AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

MEETING REVIEW

Photobacterium damselae subsp. *damselae*, a Generalist Pathogen with Unique Virulence Factors and High Genetic Diversity

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ABSTRACT *Photobacterium damselae* subsp. *damselae* causes vibriosis in a variety of marine animals, including fish species of importance in aquaculture. It also may cause wound infections in humans that can progress to a fatal outcome. Two major virulence factors are encoded within the large conjugative plasmid pPHDD1, the phospholipase D damselysin (Dly) and the pore-forming toxin phobalysin P (PhlyP). The two toxins exert hemolytic and cytolytic activities in a synergistic manner. Even though PhlyP has close homologues in many *Vibrio* species, it has unique features that differentiate it from related toxins. Dly phospholipase constitutes a singular trait of *P. damselae* subsp. *damselae* among the *Vibrionaceae*, although related toxins are found in members of the *Aeromonadaceae*. Fish farm outbreaks can also be caused by plasmidless strains. Such observations led to the characterization of two ubiquitous chromosome-encoded toxins with lesser cytolytic activity, the pore forming-toxin phobalysin C (PhlyC) and the phospholipase-hemolysin PlpV. The high genetic diversity of this pathogen deserves special attention, as it has a number of strain-specific features, including the cell envelope polysaccharide synthesis clusters. Fish outbreaks are likely caused by multiclonal populations which contain both plasmidless and pPHDD1-harboring isolates and not by well-adapted clonal complexes. Still, among such genetic heterogeneity, it is feasible to identify conserved weak points in the biology of this bacterium: the two-component regulatory system RstAB (CarSR) was found to be necessary for the maximal production of virulence factors, and its inactivation severely impaired virulence.

KEYWORDS HlyA, *Photobacterium damselae*, aquaculture, damselysin, fish pathogen, hemolysin, phobalysin

The first isolation of *Photobacterium damselae* subsp. *damselae* took place in 1971, when an "unnamed marine *Vibrio*" sp. was found responsible of a human infectious case (1). The name *Vibrio damsela* was later coined after the isolation of this same organism from skin ulcers of damselfish (*Chromis punctipinnis*) (2). After its temporary placement in the genus *Listonella*, followed by the proposal of the name *Photobacterium damsela* (3), this bacterium was renamed *P. damselae* subsp. *damselae*, sharing a species epithet with *P. damselae* subsp. *piscicida* (formerly known as *Pasteurella piscicida*) (4).

P. damselae subsp. *damselae* thrives in coastal marine ecosystems, and it has been isolated from seawater, seafood, seaweeds, and apparently healthy marine animals (5–9). However, this bacterium is best known for its pathogenic potential for a variety of marine animals, such as fish, molluscs, crustaceans, and cetaceans (10). It is nowadays considered an emerging pathogen in marine aquaculture, and numerous recent studies have reported its isolation from newly cultured species and from diverse geographical areas (11–14). In addition, it causes wound infections in humans (1, 15, 16). All these data suggest that *P. damselae* subsp. *damselae* behaves as a generalist pathogen,

Accepted manuscript posted online 12 February 2018

Citation Osorio CR, Vences A, Matanza XM, Terceti MS. 2018. *Photobacterium damselae* subsp. *damselae*, a generalist pathogen with unique virulence factors and high genetic diversity. J Bacteriol 200:e00002-18. https://doi.org/10.1128/JB.00002-18.

Editor Victor J. DiRita, Michigan State University

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capable of living as a free-swimming bacterium and as a pathogen, and with the ability to cause disease in a wide range of animal phyla.

Typical signs of *P. damselae* subsp. *damselae* infection in fish include hemorrhaged areas and ulcerative lesions on the body surface (2, 17). In humans, infections have their primary origin in wounds, which are followed by bulla formation and marked edema. Infection may progress to a necrotizing fasciitis with multiple organ failure, leading to a fatal outcome (18, 19).

pPHDD1 VIRULENCE PLASMID, THE HALLMARK OF HIGHLY PATHOGENIC ISOLATES

Early studies reported the purification of a cytolytic toxin produced in large amounts by some, but not all, *P. damselae* subsp. *damselae* isolates. This toxin caused lysis of mouse, rat, rabbit, and damselfish erythrocytes, as well as of Chinese hamster ovary cells, but was almost inactive against sheep erythrocytes (20, 21). This toxin, later dubbed damselysin (Dly), was demonstrated to be a phospholipase D with sphingomyelinase activity (22, 23). In the following years, several studies systematically reported the existence of two main sets of isolates, those exhibiting strong hemolytic properties on the one hand, and those with weak hemolytic activity on the other hand (24–26). The observation that this pathogen was indeed hemolytic for sheep red blood cells, and the fact that isolates testing negative for *dly* gene exhibited hemolytic activity, suggested early on the production of more than one cytolysin in this pathogen (23).

Between 1987 and 1989, several outbreaks of *P. damselae* subsp. *damselae* were reported in turbot (*Scophthalmus maximus*) fish farms in Galicia (northwest [NW] Spain) (27). All the isolates were strongly hemolytic, and all isolates harbored a large plasmid. One of these isolates, RM-71, was further selected as a model of a highly virulent and strongly hemolytic *P. damselae* subsp. *damselae* strain and used to unveil the genetic basis of hemolysis and virulence in this pathogen. Currently, it is known that all strongly hemolytic isolates harbor a 153-kb virulence plasmid dubbed pPHDD1 (28). This plasmid encodes the Dly toxin and a pore-forming toxin that was recently dubbed phobalysin P (PhlyP), which stands for photobacterial lysin encoded on a plasmid (29). The genes of Dly and PhlyP toxins (loci VDA000159 and VDA000160, respectively) are contiguous in the sequence of pPHDD1, but they are transcribed from divergent strands. In the light of recent studies, PhlyP and Dly cause hemolysis of erythrocytes and pathogenicity in mice and fish by acting in a synergistic fashion (30), although each toxin can individually elicit cell toxicity and pathogenicity at levels that differ depending on the type of host. pPHDD1 occurs in a fraction of *P. damselae* subsp. *damselae* strains, and it is not restricted to a unique animal host species or to a geographical origin (28, 31). The presence of pPHDD1 is easily recognized, since positive strains exhibit a wide hemolytic halo on sheep blood agar plates, whereas plasmidless strains produce a narrow halo (Fig. 1). Also, experimental inoculations clearly demonstrate that pPHDD1 strains are more virulent than plasmidless strains (12, 32). No pPHDD1-like plasmids have been so far reported in any other bacteria, suggesting that it may constitute a singular feature of this subspecies. Although some sequence stretches of pPHDD1 show homology to DNA sequences of other species of the *Vibrionaceae* family at different similarity levels, the genetic structure of pPHDD1 as a whole is unique to *P. damselae* subsp. *damselae* strains.

Damselysin and phobalysin P, two singular plasmid-encoded toxins. As mentioned above, Dly was the first virulence factor discovered in *P. damselae* subsp. *damselae* (20). Strikingly, no known homologues of Dly have been reported to date in any member of the *Vibrionaceae*. The closest homologues in the GenBank database correspond to toxins of several species of the *Aeromonadaceae* and *Pseudoalteromonadaceae* families, including pathogenic (*Aeromonas salmonicida* and *Aeromonas hydrophila*) and nonpathogenic species (*Pseudoalteromonas rubra*) (Fig. 2A).

Dly is a sphingomyelinase D that removes polar choline phosphate headgroups after hydrolysis of membrane sphingomyelin (Fig. 3). In fact, treatment of sheep erythrocytes with subnanogram amounts of Dly protects cells from lysis by some sphingomyelin-dependent cytolysins (22). The sensitivities of erythrocytes to the Dly toxin vary

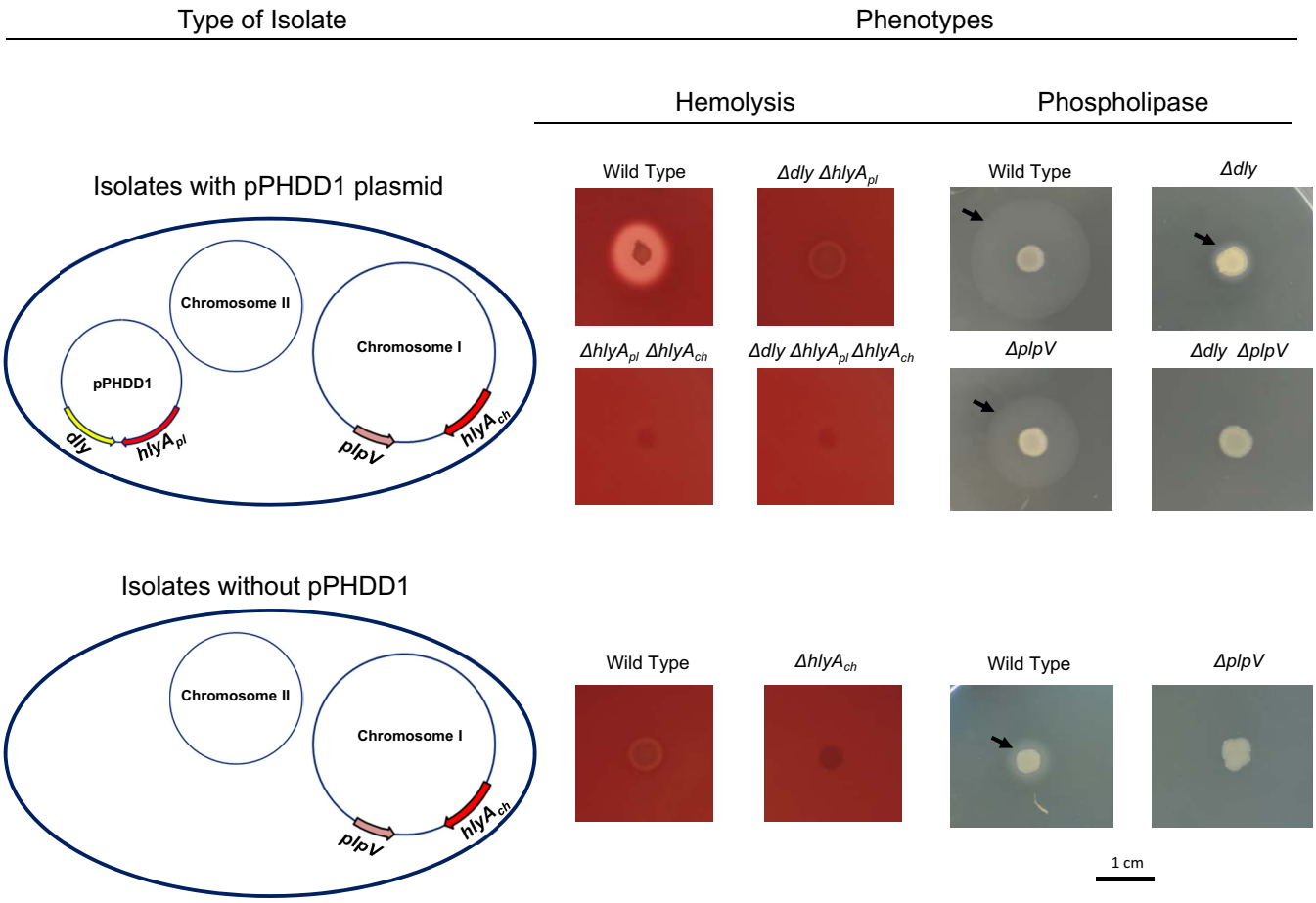

FIG 1 Hemolysis (for sheep erythrocytes) and phospholipase (in egg yolk plates) phenotypes of the two main categories of *P. damselae* subsp. *damselae* isolates. Plasmid (pPHDD1)-harboring strains produce the four cytotoxins Dly, PhlyP, PhlyC, and PlpV, whereas plasmidless strains only produce the chromosome-I-encoded cytotoxins PlpV and PhlyC. Note that Dly alone is unable to cause lysis of sheep red blood cells, but its synergistic interaction with PhlyP and PhlyC causes maximal hemolysis. A *dly* $hlyA_{pl}$ double mutant of a pPHDD1 strain exhibits a hemolytic phenotype similar to a natural plasmidless strain, and such a phenotype is due to PhlyC. The phospholipase phenotype is attributable to both Dly and PlpV, with a major contribution of Dly. Arrows indicate the border of the phospholipase halo.

depending on the animal source, being strongly hemolytic for mice but nonhemolytic for sheep red blood cells (20, 30). Recent data indicated that Dly exerts low toxicity against human epithelial HaCaT cells and zebrafish AB.9 cells (29), but information on its cytotoxicity for other cell types is still scarce.

PhlyP (initially termed $HlyA_{pl}$), encoded by the $hlyA_{pl}$ gene, is a pore-forming toxin that has homologues in many pathogenic species of *Vibrio* and *Photobacterium* (for example, *P. jeanii*), as well as in nonpathogenic species of both genera (Fig. 2B). It was demonstrated that PhlyP forms stable oligomers (likely pentamers) that are inserted into the erythrocyte membrane constituting pore complexes and requires cholesterol in order to cause hemolysis (29) (Fig. 3). PhlyP differs from the well-characterized homologous toxin VCC of *Vibrio cholerae* in several features. Even though PhlyP lacks a C-terminal lectin domain that is present in VCC, the pore size created by PhlyP is wider than that created by VCC.

In addition to causing lysis of erythrocytes, PhlyP is highly toxic for other cell types, as has been exemplified by its strong cytotoxic activity against human (HaCaT) and zebrafish (AB.9) cell lines. In this regard, PhlyP alone demonstrated to be much more toxic for these two cell types than Dly. PhlyP caused a time- and dose-dependent loss of intracellular ATP, as well as a drop in intracellular potassium ions. As a consequence, hyperphosphorylation of eIF2α, dephosphorylation of p70S6K, and a concomitant inhibition of protein synthesis and further cell damage were detected (29) (Fig. 3).

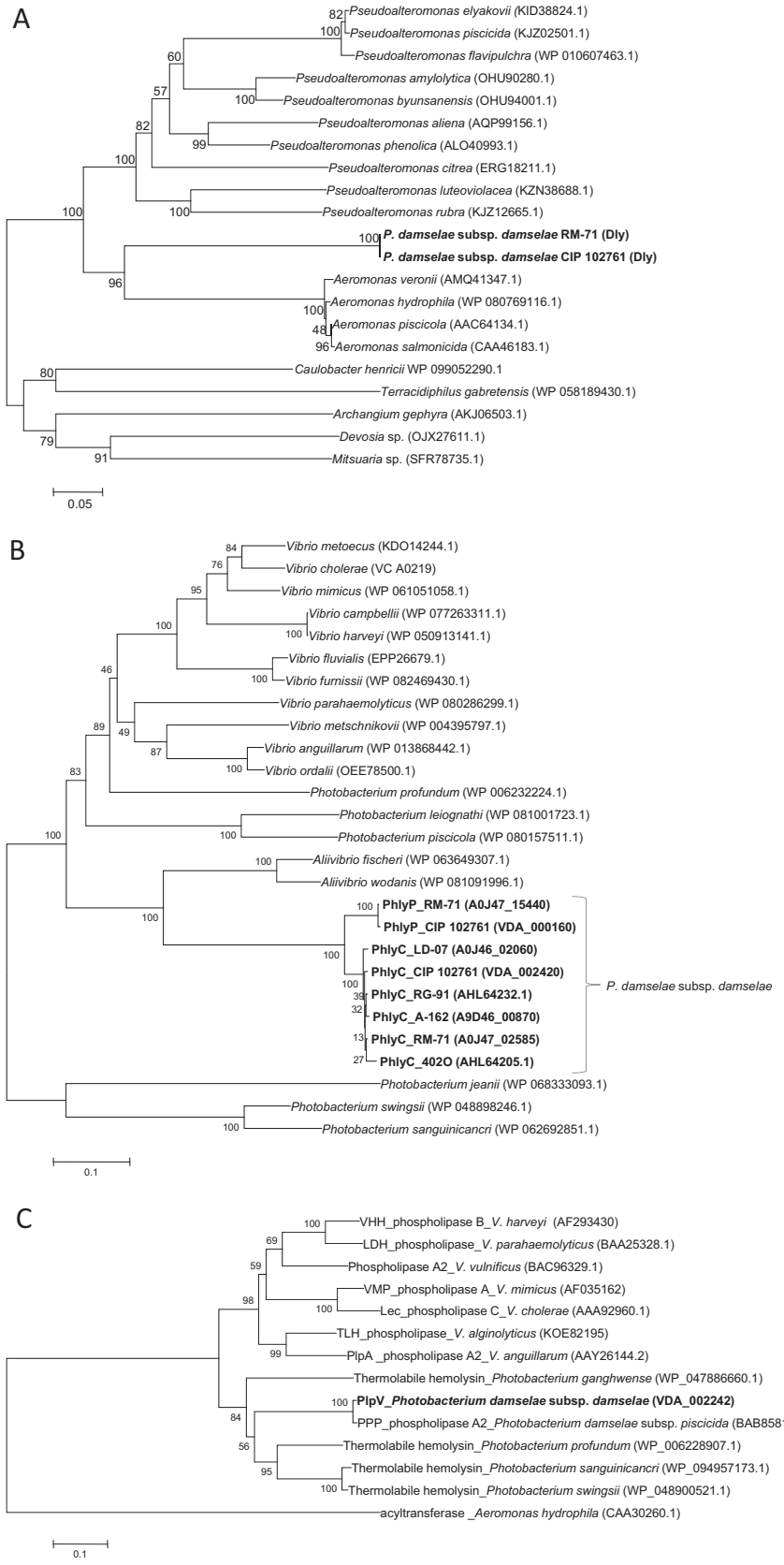

FIG 2 Phylogenetic relationships of the four *P. damselae* subsp. *damselae* cytotoxins with related toxins in other bacteria. (A) Damselysin (Dly) has no known homologues in any representative of the *Vibrio* and *Photobacterium* genera, thus constituting a singular feature of this subspecies. Interestingly, homologues

(Continued on next page)

CHROMOSOMAL VIRULENCE FACTORS

P. damselae subsp. *damselae* isolates that lack the pPHDD1 plasmid exhibit a weak hemolytic phenotype, producing narrow hemolysis halos on sheep blood agar plates. Interestingly enough, plasmidless strains constitute a large proportion of the isolates of this bacterium from disease outbreaks in sea bass and sea bream fish farms (12, 31). Recent reports have shown that although plasmidless strains are less virulent than pPHDD1 strains in a sea bass model, they do cause fish deaths in experimental inoculations, indicating that such strains encode additional virulence factors different from Dly and PhlyP (12, 32). Indeed, a previous study reported the identification in strain RM-71 of a chromosome I-borne gene, termed $hlyA_{ch}$ (locus VDA002420), that encodes a pore-forming toxin homologous to PhlyP (30). Recently, it was demonstrated that this chromosomal toxin encoded by $hlyA_{ch}$ constitutes a major virulence factor in plasmidless strains and was dubbed PhlyC (32). The PhlyP and PhlyC amino acid sequences are 92% identical (Fig. 2B), and it is likely that they share similar modes of action. PhlyC is indeed cytotoxic for fish and human cell lines, but its toxicity levels are lower than those exerted by the plasmid-encoded homologue PhlyP (29, 32). This difference is believed to be due to the amino acid residues which differ between the two toxins (an 8% divergence in amino acid sequence), although this point deserves further research. PhlyC is present in all the hemolytic *P. damselae* subsp. *damselae* isolates tested so far, irrespective of the presence of pPHDD1. A few reported isolates that are not hemolytic either lacked the $hlyA_{ch}$ gene or bore an inactivated version caused by the insertion of an insertion sequence (IS) element (12, 31). Interestingly, the $hlyA_{ch}$ gene is located within a highly variable chromosomal region, and each analyzed strain was shown to contain a unique gene repertoire in the vicinity of the $hlyA_{ch}$ gene (Fig. 4A).

Recently, it has been reported that a plasmidless strain that also lacked a functional $hlyA_{ch}$ gene (inactivated by an IS element) exhibited some degree of virulence for sea bass (12), an observation that fueled the search for additional virulence factors. As a result, it was shown that all the isolates of this pathogenic bacterium encode a phospholipase dubbed PlpV in chromosome I (locus VDA002242), with a role in hemolysis, cell toxicity, and virulence for fish, and an $hlyA_{ch}$ *plpV* double mutant of a plasmidless strain was nonvirulent for sea bass (32). PlpV exerted hemolytic activity on fish erythrocytes but not on sheep erythrocytes. The phospholipid-degrading activity of a strain producing only PlpV is much lower than that of a strain producing only Dly phospholipase (Fig. 1), although it is not clear whether this difference is due to a lower activity of PlpV or to differences in gene expression and/or enzyme secretion. Contrary to Dly, the PlpV phospholipase has close homologues in many species of the genus *Vibrio* and *Photobacterium* (32), and some homologues were reported to exhibit toxic activity for fish (33). A phylogenetic analysis suggests that PlpV might be a phospholipase of the A2 type (Fig. 2C). Thus, the virulence gene content throughout the subspecies could be summarized as follows: pPHDD1 strains encode four toxins, namely, Dly, PhlyP, PhlyC, and PlpV, whereas plasmidless strains only encode PhlyC and PlpV (Fig. 1). The four toxins are known to be secreted via the type II secretion system (T2SS) of *P. damselae* subsp. *damselae* (32, 34) (Fig. 3).

FIG 2 Legend (Continued)
exist in both pathogenic (*A. hydrophila*) and nonpathogenic (*P. rubra*) bacteria. (B) Phobalysin homologues are widespread in both pathogenic and environmental species of *Vibrio*, *Photobacterium*, and *Aliivibrio*. Also note that plasmid (PhlyP) and chromosomal (PhlyC) versions of *P. damselae* subsp. *damselae* differ in ca. 8% in their sequences and can be distinguished using a phylogenetic analysis. (C) PlpV phospholipase also has homologues in pathogenic and nonpathogenic species of the *Vibrionaceae* family, and it is closely related to phospholipases of the A2 type. Phylogenetic trees were constructed using the neighbor-joining method, and evolutionary distances (number of residue substitutions per site) were computed using the maximum composite likelihood method. 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.

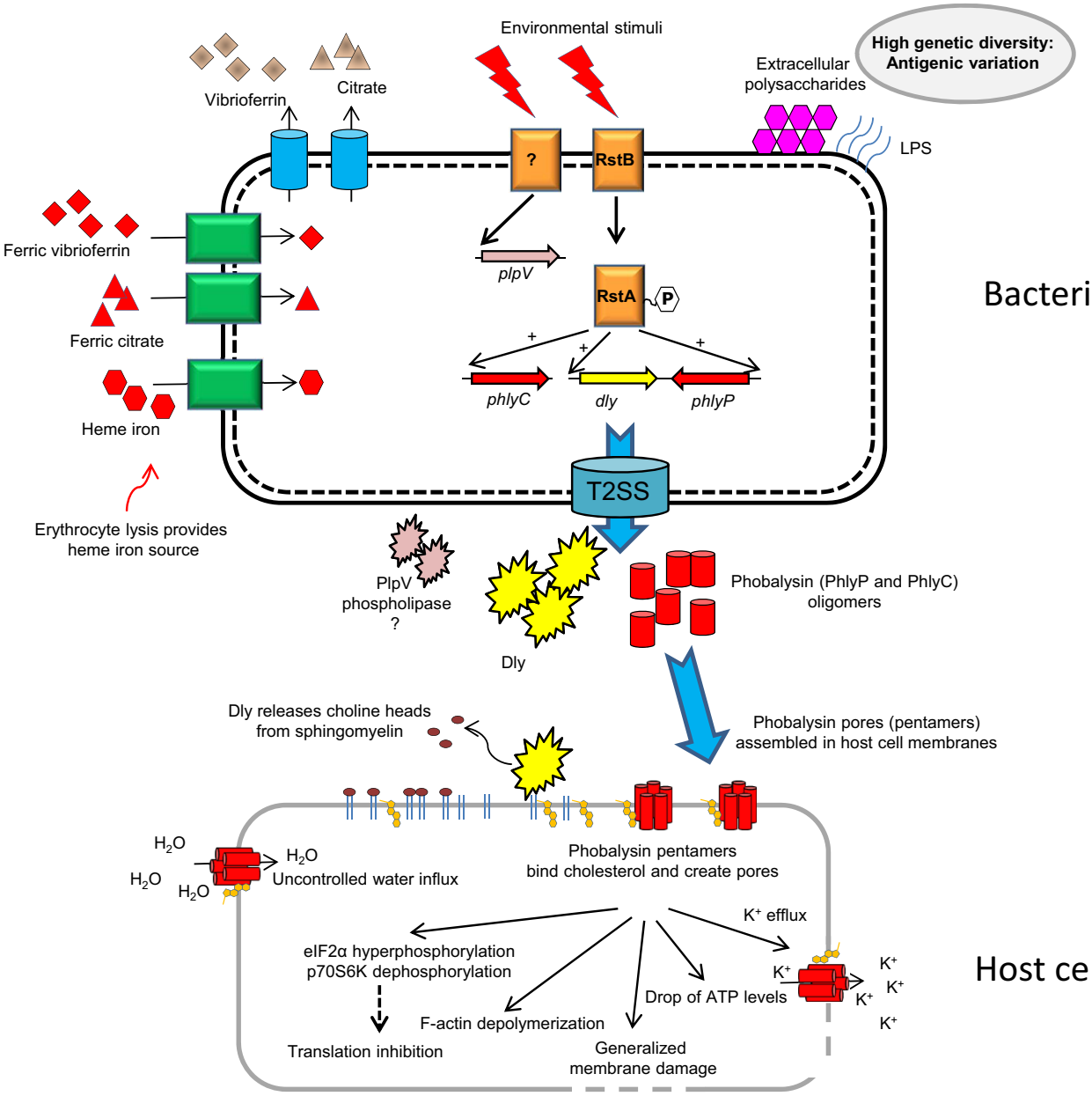

FIG 3 Diagrammatic summary of the most important features of *P. damselae* subsp. *damselae* reported to date, related to virulence and interaction with host cells. Depicted are the four main cytotoxins produced by this pathogen. PlpV phospholipase and PhlyC pore-forming toxin are encoded in chromosome I, whereas Dly sphingomyelinase and PhlyP pore-forming toxin are encoded within the pPHDD1 plasmid. The two-component regulatory system RstAB is a positive regulator of Dly, PhlyP, and PhlyC but not of PlpV. The four toxins are secreted via the type two secretion system (T2SS). It is hypothesized that, once secreted, Dly cleaves choline heads from sphingomyelin, thus allowing better access of PhlyP and PhlyC oligomers to the host cell membrane and to interaction with cholesterol molecules. PhlyP and PhlyC oligomers are thought to assemble into the host cell membrane, creating pentameric pores that cause a series of downstream effects leading to cell lysis and death. LPS, lipopolysaccharide.

INTERACTIONS BETWEEN TOXINS: SYNERGISTIC AND ADDITIVE EFFECTS HELP US UNDERSTAND TOXICITY AND VIRULENCE

Numerous studies were coincident in demonstrating that *P. damselae* subsp. *damselae* toxins exert different levels of toxicity depending on the cell type and the animal host (20, 29, 30, 35, 36). The different roles demonstrated by each toxin in virulence assays conducted with mice and fish (turbot) deserve special attention. Mutants producing Dly alone, and mutants producing PhlyP and PhlyC but not Dly, exhibited more impairment in virulence for fish than for mice. Overall, a mutation of Dly had strong consequences in virulence for fish, and virulence tests clearly suggested that full

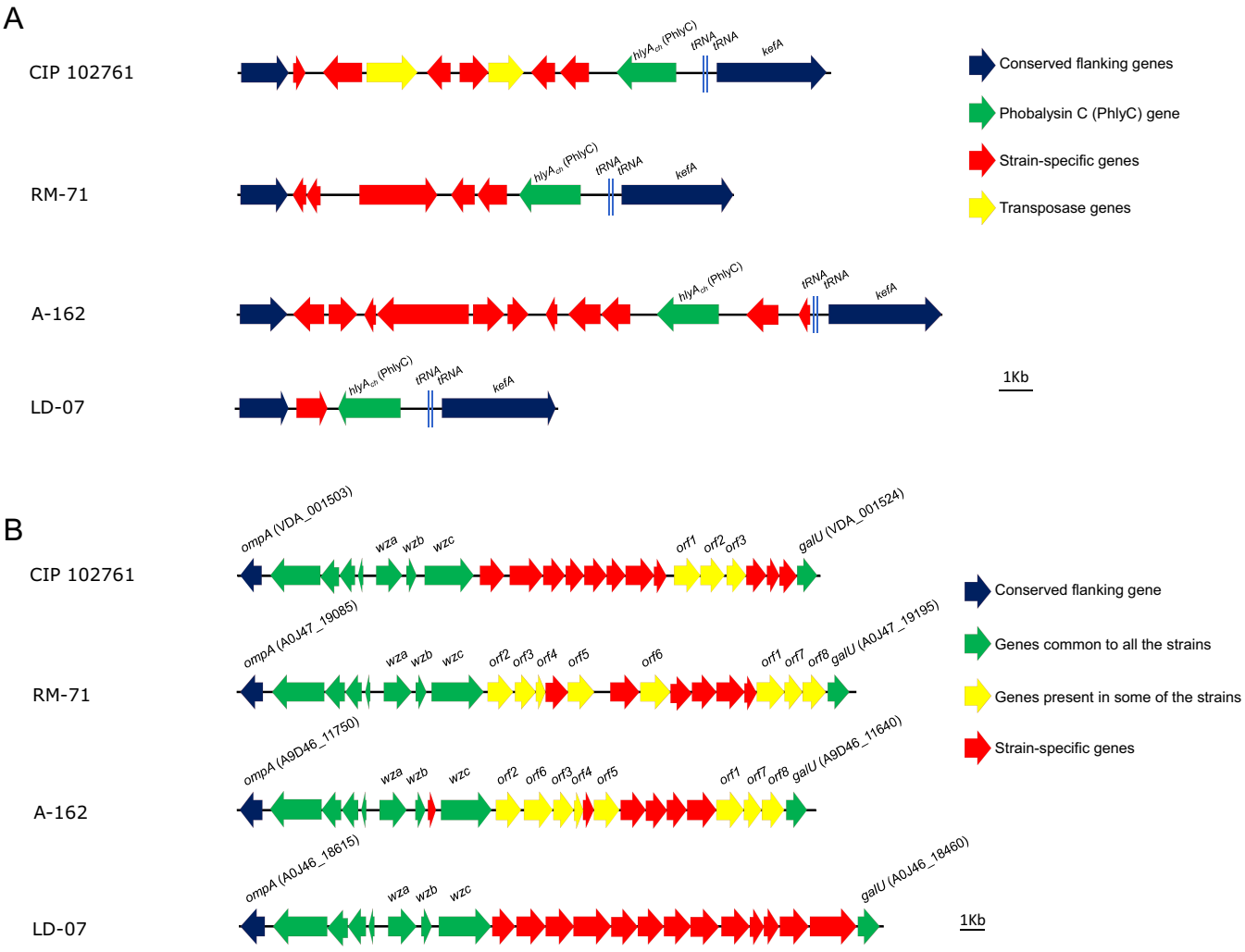

FIG 4 Gene organization in two regions of chromosome I of *P. damselae* subsp. *damselae* that show a high degree of genetic heterogeneity among isolates. Four selected strains are depicted, for which the complete genome sequence is available. (A) The genetic context of the chromosomal hemolysin PhlyC gene ($hlyA_{ch}$ locus) shows a unique gene combination in each of the strains analyzed to date and contains features that are typical of mobile DNA elements. (B) A gene cluster encoding putative functions related to extracellular capsular polysaccharides and LPS synthesis shows striking genetic diversity among isolates. Note that two strains belonging to the same serogroup (RM-71 and A-162) contain 6 unique genes each.

virulence for fish was the consequence of a synergistic effect between Dly and the two phobalysins (30). Hemolytic assays carried out using bacterial extracellular products and sheep erythrocytes also demonstrated that Dly acts in a synergistic manner with PhlyP and PhlyC, while PhlyP and PhlyC together exert an additive but not synergistic effect between the two of them (30).

The native plasmidless strains of this pathogen, which produce PhlyC but neither Dly nor PhlyP, are known to be less virulent than plasmid strains and less toxic for cells (12, 26, 32). A recent study revealed that the production of PlpV phospholipase is necessary in addition to PhlyC for the full toxicity of plasmidless strains against human and zebrafish cells and for full virulence for fish, constituting another example of how a phospholipase can enhance the virulence and toxicity of a pore-forming toxin (32).

Currently, it is not clear how the phospholipases Dly and PlpV contribute to cell damage in a synergistic manner with the pore-forming toxins PhlyP and PhlyC. The enzymatic activity of PlpV is not known at the moment, even though the *in silico* and phylogenetic analyses of its sequence suggest that it might be a phospholipase A2 (Fig. 2C). Considering that Dly is a phospholipase D capable of trimming the sphingomyelin molecule and of removing polar choline phosphate headgroups, and that PhlyP is a

cholesterol-dependent pore-forming toxin, it is hypothesized that Dly might facilitate the access of PhlyP and PhlyC to cholesterol molecules (Fig. 3). Depending on the animal and cell type and on the cholesterol and sphingomyelin cell contents, the activities of these toxins might vary. A similar mechanism is thought to occur in other synergistic interactions between hemolysins. In this sense, Dly was found to lyse sheep erythrocytes by means of a synergistic action with the staphylococcal delta-toxin (22), and a mixture of staphylococcal beta- and delta-toxins also acts synergistically (37). In the two cases, it is hypothesized that the removal of polar choline phosphate headgroups from sphingomyelin reduces the barrier to lipid bilayer access for delta-toxin, which is hydrophobic. Surely, the elucidation of the molecular basis of the synergistic processes among Dly, PlpV, and the two pore-forming toxins PhlyP and PhlyC constitutes one of the main challenges to understanding the pathogenicity of *P. damselae* subsp. *damselae*.

CYTOTOXINS AND WHAT ELSE?

The pioneering studies of Kreger (20) have not detected any protease activity in the culture supernatant fluids of *P. damselae* subsp. *damselae*. Nowadays, there is substantial evidence that *P. damselae* subsp. *damselae* is a noncaseinolytic bacterium, although a fraction of isolates encode a collagenase (ColP) that plays a minor role in virulence (32). The elucidation of the complete genome sequence of the *P. damselae* subsp. *damselae* type strain CIP 102761 uncovered the existence of a 203-kb plasmid (pPHDD203) that encodes a type III secretion system (T3SS), including a number of putative effector proteins (32). However, this T3SS coding sequence proved to be absent from the genomes of other 3 *P. damselae* subsp. *damselae* strains. In addition, a total of 96 isolates of this pathogen available in the collection of our laboratory, from diverse parts of the globe and from different hosts, all tested negative for PCR amplification of 4 gene markers of the T3SS encoded by pPHDD203 (C. R. Osorio, unpublished data), clearly indicating that this system encoded by the type strain is the exception rather than the norm in this pathogen.

Regarding the iron acquisition systems, previous studies demonstrated that this pathogen can utilize hemoglobin and ferric ammonium citrate as iron sources, and some isolates showed the ability to produce a hydroxamate siderophore (38, 39) (Fig. 3). Strains of this pathogen are known to encode a functional heme uptake system, but its role in virulence needs further investigation (40). It was recently proven that only a fraction of the *P. damselae* subsp. *damselae* isolates contain a gene cluster that guides the synthesis and utilization of vibrioferrin as a siderophore (41, 42). However, virtually all strains of this bacterium are capable of secreting endogenous citrate, which can be used for iron scavenging (42). Although previous studies reported a positive correlation between iron availability in host tissues and virulence degree (38), the specific roles of all these mentioned iron acquisition systems in virulence still remain largely uncharacterized.

A HIGHLY DIVERSE MULTICLONAL PATHOGEN

Increasing evidence suggests that *P. damselae* subsp. *damselae* outbreaks in fish farms are caused by multiclonal and preexisting natural populations that take advantage of host and environmental conditions in order to cause disease. This hypothesis is reinforced by the observation that virulent isolates are capable of survival for long periods of time in seawater microcosms at 14 to 22°C as culturable bacteria that maintain their infectivity for fish (43). Also, it has been observed that *P. damselae* subsp. *damselae* can infect new fish hosts through water, and the spread of the disease largely depended on water temperature (44). Water temperature is indeed of special importance. A few consistent reports associated the occurrence of *P. damselae* subsp. *damselae* outbreaks with unusual increases in water temperature (2, 27, 45, 46). Such temperature conditions might increase fish susceptibility to infection by *P. damselae* subsp. *damselae* genotypes already present in the environment as free-living bacteria. In fact, immersion challenges demonstrated that this pathogen induced fatal disease in fish only when temperatures were above 22°C (44). This situation

is not exclusive to *P. damselae* subsp. *damselae*, and episodes of unusually warm waters are nowadays considered a risk factor for disease outbreaks caused by species of *Vibrio* and *Photobacterium* (47, 48).

The multiclonal nature of *P. damselae* subsp. *damselae* outbreaks was well documented in a couple of studies conducted with isolates from outbreaks in Danish rainbow trout farms (26, 45). Although the pPHDD1 plasmid had not been discovered yet when these studies were carried out, it is evident from the hemolytic activity data that these outbreaks were caused by both pPHDD1 and plasmidless strains. In one of the studies, 26 isolates were classified into as many as 17 different ribotype patterns (45), and in the second study, each one of the 16 analyzed isolates yielded a distinct pulsed-field gel electrophoresis (PFGE) pattern (26), therefore demonstrating the existence of high genetic heterogeneity in the outbreaks. High levels of genetic heterogeneity were also found in isolates from sea bream and sea bass farms in the Spanish Mediterranean coast (49). A recent report has also demonstrated that outbreaks in sea bass fish farms in the Turkish Black Sea were caused by a multiclonal population of plasmidless strains (12). Such evidence suggests that the infective unit might be the population rather than a specialized clonal line. However, there is some evidence which also suggests that highly virulent clones (i.e., the pPHDD1-containing isolates) may occasionally become clonal and cause an outbreak with acute fish death. This seems to be the case for a series of outbreaks occurred in a turbot farm in Galicia (NW Spain) between 1987 and 1989, where all the isolates from the same farm contained the pPHDD1 plasmid, and all of them were antigenically related (27).

Previous studies had demonstrated that *P. damselae* subsp. *damselae* is an antigenically diverse pathogen, and as many as 7 O serogroups were identified among 16 strains analyzed, suggesting that the number of serogroups might increase significantly as the number of studied strains grew (27, 39). Definite evidence of this high genetic diversity became available after the recent comparative genomic analyses conducted with four complete genomes of virulent isolates of this pathogen, where each strain contained a large number of unique genes (32). Interestingly, these four strains belonged to three different O serogroups, namely, serogroups A (type strain, CIP 102761), B (strains RM-71 and A-162), and E (strain LD-07). Surprisingly, analysis of these four genomes reveals that each strain contains a unique combination of genes within a putative cluster for cell envelope polysaccharide synthesis. Capsular polysaccharides, along with exopolysaccharides and lipopolysaccharides, play an important role in virulence and adherence of bacterial pathogens to hosts or surfaces. The four genomes share a highly conserved region comprising the *wza*, *wzb*, and *wzc* genes, whose homologues are known to play a role in biosynthesis of colanic acid and group 1 and 4 capsules in *Escherichia coli* (50). However, the region flanked by the *wzc* and *galU* (UTP-glucose-1-P uridylyltransferase) genes is highly varied among the 4 strains, and each strain contains a large number of specific genes. This variation is particularly notorious in the genetic context of strain LD-07 (serogroup E), whereas RM-71 and A-162 (the two strains of serogroup B) share more genes in common (Fig. 4B).

Similarly, as mentioned above, the chromosome I region in the vicinity of the $hlyA_{ch}$ gene (encoding PhlyC toxin) shows a striking genetic diversity, and all the strains analyzed to date contained a unique strain-specific gene repertoire (31) (Fig. 4A). These two chromosomal regions, encoding PhlyC and cell envelope polysaccharides, respectively, constitute isolate-specific gene signatures that can be of utility in epidemiological and tracking studies.

RstB, A MASTER REGULATOR OF Dly, PhlyP, and PhlyC CYTOTOXINS

As described above, fish farm outbreaks can be caused by multiclonal populations of *P. damselae* subsp. *damselae*, with lineages that produce different virulence factors. Furthermore, different serogroups coexist, with a high probability, within an outbreak. In such a scenario, the identification of conserved weak points in the biology of this pathogen would constitute a valuable tool for the design of control measures. Recently, it has been reported that the two-component regulatory system RstAB (homologous to

the *V. cholerae* CarSR system) is necessary for the maximal production of virulence factors. The histidine kinase RstB positively regulated the expression of the three major toxins Dly, PhlyP, and PhlyC (Fig. 3), and its inactivation severely impaired virulence (51). This finding provided evidence that RstAB/CarSR-like systems regulate bacterial toxins, and that the RstAB regulon can be constituted by both chromosome and plasmid-carried genes. Recent evidence suggests that RstAB also regulate other biological processes in this marine pathogen (M. S. Terceti, X. M. Matanza, and C. R. Osorio, unpublished data). Thus, the role of the RstAB system in *P. damselae* subsp. *damselae* virulence and cell biology constitutes a promising research field in the near future.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Pathogenic *Vibrionaceae* members constitute excellent model systems to study how pathogenicity evolved from environmental ancestors and how genetic diversity-generating mechanisms shape bacterial populations. Although all *Vibrionaceae* share a similar environment (marine ecosystems with more or less intimate association with hosts), their diversity in pathobiological and adaptive gene functions is overwhelming. Not surprisingly, any given species within *Vibrionaceae* can reveal itself as a singular and rather unique biological strategy. *Photobacterium damselae* subsp. *damselae* is not an exception. In fact, well to the contrary, this bacterium has contributed to novel paradigms in microbiology. One of its major toxins, damselysin, constitutes not only a quite unique virulence factor still to characterize in depth, but it has also been recently demonstrated that its other major toxin, phobalysin P, constitutes a novel member within the small B-pore-forming toxins that was found to be different from the well-characterized *V. cholerae* VCC. The mechanism by which these toxins cooperate in a synergistic manner is still unclear, and many questions still remain. For example, why do Dly and PhlyP/PhlyC exhibit differences in toxicity depending on the cell type and animal species? What is the molecular mechanism by which Dly causes cell and tissue damage? Does Dly enzymatic activity cause the release of molecules that might elicit inflammatory responses and tissue damage? Does the infective process depend on active multiplication of *P. damselae* subsp. *damselae* in target tissues, or does it rely on the production of large amounts of toxins that are spread through the circulatory system of animal hosts? Is the variability of cell envelope polysaccharides related to adaptations to different hosts? These questions and many more remain unanswered, but future research will undoubtedly unveil some of them in the upcoming years. In a similar line of thoughts, initial analyses revealed that the *P. damselae* subsp. *damselae* RstAB regulon includes genes not previously identified in the RstAB-like regulons of closely related species (i.e., *V. cholerae*). The stimulus that triggers RstB phosphorylation in *P. damselae* subsp. *damselae* is currently unknown, and so is that for the homologous regulators described in related *Vibrio* species (52, 53). Thus, *P. damselae* subsp. *damselae* constitutes a fascinating biological model whose adaptive molecular mechanisms of biology and pathogenesis should be undoubtedly elucidated.

ACKNOWLEDGMENTS

The work our laboratory is supported by grant AGL2016-79738-R (AEI/FEDER, EU) from the State Agency for Research (AEI) of Spain and cofunded by the FEDER Programme from the European Union. The support of Xunta de Galicia (Spain) with grant GRC-2014/007 is also acknowledged.

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