and Trent, S. M. (2014). The *Vibrio cholerae* VprA-VprB two-component system controls virulence through endotoxin modification. *mBio* 5:e02283-14.

Herrero M., de Lorenzo, V., and Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol*. 172, 6557–6567.

Hirakawa H., Nishino K., Hirata T., Yamaguchi A. (2003a). Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. *J Bacteriol*. 185(6):1851-6.

Hirakawa H., Nishino K., Yamada J., *et al*. (2003b). Beta-lactam resistance modulated bythe overexpression of response reg ulators of two-component signal transduction systems in *Escherichia coli*. *J Antimicrob Chemother*. 52(4):576-82.

Hsieh Y.J., Wanner B.L. (2010). Global regulation by the seven-component Pi signaling system. *Curr. Opin. Microbiol.* 13:198–203.

Huang L., Xu W., Su Y., Zhao L., and Yan Q. (2018). Regulatory role of the RstB-RstA system in adhesion, biofilm production, motility, and hemolysis. *Microbiologyopen*. 23, e00599.

Hundenborn J., Thurig, S., Kommerell, M., Haag, H., and Nolte, O. (2013). Severe wound infection with *Photobacterium damselae* ssp. *damselae* and *Vibrio harveyi*, following a laceration injury in

marine environment: a case report and review of the literature. *Case Rep. Med.* 2013:610632.

**I**

Ishii E., Eguchi Y., Utsumi R. (2013). Mechanism of activation of PhoQ/PhoP two-component signal transduction by SafA, an auxiliary protein of PhoQ histidine kinase in *Escherichia coli*. *Biosci. Biotechnol. Biochem*. 77:814–19.

**J**

Jang J., Jung K.T., Yoo C.K., Rhie G.E. (2010). Regulation of hemagglutinin/protease expression by the VarS/VarACsrA/B/C/D system in *Vibrio cholerae*. *Microb Pathog*. 48(6):245-50.

Jang J., Jung, K. T., Park, J., Yoo, C. K., and Rhie, G. E. (2011). The *Vibrio cholerae* VarS/VarA two-component system controls the expression of virulence proteins through ToxT regulation. *Microbiology* 157, 1466–1473.

Jobling M.G., and Holmes, R.K. (1997). Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/ protease gene hap, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Mol Microbiol*. 26: 1023–1034.

Johnston C., Pegues D.A., Hueck C.J., Lee A., Miller S.I. (1996). Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. *Mol Microbiol*. 22:715e27.

Jones M.K., Oliver J.D. (2009). *Vibrio vulnificus*: Disease and pathogenesis. *Infect Immun*. 77: 1723–33.

**K**

Kanchanopas-Barnette P., Labella A., Alonso C. M., Manchado M., Castro D. and Borrego J. J. (2009). The first isolation of *Photobacterium damselae* subsp. *damselae* from asian seabass (*Lates calcarifer). FishPathol.* 44(1):47-50.

Kato A., Groisman E.A. (2004). Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor. *Genes Dev.* 18:2302–13.

Ketterer P. J. and Eaves L. E. (1992). Deaths in captive eels (*Anguila reinhardtii*) due to *Photobacterium* (*Vibrio*) *damsela*. *Aust. Vet. J.* 69(8):203-204.

Khouadja S., Lamari, F., Bakhrouf, A., and Gaddour, K. (2014). Virulence properties, biofilm formation and random amplified polymorphic DNA analysis of *Photobacterium damselae* subsp. *damselae* isolates from cultured sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). *Microb. Pathog*. 69-70, 13-19.

Kim H. R., Kim J. W., Lee M. K. and Kim J. G. (2009). Septicemia progressing to fatal hepatic dysfunction in an cirrhotic patient after oral ingestion of *Photobacterium damsela*: a case report. *Infection.* 37(6):555- 556.

Kim M.J., Kim J., Lee H.Y., Noh H.J., Lee K.H., Park S.J. (2015) Role of AcsR in expression of the acetyl-CoA synthetase gene in *Vibrio vulnificus*. *BMC Microbiol.* 12;15:86.

Kimura M., Hama, Y., Sumi, T., Asakawa, M., Rao, B.N.N., Horne, A.P., *et al*. (1994). Characterization of a deaminated neuraminic acid-containing glycoprotein from the skin mucus of the loach, *Misgurnus anguillicaudatus*. *J. Biol. Chem.* 269, 32138-32143.

Knight-Madden J. M., Barton M., Gandretti N., and Nicholson A. M. (2005). *Photobacterium damsela* bacteremia in a child with sickle-cell disease. *Pediatr. Infect. Dis. J*. 24(7):654-655.

Kreger A. S. (1984). Cytolytic activity and virulence of *Vibrio damsela*. *Infect. Immun.* 44(2):326-331.

Kreger A. S., Bernheimer, A. W., Etkin, L. A., and Daniel, L. W. (1987). Phospholipase D activity of *Vibrio damsela* cytolysin and its interaction with sheep erythrocytes. *Infect. Immun*. 55, 3209–3212.

Kosono S., Tamura M., Suzuki S., Kawamura Y., Yoshida A., *et al*. (2015). Changes in the acetylome and succinylome of *Bacillus subtili*s in response to carbon source. *PLOS ONE* 10:e0131169.

Kothary M. H. and Kreger A. S. (1985). Purification and characterization of an extracelular cytolysin produced by *Vibrio damsela*. *Infect. Immun*. 49(1):25-31.

**L**

Labella A., Vida, M., Alonso, M. C., Infante, C., Cardenas, S., López-Romalde, S., Manchado, M., and Borrego, J. J. (2006). First isolation of *Photobacterium damselae* ssp. *damselae* from cultured redbanded seabream, *Pagrus auriga* Valenciennes, in Spain. *J. Fish Dis*. 29, 175-179.

Labella A., Sanchez-Montes, N., Berbel, C., Aparicio, M., Castro, D., Manchado, M., and Borrego, J. J. (2010a). Toxicity of *Photobacterium damselae* subsp. *damselae* strains isolated from new cultured marine fish. *Dis. Aquat. Organ*. 92, 31-40.

Labella A., Manchado M., Alonso M.C., Castro D., Romalde J.L., Borrego JJ. (2010b). Molecular intraspecific characterization of

*Photobacterium damselae* ssp. *damselae* strains affecting cultured marine fish. *J Appl Microbiol*. 108:2122–2132.

Labella A., Berbel C., Manchado M., Castro D., Borrego J.J. (2011). *Photobacterium damselae* subsp. *damselae*, an emerging pathogen affecting new cultured marine fish species in southern Spain. Faruk A. and Zafer D. (Eds.), *Recent Advances in Fish Farns* (pp.135-152). New York: In Tech.

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Laub M.T., Goulian M. (2007). Specificity in two-component signal transduction pathways. *Annu. Rev. Genet*. 41:121–45.

Laville J., Voisard, C., Keel, C., Maurhofer, M., Defago, G., and Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc Natl Acad Sci USA* 89: 1562–1566.

Lenz D.H., Miller M.B., Zhu J., Kulkarni R.V., Bassler B.L. (2005). CsrA and three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. *Mol Microbiol*. 58(4):1186-202.

Lenz D.H., Bassler B.L. (2007). The small nucleoid protein Fis is involved in *Vibrio cholerae* quorum sensing. *Mol Microbiol* 63:859–871.

Le Roux F., Binesse, J., Saulnier, D., and Mazel, D. (2007). Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. *Appl. Environ. Microbiol*. 73, 777–784.

Le Roux F., Davis, B. M., and Waldor, M. K. (2011). Conserved small RNAs govern replication and incompatibility of a diverse

new plasmid family from marine bacteria. *Nucleic Acids Res*. 39, 1004–1013.

Le Roux F., Wegner, K. M., Baker-Austin, C., Vezzulli, L., Osorio, C. R., *et al*. (2015). The emergence of *Vibrio* pathogens in Europe: ecology, evolution, and pathogenesis (Paris, 11-12th March 2015). *Front. Microbiol*. 6, 830.

Liu P. V. (1957). Survey of hemolysin production among species of *pseudomonas*. *J. Bacteriol.* 74, 718–727.

Li J.D., Carroll, J., and Ellar, D.J. (1991). Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. *Nature.* 353, 815–821.

Liu M.Y., Gui G., Wei B., Preston J.F. *et al.* (1997). The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in *Escherichia coli*. *J. Biol. Chem.* 272:17502–17510.

Liu F., Liu, G., and Li, F. (2016). Characterization of two pathogenic *Photobacterium* strains isolated *from Exopalaemon carinicauda* causing mortality of shrimp. *Aquaculture* 464, 129-135.

Lippa A.M., Goulian M. (2009). Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLOS Genet.* 5:e1000788.

Louwen R., Staals, R. H. J., Endtz, H. P., van Baarlen, P., and van der Oost, J. (2014). The role of CRISPR-Cas systems in virulence of pathogenic bacteria. *Microb. Mol. Biol. Rev.* 78, 74-88.

Love M., Teebkenfisher, D., Hose, J. E., Farmer, J. J., Hickman, F. W., Fanning, G. R. (1981). *Vibrio damsela*, a marine bacterium, causes skin ulcers on the damselfish *Chromis punctipinnis*. *Science* 214, 1139–1140.

**M**

Ma K., Cao, Q., Luo, S., Wang, Z., Liu, G., Lu, C., and Liu, Y. (2018). *cas9* enhances bacterial virulence by repressing the *regR* transcriptional regulator in *Streptococcus agalactiae*. *Infect. Immun*. 86, pii:e00552-17.

Matsushita O. and Okabe A. (2001). Clostridial hydrolytic enzymes degrading extracellular components. *Toxicon*. 39(11):1769-1780.

McCleary W.R., Stock J.B. (1994). Acetyl phosphate and the activation of two-component response regulators. *J. Biol. Chem.* 269:31657–72.

Mac Donell M. T. and Colwell R. R. (1984). The nucleotide sequence of 5S ribosomal RNA from *Vibrio marinus*. *Microbiol. Sci*. 1(9):229-231.

Macfarlene R. G., Oakley C. L. and Anderson C. G. (1941). Hemolysis and the production of opalesence in serum and lecith ovitell in by the alpha toxin of *Clostridium perfringens. J. Pathol. Bacteriol.* 52:99-103.

Machado H., and Gram, L. (2017). Comparative genomics reveals high genomic diversity in the genus *Photobacterium*. *Front. Microbiol*. 8, 1204.

Mey A.R., Butz H.A., Payne S.M. (2015) *Vibrio cholerae* CsrA Regulates ToxR Levels in Response to Amino Acids and Is Essential for Virulence. *mBio.* 4;6(4):e01064.

Makarova K. S., Wolf, Y. I., Alkhnbashi O. S., Costa, F., Shah, S. A., *et al*. (2015). An updated evolutionary classification of CRISPR-Cas systems. *Nature Revs. Microbiol*. 13, 1-15.

Martin B., Granadel C., Campo N., Henard V., Prudhomme M., Claverys J.P. (2010). Expression and maintenance of ComD-ComE, the two-component signal-transduction system that controls competence of *Streptococcus pneumoniae*. *Mol. Microbiol.* 75:1513–28.

Martins P., Navarro R.V.V., Coelho F.J.R.C., Gomes N.CM. (2015). Development of a molecular methodology for fast detection of *Photobacterium damselae* subspecies in water samples. *Aquaculture* 435:137–142.

Mascher T., Helmann J.D., Unden G. (2006). Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol. Mol. Biol. Rev.* 70:910–38.

Magariños B., Romalde J.L., Bandín I., Fouz B., Toranzo A.E. (1992). Phenotypic, antigenic, and molecular characterization of *Pasteurella piscicida* strains isolated from fish. *Appl Environ Microbiol* 58:3316–3322.

Magariños B., Romalde J.L., López-Romalde S., Moriñigo M.A, Toranzo A.E. (2003). Pathobiological characterisation of *Photobacterium damselae* subsp. *piscicida* isolated from cultured sole (*Solea senegalensis*). *Bull Eur Assoc Fish Pathol* 23:183–190.

Menanteau-Ledouble, S., and Lawrence, M. L. (2013). Use of bioluminescence mutant screening for identification of *Edwardsiella ictaluri* genes involved in channel catfish (*Ictalurus punctatus*) skin colonization. *Vet. Microbiol*. 162, 724-730.

Méthot P. O. andAlizon S. (2014). What is a pathogen? Toward a process view of host-parasite interactions. *Virulence.* 5(8):775-785.

Metzger L. C., and Blokesch, M. (2016). Regulation of competence-mediated horizontal gene transfer in the natural habitat of *Vibrio cholerae*. *Curr. Opin. Microbiol.* 30, 1-7.

Miller J. H. (1992). A Short Course in Bacterial Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller V.L., Mekalanos J.J. (1987). Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. *P Natl Acad Sci USA* 81:3471–5.

Miller S.I., Kukral A.M., Mekalanos J.J. (1989) A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proceedings of the National Academy of Sciences of the United States of America. 86(13):5054–8.

Minagawa S., Ogasawara H., Kato A., Yamamoto K., Eguchi Y., *et al*. (2003). Identification and molecular characterization of the $Mg^{2+}$ stimulon of *Escherichia coli*. *J. Bacteriol*. 185:3696–702.

Mitrophanov A.Y., Groisman E.A. (2008). Positive feedback in cellular control systems. *Bio Essays* 30:542–55.

Mika F., Hengge R. (2005). A two-component phosphotransfer network involving ArcB, ArcA, and RssB coordinates synthesis and proteolysis of σS (RpoS) in *E. coli*. *Genes Dev*. 19:2770–81.

Mizuno T. (1997). Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. *DNA Res*. 28;4(2):161-8.

Minagawa S., Ogasawara H., Kato A., Yamamoto K., *et al.* (2003). Identification and molecular characterization of the $Mg^{2+}$ stimulon of *Escherichia coli*. *J Bacteriol.* 185(13):3696-702.

Mima T., Gotoh K., Yamamoto Y., *et. al*. (2018). Expression of Collagenase is Regulated by the VarS/VarA Two-Component Regulatory System in *Vibrio alginolyticus*. *J Membr Biol*. 251(1):51-63.

Mouriño S., Osorio, C. R., and Lemos, M. L. (2004). Characterization of heme uptake cluster genes in the fish pathogen *Vibrio anguillarum*. *J. Bacteriol*. 186, 6159–6167.

Mollby R. (1978). Bacterial phospholipases. Jeljaszewicz, J. and Wadstrom, T. (Eds.), Bacterial Toxins and Cell Membranes (pp. 367-424). London: Academic Press, Inc. Ltd.

Molofsky, A.B., and Swanson, M.S. (2004) Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. *Mol Microbiol* 53: 29–40.

Moolenaar G.F., van Sluis C.A., Backendorf C., van de Putte P. (1987). Regulation of the *Escherichia coli* excision repair gene *uvrC*. Overlap between the *uvrC* structural gene and the region coding for a 24 kD protein. *Nucleic Acids Res.* 15: 4273e89.

Morris J. G., Wilson R., Hollis D. G., Weaver R. E., Miller H. G., Tacket C. O., *et al.* (1982). Illness caused by *Vibrio damsela* and *Vibrio hollisae. Lance*t. 1(8284):1294-1297.

**N**

Nakamura Y., Uchihira, M., Ichimiya, M., Morita, K., and Muto, M. (2008). Necrotizing fasciitis of the leg due to *Photobacterium damsela*. *J. Dermatol*. 35(1):44-45.

Nam D., Choi. , Kweon D.H., Shin D. (2010) The RstB sensor acts on the PhoQ sensor to control expression of PhoP-regulated genes. *Mol Cells*. 30(4):363-8.

Nonaka L., Maruyama, F., Miyamoto, M., Miyakoshi, M., Kurokawa, K., and Masuda, M. (2012). Novel conjugative transferable multiple drug resistance plasmid pAQU1 from *Photobacterium damselae* subsp. *damselae* isolated from marine aquaculture environment. *Microbes Environ*. 27, 263-272.

Norsworthy A.N., Visick K.L. (2015). Signaling between two interacting sensor kinases promotes biofilms and colonization by a bacterial symbiont. *Mol. Microbiol*. 96:233–48.

Novianty R. I. and Budiarti, S. (2014). Lytic bacteriophage for *Photobacterium damselae* isolated from water environment. *Int. J. Innov. Res. Sci. Eng.* 2, 549-553.

Nurk S., Bankevich, A., Antipov, D., Gurevich, A. A., Korobeynikov, A., *et al*. (2013). Assembling genomes and mini–metagenomes from highly chimeric reads, p 158–170. In Deng M, Jiang R, Sun F, Zhang X. (eds), *Lecture notes in computer science,* vol 7821. Springer–Verlag, Berlin, Germany.

Nyholm SV, McFall-Ngai MJ. (2004). The winnowing: establishing the squid-vibrio symbiosis. *Nat Rev Microbiol.* 2(8):632–42.

**O**

Ochman H., Lawrence, J. G., and Groisman, E.A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* 405, 299-304.

Ogasawara H., Hasegawa, A., Kanda, E., Miki, T., Yamamoto, K., and Ishihama, A. (2007). Genomic SELEX search for target promoters under the control of the PhoQP-RstBA signal relay cascade. *J. Bacteriol*. 189, 4791–4799. doi: 10.1128/ JB.00319-07.

Ogasawara H., Yamamoto K., Ishihama A. (2010). Regulatory role of MlrA in transcription activation of *csgD*, the master regulator of

biofilm formation in *Escherichia coli*. *FEMS Microbiol Lett.* 312(2):160-8.

Ogut H., Uzun E. (2014). Incidence and prevalence of *Diplectanum aequans* and its influence on the fitness of juvenile sea bass (*Dicentrarchus labrax*) in the Black Sea. *Aquacult Res* 45:742–748.

Oshima T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B. L., *et al.* (2002). Transcriptome analysis of all two-component regulatory systems mutants of *Escherichia coli* K-12. *Mol. Microbiol.* 46, 281–291.

Osorio C.R., Collins M.D., Toranzo A.E., Barja J.L., Romalde J.L. 1999. 16S rRNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. *Appl Environ Microbiol* 65:2942–2946.

Osorio C. R., Toranzo, A. E., Romalde, J. L., and Barja, J. L. (2000a). Multiplex PCR assay for *ureC* and 16S rRNA genes clearly discriminates between both subspecies of *Photobacterium damselae*. *Dis. Aquat. Organ.* 40, 177-183.

Osorio C. R., and Klose, K, E. (2000b). A region of the transmembrane regulatory protein ToxR that tethers the transcriptional activation domain to the cytoplasmic membrane displays wide divergence among *Vibrio* species. *J. Bacteriol*. 182, 526-528.

Osorio C. R., Juiz-Río S. and Lemos M. L. (2010). The ABC-transporterhut CD genes of *Photobacterium damselae* subsp. *piscicida* are essential for haemutilization as iron source and are expressed during infection in fish. *J. Fish Dis.* 33(8):649-655.

Osorio C. R., Vences A., Matanza X. M. and Terceti, M. S. (2018). *Photobacterium damselae* subsp. *damselae*, a generalist pathogen with unique virulence factors and high genetic diversity. *J. Bacteriol*. (in press).

**P**

Parales R. E., and Harwood, C. S. (1993). Construction and use of a new broadhost-range *lacZ* transcriptional fusion vector, pHRP309, for Gram- bacteria. *Gene* 133, 23–30.

Park S.Y. and Groisman E.A. (2014). Signal-specific temporal response by the *Salmonella* PhoP/PhoQ regulatory system. *Mol. Microbiol*. 91:135–44.

Pedersen K., Dalsgaard, I., and Larsen, J. L. (1997). *Vibrio damsela* associated with diseased fish in Denmark. *Appl. Environ. Microbiol*. 63, 3711-3715.

Pedersen K., Skall H. F., Lassen-Nielsen A. M., Nielsen T. F., Henriksen N. H., Olesen N. J. (2008). Surveillance of health status on eight marine rainbow trout, *Oncorhynchus mykiss* (Walbaum), farms in Denmark in 2006. *J. Fish Dis.* 31 659–667.

Pedersen K., Skall, H. F., Lassen-Nielsen, A. M., Bjerrum, L., and Olesen, N. J. (2009). *Photobacterium damselae* subsp. *damselae*, an emerging pathogen in Danish rainbow trout, *Oncorhynchus mykiss* (Walbaum), mariculture. *J. Fish Dis*. 32, 465-472.

Peterson J. W. (1996). Chapter 7: Bacterial Pathogenesis. Baron S. (Ed.), *Medical Microbiology*, 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston. https://www.ncbi.nlm.nih.gov/books/NBK8526/

Peterson K. M. and Mekalanos J. J. (1988). Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. *Infect. Immun.* 56(11):2822-2829.

Peterson Kenneth M. and Patrick S. Gellings (2018). Multiple intraintestinal signals coordinate the regulation of *Vibrio cholerae* virulence determinants. *Pathogens and Disease*. 1;76(1).

Perego M., Brannigan J.A. (2001). Pentapeptide regulation of aspartyl-phosphate phosphatases. *Peptides* 22:1541–47.

Perez M. J., Rodríguez L. A. and Nieto T. P. (1998). The acetylcholinesterase ichthyo toxinis a common component in the extracelular products of *Vibrio naceae* strains. *J. Appl. Microbiol*. 84(1):47- 52.

Perez J. C., Shin, D., Zwir, I., Latifi, T., Hadley, T. J., and Groisman, E. A. (2009). Evolution of a bacterial regulon controlling virulence and $Mg^{2+}$ homeostasis. *PLoS Genet.* 5:e1000428.

Perez-Tirse J., Levine J. F. and Mecca M. (1993). *Vibrio damsela*: a cause of fulminant septicemia. *Arch. Intern. Med*. 153(15):1838-1840.

Pescaretti M., Lopez F.E., Morero R.D., Delgado M.A. (2010). Transcriptional autoregulation of the Rcs CDB phosphorelay system in *Salmonella enterica* serovar *Typhimurium*. *Microbiology* 156:3513–21.

Pescaretti M., Morero R., Delgado M.A. (2009). Identification of a new promoter for the response regulator *rcsB* expression in *Salmonella enterica* serovar *Typhimurium. FEMS Microbiol. Lett*. 300:165–73.

Pontes M.H., Smith K.L., De Vooght L., Van Den Abbeele J., Dale C. (2011). Attenuation of the sensing capabilities of PhoQ in transition to obligate insect-bacterial association. *PLOS Genet*. 7:e1002349.

Puentes B., Balado, M., Bermúdez-Crespo, J., Osorio, C. R., and Lemos, M. L. (2017). A proteomic analysis of the iron response of *Photobacterium damselae* subsp. *damselae* reveals metabolic adaptations to iron levels changes and novel potential virulence factors. *Vet. Microbiol*. 201:257-264.

Pujalte M. J., Sitja-Bobadilla A., Álvarez-Pellitero P. and Garay E. (2003). Carriage of potentially fish-pathogenic bacteria in *Sparus aurata* cultured in mediterrane an fish farms. *Dis. Aquat. Org.* 54(2):119-126.

**Q**

Quon K.C., Marczynski G.T., Shapiro L. (1996). Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* 84:83–93.

**R**

Raghavan V. and Groisman E.A. (2010). Orphan and hybrid two-component system proteins in health and disease. *Curr. Opin. Microbiol.* 13:226–31.

Raghavan V., Lowe E.C., Townsend G.E, Bolam D.N., Groisman E.A. (2014). Tuning transcription of nutrient utilization genes to catabolic rate promotes growth in a gut bacterium. *Mol. Microbiol.* 93:1010–25.

Rahme L.G., Tan M.W., Le L., Wong S.M., (1997).Tompkins R.G., Calderwood S.B., *et al*. Use of model plant hosts to identify

*Pseudomonas aeruginosa* virulence factors. *Proc Natl Acad Sci USA*.94:13245e50.

Raivio T.L. (2014). Everything old is new again: an update on current research on the Cpx envelope stress response. *Biochim. Biophys. Acta* 1843:1529–41.

Ravikumar S., Baylon M.G., Park S.J., Choi J.I (2017). Engineered microbial biosensors based on bacterial two-component systems as synthetic biotechnology platforms in bioremediation and biorefinery. *Microb Cell Fact.* 14;16(1):62.

Raivio T.L., Popkin D.L., Silhavy T.J. (1999). The Cpx envelope stress response is controlled by amplification and feedback inhibition. *J. Bacteriol*. 181:5263–72.

Rawlings N. D., Barrett A. J. and Finn R.D. (2016). Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* 44(D1):D343-D350. https://www.ebi.ac.uk/merops/

Renault T., Haffner, P. Malfondet C. and Weppe M. (1994). *Vibrio damsel*a as a pathogenic agent causing mortalities in cultured sea bass (*Lates calcarifer*). *Bull. Eur. Ass. Fish Pathol.* 14(4):117-119.

Rich J.J., Kinscherf, T.G., Kitten, T., and Willis, D.K. (1994). Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. *J Bacteriol* 176: 7468–7475.

Rio S. J., Osorio C. R., and Lemos M. L. (2005). Heme uptake genes in human and fish isolates of *Photobacterium damselae*: existence of hut Apseudo-genes. *Arch. Microbiol.* 183(5):347-358.

Rivas A. J., Balado, M., Lemos, M. L., and Osorio, C. R. (2011). The *Photobacterium damselae* subsp. *damselae* hemolysins damselysin and HlyA are encoded within a new virulence plasmid. *Infect. Immun*. 79, 4617-4627.

Rivas A. J., Balado, M., Lemos, M. L., and Osorio, C. R. (2013a). Synergistic and additive effects of chromosomal and plasmid-encoded hemolysins contribute to hemolysis and virulence in *Photobacterium damselae* subsp. *damselae*. *Infect. Immun*. 81, 3287-3299.

Rivas A.J., Lemos M.L., Osorio C.R. (2013b). *Photobacterium damselae* subsp. *damselae*, a bacterium pathogenic for marine animals and humans. *Front Microbiol* 4:283.

Rivas A. J., Labella, A., Borrego, J. J., Lemos, M. L., and Osorio, C. R. (2014). Evidences for horizontal gene transfer, gene duplication and genetic variation as driving forces of the diversity of haemolytic phenotypes in *Photobacterium damselae* subsp. *damselae*. *FEMS Microbiol. Lett*. 355, 152-162.

Rivas A. J., Von Hoven, G., Neukirch, C., Meyenburg, M., Qin, Q., Füser, S., Boller, K., Lemos, M. L., Osorio, C. R., and Husmann, M. (2015a). Phobalysin, a small ß-pore-forming toxin of *Photobacterium damselae* subsp. *damselae*. *Infect. Immun*. 83, 4335-4348.

Rivas A. J., Vences, A., Husmann, M., Lemos, M. L., and Osorio, C. R. (2015b). *Photobacterium damselae* subsp. *damselae* major virulence factors Dly, plasmid encoded HlyA, and chromosome-encoded HlyA are secreted via the type II secretion system. *Infect. Immun*. 83, 1246-1256.

Roberts D. M., Personne Y., Ollinger J. And Parish T. (2013). Proteases in *Mycobacterium tuberculosis* pathogenesis: potential as drug targets. *Future Microbiol.* 8(5):621-631.

Romeo T. (1998). Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Mol Microbiol*. 29: 1321–1330.

Romeo T., Gong M., Liu M.Y. and Brun-Zinkernagel A.M. (1993). Identification and molecular characterization of csrA, a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. *J Bacteriol*. 175: 4744– 4755.

**S**

Salyers A. and Witt D. (1994). Virulence factors that promote colonization. Salyers A. and Witt D. (Eds.), Bacterial pathogenesis: a molecular approach (pp. 30-46). Washington, D.C.: ASM Press.

Saha R., Saha N., Donofrio R. S. and Bestervelt L. L. (2013). Microbial siderophores: a mini review. *J. Basic Microbiol.* 53(4):303-317.

Saitou N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. 4, 406-425.

Schilling B., Christensen D., Davis R., Sahu A.K., Hu L.I., *et al*. (2015). Protein acetylation dynamics in response to carbon overflow in *Escherichia coli*. *Mol. Microbiol*. 98:847–63.

Schmidt H., and Hensel, M. (2004). Pathogenicity islands in bacterial pathogenesis. *Clin. Microbiol. Rev.* 17, 14-56.

Schrecke K., Jordan S., Mascher T. (2013). Stoichiometry and perturbation studies of the LiaFSR system of *Bacillus subtilis*. *Mol. Microbiol*. 87:769–88

Severi E., Hood, D.W., and Thomas, G.H. (2007). Sialic acid utilization by bacterial pathogens. *Microbiology* 153, 2817-2822.

Septer A.N., Bose J.L., Lipzen A., Martin J., Whistler C., Stabb E.V. (2015). Bright luminescence of *Vibrio fischeri* aconitase mutants reveals a connection between citrate and the Gac/Csr regulatory system. *Mol Microbiol.* 95(2):283–96.

Sharma S. R. K., Pradeep, M. A., Sadu, N., Dube, P. N., and Vijayan, K, K. (2017). First report of isolation and characterization of *Photobacterium damselae* subsp. *damselae* from cage-farmed cobia (*Rachycentron canadum*). *J. Fish Dis*. 40, 953-958.

Sherwood E.J., Bibb M.J. (2013). The antibiotic planosporicin coordinates its own production in the actinomycete *Planomonospora alba*. PNAS 110:E2500–9.

Shin J. H., Shin M. G., Suh S. P., Ryang D. W., Rew J. S. and Nolte F. S. (1996). Primary *Vibrio damsela* septicemia. *Clin. Infect. Dis*. 22(5):856- 857.

Shin D, Lee E.J., Huang H, Groisman E.A. (2006). A positive feedback loop promotes transcription surge that jump-starts *Salmonella* virulence circuit. *Science* 314:1607–9.

Shu C.J., Zhulin I.B. (2002). ANTAR: an RNA-binding domain in transcription antitermination regulatory proteins. *Trends Biochem. Sci*. 27:3–5.

Siezen R.J., and Leunissen, J.A. (1997). Subtilases: the superfamily of subtilisin-like serine proteases. *Protein Sci.* 6, 501–523.

Smith S. K., Sutton D. C., Fuerst J. A. and Reichelt J. L. (1991). Evaluation of the genus *Listonella* and reassignment of *Listonella damsela* (Love *et al.*) MacDonell and Colwell to the genus

*Photobacterium* as *Photobacterium damsela* comb. nov. With anemended description. *Int. J. Syst. Bacteriol.* 41(4):529-534.

Smith H. O., Gwinn, M. L., and Salzberg, S. L. (1999). DNA uptake signal sequences in naturally transformable bacteria. *Res. Microbiol*. 150, 603-616.

Song Y. L., Cheng W. and Wang C. H. (1993). Isolation and characterization of *Vibrio damsela* infectious for cultured shrimp in Taiwan. *J. Invertebr. Pathol*. 61(1):24-31.

Spencer-Smith, R., Roberts, S., Gurung, N., and Snyder, L. A. S. (2016). DNA uptake sequences in *Neisseria gonorrhoeae* as intrinsic transcriptional terminators and markers of horizontal gene transfer. *Microb. Genom*. 2, e000069.

Stehr F., Kretschmar M., Kröger C., Hube B. and Schäfer W. (2003). Microbial lipases as virulence factors. *J. Mol. Catal. B: Enzym*. 22(5-6):347- 355.

Stock A.M., Robinson V.L., Goudreau P.N. (2000). Two-component signal transduction. *Annu. Rev. Biochem.* 69:183–215.

Sugita H., Shinagawa Y., and Okano R. (2000). Neuraminidase-producing ability of intestinal bacteria isolated from coastal fish. *Lett. Appl. Microbiol.* 31,10-13.

Sugiura M., Aiba H., Mizuno T. (2003). Identification and classification of two-component systems that affect *rpoS* expression in *Escherichia coli*. *Biosci Biotechnol Biochem.* 67(7):1612-5.

Sun Y., Bernardy, E. E., Hammer, B. K., and Miyashiro, T. (2013). Competence and natural transformation in vibrios. *Mol. Microbiol*. 89, 583-595.

**T**

Tao Z., Shen C., Zhou S.-M., *et al.* (2018). An outbreak of *Photobacterium damselae* subsp. *damselae* infection in cultured silver pomfret *Pampus argenteus* in Eastern China. *Aquaculture* 492 201–205.

Takahashi H., Miya S., Kimura B., Yamane K., Arakawa Y., and Fujii T. (2008). Difference of genotypic and phenotypic characteristics and pathogenicity potential of *Photobacterium damselae* subsp. *damselae* between clinical and environmental isolates from Japan. *Microb. Pathog*. 45, 150-158.

Tamura K., Nei, M., and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA* 101, 11030-11035.

Tamura K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol*. 30, 2725-2729.

Terceti M. S., Ogut, H., and Osorio C.R. (2016). *Photobacterium damselae* subsp. *damselae*, an emerging fish pathogen in the Black Sea: evidence of a multiclonal origin. *Appl. Environ. Microbiol*. 82, 3736-3745.

Terceti M. S., Rivas, A. J., Alvarez, L., Noia, M., Cava, F., and Osorio C.R. (2017). *rstB* regulates expression of the *Photobacterium damselae* subsp. *damselae* major virulence factors Damselysin, Phobalysin P and Phobalysin C. *Front. Microbiol*. 8, 582.

Terceti M. S., Vences A., Matanza X. M., Dalsgaard I., Perdersen K., Osorio C. R. (2018a). Molecular epidemiology of *Photobacterium damselae* subsp. *damseale* outbreaks in marine raibow trout farms reveals extensive horizontal gene transfer and high genetic diversity. *Front. Microbiol*. 9, 2155.

Terceti M. S., Vences A., Matanza X.M., Barca A.V., Noia M., Lisboa J., Santos N.M.S., Vale A., Osorio C. R. (2018b). Mutation of the RstAB two-component regulatory system impairs virulence, motility, cell morphology, antimicrobial resistance and production of type II secretion system-dependent factors in the fish and human pathogen *Photobacterium damselae* subsp. *damselae*.**( submitted for publication).**

Thyssen A., Grisez L., van Houdt R., and Ollevier F. (1998). Phenotypic characterization of the marine pathogen *Photobacterium damselae* subsp. *piscicida. Int. J. Syst. Bacteriol.* 48(Pt 4):1145-1151.

Timur G., Timur M., Akayli T., Korun J., Erkan M. (2008). The first observation of viral erythrocytic infection associated with pathogenic bacteria and some ectoparasites in cultured Mediterranean sea bass (*Dicentrarchus labrax* L.) in Turkey. *Bull Eur Assoc Fish Pathol* 28:58–65.

Tran T. K., Han Q. Q., Shi Y., and Guo, L. (2016). A comparative proteomic analysis of *Salmonella typhimurium* under the regulation of the RstA/RstB and PhoP/PhoQ systems. *Biochim. Biophys*. Acta 1864, 1686–1695.

Trajtenberg F., Albanesi D., Ruetalo N., Botti H., Mechaly A.E., *et al.* (2014). Allosteric activation of bacterial response regulators: The role of the cognate histidine kinase beyond phosphorylation. *mBio* 5:e02105.

Travis J., Potempa J. and Maeda H. (1995). Are bacterial proteinases pathogenic factors? *Trends Microbiol*. 3(10):405-407.

Tsou A.M., Liu Z., Cai T., Zhu J. (2011). The VarS/VarA two-component system modulates the activity of the *Vibrio cholerae* quorum-sensing transcriptional regulator HapR. *Microbiology.* 157 (Pt 6):1620-8.

Tu K.C, Long T., Svenningsen S.L., Wingreen N.S., Bassler B.L. (2010). Negative feedback loops involving small regulatory RNAs precisely control the *Vibrio harveyi* quorum-sensing response. *Mol. Cell* 37:567–79.

**U**

Uzun E., and Ogut H. (2015). The isolation frequency of bacterial pathogens from sea bass (*Dicentrarchus labrax*) in the Southeastern Black Sea. *Aquaculture* 437, 30-37.

**V**

Vakulskas C.A., Potts A.H., Babitzke P., Ahmer B.M., Romeo T. (2015). Regulation of bacterial virulence by Csr (Rsm) systems. *Microbiol Mol Biol Rev*. 79:193–224.

Valverde C., Heeb, S., Keel, C., and Haas, D. (2003). RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. *Mol Microbiol 50*: 1361– 1379.

Vaseeharan B., Sundararaj S., Murugan T., and Chen J.C. (2007). *Photobacterium damselae* ssp *damselae* associated with diseased black tiger shrimp *Penaeus monodon* Fabricius in India. *Lett. Appl. Microbiol*. 45, 82-86.

Verhamme D.T., Arents J.C., Postma P.W., Crielaard W, Hellingwerf K.J. (2002). Investigation of in vivo cross-talk between key two-component systems of *Escherichia coli. Microbiology.* 148:69–78.

Vences A., Rivas A. J., Lemos M. L., Husmann M., and Osorio C. R. (2017). Chromosome-encoded hemolysin, phospholipase, and collagenase in plasmidless isolates of *Photobacterium damselae*

subsp. *damselae* contribute to virulence for fish. *Appl. Environ. Microbiol*. 83, pii: e00401-17.

Vera P., Navas J.I., and Fouz B. (1991). First isolation of *Vibrio damsela* from sea bream (*Sparus aurata*). *Bull. Eur. Ass. Fish Pathol*. 11, 112-113.

**W**

Wang F.F., Deng C.Y., Cai Z., Wang T., Wang L., *et al*. (2014). A three-component signalling system finetunes expression kinetics of HPPK responsible for folate synthesis by positive feedback loop during stress response of *Xanthomonas campestris. Environ. Microbiol.* 16:2126–44.

Weilbacher T., Suzuki, K., Dubey, A.K., Wang, X., Gudapaty, S., Morozov, I., *et al*. (2003). A novel sRNA component of the carbon storage regulatory system of *Escherichia coli*. *Mol Microbiol*. 48: 657–670.

Whistler C.A. and Ruby E.G. (2003). GacA regulates symbiotic colonization traits of *Vibrio fischeri* and facilitates a beneficial association with an animal host. *J Bacteriol*. 185(24):7202–12.

Whitaker W. B., Parent, M. A., Boyd, A., Richards, G. P., and Boyd, E. F. (2012). The *Vibrio parahaemolyticus* ToxRS regulator is required for stress tolerance and colonization in a novel orogastric streptomycin-induced adult murine model. *Infect. Immun.* 80, 1834–1845.

Witte G., Hartung S., Buttner K., Hopfner K.P. (2008). Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. *Mol Cell.*30, 167-178.

Wong S.M., Carroll, P.A., Rahme, L.G., Ausubel, F.M., and Calderwood, S.B. (1998). Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two component family of response regulators. *Infect Immun*. 66: 5854–5861.

Wong S.M., Carroll P.A., Rahme L.G., *et al*., (1998). Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two-component family of response regulators. Infect Immun. 66(12):5854-61.

Wu L. N., Lin X. M., Wang F. P., Ye D. Z., Xiao X., Wang S. Y., *et al*. (2006). OmpW and OmpV are required for NaCl regulation in *Photobacterium damsela*. *J. Proteome Res.* 5(9):2250-2257.

**X**

Xu J., Bjursell M.K., Himrod J., Deng S., Carmichael L.K., *et al*. (2003). A genomic view of the human *Bacteroides thetaiotaomicron* symbiosis. *Science* 299:2074–76.

Xu J., Chiang H.C., Bjursell M.K., Gordon J.I. (2004). Message from a human gut symbiont: Sensitivity is a prerequisite for sharing. *Trends Microbiol.* 12:21–28.

**Y**

Yamamoto K., Hirao K., Oshima T., *et al*., (2005). Functional characterization in vitro of all two-component signal transduction systems from *Escherichia coli*. *J Biol Chem*. 14;280(2):1448-56.

Yamane K., Asato J., Kawade N., Takahashi H., Kimura B., and Arakawa Y. (2004). Two cases of fatal necrotizing fasciitis caused by *Photobacterium damsela* in Japan. *J. Clin. Microbiol.* 42, 1370–1372.

Yuen K. Y., Ma L., Wong S. S. Y. and Ng W. F. (1993). Fatal necrotizing fasciitis due to *Vibrio damsela*. Scand. *J. Infect. Dis*. 25(5):659-661.

## Z

Zhang X. J., Qin G. M., Bing X. W., Yan B. L. and Bi K. R. (2011). Phenotypic and molecular characterization of *Photobacterium damselae*, a pathogen of the cultured tongue sole *Cynoglossuss emilaevis* in China. *N. Z. J. Mar. Freshw. Res.* 45(1):1-13.

Zhang Y. Z., Ran L. Y., Li C. Y. and Chen X. L. (2015). Diversity, structures, and collagen- degrading mechanisms of bacterial collagenolytic proteases. *Appl. Environ. Microbiol.* 81(18):6098-6107.

Zhao D. H., Sun, J.J., Liu, L., Zhao, H. H., Wang, H. F., Liang, L. Q., and Liu, L. B. (2009). Characterization of two phenotypes of *Photobacterium damselae* subsp. *damselae* isolated from diseased juvenile *Trachinotus ovatus* reared in cage mariculture. *J. World Aquacult.* Soc. 40, 281-289.

Zhu S., Kojima S., Homma M. (2013). Structure, gene regulation and environmental response of flagella in *Vibrio*. *Front Microbiol* 4:410.

Zwir I., Latifi T., Perez J.C., Huang H., Groisman E.A. (2012). The promoter architectural landscape of the *Salmonella* PhoP regulon. *Mol. Microbiol*. 84:463–85.

Zwir I., Yeo W.S., Shin D., Latifi T., Huang H., Groisman E.A. (2014). Bacterial nucleoid-associated protein uncouples transcription levels from transcription timing. *mBio* 5:e01485–14.

Zhu J., and Mekalanos J.J. (2003) Quorum sensingdependent biofilms enhance colonization in *Vibrio cholerae*. *Dev Cell.* 5: 647–656.

Zhu J., Miller, M.B., Vance, R.E., Dziejman, M., Bassler, B.L., and Mekalanos, J.J. (2002). Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA*. 99: 3129–3134.

# ANEXO

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

# ANEXO I

## ASPECTOS ÉTICOS

- En esta tesis se incluyen estudios que utilizan peces como animales de experimentación, en cumplimiento con el RD 53/2013.
- El proyecto de investigación se titula "Inoculaciones experimentales de peces con bacterias patógenas y con proteínas bacterianas", del que es investigador responsable el Prof. Dr. Carlos Rodríguez Osorio, tutor y director de la presente tesis doctoral.
- El número de autorización de este proyecto de experimentación animal es: **15004/14/003** (se adjunta copia de la autorización).
- El centro de usuario en el que se realizó el trabajo, es el Animalario Experimental de la Facultad de Biología de la Universidade de Santiago de Compostela, con código de centro: **15004AE: ES150780263301**.
- Los experimentos con peces fueron realizados por el Prof. Dr. Carlos Rodríguez Osorio, Tutor y Director de la presente tesis.
- Se adjuntan los Certificados de Capacitación en las Categorías "C" y "D" del Prof. Dr. Carlos Rodríguez Osorio.

XUNTA DE GALICIA
DELEGACIÓN TERRITORIAL
DA CORUÑA
Xefatura Territorial da Consellería do
Medio Rural e do Mar

Edificio administrativo Monelos
Vicente Ferrer, 2
15071 A Coruña

galicia



José Manuel Cifuentes
Departamento de Anatomía
Facultade de Veterinaria
Campus Universitario
27002 Lugo

Ref.: JLPV/lld

NOTIFICACIÓN DE RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTOS DE EXPERIMENTACIÓN ANIMAL

**Expediente núm.:** 15004/14/003
**Interesado:** Carlos Rodríguez Osorio
**Procedemento:** resolución de autorización
**Data de inicio:** 11-12-2014
**Forma de inicio:** solicitude do interesado

Notifícolle que con data 23 de decembro de 2014, o xefe territorial da Consellería do Medio Rural e do Mar, por ausencia a xefa do Servizo Xurídico Administrativo, emitiu unha resolución de autorización de proxectos de experimentación animal, cuxo texto íntegro é o seguinte:

ANTECEDENTES

O interesado, como representante do centro da Facultade de Bioloxía (Universidade de Santiago de Compostela), presentou con data 11-12-2014 e rexistro de entrada 140511 RX 1550666, unha solicitude para a realización do proxecto de experimentación animal, cuxos datos se detallan a continuación:

**Denominación do proxecto:** Inoculacións experimentais de peixes con bacterias patóxenas e con proteínas bacterianas
**Nome do centro usuario:** Animalario experimental da Facultade Bioloxía (USC)
**Persoa responsable do proxecto:** Carlos Rodríguez Osorio
**Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo):** Facultade de Bioloxía (USC)
**Clasificación do proxecto :** Tipo I ☐ Tipo II ☐ Tipo III ☒

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1. O Real decreto 53/2013, de 1 de febreiro (BOE núm. 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

2. O artigo 89 da Lei 30/1992, de 26 de novembro, do réxime xurídico das administracións públicas e do procedemento administrativo común (BOE núm. 285, 27 de novembro de 1992), modificada pola Lei 4/1999, de 14 de xaneiro, establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.

XUNTA DE GALICIA
DELEGACIÓN TERRITORIAL
DA CORUÑA
Xefatura Territorial da Consellería do
Medio Rural e do Mar

Edificio administrativo Monelos
Vicente Ferrer, 2
15071 A Coruña

galicia

3. O Servizo de Gandaría da Coruña revisou a documentación achegada na solicitude e o resultado favorable da avaliación do proxecto, realizada polo órgano habilitado Sección de Experimentación animal do Comité de Bioética da Universidade de Santiago de Compostela.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co artigo 11 do Decreto 245/2009 de 3 de abril, polo que se regulan as delegacións territoriais da Xunta de Galicia e o Decreto 46/2012, de 19 de xaneiro, polo que establece a estrutura orgánica da Consellería do Medio Rural e do Mar e do Fondo Galego de Garantía Agraria.

De acordo con todo o indicado, RESOLVO:

1. Autorizar o proxecto solicitado.
2. Notificarlle esta resolución ao interesado.

O mencionado proxecto deberá someterse a unha avaliación retrospectiva que se realizará no prazo de tres anos.

A autorización deste proxecto terá unha duración de 5 anos, transcorridos os cales, deberá ser renovada esta autorización.

A citada autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandaría.

Esta autorización poderá ser suspendida, no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala, previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non pon fin á vía administrativa, poderá interpor recurso de alzada ante a conselleira de Medio Rural e do Mar da Xunta de Galicia no prazo dun mes contado a partir da recepción da notificación da presente resolución, conforme coa Lei 30/1992, do 26 de novembro, (BOE núm.: 285, 27 de novembro de 1992), de réxime xurídico das administracións públicas e do procedemento administrativo común na súa redacción dada pola Lei 4/1999, do 13 de xaneiro.

A Coruña, 26 de decembro de 2014
O xefe do Servizo de Gandaría

XUNTA DE GALICIA · CONSELLERÍA DO MEDIO RURAL E DO MAR · XEFATURA TERRITORIAL DA CORUÑA · DELEGACIÓN TERRITORIAL DA CORUÑA

SERVIZO DE GANDARÍA

Eugenio Romero Senande

DE ASÍS A COMPOSTELA · 1214 · 2014 ·

VICERREITORADO DE INVESTIGACIÓN
E INNOVACIÓN
Oficina de Investigación e Tecnoloxía

Edificio CACTUS – Campus universitario sur
15782 Santiago de Compostela
Tel. 981 547 040 - Fax 981 547 077
Correo electrónico: citiinfo@usc.es
http://imaisd.usc.es

**Informe del Comité de Ética de Experimentación Animal (CEEA) de los centros usuarios de animales de experimentación de la USC en el Campus de Santiago**

El CEEA de los centros usuarios de animales de experimentación de la USC en el Campus de Santiago, tras evaluar el Proyecto titulado "Inoculacións experimentais de peixes con bacterias patóxenas e con proteínas bacterianas" del que es Investigador Responsable D. Carlos Rodríguez Osorio, acordó con fecha 14 de Noviembre de 2014 emitir

INFORME FAVORABLE

para la realización de dicho proyecto, así como los procedimientos que incluye, en las instalaciones del establecimiento usuario Animalario de la Facultad de Biología, con número de registro ES150780263301, y siempre que, en cumplimiento del RD 53/2013, se obtenga la correspondiente autorización administrativa.

En Santiago, a 14 de Noviembre de 2014

Fdo. El secretario

Fdo.: El presidente

| | | |
|---|---|---|
| Responsable administrativo: | Nombre: Raúl Vieira Miguel<br>Cargo: Director de la RIAIDT de la USC | V.ºB.º Firma y sello |

VICERREITORÍA DE INVESTIGACIÓN
E INNOVACIÓN
Oficina de Investigación e Tecnoloxía
Servizo de Convocatorias e Recursos Humanos de I+S
Edificio CACTUS – Campus Vida
15782 Santiago de Compostela
Tel. 981 547 040 - Fax 981 547 077
Correo electrónico: citinfo@usc.es
http://imaisd.usc.es

**JOSÉ MANUEL CIFUENTES MARTÍNEZ, PROFESOR TITULAR DE UNIVERSIDAD Y PRESIDENTE DEL COMITÉ DE BIOÉTICA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA**, cuya Sección de Experimentación animal ha sido designada como Órgano habilitado para la evaluación de proyectos de experimentación animal por resolución de la Xunta de Galicia, con fecha 11 de noviembre de 2013, de acuerdo con lo exigido por el RD 53/2013 de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia,

**INFORMA:**

Que el proyecto de investigación titulado: **"Inoculaciones experimentales de peces con bacterias patógenas y con proteínas bacterianas"** del que es investigador responsable D. **Carlos Rodríguez Osorio,** ha sido examinado por el Comité de Bioética de esta Universidad, Sección de Experimentación Animal, reunido el nueve de diciembre del presente año, llegando a las siguientes conclusiones:

- Con respecto a su finalidad, se trata de un proyecto de investigación traslacional o aplicada cuyo objetivo es la identificación de toxinas bacterianas y otros sistemas que causan citotoxicidad y muerte en peces por la bacteria patógena *Photobacterium damselae*.
- Con respecto a los requisitos de las 3Rs, no cabe la posibilidad de reemplazo ya que no es posible demostrar si la virulencia de las bacterias está total o parcialmente abolida de otra forma que no sea con una infección experimental de las especies modelo. El estudio en cultivo celular es solo una aproximación que nos indicará la disminución de la toxicidad pero no evita el uso de animales para completar el estudio y demostrar taxativamente que la cepa es avirulenta. En cuanto al principio de reducción, el grupo ha realizado ensayos previos para calcular el número mínimo de peces a utilizar, que asegure un número de muestras representativo y un resultado fiable de las pruebas. Finalmente, a tenor del procedimiento descrito, se han elegido las especies modelo más susceptible para las bacterias estudiadas, y de las que ya se conocen las dosis a emplear, además de conocer de antemano que las cepas salvajes son virulentas para estos modelos animales, cumpliéndose así el principio de refinamiento. Además, los sujetos experimentales estarán alojados en un centro usuario registrado por lo que la manipulación, manejo y supervisión de los animales antes y durante los procedimientos será llevada a cabo por personas capacitadas. El grupo investigador lo componen personas con categoría B y C lo que asegura su preparación para realizar los procedimientos de manera adecuada.
- La clasificación de los tres procedimientos incluidos en el proyecto en función de su grado de severidad es de "severo" (estudios de patogenicidad, protección de vacuna).
- Con respecto al balance de los daños y los beneficios, la ejecución del procedimiento incluido en este proyecto revertirá en el desarrollo potencial de nuevos vacunas. Concretamente en la actualidad no existe ningún método de control totalmente efectivo contra las enfermedades bacterianas causadas por *Photobacterium damselae* en peces. La determinación de los mecanismos de virulencia y la identificación de las cepas con capacidad de vacuna y de proteínas antigénicas es fundamental para conocer la forma en que la bacteria causa la enfermedad en el huésped. Una vez estudiados esos mecanismos será posible diseñar estrategias de prevención eficaces. Valorando ambos aspectos: daños a los animales y posibles beneficios a los pacientes, este Comité considera que los beneficios que podría aportar este proyecto compensan el sacrificio de los animales empleados.
- Con respecto al uso de puntos finales humanitarios: el investigador responsable indica que los animales serán eutanasiados anticipadamente cuando: se observen peces moribundos, para

VICERREITORÍA DE INVESTIGACIÓN
E INNOVACIÓN
Oficina de Investigación e Tecnoloxía
Servizo de Convocatorias e Recursos Humanos de I+S
Edificio CACTUS – Campus Vida
15782 Santiago de Compostela
Tel. 981 547 040 - Fax 981 547 077
Correo electrónico: citiinfo@usc.es
http://imaisd.usc.es

evitar el sufrimiento, estos serán retirados a un pequeño tanque en el que se mezcla agua de mar con una sobredosis de anestésico (MS-222, 160mg/L) y se confirmará la eutanasia por desangrado. El destino final de los animales sobrevivientes es la eutanasia por los medios anteriormente descritos

- Se han examinado las situaciones y excepciones previstas en el punto e) del artículo 34. 2 encontrando que ninguna de ellas es aplicable en este proyecto.
- El proyecto pertenece al tipo III y como tal debe ser sometido a una evaluación retrospectiva. Este Comité considera que dicha evaluación debería efectuarse a los tres años de la concesión de la autorización.

Por todas estas razones, este Comité acordó emitir un **INFORME FAVORABLE.**

En la evaluación de este proyecto NO HA EXISTIDO CONFLICTO DE INTERESES.

Y para que así conste se expide el presente documento en Lugo, a diez de diciembre del año dos mil catorce.

XUNTA DE GALICIA
CONSELLERÍA DO MEDIO RURAL
E DO MAR

galicia

| PROCEDEMENTO | CÓDIGO DO PROCEDEMENTO | DOCUMENTO |
|---|---|---|
| SOLICITUDE DE REALIZACIÓN DE PROXECTO DE EXPERIMENTACIÓN ANIMAL | 15004/14/003 | SOLICITUDE |

## Datos do centro

NOME DO CENTRO USUARIO: Animalario experimental Facultade de Bioloxía

CÓDIGO DO CENTRO:__ 15004AE; ES150780263301

ESTABLECEMENTO ONDE SE REALIZARÁN OS PROCEDEMENTOS DO PROXECTO:_ Animalario experimental Facultade de Bioloxía

## Datos da persoa que realiza a solicitude

| NOME E APELIDOS: Carlos Rodríguez Osorio | | | | DNI 33292553F |
|---|---|---|---|---|
| ENDEREZO Instituto de Acuicultura<br>R/Constantino Candeira s/n<br>Campus Vida | LOCALIDADE<br>Santiago de Compostela | PROVINCIA<br>A Coruña | CONCELLO<br>Santiago de Compostela | |
| CÓDIGO POSTAL<br>15782 | TELÉFONO<br>881816050 | TELÉFONO MÓBIL<br>661298211 | FAX<br>881816047 | CORREO ELECTRÓNICO<br>cr.osorio@usc.es |
| ~~EN CALIDADE DE: USUARIO~~ / PERSOA RESPONSABLE DO PROXECTO (Ríscquese o que non proceda) | | | | |

## Datos do proxecto

DENOMINACIÓN DO PROXECTO AUTORIZADO:

Inoculacións experimentais de peixes con bacterias patóxenas e con proteínas bacterianas

TIPO DE PROXECTO: ☐ TIPO I ☐ TIPO II x TIPO III

PERSOA RESPONSABLE DO PROXECTO: Carlos Rodríguez Osorio

FINALIDADE DO PROXECTO (ALGÚN DOS CONTIDOS NO ARTIGO 5 DO REAL DECRETO 53/2013)

B) Investigación traslacional o aplicada:
- La prevención, profilaxis, diagnóstico o tratamiento de enfermedades en los animales

## Documentación achegada

x INFORME DO COMITÉ DE ÉTICA DE EXPERIMENTACIÓN ANIMAL DO CENTRO

x COPIA DA SOLICITUDE DE AVALIACIÓN DO PROXECTO ANTE O ÓRGANO HABILITADO QUE CONTÉN POLO MENOS A INFORMACIÓN REQUIRIDA

REXISTRO XERAL DA XUNTA DE GALICIA
REXISTRO DO EDIFICIO ADMINISTRATIVO DE LUGO
LUGO
Data 11/12/2014 09:19:53
ENTRADA 140511 / RX 1550666

XUNTA DE GALICIA
CONSELLERÍA DO MEDIO RURAL
E DO MAR

galicia

| |
|---|
| NOS APARTADOS 4 A 14 DO ANEXO X DO REAL DECRETO 53/2013 |
| x VISTO BÓ DO RESPONSABLE ADMINISTRATIVO DO CENTRO USUARIO |
| x RESUMO NON TÉCNICO (SÓ NOS PROXECTOS DE TIPO II E III) |

O prazo de resolución e notificación da resolución deste procedemento é de 40 días hábiles. Este período inclúe a avaliación do proxecto, que debe ser favorable. Nos proxectos Tipo II e III e cando estea xustificado pola complexidade ou a natureza multidisciplinaria do proxecto, poderase ampliar este prazo unha vez, por un período adicional non superior a 15 días hábiles.

| | |
|---|---|
| LEXISLACIÓN APLICABLE<br><br>Real decreto 53/2013, de 1 de febreiro (BOE nº 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia. | REXISTRADO (A cubrir pola Administración) |
| | En Santiago de Compostela, a 5 de Decembro de 2014<br><br>Prof. Dr. Carlos Rodríguez Osorio<br><br>SINATURA DO SOLICITANTE/REPRESENTANTE LEGAL |

DEPARTAMENTO TERRITORIAL DA CONSELLERIA DO MEDIO RURAL E DO MAR DE A CORUÑA
SERVIZO DE GANDERÍA

XUNTA DE GALICIA
CONSELLERÍA DO MEDIO RURAL
Dirección Xeral de Ordenación e Produción Forestal

Camiño Francés, 10
15703 Santiago de Compostela
Tlf: 981 54 66 57
Fax: 981 54 66 51

galicia

Carlos Rodríguez Osorio
Instituto de Acuicultura
Campus Vida
Universidad de Santiago de Compostela
Santiago de Compostela
A Coruña



**Asunto:** Certificado/s de capacitación en materia de protección de animais utilizados, criados ou subministrados con fins de experimentación e outros fins científicos, incluíndo a docencia.

Recibida e revisada a documentación da solicitude de recoñecemento da capacitación en materia de protección de animais utilizados, criados ou subministrados con fins de experimentación e outros fins científicos, incluíndo a docencia conforme coa Orde ECC/566/2015 de 20 de marzo, procedemos a enviarlle o/s seu/s certificado/s.

Santiago de Compostela, 11 de setembro de 2017

A subdirectora xeral de Formación e Innovación Agroforestal

María José Cortés Jiménez

XUNTA DE GALICIA
CONSELLERÍA DO MEDIO RURAL
Dirección Xeral de Ordenación Forestal

San Lázaro, s/n
15781 Santiago de Compostela
Teléfono: 981 546 108 Fax: 981 546 101
mediorural.montesgalicia@xunta.es

galicia

**Certificado de capacitación en materia de protección de animais utilizados, criados ou subministrados con fins de experimentación e otros fins científicos, incluíndo a docencia conforme coa Orde ECC/566/2015 de 20 de marzo.**

*Certificado de capacitación en materia de protección de animales utilizados, criados o suministrados con fines de experimentación y otros fines científicos, incluyendo la docencia conforme con la Orden ECC/566/2015 de 20 de marzo.*

**1. IDENTIFICACIÓN**

1.1. Apelidos / *Apellidos / Surname:*

**RODRÍGUEZ OSORIO**

| 1.2. Nome / *Nombre / First names:* | DNI /*DNI / Identity card number:* |
|---|---|
| **CARLOS** | **33292553F** |

| 1.3. Categoría/*Categoría/Category:* | 1.4. Especies/*Especies/Species:* | 1.5. Válido ata/ *válido hasta/expires:* |
|---|---|---|
| "C" | *Todas* | ***2.04.2023*** |

**2. Nº DO CERTIFICADO / *Nº DEL CERTIFICADO / CERTIFICATE NUMBER***

**c042**

**3.ORGANISMO QUE EXPIDE O CERTIFICADO / *ORGANISMO QUE EXPIDE EL CERTIFICADO / BODY ISSUING THE CERTIFICATE:***

3.1. Nome e enderezo do organismo que expide o certificado / *Nombre y dirección del organismo que expide el certificado / Name and address of the body issuing the certificate:*

**Dirección Xeral de Ordenación Forestal**
**CONSELLERÍA DO MEDIO RURAL – XUNTA DE GALICIA**
San Lázaro, s/n
15781 Santiago de Compostela
A Coruña (España)

| 3.2. Teléfono / *Teléfono / Telephone:* | 3.3. Fax / *Fax / Fax:* | 3.4. Correo electrónico / *Correo electrónico / Email:* |
|---|---|---|
| 981 546 654 | 981 546 651 | formacion.cmrm@xunta.es |

| 3.5. Data / *Fecha / Date:* | 3.6. Lugar / *Lugar / Place:* |
|---|---|
| 8/09/2017 | **Santiago de Compostela** |

| 3.7. Nome e sinatura / *Nombre y firma / Name and signature* | 3.8. Selo / Sello / Stamp |
|---|---|
| Asdo.: **Tomás Fernández-Couto Juanas**<br>**Director Xeral de Ordenación Forestal** | XUNTA DE GALICIA - DIRECCIÓN XERAL DE ORDENACIÓN FORESTAL - CONSELLERÍA DO MEDIO RURAL |

# *MÓDULOS FUNDAMENTAIS OU TRONCAIS E ESPECÍFICOS CORRESPONDENTES Á CATEGORÍA "C" REALIZACIÓN DOS PROCEDEMENTOS – ORDE ECC/566/2015, DE 20 DE MARZO*

*MÓDULOS FUNDAMENTAIS OU TRONCAIS*

*1.- Lexislación nacional (1 hora).*

*2.- Ética, benestar animal e as "tres erres", nivel 1 (2 horas).*

*3.- Bioloxía básica e adecuada, nivel 1 (3 horas).*

*4.- Coidado, saúde e manexo dos animais, nivel 1 (5 horas).*

*5.- Recoñecemento do dolor, o sufrimento e a angustia ( 3 horas).*

*6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)*

*MÓDULOS ESPECÍFICOS DA CATEGORÍA "C"*

*1.- Bioloxía básica e adecuada, nivel 2 (3 horas)*

*2.- Procedementos minimamente invasores sen anestesia, nivel 1 (5 horas)*

*3.- Procedementos minimamente invasores sen anestesia, nivel 2 (10 horas)*

*4.- Anestesia para procedementos menores (5 horas)*

*5.- Anestesia avanzada para intervencións cirúrxicas ou procedementos prolongados ( 8 horas)*

*6.- Principios de cirurxía (5 horas)*

.....

*MÓDULOS FUNDAMENTALES O TRONCALES*

*1.- Legislación nacional (1 hora).*

*2.- Ética, bienestar animal y las "tres erres", nivel 1 (2 horas).*

*3.- Biología básica y adecuada, nivel 1 (3 horas).*

*4.- Cuidado, salud y manejo de los animales, nivel 1 (5 horas).*

*5.- Reconocimiento del dolor, el sufrimiento y la angustia ( 3 horas).*

*6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)*

*MÓDULOS ESPECÍFICOS DE LA CATEGORÍA "C"*

*1.- Biología básica y adecuada, nivel 2 (3 horas)*

*2.- Procedimientos mínimamente invasivos sin anestesia, nivel 1 (5 horas)*

*3.- Procedimientos mínimamente invasivos sin anestesia, nivel 2 (10 horas)*

*4.- Anestesia para procedementos menores (5 horas)*

*5.- Anestesia avanzada para intervenciones quirúrgicas o procedimientos prolongados ( 8 horas)*

*6.- Principios de cirugía (5 horas)*

XUNTA DE GALICIA
CONSELLERÍA DO MEDIO RURAL
Dirección Xeral de Ordenación Forestal

San Lázaro, s/n
15781 Santiago de Compostela
Teléfono: 981 546 108 Fax: 981 546 101
mediorural.montesgalicia@xunta.es

galicia

**Certificado de capacitación en materia de protección de animais utilizados, criados ou subministrados con fins de experimentación e otros fins científicos, incluíndo a docencia conforme coa Orde ECC/566/2015 de 20 de marzo.**

*Certificado de capacitación en materia de protección de animales utilizados, criados o suministrados con fines de experimentación y otros fines científicos, incluyendo la docencia conforme con la Orden ECC/566/2015 de 20 de marzo.*

**1. IDENTIFICACIÓN**

1.1. Apelidos / *Apellidos / Surname:*

**RODRÍGUEZ OSORIO**

| 1.2. Nome / *Nombre / First names:* | DNI / *IDNI / Identity card number:* |
|---|---|
| **CARLOS** | **33292553F** |

| 1.3. Categoría/*Categoría/Category:* | 1.4. Especies/*Especies/Species:* | 1.5. Válido ata/ *válido hasta/expires:* |
|---|---|---|
| "d" | *Todas* | ***2.04.2023*** |

**2. Nº DO CERTIFICADO / *Nº DEL CERTIFICADO / CERTIFICATE NUMBER***

**d025**

**3.ORGANISMO QUE EXPIDE O CERTIFICADO / *ORGANISMO QUE EXPIDE EL CERTIFICADO / BODY ISSUING THE CERTIFICATE:***

3.1. Nome e enderezo do organismo que expide o certificado / *Nombre y dirección del organismo que expide el certificado / Name and address of the body issuing the certificate:*

**Dirección Xeral de Ordenación Forestal**
**CONSELLERÍA DO MEDIO RURAL – XUNTA DE GALICIA**
San Lázaro, s/n
15781 Santiago de Compostela
A Coruña (España)

| 3.2. Teléfono / *Teléfono / Telephone:* | 3.3. Fax / *Fax / Fax:* | 3.4. Correo electrónico / *Correo electrónico / Email:* |
|---|---|---|
| 981 546 654 | 981 546 651 | formacion.cmrm@xunta.es |

| 3.5. Data / *Fecha / Date:* | 3.6. Lugar / *Lugar / Place:* |
|---|---|
| 8/09/2017 | **Santiago de Compostela** |

3.7. Nome e sinatura / *Nombre y firma / Name and signature*

Asdo.: **Tomás Fernández-Couto Juanas**
**Director Xeral de Ordenación Forestal**

3.8. Selo / Sello / Stamp

XUNTA DE GALICIA - DIRECCIÓN XERAL DE ORDENACIÓN FORESTAL - CONSELLERÍA DO MEDIO RURAL

# *MÓDULOS FUNDAMENTAIS OU TRONCAIS E ESPECÍFICOS CORRESPONDENTES Á CATEGORÍA "D" DESEÑO DOS PROXECTOS E PROCEDEMENTOS – ORDE ECC/566/2015, DE 20 DE MARZO*

*MÓDULOS FUNDAMENTAIS OU TRONCAIS*

*1.- Lexislación nacional (1 hora).*

*2.- Ética, benestar animal e as "tres erres", nivel 1 (2 horas).*

*3.- Bioloxía básica e adecuada, nivel 1 (3 horas).*

*4.- Coidado, saúde e manexo dos animais, nivel 1 (5 horas).*

*5.- Recoñecemento do dolor, o sufrimento e a angustia ( 3 horas).*

*6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)*

*MÓDULOS ESPECÍFICOS DA CATEGORÍA "D"*

*1.- Ética, benestar animal e as "tres erres", nivel 2 (10 horas)*

*2.- Fundamentos de bioloxía e fisioloxía animal (20 horas)*

*3.- Procedementos minimamente invasores sen anestesia, nivel 1 (5 horas)*

*4.- Deseño de proxectos e procedementos, nivel 1 (5 horas)*

*5.- Deseño de proxectos e procedementos, nivel 2 (10 horas)*

*.....*

*MÓDULOS FUNDAMENTALES O TRONCALES*

*1.- Legislación nacional (1 hora).*

*2.- Ética, bienestar animal y las "tres erres", nivel 1 (2 horas).*

*3.- Biología básica y adecuada, nivel 1 (3 horas).*

*4.- Cuidado, salud y manejo de los animales, nivel 1 (5 horas).*

*5.- Reconocimiento del dolor, el sufrimiento y la angustia ( 3 horas).*

*6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)*

*MÓDULOS ESPECÍFICOS DE LA CATEGORÍA "D"*

*1.- Ética, bienestar animal y las "tres erres", nivel 2 (10 horas)*

*2.- Fundamentos de biología y fisiología animal (20 horas)*

*3.- Procedimientos minimamente invasores sin anestesia, nivel 1 (5 horas)*

*4.- Diseño de proyectos y procedimientos, nivel 1 (5 horas)*

*5.- Diseño de proyectos y procedimientos, nivel 2 (10 horas)*

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA