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The two-component system RstAB regulates production of a polysaccharide capsule with a role in virulence in the marine pathogen *Photobacterium damselae* subsp. *damselae*

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Summary

The marine bacterium *Photobacterium damselae* subsp. *damselae* (*Pdd*) causes disease in marine animals and humans. Previous studies demonstrated that mutation of the two-component system RstAB strongly impacts virulence of this pathogen, but the RstAB regulon has not been thoroughly elucidated. We here compared the transcriptomes of *Pdd* RM-71 and Δ*rstA* and Δ*rstB* derivatives using RNA-seq. In accordance with previous studies, RstAB positively regulated cytotoxins Dly, PhlyP and PhlyC. This analysis also demonstrated a positive regulation of outer membrane proteins, resistance against antimicrobials and potential virulence factors by this system. Remarkably, RstAB positively regulated two hitherto uncharacterised gene clusters involved in the synthesis of a polysaccharide capsule. Presence of a capsular layer in wild-type cells was confirmed by transmission electron microscopy, whereas *rstA* and *rstB* mutants were non-capsulated. Mutants for capsule synthesis genes, *wza* and *wzc* exhibited acapsular phenotypes, were impaired in resistance against the bactericidal action of fish serum and mucus, and were strongly impaired in virulence for fish, indicating a major role of capsule in virulence. Collectively, this study demonstrates that RstAB is a major positive regulator of key virulence factors including a polysaccharide capsule essential for full virulence in a pathogenic *Photobacterium*.

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Introduction

The family *Vibrionaceae* includes a diverse group of bacterial species that thrive in marine and estuarine habitats, and some members are important pathogens for marine animals and for humans (Thompson *et al*., 2004; Baker-Austin *et al*., 2018). *Photobacterium damselae* subsp. *damselae* (hereafter dubbed *Pdd*), a member of this family, is a generalist pathogen that causes disease in marine animals and humans (Osorio *et al*., 2018). It is an emerging pathogen in marine aquaculture, causing financial losses in fish species of high commercial value and has a special incidence during warm summer months (Fouz *et al*., 1992; Pedersen *et al*., 1997; Pedersen *et al*., 2009; Uzun and Ogut, 2015; Essam *et al*., 2016; Mahmoud *et al*., 2017). Temperatures around 25°C favour *Pdd* growth and enhance the expression of virulence-related functions, thus contributing to the onset of outbreaks (Matanza and Osorio, 2018). Recent reports have raised the alarm about the isolation of this pathogen as a causative agent of disease in shrimp farms in far-off areas around the globe (Aguilera-Rivera *et al*., 2019; Sumithra *et al*., 2019; Bachand *et al*., 2020; Singaravel *et al*., 2020; Wang *et al*., 2020). *Pdd* can also infect humans through wounds exposed to the marine environment or inflicted while handling fish, tools and other objects in contact with seawater, and causes a very aggressive type of necrotising fasciitis with an elevated risk of complication into a fulminant death (Clarridge and Zighelboim-Daum, 1985; Yamane *et al*., 2004; Alhemairi *et al*., 2015). The geographical distribution of human cases was limited to warm coastal areas of the United States, Australia, Jamaica and Japan, among others (Hundenborn *et al*., 2013). However, it has been recently isolated for the first time from human infections in the European continent, in the Atlantic and the

Mediterranean basins, in Greece (Chochlakis *et al*., 2019), Portugal (Guimaraes *et al*., 2020) and Spain (Schröttner *et al*., 2020). *Pdd* likely behaves as an accidental and opportunistic pathogen for humans since a temperature of 37°C constitutes a stressful condition for this bacterium (Matanza and Osorio, 2020).

Pdd produces several cytotoxins that constitute important virulence factors. The phospholipase D damselysin (Dly) and the pore-forming toxin phobalysin P (PhlyP) are encoded in the virulence plasmid pPHDD1 (Rivas *et al*., 2011; Rivas *et al*., 2013; Rivas *et al*., 2015a). The chromosome I-encoded pore-forming toxin phobalysin C (PhlyC) and the phospholipase PlpV also play a role in virulence and cell toxicity (Vences *et al*., 2017). *Pdd* triple mutants for the genes encoding Dly, PhlyP and PhlyC cytotoxins are non-virulent for fish and mice (Rivas *et al*., 2013). These cytotoxins are secreted via the type II secretion system (T2SS) (Rivas *et al*., 2015b; Vences *et al*., 2017). Phylogenetic, phenotypic and molecular studies revealed a high heterogeneity in virulence markers and the multiclonal nature of *Pdd* isolates from aquaculture outbreaks (Pedersen *et al*., 1997; Pedersen *et al*., 2009; Terceti *et al*., 2016; Terceti *et al*., 2018). In addition, it is of relevance that *Pdd* is an antigenically diverse pathogen with at least seven O serogroups reported (Fouz *et al*., 1992; Fouz *et al*., 1997).

Given this high genetic heterogeneity, it is of special importance to identify ubiquitous mechanisms that control virulence and host colonization in this pathogen. Two-component regulatory systems (TCSs) govern gene expression driven by changes in the environment and typically consist of a sensor histidine kinase (HK) at the cell membrane, and a response regulator (RR) at the cytoplasm that binds DNA and regulates gene expression (Stock *et al*., 2000). The TCS RstAB consisting of the HK RstB and the RR RstA was identified as a positive regulator of virulence in *Pdd*, and transcriptional activity of the cytotoxin genes was strongly impaired in an *rstB* mutant (Terceti *et al*., 2017). Furthermore, mutants in either gene of this system exhibited a major impairment in secretion of some T2SS-dependent proteins (Terceti *et al*., 2019) and in virulence for fish (Terceti *et al*., 2017; Terceti *et al*., 2019). Despite all the currently available information, the RstAB regulon remains incompletely elucidated. Genes encoding a TCS homologous to RstAB exist in most *Vibrio* and *Photobacterium* species (Terceti *et al*., 2019). However, to date, this regulatory system has been subject of investigation in only two species of this family besides *Pdd*, which include *Vibrio cholerae* (Bilecen and Yildiz, 2009; Herrera *et al*., 2014; Bilecen *et al*., 2015) and *V. alginolyticus* (Huang *et al*., 2018).

In this study, we compared the global transcriptional profile of *Pdd* wt RM-71 versus *rstA* and *rstB* mutants through RNA-sequencing. This analysis confirmed previous evidence on the role of this TCS as a major positive regulator of plasmid and chromosome-encoded cytotoxins. Most notably, *rstA* and *rstB* mutants exhibited a very strong downregulation of two hitherto uncharacterised, divergently transcribed gene clusters for the biogenesis of a polysaccharide capsule. The phenotypic characterization of mutants of the RstAB system genes *rstA* and *rstB*, and of putative capsular genes *wza*, *wzc* and *yjbH* has here disclosed the existence of a capsule and its crucial role for full virulence in this life-threatening human and animal pathogen. Furthermore, the identification of the RstAB regulon has revealed other genes putatively involved in the pathogenic process. The ubiquitous presence of RstAB homologous systems in the family *Vibrionaceae* will surely warrant further studies on the role of this TCS in virulence in other important pathogens for animals and humans.

Results

The RstAB system is a major positive regulator of genes encoding virulence factors in Pdd

In two recent studies, it was reported that the TCS RstAB is a positive regulator of the three main cytotoxins produced by *Pdd*, and mutants in *rstA* and *rstB* genes are strongly impaired in virulence in a fish infection model. The genes encoding the HK RstB (VDA_000601) and the RR RstA (VDA_000600) are linked in the *Pdd* genome and are predicted to constitute a cognate pair (Terceti *et al*., 2017; Terceti *et al*., 2019). To gain a deeper insight into the RstAB regulon in this pathogen, we conducted an RNA-seq analysis of *Pdd* RM-71 wt and its derivative deletion mutants Δ*rstB* and Δ*rstA*. Principal component analysis demonstrated a clear distinction of the three biological replicates of each strain (Fig. 1). Genes exhibiting a fold change value (FC) higher than 1.5 or lower than −1.5, and a *P*-value adjusted by false discovery rate (FDR) <0.05, were considered differentially expressed genes (DEGs). From the comparison between the wt and the Δ*rstB* mutant, the number of genes that underwent a differential expression was 747 (257 downregulated and 490 upregulated relatively to the wt) (Supplementary Table S1). Likewise, 397 genes were differentially expressed in the Δ*rstA* mutant (230 downregulated and 167 upregulated with respect to the wt) (Supplementary Table S2). There was a great difference in the range of FC values between downregulated and upregulated genes. As an example, the top 20 downregulated genes in the Δ*rstA* and Δ*rstB* mutants exhibited acute changes of gene expression with respect to the wt, with FC values ranging between −100 and −1400 (Δ*rstA*), and between −41 and −1509 (Δ*rstB*). On the contrary, the FC value ranges of the top 20 upregulated genes in Δ*rstA* and

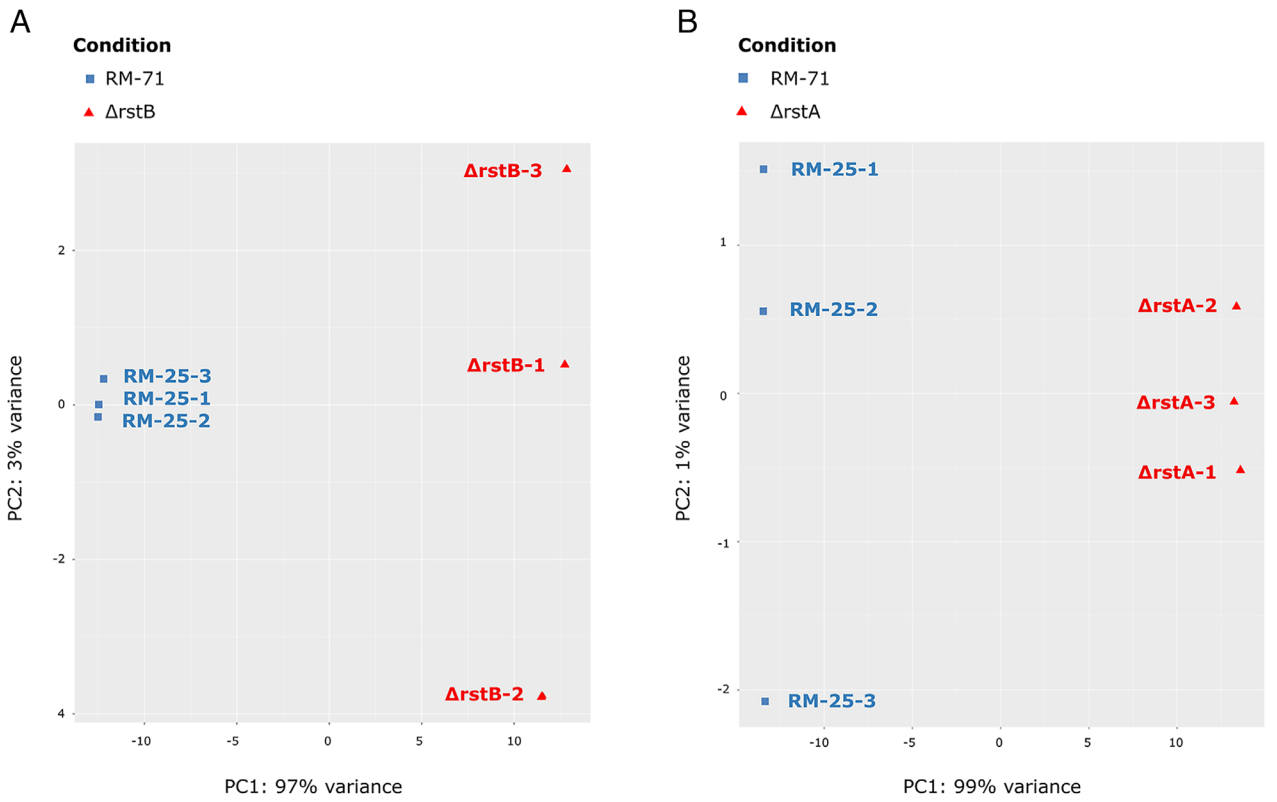

Fig. 1. Hierarchical clustering of all genes by principal component analysis (PCA) demonstrates clear separation of the sample groups (three biological replicates per group) when comparing wild type strain (RM-71) transcriptome to that of deletion mutants Δ*rstB* (A) or Δ*rstA* (B).

Δ*rstB* mutants were as low as 4–10 and 4–6.8 respectively. Overall, >90% of the genes whose expression is upregulated in the Δ*rstA* and Δ*rstB* mutants exhibited very low degrees of differential expression with respect to the wt and the FC values were very close to the threshold for being considered as DEGs (Supplementary Tables S1 and S2).

The number of DEGs exclusively downregulated in either the Δ*rstB* or the Δ*rstA* mutant was 129 and 102 genes respectively. As expected for a putative cognate pair, there was a substantial overlap between the RstB and RstA regulons, represented by 128 genes downregulated in both mutants, and these genes will constitute the focus of the present study (Fig. 2A).

Corroborating previous observations (Terceti *et al*., 2019), the RstAB regulon included chromosome- and plasmid-encoded genes with either a demonstrated or a predicted role in virulence, indicating that RstAB is a major regulator of virulence in *Pdd*. The top downregulated genes are listed in Table 1, and the FC values correspond to the comparison between Δ*rstA* versus wt. These genes were classified into functional categories that include synthesis of cell envelope polysaccharides, cytotoxins, the T2SS, resistance to antimicrobial agents, survival within the host, outer membrane proteins and other functions with potential roles in virulence. A heat map comparing expression in Δ*rstA* versus wt, Δ*rstB* versus wt and Δ*rstA* versus Δ*rstB* was constructed to illustrate the $\log_2$ FC of selected virulence-related genes of the RstAB regulon (Fig. 2B). In general, there is a common downregulation (represented by green colour) of virulence-related genes in the RstAB system mutants. The comparison between Δ*rstA* and Δ*rstB* regulons is also presented, showing that the vast majority of genes do not show differences (denoted with a black colour), indicative of a co-regulation of such genes by the cognate pair RstAB. The slight level of different regulation levels in a reduced number of genes between the two mutants (namely, VDA_001503 and A0J47_019170, depicted in yellow colour) is largely explained by the very low expression levels of those genes in both mutants.

RNAseq data confirm the RstAB-dependent regulation of the Pdd *cytotoxins and additional T2SS-dependent proteins*

The transcriptomics data showed a strong downregulation of the three cytotoxins Dly, PhlyP and PhlyC

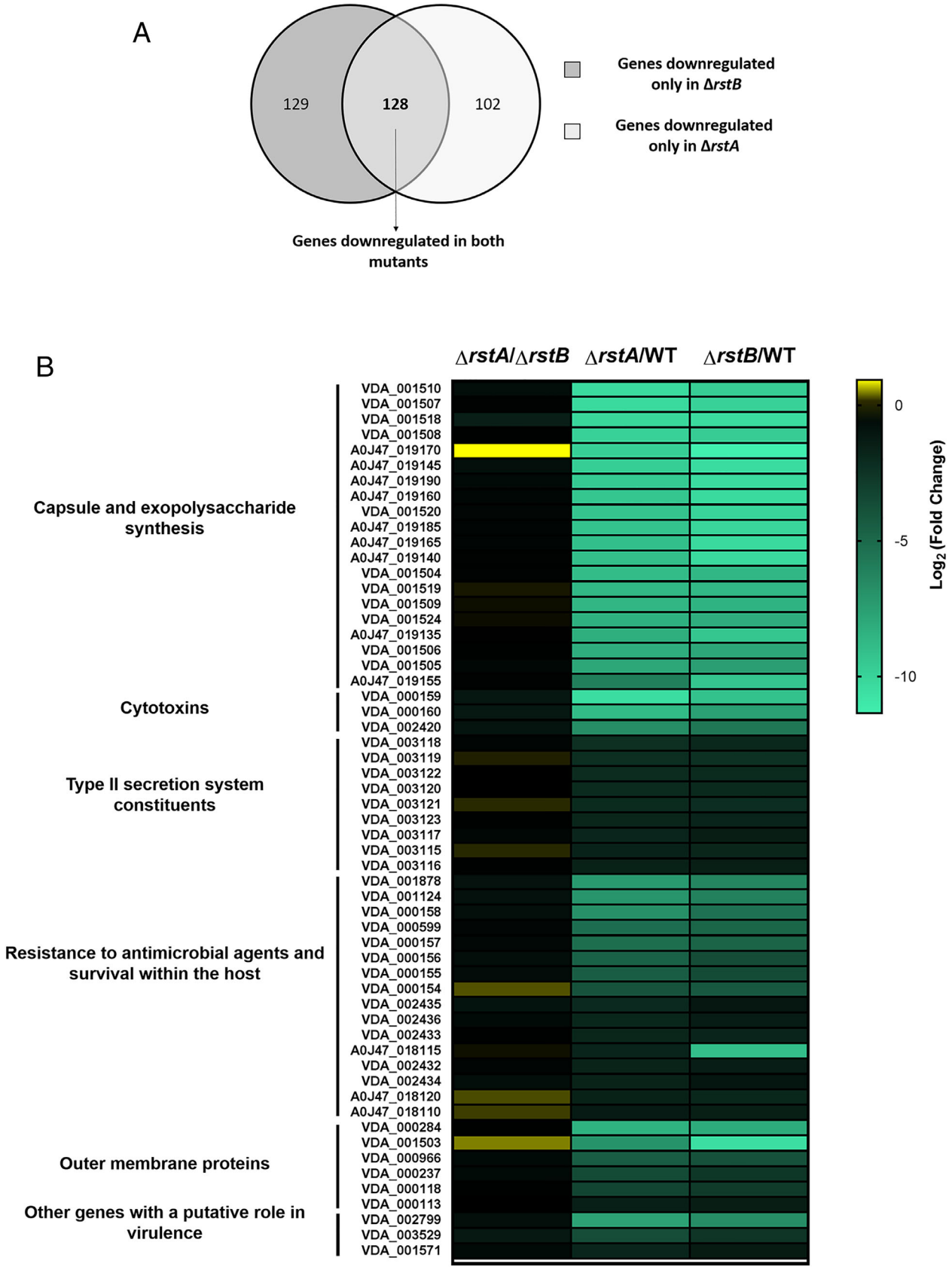

Fig. 2. Legend on next page.

in *rstA* and *rstB* mutants. Notably, the *dly* gene (VDA_000159) encoding damselysin (Dly) toxin was the top most downregulated gene in the Δ*rstA* transcriptome, with a remarkable FC value of −1408 (Table 1). The two phobalysin toxin (PhlyP and PhlyC) genes exhibited FC of −465 (plasmid-borne $hlyA_{pl}$ gene VDA_000160 encoding PhlyP) and −102 (chromosome-borne $hlyA_{ch}$ gene VDA_002420 encoding PhlyC). These results corroborate the data from previous studies that reported a downregulation of cytotoxin gene promoters in a *rstB* mutant background (Terceti *et al*., 2017), as well as the impairment in secretion of the three cytotoxins in *rstA* and *rstB* mutants (Terceti *et al*., 2019). In addition, it was here corroborated a strong downregulation in the *rstA* mutant, of five genes encoding proteins known to be secreted by the T2SS in *Pdd* RM-71 (Terceti *et al*., 2019), and whose biological roles await further investigation. The two genes VDA_002799 and A0J47_007530 encode a predicted δ-endotoxin and a small protein of unknown function respectively (Table 1). These two genes occur in a fraction of *Pdd* strains and are located within a region of the *Pdd* chromosome that exhibits a high genetic variability (Terceti *et al*., 2018). The T2SS-dependent hypothetical proteins VDA_000112, VDA_000358 and trypsin-like A0J47_009785, are also under the control of RstAB (Table 1). Since *dly*, $hlyA_{pl}$ and $hlyA_{ch}$ are among the most highly expressed genes of the whole *Pdd* transcriptome (Matanza and Osorio, 2018; Matanza and Osorio, 2020), it can be concluded that the RstAB system is a major regulator of the secretome in this pathogenic bacterium. The regulation of T2SS genes themselves remains largely uncharacterised. We here found that the expression of T2SS genes displays some level of downregulation in *rstA* and *rstB* mutants (Table 1). Of note, the enormous leap between the FC values of toxin genes (FC of −1408 in *dly* gene) and of structural T2SS genes (*eps* genes) (FC values ranging from −3 to −4.8) further demonstrates that the absence of cytotoxin secretion in mutants for the RstAB system is mainly explained by the downregulation of cytotoxin genes themselves and not by the downregulation of the T2SS genes.

The RstAB regulon includes genes encoding outer membrane proteins, porins, and proteins for survival within hosts and for resistance to antimicrobials

The analysis of genes whose expression is downregulated in RstAB system mutants has unveiled a set of genes not yet characterized with potential roles in virulence (Table 1). Among these genes are outer membrane proteins and porins, found to be strongly downregulated in RstAB system mutants. The most downregulated gene in this category, VDA_000284 (FC value of −338), encodes an outer membrane protein of unknown function. VDA_001503 and VDA_000966 encode two distinct OmpA-like outer membrane proteins, whose homologues constitute virulence factors in some species, being involved in invasion and immune evasion in *Escherichia coli* (Weiser and Gotschlich, 1991), and in adherence to epithelia and biofilm formation in *Acinetobacter baumannii* (Gaddy *et al*., 2009). In other *Photobacterium* species VDA_001503 homologous proteins are upregulated under UVB radiation (Matallana-Surget *et al*., 2012) and high pressure (Le Bihan *et al*., 2013). pPHDD1-encoded OmpU (VDA_000113) homologous proteins participate in colonization, adhesion and immune evasion in other pathogens of the family *Vibrionaceae* (Goo *et al*., 2006; Duperthuy *et al*., 2011; Lynch *et al*., 2019). In a recent study, we have shown that expression of this gene is enhanced at temperatures that favour the onset of outbreaks in fish farms (Matanza and Osorio, 2018).

Genes related to survival within the host and resistance to antimicrobial agents are also under control of RstAB (Table 1). A five-gene cluster (VDA_000154 to VDA_000158) that maps to virulence plasmid pPHDD1 encodes TolC protein, AcrAB and two proteins of a complex for multidrug efflux pumping (Du *et al*., 2014). Although their role in the biology of *Pdd* remains uncharacterised, expression of these genes is enhanced at 25°C when compared to 15°C (Matanza and Osorio, 2018). The Tol-Pal system (VDA_002432-VDA_002436), also downregulated in RstAB system mutants, is a periplasmic protein complex that has many functions in Gram-negative bacteria such as outer membrane integrity, colicin tolerance and survival within host cells (Gerding *et al*., 2007; Hirakawa *et al*., 2019). Our analysis also showed the downregulation of VDA_000599

Fig. 2. RNA sequencing reveals the strong association between the histidine kinase RstB and the response regulator RstA in the regulation of numerous genes related to virulence.
A. Graphical representation of the number of downregulated genes in Δ*rstA* and/or Δ*rstB* mutants.
B. Heatmap showing the differential expression of virulence-related genes in pairwise comparisons of the wt, Δ*rstB* and Δ*rstA* mutants of *Pdd*. Gene expression is plotted as log2 fold change. Genes were attributed to functional categories: capsule and exopolysaccharide synthesis, cytotoxins, type II secretion system constituents, resistance to antimicrobial agents and survival within the host, outer membrane proteins, and other genes with a putative role in virulence. VDA codes correspond to the annotation in the genome of the *Pdd* type strain CIP102761 and A0J47 codes correspond to the annotation in the *Pdd* RM-71 genome.

Table 1. List of top DEGs downregulated by the RstAB system.

Gene ID	Product/function	Fold change (FC)	*P*-value	Location
Virulence-related genes and proteins				
Capsule and exopolysaccharide synthesis				
VDA_001510	Wzc	−1299.9	7.88E-269	ChrI
VDA_001507	Hypothetical protein YjbE	−1239.58	0	ChrI
VDA_001518	UDP-glucose dehydrogenase	−1156.87	4.26E-207	ChrI
VDA_001508	Wza	−955.43	2.99E-268	ChrI
A0J47_019170	Glycosyltransferase group 1	−911.3	8.98E-214	ChrI
A0J47_019145	Aminotransferase	−862.95	2.01E-185	ChrI
A0J47_019190	EPS biosynthesis	−772.88	9.55E-169	ChrI
A0J47_019160	Glycosyltransferase	−673.59	1.20E-159	ChrI
VDA_001520	Glucose-1-phosphate thymidylyltransferase	−633.83	5.39E-179	ChrI
A0J47_019185	Glycosyltransferase	−588.63	9.49E-151	ChrI
A0J47_019165	Polysaccharide pyruvyl transferase	−541.87	5.24E-134	ChrI
A0J47_019140	Acetyltransferase	−518.56	4.09E-141	ChrI
VDA_001504	YjbH	−488.95	4.19E-197	ChrI
VDA_001519	dTDP-glucose 4,6-dehydratase	−429.72	1.42E-171	ChrI
VDA_001509	Wzb	−396.09	8.41E-166	ChrI
VDA_001524	UTP-glucose-1-phosphate uridylyltransferase	−331.37	9.34E-146	ChrI
A0J47_019135	dTDP-6-deoxy-3,4-keto-hexulose isomerase	−301.71	1.93E-111	ChrI
VDA_001506	YjbF	−298.77	1.72E-139	ChrI
VDA_001505	YjbG	−232.8	1.76E-123	ChrI
A0J47_019155	O-antigen translocase	−64.72	2.08E-38	ChrI
Cytotoxins				
VDA_000159	Damselysin	−1408.26	0	pPHDD1
VDA_000160	PhlyP	−465.66	0	pPHDD1
VDA_002420	PhlyC	−102.4	0	ChrI
Type II secretion system constituents				
VDA_003118	EpsH	−4.83	3.35E-136	ChrI
VDA_003119	EpsG	−4.69	1.30E-263	ChrI
VDA_003122	EpsD	−4.32	5.45E-179	ChrI
VDA_003120	EpsF	−4.16	2.28E-158	ChrI
VDA_003121	EpsE	−4.12	4.03E-203	ChrI
VDA_003123	EpsC	−3.54	1.92E-175	ChrI
VDA_003117	EpsJ	−3.44	1.27E-111	ChrI
VDA_003115	EpsL	−3.11	6.58E-24	ChrI
VDA_003116	EpsK	−3.07	3.92E-120	ChrI
Resistance to antimicrobial agents and survival within the host				
VDA_001878	DUF535, VirK-like	−159.9	0	ChrI
VDA_001124	DUF535, VirK-like	−135.39	0	ChrII
VDA_000158	AcrA/MacA-like membrane fusion protein	−115.36	0	pPHDD1
VDA_000599	Phosphoethanolamine lipid A transferase, EptA-like	−37.36	0	ChrII
VDA_000157	Outer membrane protein TolC	−36.94	0	pPHDD1
VDA_000156	ABC-type antimicrobial peptide transport system permease component	−24.9	0	pPHDD1
VDA_000155	ABC-type antimicrobial peptide transport system permease component	−21.67	0	pPHDD1
VDA_000154	Macrolide export ATP-binding/permease protein MacB	−15.09	0	pPHDD1
VDA_002435	TolA protein	−3.95	1.69E-170	ChrI
VDA_002436	MotA/TolQ/ExbB proton channel family protein	−3.59	1.80E-161	ChrI
VDA_002433	TolAB	−3.55	1.98E-121	ChrI
A0J47_018115	Bacterial toxin 44	−3.3	1.08E-69	pPHDD1
VDA_002432	TolAB	−3.22	1.07E-178	ChrI
VDA_002434	TolAB	−3.18	6.85E-170	ChrI
A0J47_018120	PAAR domain-containing protein	−3.07	1.00E-61	pPHDD1
A0J47_018110	hypothetical protein	−2.4	7.08E-17	pPHDD1
Outer membrane proteins and porins				
VDA_000284	Outer membrane protein	−338.06	0	ChrII
VDA_001503	OmpA	−125.49	1.40E-65	ChrI
VDA_000966	Outer membrane protein	−21.77	0	ChrII
A0J47_015910	Outer membrane protein	−11.91	1.85E-34	ChrII
VDA_000118	Outer membrane protein	−9.63	6.71E-32	pPHDD1
VDA_000113	OmpU	−2.83	4.49E-65	pPHDD1
Other genes with a putative role in virulence				
VDA_000358	Hypothetical protein, T2SS-dependent	−349.15	0	ChrII
VDA_002799	Delta endotoxin, T2SS-dependent	−206.86	0	ChrI

(Continues)

Table 1. Continued

Gene ID	Product/function	Fold change (FC)	*P*-value	Location
VDA_003529	Protease DegP	−12.73	2.23E-63	ChrI
A0J47_007530	Hypothetical protein, T2SS-dependent	−12.34	7.28E-17	ChrI
VDA_000112	Hypothetical protein, T2SS-dependent	−9	5.70E-223	pPHDD1
VDA_001571	CAAX protease self-immunity	−3.57	1.49E-125	ChrI
Other genes and proteins				
VDA_000598	Hypothetical protein	−108.43	1.56E-301	ChrII
VDA_001132	Hypothetical protein	−74.92	0	ChrII
A0J47_009785	Trypsin	−69.92	0	ChrII
VDA_002544	Gamma-Crystallin-like superfamily	−54.19	0	ChrI
VDA_000787	Sodium-dependent transporter	−10.21	0	ChrII
VDA_000563	Beta-ketoadipate enol-lactone hydrolase	−9.11	0	ChrII
VDA_000359	Thioredoxin	−8.7	0	ChrII
VDA_000351	Cysteine desulfurase	−8.1	3.19E-185	ChrII
VDA_000943	Hypothetical protein	−5.91	3.73E-30	ChrII
VDA_001932	Hypothetical protein	−5.38	2.72E-65	ChrI
VDA_000942	Mox-R like protein	−5.28	1.70E-23	ChrII
VDA_001051	Hypothetical protein	−5.27	3.39E-268	ChrII
VDA_003431	Hypothetical protein	−5	3.02E-175	ChrI
VDA_002368	Putative ATP-binding component of a transport system	−4.68	3.52E-33	ChrI
VDA_000564	Acetyltransferase	−4.48	4.50E-76	ChrII
VDA_000944	BatA protein	−4.16	1.65E-64	ChrII
VDA_001026	Hypothetical protein	−3.94	7.09E-106	ChrII
VDA_000520	Hypothetical protein	−3.94	6.91E-120	ChrII
VDA_001543	Hypothetical response regulator	−3.47	9.63E-184	ChrI
VDA_001621	Alpha-aspartyl dipeptidase	−3.39	6.66E-167	ChrI
VDA_000544	Heavy metal-binding domain-containing protein	−3.38	6.07E-60	ChrII
VDA_002181	Hypothetical protein	−3.34	3.92E-106	ChrI
VDA_000877	Hypothetical nitroreductase	−3.31	2.52E-190	ChrII
VDA_000979	HNH nuclease	−3.12	5.99E-60	ChrII
VDA_000757	Cyclic diguanylate phosphodiesterase	−3.05	2.61E-13	ChrII
VDA_002217	Hypothetical protein	−3.05	2.17E-182	ChrI
VDA_000575	DUF4344	−2.91	5.86E-09	ChrII
A0J47_002580	AraC family transcriptional regulator	−2.63	6.72E-38	ChrI
A0J47_001760	Hypothetical protein	−2.01	8.65E-20	ChrI
A0J47_000295	HNH endonuclease	−1.96	8.44E-13	ChrI

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

homologous to phosphoethanolamine lipid A transferase EptA (VCA1102) of *V. cholerae* which contributes to polymyxin B resistance (Herrera *et al*., 2017).

Also in this set of downregulated genes in RstAB mutants, DUF535 (domain of unknown function 535)-containing proteins (VDA_001878, VDA_001124) were identified. Of note, the homologous protein in *Salmonella enterica*, dubbed VirK, was found to be a major virulence factor influencing long-term survival in the host (Spencer *et al*., 2010), and the homologous protein in *V. cholerae* is induced during early infection (LaRocque *et al*., 2005). In our data set, we also found VDA_003529 with high identity to *V. cholerae* protease DegP (VC0566) which affects biofilm formation, intestinal colonization and correct function of the T2SS (Altindis *et al*., 2014). Furthermore, the protease encoded by VDA_001571 is homologous to an *E. coli* virulence factor that modulates host immune response (Jandu *et al*., 2009). Three RstAB-regulated, pPHDD1 plasmid-encoded genes related to the type VI secretion system (T6SS) (Table 1) are putatively involved in defence against other bacteria and their expression is favoured at 25°C versus 15°C (Matanza and Osorio, 2018).

The RstAB system strongly regulates the expression of genes involved in capsular polysaccharide synthesis

One of the most remarkable findings of this study was the observation that mutation of either *rstA* or *rstB* caused a drastic downregulation of 21 genes predicted to encode functions for synthesis of extracellular and capsular polysaccharides (Fig. 2B). Thirteen of these genes exhibited a >500-fold downregulation in their expression in RstAB mutants compared to the parental strain, achieving dramatic FC values of >1000-fold downregulation in three genes (Table 1). These genes map to two divergently transcribed gene clusters located in a 25 kb contig of the draft genome sequence of *Pdd*

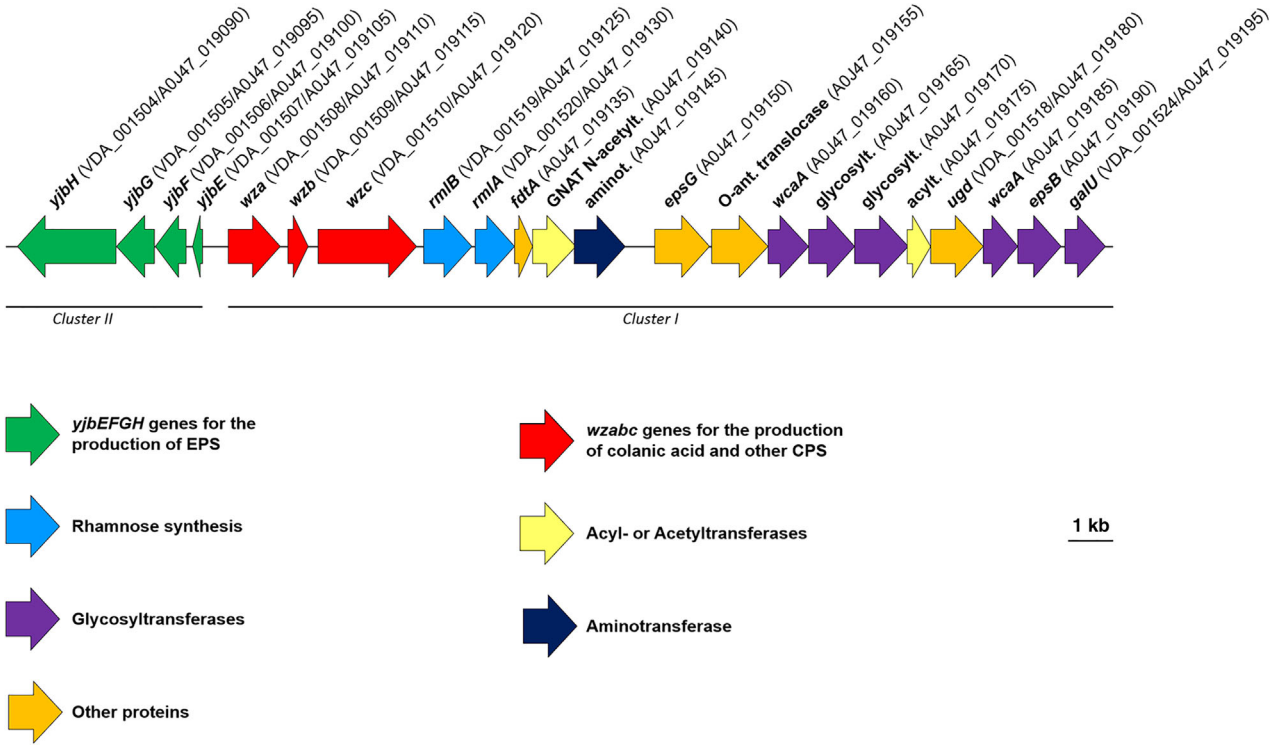

Fig. 3. Scheme depicting the two divergently transcribed gene clusters (clusters I and II) that encode functions related to synthesis of capsular and extracellular polysaccharides, and that exhibit a strong downregulation in RstAB system mutants. VDA codes correspond to the annotation of the *Pdd* type strain CIP102761 genome, and A0J47 codes correspond to the annotation of the *Pdd* RM-71 genome.

RM-71 (GenBank Acc. No. LYBT01000063.1) and were here dubbed as cluster I and cluster II (Fig. 3). Recent studies have revealed that this genomic region is highly variable among *Pdd* isolates, containing many strain-specific genes (Osorio *et al*., 2018; Terceti *et al*., 2018).

Similarity searches clearly suggest that the genes of these two clusters are involved in the synthesis and export of a polysaccharide capsule. The first three genes of cluster I, *wza*, *wzb* and *wzc*, are homologous to three *E. coli* genes that encode the export system for biosynthesis of colanic acid and group 1 and 4 capsules (Whitfield, 2006) (*wza*: 65.62% identity; *wzb*: 76.84% id.; *wzc*: 66.24% id.). Wza is an outer membrane protein responsible for the export of capsular polysaccharides from the periplasm to the cell surface (Dong *et al*., 2006). Wzc is a tyrosine kinase while Wzb is the cognate phosphatase that regulates the phosphorylation state of Wzc (Temel *et al*., 2013). In addition, a number of genes encoding sugar transferases for capsule and polysaccharide synthesis are found downstream *wza*, *wzb* and *wzc* (Fig. 3). The divergently transcribed cluster II comprises genes *yjbEFGH*. Homologues of these genes have received scarce attention so far. Mutants for these genes have been reported to exhibit alterations of colony morphology and in the production of an extracellular polysaccharide in *E. coli* (Ferrières *et al*., 2007), but their specific function remains unknown.

Mutants in rstA*,* rstB*,* wza *and* wzc *genes are impaired in capsule production*

Capsule production in *Pdd* has not been described so far. The strong downregulation of two putative capsular gene clusters observed here in RstAB system mutants prompted us to conduct studies that revealed the production of a virulence-related capsule in *Pdd*. To assess the presence of a capsule in *Pdd* RM-71, we investigated the differences between wild type and Δ*rstA* and Δ*rstB* mutant strains by transmission electron microscopy (TEM). Ultrathin sections of the parental strain RM-71 revealed the presence of a ~70 nm electron-dense capsular layer outside the outer membrane (Fig. 4). This layer was absent in mutants Δ*rstA* and Δ*rstB*. To demonstrate that the putative capsular gene clusters downregulated in RstAB mutants are involved in capsule production, we constructed in-frame deletion mutants by allelic exchange, of three selected genes, *wza* and *wzc* in cluster I, and *yjbH* in the divergently transcribed cluster II depicted in Fig. 3. When analysed by TEM, Δ*wza* and Δ*wzc* mutants were shown to be acapsulated (Fig. 4),

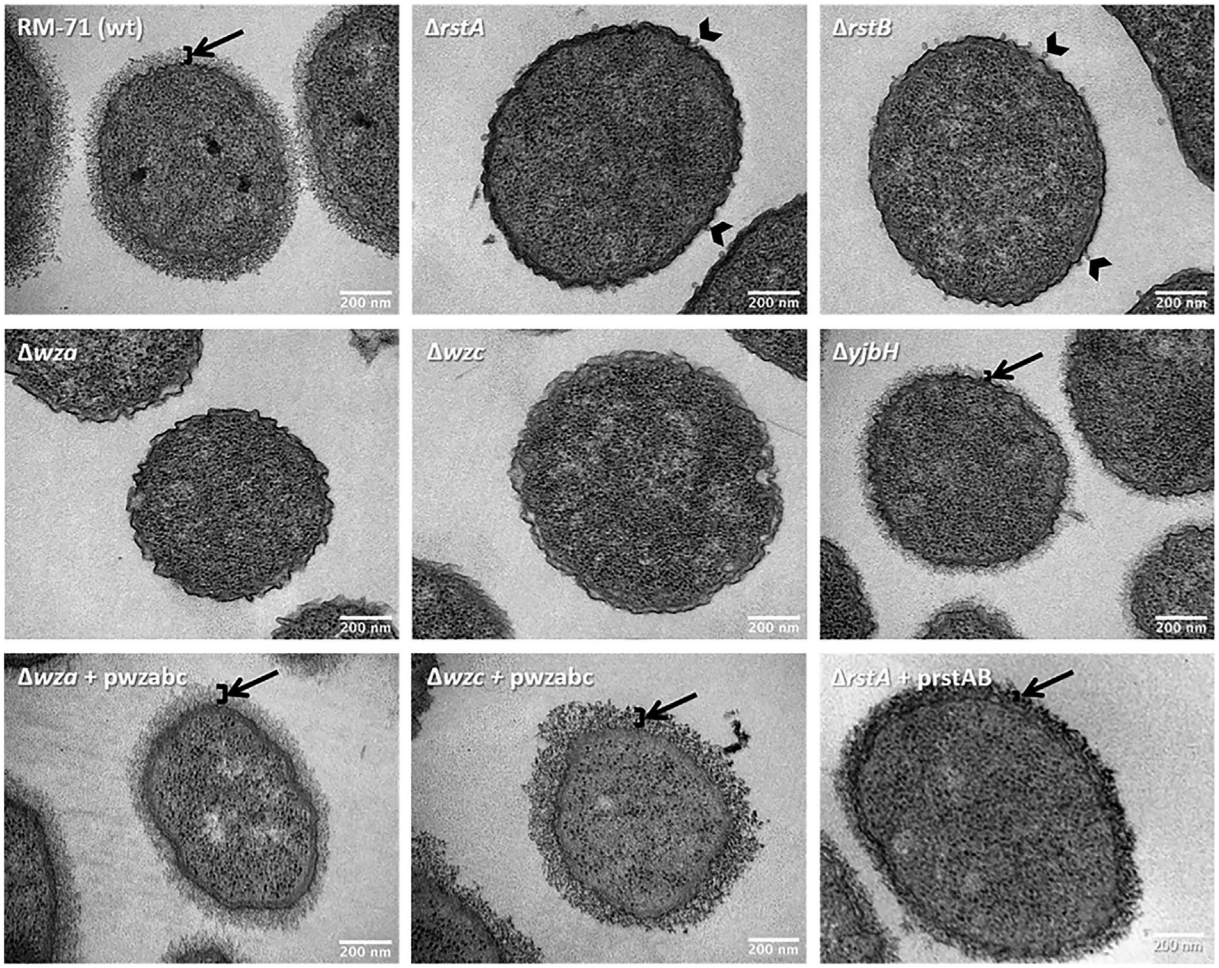

Fig. 4. Evidence of capsule production in *Pdd* RM-71 wt, and of impaired capsule synthesis in Δ*rstA*, Δ*rstB*, Δ*wza*, Δ*wzc* and Δ*yjbH* mutants by transmission electron microscopy. The black thin arrows show the electron-dense capsular layer. Δ*rstA*, Δ*rstB*, Δ*wza* and Δ*wzc* mutants lack capsule layer, whereas Δ*yjbH* mutant exhibits a reduced capsule. Black arrowheads point at small projections around the cell surface of acapsular mutants of the RstAB system (Δ*rstA*, Δ*rstB*) that might correspond to outer membrane vesicles. Complementation of structural mutants Δ*wza* and Δ*wzc*, and of regulatory mutant Δ*rstA* with the plasmid-encoded parental genes restores capsule production.

whereas the Δ*yjbH* mutant exhibited a capsule, although thinner than the one of the wt (Figs 4 and 5). Interestingly, Δ*rstA* and Δ*rstB* mutants displayed small projections and vesicles at the cell surface or in the close vicinity of the cells, which were not observed in the parental strain, and in Δ*wza*, Δ*wzc* and Δ*yjbH* mutants. These projections might correspond to outer membrane vesicles (OMVs) and surely will deserve further study. While production of OMVs has not been reported to date in *P*. *damselae*, its production by species of the family *Vibrionaceae* is being increasingly reported in recent years, and they pose a major interest as deliverers of virulence factors (Zingl *et al*., 2021).

Complementation of the two structural mutants Δ*wza* and Δ*wzc* and of the regulatory mutant Δ*rstA* with plasmids expressing the parental genes (pwzabc and prstAB respectively) restored capsule biogenesis (Fig. 4). Altogether, all these results demonstrate that *wza* and *wzc*

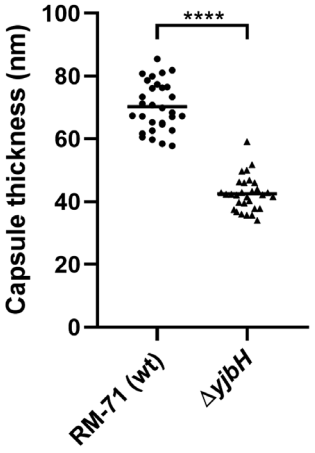

Fig. 5. Analysis of capsule thickness measuring data in *Pdd* RM-71 wt and the respective Δ*yjbH* mutant. Capsule was significantly thinner in the Δ*yjbH* mutant (*P*-value <0.0001).

genes are essential for capsule biosynthesis in *Pdd*, and clearly suggest that the acute downregulation observed in these genes in RstAB system mutants causes, at least in part, the absence of capsule in Δ*rstA* and Δ*rstB* strains.

Mutation of either wza *or* wzc*, but not* yjbH*, increases biofilm formation*

Biofilms play a significant role in bacterial environmental survival and also in pathogenicity and antimicrobial resistance. Mutations that impair the production of extracellular and capsular polysaccharides may impact biofilm formation, either by increasing biofilm or by impairing its formation (Joseph and Wright, 2004; Wu *et al*., 2011; Yi *et al*., 2017). The ability to form biofilm has been scarcely studied in *Pdd* so far. Here, the parental strain RM-71 and Δ*wza*, Δ*wzc*, Δ*yjbH*, Δ*rstA* and Δ*rstB* mutants were screened for their ability to form a biofilm using a crystal violet assay. Notably, biofilm formation was inversely related to capsule production as capsule-deficient mutants (Δ*wza*, Δ*wzc*, Δ*rstA* and Δ*rstB*) presented a significantly greater capacity to form biofilm than the parental strain and Δ*yjbH* mutant (*P*-value <0.0001 in Kruskal–Wallis test) (Fig. 6**)**.

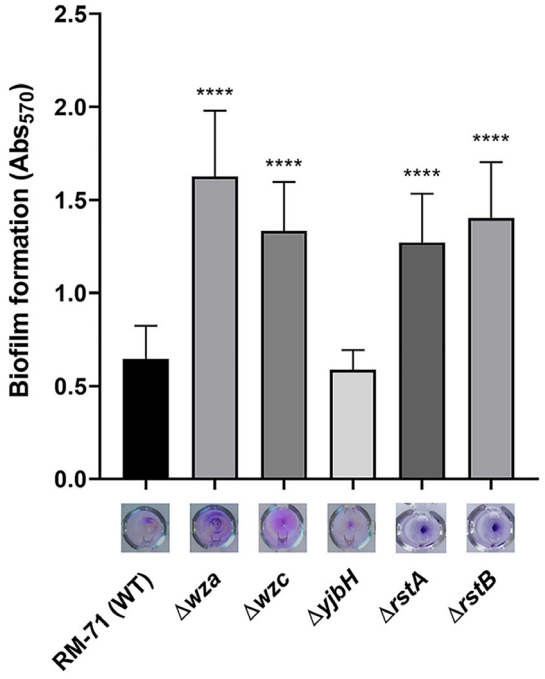

Fig. 6. Biofilm formation of RM-71 wt strain and deletion mutants Δ*wza*, Δ*wzc*, Δ*yjbH*, Δ*rstA* and Δ*rstB*. Biofilm formation was quantified as crystal violet absorbance at 570 nm. The data summarize three independent experiments (**** = *P*-value <0.0001). A representative image of biofilm formation on 96-well plates is shown.

Acapsular mutants are impaired in virulence for fish and survival in fish mucus and serum

Previous studies reported that mutation of *rstA* or *rstB* strongly impaired virulence of *Pdd* for fish (Terceti *et al*., 2017; Terceti *et al*., 2019). In the present study, we show that mutants in the RstAB system genes undergo a drastic downregulation, not only of the three cytotoxins Dly, PhlyP and PhlyC but also of the capsule synthesis genes. Of note, Δ*wza*, Δ*wzc* and Δ*yjbH* mutants exhibited haemolytic and phospholipase phenotypes similar to RM-71 wt, which demonstrates that capsule mutants do not have a defect in production or secretion of those cytotoxins (Fig. 7A). In addition, we corroborated that all the mutant strains analysed in this study were not affected in swimming motility with respect to parental strain (Fig. 7B). Similarly, mutant strains did not display any impairment in growth dynamics under different conditions of salinity and temperature in comparison to the RM-71 wt strain (Fig. 7C). In order to study the impact of mutations in *wza*, *wzc* and *yjbH* genes and, hence, to assess the contribution of capsule to virulence of *Pdd* for fish, we conducted virulence tests using turbot, which is the natural source of isolation of the highly virulent strain RM-71 (Fouz *et al*., 1992), and gilthead sea bream, a fish species that also constitutes a host for *Pdd* (Vera *et al*., 1991). Remarkably, the results showed a major impairment in virulence in the two mutants Δ*wza* and Δ*wzc* with respect to wt RM-71, whereas Δ*yjbH* mutant was not affected with respect to the wt. In addition, as reported in previous studies, inoculation of the Δ*rstA* and Δ*rstB* mutants did not cause any fish mortality (Fig. 8A). Collectively, these results provide strong evidence that the attenuation in virulence observed in Δ*wza* and Δ*wzc* mutants is attributable to the absence of capsule.

Next, to gain an insight into the role of the *Pdd* polysaccharide capsule in resistance against fish defensive mechanisms, survival assays of parental RM-71 and deletion mutants Δ*wza*, Δ*wzc*, Δ*yjbH*, Δ*rstA* and Δ*rstB* in turbot serum and mucus were performed. Results showed that the number of culturable cells of Δ*rstA* and Δ*rstB* was significantly lower (*P*-value <0.0001) than that of RM-71 and Δ*yjbH* cells after 1.5 h in presence of serum (Fig. 8B). In addition, the two capsule structural mutants Δ*wza* and Δ*wzc* were significantly (*P*-value <0.0001) impaired compared to RM-71 and Δ*yjbH* after 3 h in presence of serum (Fig. 8B). Of note, no cell counts could be detected for the regulatory mutants Δ*rstA* and Δ*rstB* after 3-h exposure.

Concerning mucus survival, all the acapsular mutants exhibited a significant impairment compared to RM-71 after 4.5 h in presence of mucus (*P*-value <0.0001). Similar to serum experiments, culturability of Δ*yjbH* in turbot mucus remained unaffected in comparison to RM-71 in

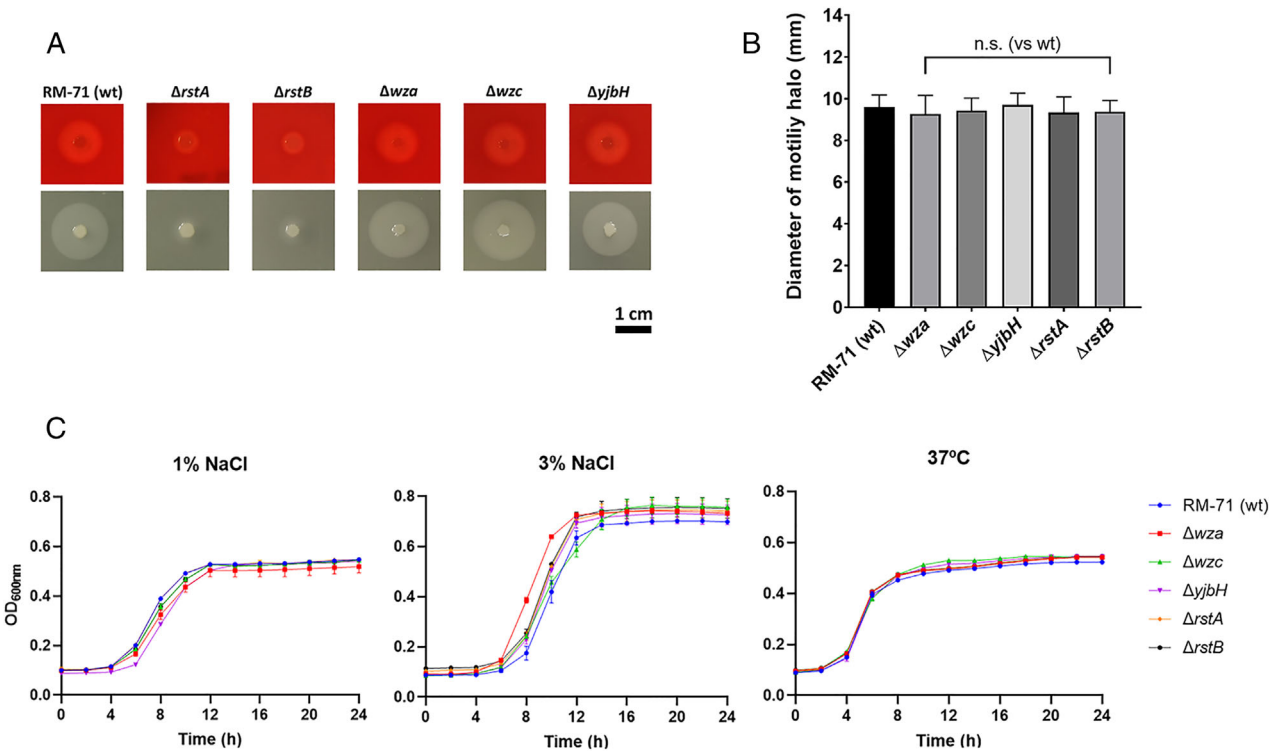

Fig. 7. Mutants Δ*wza*, Δ*wzc* and Δ*yjbH* show normal cytotoxin production, motility and growth.
A. Haemolytic (top panel) and phospholipase (bottom panel) phenotypes of *Pdd* wt RM-71 and its derivative mutants Δ*rstA*, Δ*rstB*, Δ*wza*, Δ*wzc* and Δ*yjbH*. Scale bar, 1 cm. Haemolytic activity is attributable to the synergistic and additive effects among cytotoxins Dly, PhlyP and PhlyC, whereas phospholipase activity is mainly attributable to Dly toxin (a phospholipase-D). Note that haemolysis and phospholipase activities are impaired in Δ*rstA* and Δ*rstB* mutants, whereas structural capsule gene mutants exhibit wt phenotypes.
B. Quantitative measurements of swimming motility phenotypes of wt and mutant strains in motility agar. Haloes (in mm) were measured for 15 independent inocula per strain, and mean data with standard deviation are shown. A one-way ANOVA analysis revealed no significant differences among groups.
C. Growth of mutants in capsule synthesis genes Δ*wza*, Δ*wzc* and Δ*yjbH* and in regulatory mutants Δ*rstA* and Δ*rstB* is not impaired at different conditions of salinity and temperature. Mean data of three independent experiments are shown.

all the exposure times tested (Fig. 8B). Collectively, all these results show that although the acapsular structural mutants Δ*wza* and Δ*wzc* produce wild-type levels of haemolysins and do not exhibit any detectable impairment in swimming motility and in growth under different temperatures and NaCl concentrations, the production of a capsule plays a role in bacterial resistance against fish defence mechanisms and is required for full *Pdd* virulence for fish. The observation that regulatory mutants Δ*rstA* and Δ*rstB* show a greater impairment in serum resistance than the capsule mutants Δ*wza* and Δ*wzc* suggests that other genes of the RstAB regulon in addition to capsule biogenesis functions may play a role in resistance against host defences.

Discussion

TCSs are key bacterial mechanisms involved in adaptation to environmental changes (Stock *et al*., 2000). RstAB is a TCS identified as a strong positive regulator of cytotoxin genes in *Pdd* (Terceti *et al*., 2017; Terceti *et al*., 2019). In the present study, we have characterized the RstAB regulon in *Pdd*, and showed that RstAB positively regulates key virulence factors including a polysaccharide capsule. This study has also unveiled yet-uncharacterised genes that represent a promising field of study for the understanding of the pathogenicity of this generalist pathogen. The HK RstB and the RR RstA are predicted to act as a cognate pair (Terceti *et al*., 2017; Terceti *et al*., 2019). However, the RstA and the RstB regulons of *Pdd* do not completely overlap. Absence of complete overlapping between HK and RR has been reported in various TCS, and is in part explained by cross-regulation between noncognate partners of different TCS (Rabin and Stewart, 1993; Matsubara *et al*., 2000; Howell *et al*., 2006; Gangaiah *et al*., 2017; Guckes *et al*., 2017).

Expression of *rstAB* genes is under the control of the Mg^{2+}-sensing TCS PhoPQ in *E. coli* and in *S. enterica* (Ogasawara *et al*., 2007; Perez *et al*., 2009), and the *V.*

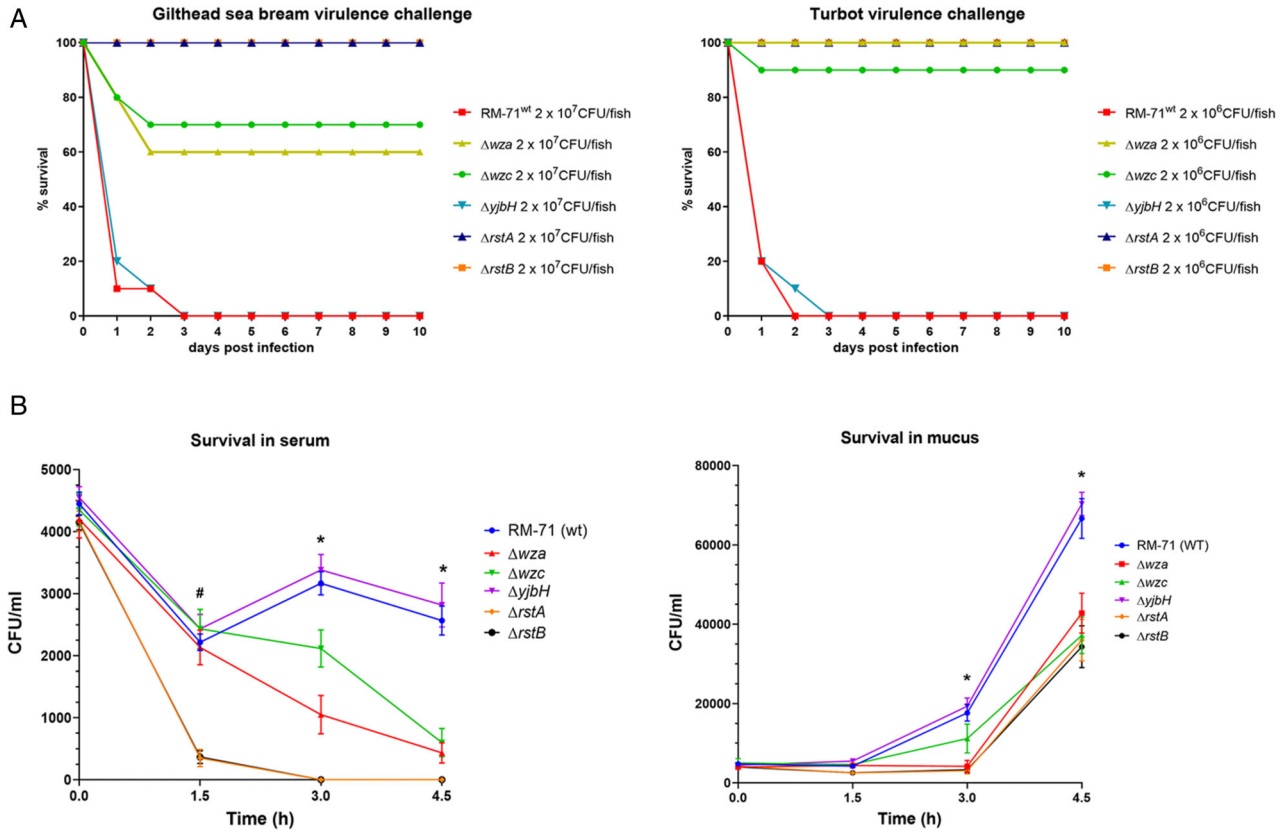

Fig. 8. A. Survival (%) of turbot and gilthead sea bream fish after intraperitoneal injection of *Pdd* RM-71 and mutants. Deletion of *rstA*, *rstB*, *wza* or *wzc* impaired virulence for fish. The respective control fish group (10 fish inoculated with 0.1 ml of sterile 0.85% NaCl solution) did not register any mortalities (data not shown). A total of 10 fish were inoculated per strain.
B. Deletion of *rstA*, *rstB*, *wza* and *wzc* impairs survival of *Pdd* in turbot serum and mucus. Data are shown as means ± standard deviation. # indicates significant differences between the wt and Δ*rstA* and Δ*rstB* mutants. Asterisk indicates significant differences between the wt and Δ*wza*, Δ*wzc*, Δ*rstA* and Δ*rstB* mutants.

cholerae rstAB homologous genes (*carSR*) were identified as downregulated genes in response to an increase in external [Ca^{2+}] (Bilecen and Yildiz, 2009). However, the specific stimuli that trigger RstB autophosphorylation as such have not been identified so far. In addition, the conditions that influence transcription of *rstAB* genes are poorly investigated, although previous studies indicate that temperature changes do not affect expression of *rstAB* genes (Matanza and Osorio, 2018; Matanza and Osorio, 2020).

Transcriptomic studies of homologous RstAB regulons are very scarce, being limited, to the best of our knowledge, to *V. cholerae* (Bilecen and Yildiz, 2009) and *Escherichia coli* (Oshima *et al*., 2002; Liu *et al*., 2019; Gao *et al*., 2020). Additional studies have identified RstAB-regulated genes by other experimental approaches including overexpression and deletion of *rstAB* (Cabeza *et al*., 2007; Ogasawara *et al*., 2007). In *E. coli*, RstAB regulates iron acquisition, acid resistance, biofilm formation, adherence and virulence (Oshima *et al*., 2002; Ogasawara *et al*., 2007; Ogasawara *et al*., 2010; Gao *et al*., 2015; Liu *et al*., 2019; Gao *et al*., 2020), whereas some of the functions impacted by RstAB in *Salmonella* include pyrimidine metabolism, iron acquisition and acid resistance (Tran *et al*., 2016; Torrez Lamberti *et al*., 2019). Inactivation of RstAB caused virulence reduction in *Yersinia pseudotuberculosis* (Flamez *et al*., 2008), avian pathogenic *E. coli* (Gao *et al*., 2015) and *Edwardsiella ictaluri* (Menanteau-Ledouble and Lawrence, 2013), providing evidence that this regulatory system governs virulence-related functions in diverse species of the *Enterobacteriaceae*.

TCSs homologous to RstAB exist in virtually all *Vibrio* and *Photobacterium* species (Terceti *et al*., 2019). However, apart from the *Pdd* system studied here, only the *V. cholerae* and *Vibrio alginolyticus* homologous systems have received some attention so far. In *V. cholerae*, CarSR negatively regulates biofilm formation and the VPS exopolysaccharide encoded by *vps* genes (Bilecen and Yildiz, 2009), whereas it positively regulates *almEFG* genes involved in lipid A modification thus enhancing polymyxin B resistance (Herrera *et al*., 2014; Bilecen

et al., 2015). In *V. alginolyticus*, *rstA* and *rstB* gene silencing caused effects on motility, adhesion, biofilm formation and haemolytic activity, but the genes responsible for these phenotypes have not been identified (Huang *et al*., 2018). Last, the *rstA* homologue in *Vibrio vulnificus* CMCP6 (locus VV2_1414) was upregulated during human infection with respect to *in vitro* growth conditions (Bisharat *et al*., 2013), but the RstAB regulon in this important human and animal pathogen remains unstudied so far.

Thus, the *Pdd* RstAB system represents a singular case of RstAB-mediated positive regulation of cytotoxins and of a polysaccharide capsule. Figure 9 depicts a scheme of genes and functions positively regulated by RstAB in *Pdd* RM-71. Cytotoxins Dly, PhlyP and PhlyC are crucial virulence factors for fish and mice (Rivas *et al*., 2011; Rivas *et al*., 2013) and are expressed at very high levels in the bacterial cell (Matanza and Osorio, 2018). The downregulation of cytotoxin genes in RstAB system mutants is in accordance with previous findings which demonstrated that deletion of *rstB* and *rstA* impaired haemolytic activity (Terceti *et al*., 2017; Terceti *et al*., 2019). RstAB was here found to control the expression of outer membrane proteins that constitute a promising research topic, as homologues of these proteins are important virulence factors in other bacteria (Weiser and Gotschlich, 1991), and have been used in the design of vaccines against diseases caused by aquaculture threatening bacteria as *Vibrio parahaemolyticus*, *V. alginolyticus* and *Edwardsiella tarda* (Li *et al*., 2010; Cheng *et al*., 2018).

This study has unveiled for the first time the presence of a polysaccharide capsule around *Pdd* cells. RstAB

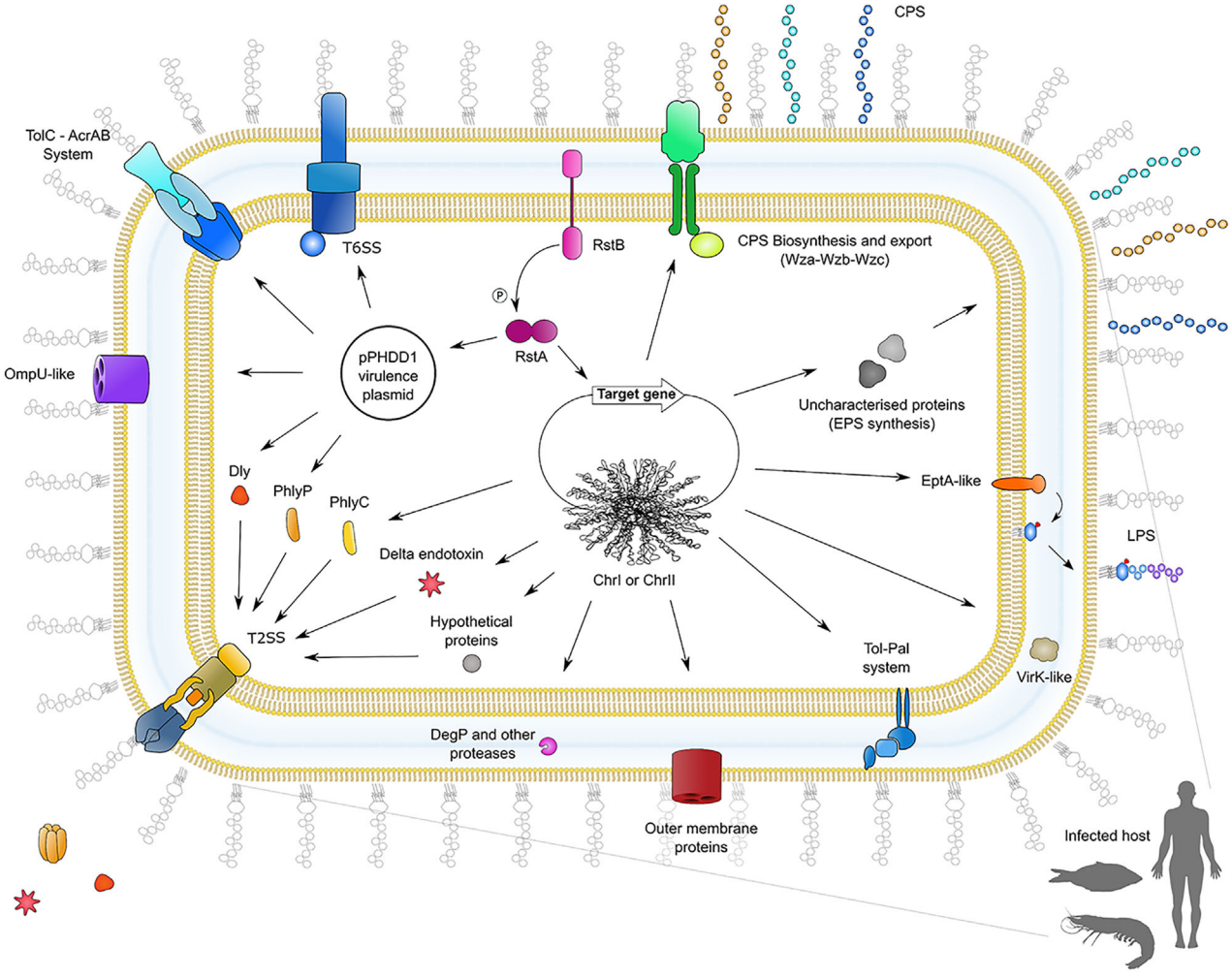

Fig. 9. The two-component system RstAB is a major positive regulator of virulence-related functions in *Pdd*. The RstAB regulon comprises functions encoded within the two chromosomes and within the virulence plasmid pPHDD1. Regulated functions include the type II secretion system (T2SS)-dependent cytotoxins Dly, PhlyP and PhlyC and a putative δ-endotoxin; outer membrane proteins, T6SS, Tol-Pal system, TolC-AcrAB efflux pump for antimicrobial resistance, and lipid-A modification protein EptA among others. Notably, RstAB is a major regulator of functions related to synthesis and export of capsular polysaccharides with a major role in virulence.

system mutants showed a strong downregulation of two divergently transcribed clusters of hitherto uncharacterised capsule synthesis genes, organized within a highly variable genetic region in *Pdd* genomes (Osorio *et al*., 2018; Terceti *et al*., 2018). TEM assays showed that mutation of *rstA*, *rstB*, *wza* or *wzc* abolished capsule formation, whereas mutation of *yjbH* caused the production of a thinner capsular layer. Wza, Wzb and Wzc constitute an exportation system for colanic acid and group 1 and 4 capsules (Whitfield, 2006). *yjbEFGH* genes are involved in the production of an extracellular polysaccharide in *E. coli* that alters colony morphology (Ferrières *et al*., 2007) and plays a role in adaptation to osmotic stress (Ionescu *et al*., 2008; Ionescu and Belkin, 2009). In *V. parahaemolyticus*, deletion of *yjbH* leads to overexpression of K-antigen polysaccharides (Chen *et al*., 2010). However, the role of YjbH protein in bacterial capsule synthesis remains poorly studied so far and will need further investigation.

Biofilms consist of cells embedded within a matrix of extracellular polysaccharides, proteins and extracellular nucleic acids (Costerton *et al*., 1995) and can enhance resistance to host immune system and antibacterial agents (Donlan and Costerton, 2002). In this work, we observed that biofilm formation was higher in Δ*wza* and Δ*wzc* compared to the wt. This finding is in agreement with previous observations in other pathogenic bacteria as *V. vulnificus* (Joseph and Wright, 2004) and *Riemerella anatipestifer* (Yi *et al*., 2017). Still, other studies showed decreased capacity to form biofilm by *Klebsiella pneumoniae* capsule mutants (Wu *et al*., 2011). Our results suggest that capsular polysaccharides may inhibit biofilm formation in *Pdd*, and acapsular mutants would expose adhesive molecules at the cell surface.

Capsules may mediate a direct interaction between bacteria and host defences (Moxon and Kroll, 1990). Capsular polysaccharides can mask structures at the cell surface that would otherwise activate the immune system of the host, and thus prevent complement attack and phagocytosis (Merino and Tomás, 2015). The present study revealed that survival in fish serum is lower in capsule structural mutants Δ*wza* and Δ*wzc* and in regulatory mutants Δ*rstA* and Δ*rstB*. In accordance with our results, a *V. vulnificus* Δ*wza* mutant was not able to survive within human serum (Carda-Diéguez *et al*., 2018). A previous study showed that *Pdd* RM-71 was resistant to the antimicrobial action of fish skin mucus and had a strong ability to adhere to it (Fouz *et al*., 2000), and in the present study, we have provided evidence that capsule deficiency impairs proliferation in mucus. Taken together, these results indicate that *Pdd* capsule plays a role in evasion from fish host defences. We show here that mutations of *wza* and *wzc* strongly impair virulence for turbot and sea bream, demonstrating the important contribution of capsule to pathogenicity. These findings are in agreement with previous studies showing that these genes play a role in capsule synthesis and virulence in human and fish pathogens such as *A. baumannii* (Niu *et al*., 2020), *K. pneumoniae* (Lin *et al*., 2017), *Vibrio anguillarum* (Croxatto *et al*., 2007; Weber *et al*., 2010) and *V. alginolyticus* (Hernández-Robles *et al*., 2016). In our results, the virulence attenuation observed in *wza* and *wzc* mutants was not a consequence of a growth defect, impaired production of haemolysins or altered motility, since mutants exhibited wild type phenotypes for all these traits.

Concluding remarks

In conclusion, this study demonstrates that the TCS RstAB is a positive regulator of key virulence factors in *Pdd*. Remarkably, the present work has disclosed the production by *Pdd* of a RstAB-regulated polysaccharide capsule that plays a role in resistance to host fish defences and is required for full virulence for fish. These results are expected to boost the design of future strategies for the control of animal and human infections caused by *Pdd*. In addition, the ubiquitous presence of RstAB-like systems in species of the family *Vibrionaceae* will surely inspire future research on the role of this TCS in virulence in other important pathogens for animals and humans.

Experimental procedures

Bacterial strains, plasmids and standard culture conditions

Bacterial strains and plasmids used and constructed in the present study are described in Table 2. *Pdd* strains were grown at 25°C on tryptic soy agar (TSA) and tryptic soy broth (TSB) supplemented with additional 0.5% NaCl so that the final salt concentration was 1%. The salt-supplemented media were dubbed TSA-1 and TSB-1 respectively. *Escherichia coli* strains were cultured on Luria–Bertani (LB) agar or LB broth at 37°C. When required, kanamycin (Kn) and chloramphenicol (Cm) were supplied at final concentrations of 50 μg ml^{-1} and 12 μg ml^{-1}, respectively.

RNA sequencing

For RNA extraction, cells from three independent precultures of the wild type strain RM-71 and deletion mutants Δ*rstA* and Δ*rstB* were cultivated in 10 ml TSB-1 in 100 ml flasks until they achieved an OD_{600} of 0.55. Cultures were instantly subjected to a treatment with the

Table 2. List of bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference/source
Strains		
P. damselae* subsp. *damselae		
RM-71	Isolated from turbot; carries pPHDD1 plasmid	Fouz *et al*. (1992)
MT151	RM-71 Δ*rstB*	Terceti *et al*. (2017)
MT319	RM-71 Δ*rstA*	Terceti *et al*. (2019)
XMF215	RM-71 Δ*wza*	This study
XMF222	RM-71 Δ*wzc*	This study
XMF333	RM-71 Δ*yjbH*	This study
XMF363	XMF215 with pwzabc (complemented mutant); chloramphenicol resistance	This study
XMF324	XMF222 with pwzabc (complemented mutant); chloramphenicol resistance	This study
XMF376	MT319 with prstAB (complemented mutant); chloramphenicol resistance	This study
E. coli		
DH5α	Cloning strain	Laboratory stock
S17-1-λpir	RP4-2 (Km::Tn7, Tc::Mu-1) *pro*-82 λ*pir recA1 endA1 thiE1 hsdR17 creC510*	Herrero *et al*. (1990)
Plasmids		
pNidkan	Suicide vector derived from pCVD442, with kanamycin resistance	Mouriño *et al*. (2004)
pMRB24	Cloning vector, mob, chloramphenicol resistance	Le Roux *et al*. (2011)

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

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

FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) program was used for quality control of raw reads as previously described (Matanza and Osorio, 2018). The raw pair-end reads were mapped against the reference genome of the *Pdd* type strain CIP102761 (GenBank accession number NZ_ADBS00000000.1) to provide better localisation of each gene in either chromosomes or plasmids, as this type strain genome is entirely closed. Reads not mapping to the type strain genome, corresponding to unique genes of RM-71 strain with respect to CIP102761, were mapped against the sketched genome sequence of strain RM-71 (GenBank Acc. No. NZ_LYBT00000000.1) via Bowtie2 (Langmead and Salzberg, 2012) v2.2.6 algorithm. A number of quality control steps were conducted. Very low-quality reads were excluded by Samtools (Li *et al*., 2009) and Picard Tools softwares (http://broadinstitute.github.io/picard/). Furthermore, one key determinant for sequencing processes is the GC content of samples, which was found to be normal (distribution between 40% and 60%) in our assay. The distribution of duplicates was also assessed to confirm their normal small proportion. The genetic quantification process was accomplished by the HTSeq (Anders *et al*., 2015) software (0.6.1 version).

Concordance between each strain sample was assessed using the statistics program R through a correlation and distance study, taking the whole transcriptome normalized by the size of the library. Differential expression analysis was conducted by DESeq2 (Love *et al*., 2014) method (1.18.1 version). The analysis of DEGs was evaluated by statistical packages developed by Python and R, with the use of the DESseq2 (Love *et al*., 2014) algorithm which applies a differential negative binomial distribution for the statistical significance. A Python script designed at Sistemas Genómicos (Valencia, Spain) was applied to create a data matrix for each group condition with the counts derived from HTSeq count for each sample (each of the three replicates of each strain). Those genes with FC values lower than −1.5 or higher than 1.5 and a *P*-value corrected by FDR

≤0.05 were regarded as differentially expressed. FPKM (Fragments per kilobase per million fragments mapped) values determined with Cufflinks v2.2.1 (Langmead and Salzberg, 2012) were used to obtain the expression of each specific gene.

Allelic-exchange deletion mutant construction and gene complementation

The deletion mutants for *rstA* (VDA_000600) and *rstB* (VDA_000601) genes were constructed in two previous studies (Terceti *et al*., 2017; Terceti *et al*., 2019). In this study, we have constructed non-polar deletion mutants for the capsular genes *wza* (VDA_001508), *wzc* (VDA_001510) and *yjbH* (VDA_001504), following an allelic exchange methodology. Approximately, 2 kb DNA fragments upstream and downstream of each gene were obtained by PCR amplification and subsequently ligated so that the reconstructed allele underwent an in-frame deletion of >90% of its coding sequence. Primers used are listed in Supplementary Table S3 and Hi-Fidelity Kapa *Taq* was employed for amplifications. For allelic exchange, Kn^R suicide vector pNidKan, bearing the sucrose sensitivity gene *sacB* and the *pir*-dependent R6K ori was used. The plasmid constructs carrying the deleted alleles were moved from *E. coli* S17-1-λpir into *Pdd* RM-71 by conjugation during 48 h on TSA plates containing seawater instead of distilled water. Cells were suspended in TSB-1, and 100-μl aliquots of serial decimal dilutions were seeded on thiosulfate-citrate-bile-sucrose (TCBS) agar plates containing Kn to select a first recombination event. Next, Kn-resistant colonies were selected on TSA plates with 15% (wt./vol.) sucrose to select a second recombination event. The *Pdd* mutant strains generated in this study are listed in Table 2. The genome region involved in the deletion of each gene was subjected to DNA sequencing to verify that deletions were nonpolar. For gene complementation, ORF sequences of *wzabc* and *rstAB* gene clusters were amplified with their corresponding promoter sequences by PCR using Hi-Fidelity Kapa *Taq*, cloned into the Cm^R vector pMRB24 and mobilized from *E. coli* S17-1-λpir into their respective mutant strains Δ*wza* (XMF215), Δ*wzc* (XMF222) and Δ*rstA* (MT245).

Transmission electron microscopy

For TEM, *Pdd* strains were grown in TSA-1 for 24 h at 25°C and then suspended in TSB-1 at an OD_{600} nm ~0.5, serial diluted, spread on TSA-1 plates and grown overnight at 25°C, in order to obtain isolated colonies. 10 ml of a fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde and 0.075% ruthenium red in 0.1 M cacodylate buffer pH 7.4 was poured on each plate and after 3 h at room temperature, colonies were gently detached from the agar, washed three times with 0.1 M cacodylate buffer pH 7.4 containing 0.075% ruthenium red and fixed for 1 h with 1% osmium tetroxide, 0.075% ruthenium red in 0.1 M cacodylate buffer pH 7.4. Colonies were washed with 0.1 M cacodylate buffer pH 7.4 containing 0.075% ruthenium red, dehydrated through a graded series of ethanol and embedded in Epon resin. Ultrathin sections (40–60 nm thickness) were obtained on an RMC Ultramicrotome (PowerTome) using Diatome diamond knives, contrasted with 2% uranyl acetate for 5 min and observed under a JEOL JEM 1400 TEM. Images were digitally recorded using a CCD digital camera Orius 1100 W (Gatan). Capsule thickness of the RM-71 wild type (wt) and *yjbH* strains was determined by measuring the capsule of 30 cells per strain using the Fiji software (ImageJ version 1.51n) (Schindelin *et al*., 2012). For each individual cell, the capsule was measured at six different points and the capsule thickness for each cell was calculated as the average of the six measurements. Data were subjected to an unpaired *t*-test with Welch's correction.

Assays of haemolysis and phospholipase activities

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

Motility assays

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

Growth assays under different conditions of salinity and temperature

Pdd RM-71 (wt), Δ*wza*, Δ*wzc*, Δ*yjbH*, Δ*rstA* and Δ*rstB* mutants were grown in TSB-1 to obtain exponentially growing precultures with an OD_{600} of 0.3. Then, 1:100 dilutions of each preculture were grown in 100 μl TSB with a final NaCl concentration of 1% or 3% at 25°C or in TSB-1 at 37°C in 96-well plates (Costar), with three replicates per strain. The optical density (OD_{600}) was measured at 2 h intervals using the spectrophotometer Epoch2 microplate reader (Biotek). Mean data of three independent experiments are shown. The two assayed salinity conditions were selected in order to obtain a picture of *Pdd* growth at NaCl conditions that mimic the salinity of fish internal medium (1%) and the planktonic lifestyle in seawater (3%). It was also assessed whether mutant strains had a different response to the stressful condition of cultivation at 37°C (Matanza and Osorio, 2020) in comparison to 25°C (optimal temperature for *Pdd* growth) (Fouz *et al*., 2000; Matanza and Osorio, 2018).

Biofilm formation

Pdd parental strain RM-71 and mutant strains were grown overnight at 25°C in M9 minimal medium (Miller, 1972) supplemented with 0.2% (wt./vol.) Casamino Acids (Bacto) (CM9), 0.5% glucose (wt./vol.) and NaCl adjusted to a final concentration of 1%. These overnight cultures were diluted to an OD_{600} of 0.3 and aliquots of 100 μl of each strain were allowed to grow statically in 96-well plates (Deltalab) for 24 h. Subsequently, the cultures were gently removed, and each well was washed thrice with sterile phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, KH_2PO_4 2 mM, pH 7.4) to remove any poorly adherent cells. Biofilms were stained by adding crystal violet at a concentration of 0.1% (wt./vol.; Panreac) followed by incubation at room temperature for 30 min. Biofilms were quantified by solubilization with ethanol and measurement of absorbance at 570 nm (A570). Experiments were carried out three times with six replicas per strain per time. One-way ANOVA for non-parametric data (Kruskal–Wallis test) was used to analyse the data.

Survival in fish serum and mucus

The sensitivity of the different *Pdd* strains to turbot (*Scophthalmus maximus*) serum or mucus was assessed with bacteria grown in microtiter plates, as described in previous studies (Sanjuán and Amaro, 2004), with modifications. Mucus was collected from 250 g fish by gently scraping the skin and subsequently centrifuged for 5 min at 2655*g* to remove particulate material. The supernatant was dissolved in sterile saline solution (0.85%) in a ratio of 1:1, filter-sterilized onto 0.22 μm and stored at −20°C before use. Serum was aseptically obtained by blood extraction through a caudal vein puncture from a 2-year old (ca. 2 kg weight) adult turbot, and was allowed to clot overnight at 4°C. Serum was stored at −20°C until use.

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

Virulence challenge

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

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Author Contributions

X.M.M. and C.R.O. conceived the study, designed the experiments, analysed and interpreted the data and wrote the manuscript. X.M.M. and L.L.-S. performed the experimental work, analysed and interpreted the data and elaborated tables and figures. A.d.V. performed the TEM analyses and contributed to data interpretation. All authors discussed the results and approved the submitted manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Fold Change Values and other data for Differentially expressed genes (DEG) in the comparison between *Pdd* RM71 wt and the *rstB* mutant Transcriptomes

Table S2. Fold Change Values and other data for Differentially expressed genes (DEG) in the comparison between *Pdd* RM71 wt and the *rstA* mutant Transcriptomes.

Table S3. Oligonucleotides used in this study.