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The *Photobacterium damselae* subsp. *damselae* Hemolysins Damselysin and HlyA Are Encoded within a New Virulence Plasmid[▽]

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***Photobacterium damselae* subsp. *damselae* (formerly *Vibrio damsela*) is a marine bacterium that causes infections and fatal disease in a wide range of marine animals and in humans. Highly hemolytic strains produce damselysin (Dly), a cytolysin encoded by the *dly* gene that is lethal for mice and has hemolytic activity. We found that Dly is encoded in the highly hemolytic strain RM-71 within a 153,429-bp conjugative plasmid that we dubbed pPHDD1. In addition to Dly, pPHDD1 also encodes a homologue of the pore-forming toxin HlyA. We found a direct correlation between presence of pPHDD1 and a strong hemolytic phenotype in a collection of *P. damselae* subsp. *damselae* isolates. Hemolysis was strongly reduced in a double *dly hlyA* mutant, demonstrating the role of the two pPHDD1-encoded genes in hemolysis. Interestingly, although single *hlyA* and *dly* mutants showed different levels of hemolysis reduction depending on the erythrocyte source, hemolysis was not abolished in any of the single mutants, suggesting that the hemolytic phenotype is the result of the additive effect of Dly and HlyA. We found that pPHDD1-encoded *dly* and *hlyA* genes are necessary for full virulence for mice and fish. Our results suggest that pPHDD1 can be considered as a driving force for the emergence of a highly hemolytic lineage of *P. damselae* subsp. *damselae*.**

Photobacterium damselae subsp. *damselae* (formerly *Vibrio damsela*) is a halophilic bacterium associated with marine environments that was initially isolated in 1981 as the causative agent of skin ulcers in damselfish (34). It is a primary pathogen causing ulcers and hemorrhagic septicemia in a variety of marine species as sharks, dolphins, and shrimps, as well as wild and cultivated fish (18, 20, 45). In addition, this pathogen can cause fatal infections in humans. Most of the reported infections in humans have their origin in wounds inflicted during the handling of fish, exposure to seawater and marine animals, and ingestion of raw seafood (1, 2, 26, 38, 53). In some of the human cases the infection progresses into an extreme variant of a highly severe necrotizing fasciitis that advances following a very aggressive course leading to a fatal outcome (7, 53).

P. damselae subsp. *damselae* shares species level status with *P. damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*), the causative agent of fish pasteurellosis (21). Although subsp. *damselae* is pathogenic for marine animals and humans, subsp. *piscicida* is only pathogenic for fish, it does not grow at 37°C and lacks observable hemolytic activity on blood agar plates (36, 41, 42). Very little is known about the virulence factors that enable *P. damselae* subsp. *damselae* to cause septicemia in aquatic animals and humans. A correlation was initially observed between the ability of *P. damselae* subsp. *damselae* to cause disease in mice and to produce large amounts of a cytolytic toxin that was later named damselysin (28) (hereafter referred to as Dly). Partially purified Dly preparations from culture supernatants showed to be active against erythrocytes of 16 species of homeotherm animals, with rat and mouse being the more sensitive ones (28). Other studies reported that the extracellular products of this bacterium also had hemolytic activity on turbot erythrocytes (16). A deeper characterization of Dly showed that it has phospholipase D activity against sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine (10, 29). The main molecular activity of Dly consists of the removal of the polar choline groups of choline-containing membrane lipids. Dly was found to enhance the hemolytic effect of staphylococcal delta-toxin by removing the polar choline phosphate head group of sphingomyelin (29), which constituted the first evidence that Dly can act synergistically with hemolysins produced by other cells. The Dly toxin gene, *dly*, was cloned (9), and its sequence was determined (GenBank accession no. L16584). However, the genomic context of *dly* gene remained elusive, and the initial observation that highly hemolytic strains yielded spontaneous mutants with markedly reduced hemolytic activity which had lost *dly* gene, along with extensive flanking sequences, suggested that this gene might be located on a mobile element (9).

Thin-layer isoelectric focusing assays showed one major and two minor components with hemolytic activity in *P. damselae* subsp. *damselae* supernatants (27). These observations suggested that other hemolysins in addition to Dly might be produced by this subspecies. The degree of hemolysis varies among *P. damselae* subsp. *damselae* isolates. Two main distinct hemolytic phenotypes could be observed on blood agar plates, with strains showing a large hemolysis halo (LH) and strains producing a small hemolysis halo (SH), although the type strain ATCC 33539 can be described as moderately hemolytic (MH) (7, 9, 16, 30, 42). Early studies demonstrated that the *P. damselae* subsp. *damselae* strains showing the highest values of hemolytic activity were also those more virulent for mice (28). In addition, it was later demonstrated that the strongly hemo-

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TABLE 1. Strains and plasmids used and constructed in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
P. damselae subsp. *damselae*		
RM-71	Wild type, isolated from turbot (*Psetta maxima*), strongly hemolytic	18
AR57	RM-71 derivate, spontaneous rifampin-resistant mutant; Rf^r	This study
AR64	AR57 with in-frame deletion of *dly* gene	This study
AR133	AR57 with in-frame deletion of *hlyA* gene	This study
AR78	AR57 with in-frame deletion of *dly-hlyA* genes	This study
AR61	AR57 with a first cross-over of suicide vector for *dly* mutant construction; Km^r	This study
P. damselae subsp. *piscicida*		
PC554.2	Nonhemolytic; Tc^r	35
AR83	PC554.2 transconjugant that acquired pPHDD1 from AR61 and further selected for suicide plasmid loss	This study
E. coli		
XL1-Blue MR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
DH5-α	Cloning strain, *recA*	Laboratory stock
S17-1 λ*pir*	*recA thi pro* Δ*hsdR hsdM* $^+$ RP4-2-Tc:Mu-Km:Tn*7* λ*pir*; Tp^r Sm^r	Laboratory stock
Plasmids		
pKD4	Template for Km^r gene	11
pNidKan	Suicide vector, derived from pCVD442; Km^r	39
pHRP309	*lacZ* reporter plasmid, mob; Gm^r	44
pWKS30	Low-copy-number cloning vector; Ap^r	51
pACYC184	Low-copy-number cloning vector; Tc^r	Stratagene
pAJR38	pHRP309 with *hlyA* gene from RM-71	This study
pAJR27	pWKS30 with *hlyA* gene from RM-71	This study
pAJR29	pACYC184 with *dly* gene from RM-71	This study

[a] Rf^r, rifampin resistance; Tc^r, tetracycline resistance; Gm^r, gentamicin resistance; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Km^r, kanamycin resistance; Tp^r, trimethoprim resistance.

lytic strains against mouse erythrocytes were also those that hybridized to a *dly* DNA probe (9). Interestingly, both fish and human isolates were represented among the strongly hemolytic and highly virulent strains (28). Actually, previous studies reported that the human isolate CDC2227-81 and the fish isolate RM-71 showed almost identical 50% lethal doses for mice, whereas RM-71 was more virulent for fish than CDC2227-81 (17). Other studies reported that strains lacking *dly* gene still showed virulence for mice and fish, indicating that *dly* is not a prerequisite for virulence in this bacterium (16, 30, 42). In addition, the extracellular products of virulent strains regardless of presence of *dly* gene were lethal for fish and mice, as well as cytotoxic for homeotherm and poikilotherm cell lines (16, 30), suggesting that other virulence factors than Dly might play a role in the pathogenesis of this bacterium, but their nature remains unknown.

A significant diversity in plasmid content has been demonstrated in *P. damselae* subsp. *damselae* strains. Some studies reported that highly hemolytic strains harbor a plasmid of ca. 90 to 100 MDa (150 to 170 kb) that is absent in the weakly hemolytic strains (18, 47). Plasmids have been found associated to virulence in *P. damselae* subsp. *piscicida* (13), *V. anguillarum* (12), and *V. nigripulchritudo* (31), among others.

In the present study we sequenced and characterized pPHDD1, a novel 153-kb plasmid in *P. damselae* subsp. *damselae* strain RM-71 that carries *dly* gene. In addition to Dly, pPHDD1 encodes a pore-forming toxin hemolysin of the HlyA family. We provide evidence that Dly and HlyA contribute to the hemolysis and the virulence of *P. damselae* subsp. *damselae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains used here and those derived from the present study are listed in Tables 1 and 2. The plasmids are listed in Table 1. *P. damselae* subsp. *damselae* strains were subjected to standard biochemical tests to corroborate their taxonomic position and PCR tested for subspecies-specific gene markers (43). *P. damselae* subsp. *damselae* cells were routinely grown at 25°C on tryptic soy agar supplemented with 1% NaCl (TSA-1) or trypic soy broth supplemented with 1% NaCl (TSB-1). Sheep blood agar plates (Oxoid) were used for conjugative matings and hemolysis assays. Human, rat, and turbot blood was aseptically collected and added to TSA-1 at a final concentration of 5% (vol/vol) to obtain human, rat, and turbot blood agar, respectively. For hemolysis assays on agar plates, a single colony of each strain grown on a TSA-1 plate was picked with the tip of a rounded wooden pick and seeded on the blood agar plate, and pictures were taken after 15 h of incubation at either 25 or 37°C. *Escherichia coli* strains were routinely grown at 37°C in Luria-Bertani (LB) broth and LB agar, supplemented with antibiotics when appropriate. Antibiotics were used at the following final concentrations: kanamycin at 50 $\mu g\ ml^{-1}$, ampicillin sodium salt at 50 $\mu g\ ml^{-1}$, tetracycline at 4 $\mu g\ ml^{-1}$, gentamicin at 15 $\mu g\ ml^{-1}$, and rifampin at 50 $\mu g\ ml^{-1}$.

Cosmid library construction, DNA sequencing, and annotation. Genomic DNA of *P. damselae* subsp. *damselae* RM-71 was purified using a genome DNA kit (Qbiogene). DNA was partially digested with Sau3AI and ligated into the compatible BamHI site of alkaline phosphatase-treated SuperCos 1 cosmid vector (Stratagene). The ligated products were packaged into bacteriophage lambda particles using an *in vitro* packaging kit (Gigapack III Gold packaging extract; Stratagene) and introduced into *E. coli* XL1-Blue MR cells. The nucleotide sequences of cosmid DNA were determined using a 454 GS-FLX platform (Roche) and assembled using Newbler software (Roche). Sequences were further analyzed with the online BLAST facility of the National Center for Bio-

TABLE 2. PCR detection of pPHDD1 gene markers within the *P. damselae* subsp. *damselae* strain collection[a]

P. damselae subsp. *damselae* strain	Source	Presence (+) or absence (–) of various pPHDD1 markers									Hemolytic halo[b]
		dly	*hlyA*	*parA*	*orf2*	*repA*	*vep07*	*tolC*	*tadC*	*rcpA*	
RM-71	Turbot, Spain	+	+	+	+	+	+	+	+	+	LH
RG-91	Turbot, Spain	+	+	+	–	–	+	+	+	+	LH
RG-153	Turbot, Spain	+	+	+	–	–	+	+	+	+	LH
RG-214	Turbot, Spain	+	+	+	–	–	+	+	+	+	LH
CDC2227-81	Human, United States	+	+	+	–	–	+	+	+	+	LH
ATCC 33539	Damselfish, United States	+	+	+	–	–	+	+	+	+	MH
LD-07	Seabream, Spain	–	–	–	–	–	–	–	–	–	SH
340	Seawater, Spain	–	–	–	–	–	–	–	–	–	SH
309	Mussel, Spain	–	–	–	–	–	–	–	–	–	SH
158	Eel, Belgium	–	–	–	–	–	–	–	–	–	SH
162	Eel, Belgium	–	–	–	–	–	–	–	–	–	SH
PG801	Shrimp, Taiwan	–	–	–	–	–	–	–	–	–	SH
192	Dolphin, United States	–	–	–	–	–	–	–	–	–	SH
238	Dolphin, United States	–	–	–	–	–	–	–	–	–	SH
ATCC 35083	Brown shark, United States	–	–	–	–	–	–	–	–	–	SH
J3G801	Shrimp, Taiwan	–	–	–	–	–	–	–	–	–	NH
PC586.1	Seabream, Spain	–	–	–	–	–	–	–	–	–	SH

[a] *orf2* and *repA* are gene markers of the two RM-71-specific insertions A and B, respectively.
[b] LH, large hemolytic halo; MH, medium hemolytic halo; SH, small hemolytic halo; NH, no hemolytic halo.

technology Information (NCBI; http://www.ncbi.nlm.nih.gov). Annotation of plasmid was carried out with the RAST server (3), and a plasmid graphical map was generated with the CGView Server (22). G+C content analyses were conducted using the Artemis comparison tool (6). Protein domains were searched using the Pfam database (http://pfam.sanger.ac.uk/). The nucleotide sequence of pPHDD1 determined in the present study was deposited in GenBank under accession number NC_014653.

Plasmid DNA. Plasmid DNA was extracted from *P. damselae* subsp. *damselae* following a modification of a previously described method (54). Cells were harvested from a 1-ml overnight culture in tryptic soy broth. Pelleted cells were resuspended in 300 μl of TENS solution (0.09 N NaOH and 0.45% sodium dodecyl sulfate in Tris-EDTA buffer) plus 170 μl of 3 M sodium acetate (pH 5.2). This mixture was incubated on ice and centrifuged. Plasmid DNA in the supernatant was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with ethanol, and resuspended in ca. 20 to 40 μl of Tris-EDTA buffer with RNase (20 μg/ml). DNA samples mixed with loading buffer were electrophoresed through 0.7% agarose gels (type II; Sigma). Plasmid molecular sizes were estimated using the reference plasmids from *E. coli* 39R861 (plasmids of 154, 66.2, 37.6, and 7.4 kb) (48).

Mapping of pPHDD1 replication origin. A pPHDD1 2,698-bp region (positions 94549 to 97247 in the annotated sequence) containing *repB* gene (open reading frame 105 [ORF105]) and the putative iteron sequences was PCR amplified with oligonucleotides containing BamHI sites and ligated to the BamHI-cut PCR-amplified kanamycin resistance gene from plasmid pKD4 (11). The ligation reaction was electroporated into *E. coli* DH5-α cells, and kanamycin-resistant colonies were selected. The plasmid DNA from a kanamycin-resistant colony was purified and subjected to DNA sequencing.

Conjugation. Conjugations were performed by agar plate matings. Exponential growing cells of donor and recipient strains were mixed, a drop (100 μl) placed directly onto a sheep blood agar plate, followed by incubation at 25°C for 3 days. Cells were scraped off the plate and resuspended in TSB-1, and 100-μl aliquots of serial decimal dilutions were spread onto TSA-1 plates with the corresponding antibiotic combinations to select for donors and transconjugants. The frequency of conjugal transfer, when necessary, was calculated as the number of transconjugants per donor cell.

Mutant construction and gene complementation. Single and double nonpolar deletions were constructed by using PCR amplification of the amino- and carboxy-terminal fragments of each gene which, when fused together, would result in an in-frame deletion. Amplification was carried out using Hi-Fidelity Kapa Taq (Kapa). Allelic exchange was performed as previously described using the Km^r suicide vector pNidKan, containing *sacB* gene conferring sucrose sensitivity and R6K *ori* requiring the *pir* gene product for replication (39). The plasmid constructions containing the deleted alleles were mated from *E. coli* S17-1 λ*pir* into a rifampin-resistant derivative (AR57) of *P. damselae* subsp. *damselae* RM-71, selecting for kanamycin resistance for plasmid integration and subsequently for sucrose resistance (15% [wt/vol]) for a second recombination event. This led to the obtention of *P. damselae* subsp. *damselae dly*, *hlyA*, and double *dly hlyA* mutant strains (Table 1). The presence of the correct alleles was confirmed by PCR. For complementation, *dly* and *hlyA* ORFs, together with their respective promoter sequences, were PCR amplified with Hi-Fidelity Kapa Taq, cloned into pHRP309 vector and mobilized from *E. coli* S17-1 λ*pir* into the respective *P. damselae* subsp. *damselae dly* and *hlyA* mutants, as well as into *P. damselae* subsp. *damselae* ATCC 33539.

Curation of pPHDD1. Curation of pPHDD1 was attempted by using RM-71 derivative strains containing a first crossover of the suicide vector constructions used for generation of the *hlyA* and the *dly* mutants (Table 1). This approach takes advantage of the inability of the suicide vector pNidKan to replicate in *P. damselae* subsp. *damselae* and of its positive (Km^r) and negative (sucrose sensitivity) selectable properties. After several passages on LB without selection, the first crossover strains were grown on LB agar with 15% sucrose, and colonies were tested for kanamycin sensitivity. Colonies that had lost the suicide plasmid were further tested on sheep blood agar plates for hemolysis reduction, as well as for lack of pPHDD1 markers by PCR, until pPHDD1-negative clones were found. As explained below, curation of pPHDD1 was a very rare event that could only be achieved from first crossover strains that had undergone additional mutations or DNA sequence loss that altered the hemolytic phenotype and rendered the strain unable to grow at 37°C.

Mice and fish virulence assays. Virulence assays were carried on with BALB/c mice (6 to 8 weeks old, 26 to 30 g), as well as with turbot (*Psetta maxima*) (average weight, 15 g), in groups of five animals. The inoculum was prepared by suspending several colonies from a 24-h TSA-1 culture into saline solution to achieve the turbidity of a no. 2 McFarland standard. Mice were inoculated at the tail vein with 50 μl of a 2.5 μM hemoglobin solution (8 μg hemoglobin per mouse) 2 h before inoculation with the bacterial suspension, as previously described (19). Mice were inoculated intravenously at the tail vein, and turbot were inoculated intraperitoneally, with 0.1 ml of 10-fold serial dilutions of the bacterial suspensions and the actual number of injected CFU was determined by plate count on TSA-1. The final doses assayed corresponded to 2.1×10^6 and 2.1×10^5 bacterial cells per mice and 2.1×10^4 and 2.1×10^3 bacterial cells per fish. The mortalities were recorded daily for 3 days (mice) and 4 days (turbot), and the degree of virulence was expressed as percent values.

RESULTS

Diversity of hemolytic phenotypes in *P. damselae* subsp. *damselae* isolates and correlation with *dly* gene presence. We conducted a hemolysis screening on sheep blood agar for a collection of 17 *P. damselae* subsp. *damselae* strains from different origins (Table 2), and four distinct phenotypes were found. Among the 17 strains, 6 showed a large hemolysis halo

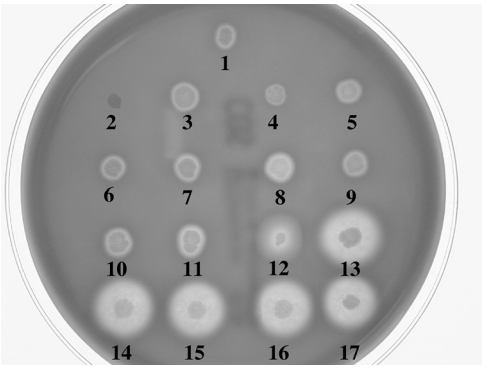

FIG. 1. Hemolytic phenotypes of *P. damselae* subsp. *damselae* strains on sheep blood agar. Strains (spot number, strain): 1, PC586.1; 2, J3G801; 3, ATCC 35083; 4, 238; 5, 192; 6, PG801; 7, 162; 8, 158; 9, 309; 10, 340; 11, LD-07; 12, ATCC 33539; 13, CDC2227-81; 14, RM-71; 15, RG-214; 16, RG-153; 17, RG-91. Four distinct phenotypes are recognized: large hemolysis (LH) (strains at spots 13 to 17), moderate hemolysis (MH) (strain 12), small hemolysis (SH) (strains at spot 1 and spots 3 to 11) and no hemolysis (NH) (strain at spot 2). The strains at spots 12 to 17 are positive for pPHDD1 markers.

(LH), one was nonhemolytic (NH), one showed moderate hemolysis (MH), and the remaining 9 strains yielded a small hemolytic halo (SH) on sheep blood agar plates (Fig. 1 and Table 2). In order to assess the correlation between *dly* gene presence and hemolytic phenotype, we PCR tested the strain collection using specific primers for *dly* gene (42). We found that the strains positive for *dly* (strains 12 to 17) ((Fig. 1 and Table 2) showed a hemolytic halo whose radius was ca. 5 to 10 times larger than that produced by the *dly*-negative strains (Fig. 1). In light of these results, the presence of *dly* is linked to the ability of *P. damselae* subsp. *damselae* to cause a large hemolytic halo on blood agar plates (Fig. 1 and Table 2), whereas the small hemolytic halo is characteristic of strains that lack *dly*.

Damselysin is encoded on a large plasmid in *P. damselae* subsp. *damselae* RM-71. In order to characterize the genetic context of *dly*, we constructed a cosmid library of the highly hemolytic strain RM-71. This strain was selected because of its strong hemolytic phenotype and because it was reported to be as virulent for mice as the clinical strain CDC2227-81 and more virulent for fish (17). A total of 316 clones were streaked on sheep blood agar and two beta-hemolytic cosmids that tested positive by PCR for *dly* gene were identified. We found five overlapping cosmids whose 5′ and 3′ end sequences tested negative by PCR in *dly*-negative strains and accounted for a circular structure. The five cosmids were subjected to 454 DNA sequencing, yielding a 153,429-bp circular molecule that constituted a novel *P. damselae* subsp. *damselae* plasmid which was dubbed pPHDD1 and that contains *dly* gene (Fig. 2). In order to have physical evidence of pPHDD1, plasmid DNA was isolated from three *dly*-positive strains (RM-71, ATCC 33539, and RG-91) and from one *dly*-negative isolate (PG801). An ~150-kb plasmid band (100 MDa) was evident in agarose gels in the three *dly*-positive strains but not in the *dly*-negative strain (data not shown).

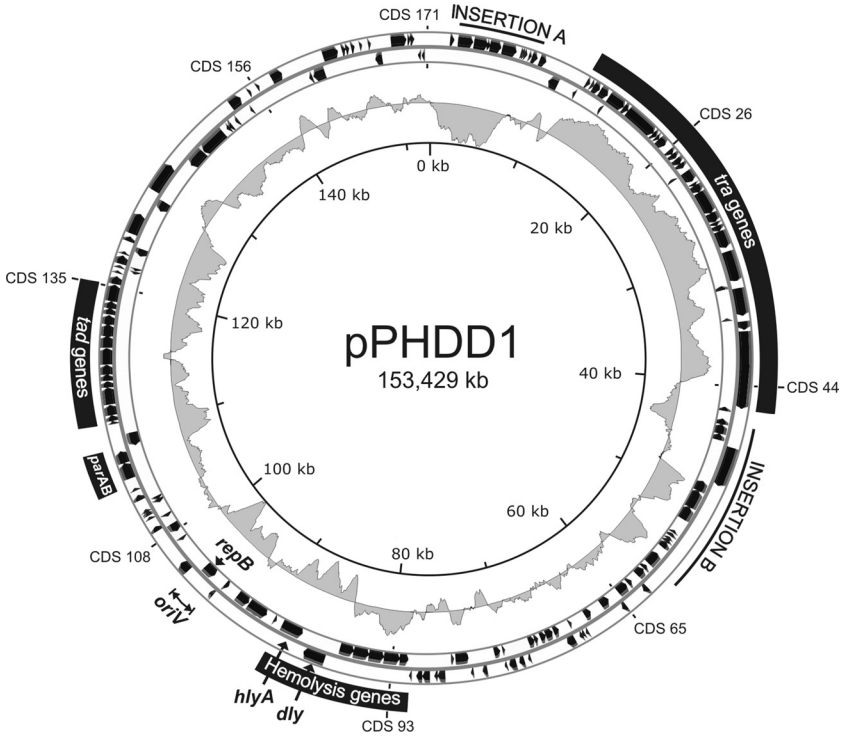

FIG. 2. Circular representation of the *P. damselae* subsp. *damselae* pPHDD1 plasmid. The first circle (i.e., the outermost) represents pPHDD1 ORFs (in black, two rows of arrows corresponding to each of the two DNA strands, respectively), and specific genes, replication origin (*oriV*) as well as functional modules and insertions are highlighted. The next circle (moving inward) shows the G+C percent variation. The innermost circle shows the nucleotide positions in 20-kb intervals.

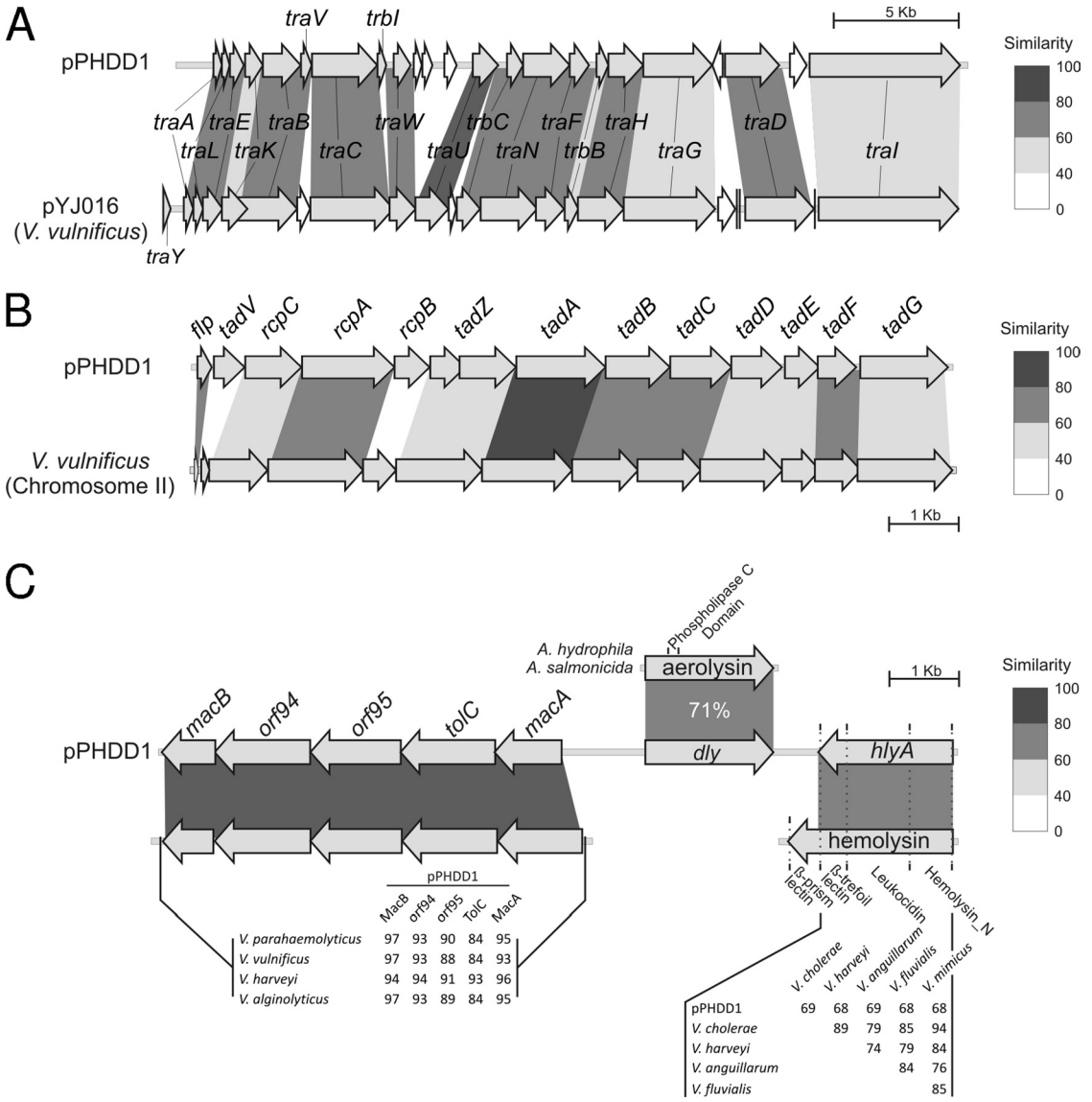

FIG. 3. Physical maps of *tra* (A), *tad* (B), and hemolysin (C) clusters of *P. damselae* subsp. *damselae* pPHDD1 plasmid compared to related genes in other species of marine bacteria. pPHDD1 *tra* genes without counterpart in the species being compared are filled in white. The percent similarity refers to the amino acid sequences of the predicted proteins and is represented in similarity intervals in grayscale tones according to the scale depicted at the right of the figures. In panel C, similarity percent values between pairs of species are shown either in the shaded region (for Dly) or in data matrices.

Genetic structure of pPHDD1. The complete sequence of pPHDD1 consists of 153,429 bp and encodes 172 predicted ORFs (Fig. 2). The average G+C content was 37.9%, which is comparable to the G+C content of the *P. damselae* subsp. *damselae* reference strain genome (40%). The G+C content distribution is heterogeneous along the plasmid (Fig. 2), varying from 62.5 to 11.6% using a 120-bp window. Five modules can be highlighted in pPHDD1: a replication module, a partitioning module, a conjugation machinery module, a *tad* (tight adherence) module, and a hemolysin module (Fig. 2).

The nucleotide sequence upstream and downstream of the *repB* gene shows a high A+T value, and downstream of the *repB* there are three directed tandem repeats of the 9-mer TAAGATCTA that might correspond to iterons. These data suggest that the region surrounding *repB* might contain the pPHDD1 replication origin. In support of this, we found that a PCR-amplified 2.6-kb region that included *repB*, and the putative iteron sequences ligated to a kanamycin resistance gene was capable of independent replication into *E. coli* DH5-α (data not shown). The putative partitioning module (*parA* and *parB* genes) bears similarity to the *par* genes of *Enterobacteriaceae* plasmids. No evident addiction system genes, such as toxin/antitoxin genes, were found in pPHDD1 sequence by homology search.

pPHDD1 contains a set of *tra* genes encoding homologues of proteins of type IV secretion systems described in plasmids of other marine bacteria (Fig. 3A), suggesting that pPHDD1 might be conjugative. In this regard, we were able to detect

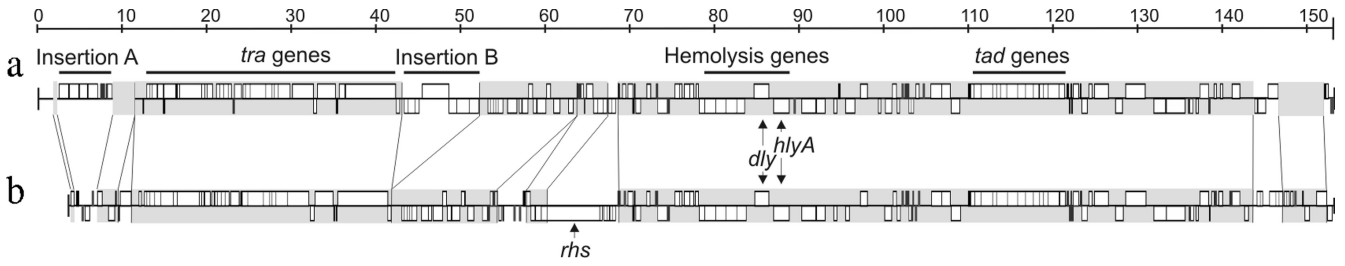

FIG. 4. Conservation of synteny between *P. damselae* subsp. *damselae* RM-71 pPHDD1 plasmid (a) and contig 55 from *P. damselae* subsp. *damselae* ATCC 33539 (GenBank accession no. NZ_ADBS01000004) (b). Conserved blocks of synteny between the two sequences are indicated in gray. Vertical and diagonal lines connect regions of synteny that are separated by insertions specific to one of the two molecules. Two main pPHDD1 insertions (insertions A and B), functional modules (*tra*, *tad*, and hemolysis clusters), and gene names (*rhs*, *dly*, and *hlyA*) are highlighted.

conjugative transfer of a marked version of this plasmid to *P. damselae* subsp. *piscicida* although at a very low frequency (see below). pPHDD1 also contains a complete set of *tad* (tight adherence) genes that likely encode the biogenesis of the Flp subfamily of pili and show synteny with *tad* clusters of *Vibrio* species (Fig. 3B). These pili, initially described in *Aggregatibacter actinomycetemcomitans* as mediators of a tight-adherence phenotype, are involved in adhesion to abiotic surfaces and host cell attachment (49).

One of the most notable aspects of pPHDD1 is the presence of a hemolysin cluster that contains five genes of a putative secretion system, as well as two hemolysin genes that encode the previously characterized Dly toxin and a hitherto uncharacterized pore-forming toxin HlyA, respectively (Fig. 3C). Although the activity of Dly was known for the past 2 decades, the complete sequence of *dly* gene remained unpublished until now. We found that the amino acid sequence of Dly bears little homology to known proteins and shows no conserved domains other than a putative phospholipase C domain. Only two homologues of Dly exist in databases: the phospholipase C aerolysins of *Aeromonas hydrophila* (37) and *A. salmonicida* (24), respectively (Fig. 3C). The gene for HlyA lies downstream of *dly* and is transcribed from the opposite strand. HlyA has similarity with HlyA pore-forming toxins with hemolytic activity described in *Vibrio* species, with similarity values ranging from 68 to 69% (Fig. 3C). HlyA hemolysins are predicted to form heptameric pore structures into the erythrocyte membrane, altering its permeability (23). A Pfam database search predicted in *P. damselae* subsp. *damselae* HlyA three conserved domains that are shared with HlyA of *Vibrio cholerae* (23, 40, 52) and other *Vibrio* species, albeit some *Vibrio* HlyAs contain an additional fourth domain at their C terminus that is absent in *P. damselae* (Fig. 3C).

The five genes upstream *dly* encode a putative secretion system that might be involved in the secretion of the two hemolysins. This system includes an inner membrane ATPase component homologous to *macB* (32), two ATP-binding/permease components, a membrane fusion protein, and TolC, respectively. In *E. coli*, TolC is specifically required for HlyA secretion (50). Interestingly, highly conserved homologues of these five proteins are found in several *Vibrio* genomes (Fig. 3C). It is noteworthy that these homologues have chromosomal locations in the *Vibrio* species and are not closely linked to Dly and HlyA homologues (data not shown).

Distribution and variability of pPHDD1 among *P. damselae* subsp. *damselae* isolates. While the present study was under way, the genome sequence of the *P. damselae* subsp. *damselae* type strain ATCC 33539 was made available in public databases under several separate contigs (GenBank accession number ADBS00000000). One contig (contig 55) of 149,928 bp showed a high degree of synteny with the pPHDD1 sequence that we report here (strain RM-71). However, we found some differences between these two sequences, pPHDD1 being 3,501 bp larger (Fig. 4). Contig 55 harbors an *rhs* gene that is absent from pPHDD1, and two DNA regions of pPHDD1 are absent from the ATCC 33539 contig 55. These two DNA regions were dubbed insertion A (6.6 kb) and insertion B (11 kb), respectively (Fig. 2 and 4). Insertion A has a G+C content lower than the average of the plasmid and comprises 10 truncated ORFs that constitute pseudogenes. Insertion B comprises 11 predicted ORFs, two of them encoding distinct phage integrases with low homology to *Shewanella* integrases. Interestingly, we found that insertion B is unstable since a spontaneous deletion of a large part of it was observed in a transconjugant *P. damselae* subsp. *piscicida* clone that received pPHDD1 by conjugative transfer (see below). To get an insight into the molecular mechanism underlying this loss of DNA sequences, the region involved in the excision was PCR amplified and sequenced. We found that the excised region corresponded to a 9,202-bp sequence flanked by a perfect 12-bp DNA direct repeat (CGTGGGGTGTCA). It is tempting to speculate that this direct repeat might be the target of one of the integrases encoded by *orf51* and *orf52*.

In order to get an insight into the genetic diversity among pPHDD1-like plasmids, we conducted a PCR screening of seven interspersed pPHDD1 gene markers—*dly*, *hlyA*, *parA*, *vep07*, *tolC*, and the two Tad genes *rcpA* and *tadC*—in the collection of *P. damselae* subsp. *damselae* strains. Of the 17 strains tested, 6 gave a positive result for the seven markers, whereas the remaining 11 strains tested negative (Table 2). These results suggest the genetic linkage between the assayed genes and show the existence of differential distribution of pPHDD1-like plasmids among strains of this pathogen. Moreover, the three strains in which physical presence of pPHDD1 was demonstrated by gel electrophoresis (see above) tested positive for the markers, whereas PG801 strain tested negative, confirming the direct relationship between the presence of the ~150-kb plasmid band and pPHDD1 markers. As expected,

we found a correlation between presence of pPHDD1 markers and the production of a large or moderate hemolytic halo in the strains that had previously tested positive for the *dly* gene by PCR (strains 12 to 17) (Fig. 1, Table 2). When we PCR tested two pPHDD1 genes (*repA* and *orf2*) that are part of the two DNA insertions A and B, respectively, all of the strains except RM-71 yielded a negative result (Table 2), indicating that insertions A and B are unique to strain RM-71.

pPHDD1 confers hemolytic activity to *P. damselae* subsp. *piscicida* upon conjugative transfer. Further evidence of the pPHDD1 involvement in the hemolysis activity of *P. damselae* could be gained by attempting to transfer pPHDD1 by conjugation into a strain of the nonhemolytic subspecies *P. damselae* subsp. *piscicida* and analyzing the phenotypic changes in the recipient. As described above, pPHDD1 encodes a set of *tra* genes, suggesting that this plasmid is conjugative. We mated the kanamycin-labeled *P. damselae* subsp. *damselae* AR61 (Table 1) as a donor and the tetracycline-resistant *P. damselae* subsp. *piscicida* PC554.2 as a recipient. As a result, tetracycline/kanamycin-resistant *P. damselae* subsp. *piscicida* transconjugants were isolated at a low frequency of $\sim 10^{-8}$. These transconjugants showed a reduced hemolysis due to the fact that they contained the insertion of the suicide plasmid within the *hlyA* gene. We therefore selected for suicide plasmid loss on sucrose plates and searched for clones that had lost the suicide vector but that restored the wild-type gene. Doing this, we isolated the kanamycin-sensitive *P. damselae* subsp. *piscicida* AR83. This strain tested positive by PCR for eight pPHDD1 gene markers, as well as for several subsp. *piscicida*-specific gene markers (data not shown). This indicates that pPHDD1 is an independent replicon capable of undergoing conjugative transfer. We found that AR83 produced hemolysis on sheep blood agar plates (Fig. 5), although the halo was ca. 63% of that produced by subsp. *damselae* parental strain AR57 (rifampin-resistant derivative of RM-71). The hemolysis caused by AR83 on human and rat blood agar plates was very weak compared to AR57, suggesting that either additional factors non-pPHDD1 encoded are involved in the production of the strong hemolytic phenotype showed by AR57 or that the expression of the hemolytic determinants in subsp. *piscicida* cells does not achieve the optimal conditions.

HlyA and Dly contribute to hemolysis in *P. damselae* subsp. *damselae*. To date, the hemolytic activity of *P. damselae* subsp. *damselae* has been explained exclusively on the basis of *dly* gene, having been reported a direct relationship between highly hemolytic *P. damselae* subsp. *damselae* strains and the presence of the *dly* gene (9). However, our finding of an HlyA family hemolysin gene in pPHDD1 plasmid raises the question whether Dly is the only responsible of hemolysis or whether HlyA also plays a role. Based on previously described homologues in vibrios, the *P. damselae* subsp. *damselae* HlyA is predicted to be a protein with hemolytic activity. In order to unravel the contribution of Dly and HlyA, we analyzed single *dly* (AR64), single *hlyA* (AR133), and double *dly hlyA* (AR78) knockout mutants.

Since *P. damselae* subsp. *damselae* is a marine bacterium that causes disease in marine animals but is also known to cause opportunistic disease and death in humans, we evaluated the hemolytic phenotype on homeotherm (sheep, human, and rat) and poikilotherm (turbot) blood sources. Hemolysis was assayed at 25°C for the four blood types (Fig. 6A) and also at 37°C in two homeotherm blood types (Fig. 6B). We observed that each erythrocyte source had a different susceptibility to be hemolyzed by the parental strain. Turbot and rat erythrocytes, followed by sheep blood, showed the largest hemolysis halos at 25°C after 15 h, whereas human blood showed low susceptibility. Interestingly, when the assays were carried out at 37°C, a larger but less translucent halo was observed, and this halo disappeared in the *dly* mutants, which suggested that it is due to Dly (Fig. 6B).

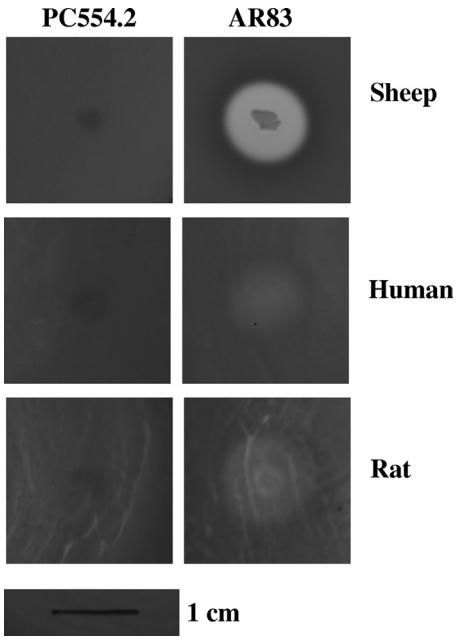

FIG. 5. Hemolytic activity in sheep, human, and rat blood agar plates of *P. damselae* subsp. *piscicida* PC554.2 and AR83 (the transconjugant that acquired pPHDD1 after conjugative transfer). Scale bar, 1 cm.

Although different results were observed with the *dly* mutant depending on the erythrocyte source, we found that mutation of *dly* did not completely abolish hemolysis in any of the four erythrocyte sources. This indicates that Dly is not the only cause of the hemolytic phenotype in pPHDD1-harboring strains, although it clearly contributes to the production of a phenotype of large hemolytic halo (LH). We therefore wanted to test the contribution of *hlyA* to hemolysis. Interestingly, mutation of *hlyA* caused only a slight reduction in the radius of the hemolysis halo on the four blood sources assayed. Based on these results we can propose that hemolysis in *P. damselae* subsp. *damselae* is mainly due to the sum of the contributions of Dly and HlyA. In order to demonstrate this hypothesis, we assayed the effect of the deletion of the two hemolysis genes. As expected, the *hlyA dly* double mutant (AR78) showed a >80% reduction in the hemolytic halo on sheep, rat, human, and turbot blood, which decreased to levels similar to those observed for the pPHDD1-negative strains on sheep erythrocytes (Fig. 6; see also Fig. 1). These results suggest the existence of an additive effect between Dly and HlyA to produce hemolysis and also suggest that Dly is a major contributor to hemolysis on rat, human, and turbot erythrocytes.

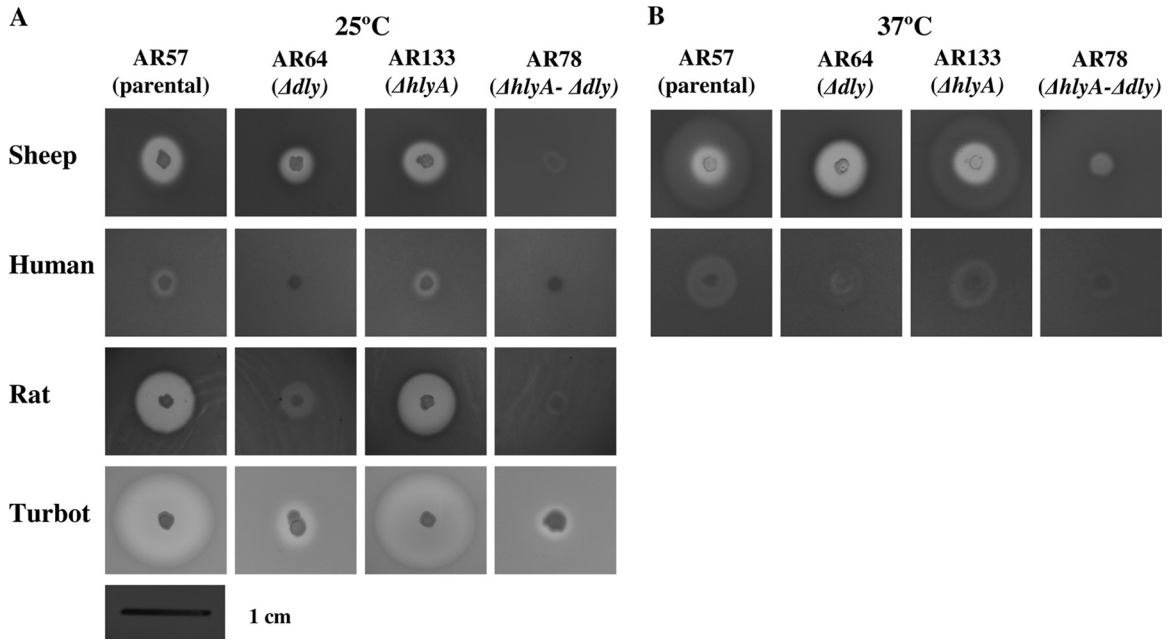

FIG. 6. Hemolytic activity in sheep, human, rat, and turbot blood agar plates of *P. damselae* subsp. *damselae* parental strain and mutants at 25°C (A) and 37°C (B). Strains: AR57 (parental strain), AR64 (Δ*dly*), AR133 (Δ*hlyA*), AR78 (Δ*dly* Δ*hlyA* double mutant). Pictures were taken after 15 h of growth. Scale bar, 1 cm.

In order to gain additional information on the contribution of Dly and HlyA to the hemolytic phenotype and to assess whether the two hemolysins show a synergistic as well as an additive effect, we assayed the hemolytic phenotype conferred by each individual gene and by the two genes together to *E. coli* DH5-α. Interestingly, *E. coli* cells harboring either *hlyA* or *dly* gene showed small hemolytic halos on sheep blood agar plates (Fig. 7A), the halo of the *hlyA* gene being more translucent than that conferred by *dly*. Interestingly, when the two genes were introduced into *E. coli*, the hemolytic halo produced was significantly larger than the mere addition of the two individual halos (Fig. 7A). This result suggests the existence of a synergistic effect between the two hemolysins.

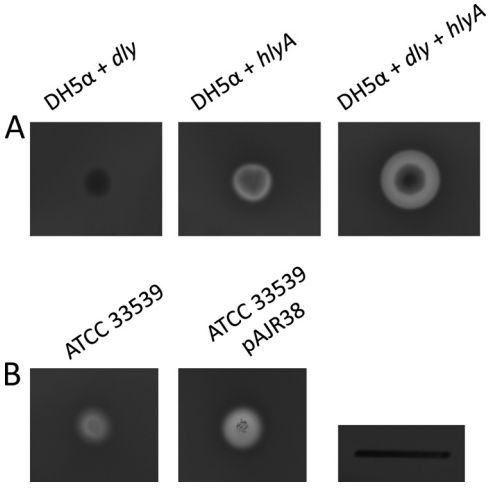

FIG. 7. (A) Complementation of *E. Coli* DH5-α with *dly* and *hlyA* genes of strain RM-71, cloned in plasmids pAJR29 and pAJR27. (B) Complementation of *P. damselae* subsp. *damselae* ATCC 33539 with the *hlyA* gene of strain RM-71, cloned in plasmid pAJR38. Scale bar, 1 cm.

It is interesting that the type strain ATCC 33539 is positive for pPHDD1 markers, while it only shows moderate hemolysis (MH) on sheep blood agar, whereas all of the other pPHDD1-positive strains show a large hemolysis (LH) halo (Fig. 1). We compared the nucleotide sequences of *hlyA* genes between RM-71 and ATCC 33539 and found a number of amino acid substitutions (data not shown). Hence, we hypothesized that ATCC 33539 might produce a protein with reduced hemolytic activity. We therefore conjugally transferred into ATCC 33539 a plasmid (pAJR38) containing the cloned *hlyA* gene from RM-71 and found a restoration of the hemolytic phenotype at levels similar to those of RM-71 (Fig. 7B). This observation suggests that the moderate hemolytic halo of the type strain could be in part due to substitutions in the ATCC 33539 HlyA sequence with respect to RM-71 HlyA.

We tried to cure pPHDD1 from AR57 derivatives containing the first crossover insertions of the suicide plasmids used to obtain the *dly* and *hlyA* mutants (see Materials and Methods), but all of the attempts were unsuccessful. We finally obtained to some extent a first crossover insertion for the *hlyA* mutant construction that, when selecting for loss of the complete pPHDD1-suicide plasmid cointegrate, yielded cured colonies at a very high frequency (data not shown). However, we noted that this first crossover insertion was unable to grow at 37°C and showed a considerable reduction in the hemolytic activity that could not be restored by complementation with *dly* and *hlyA* (data not shown). Thus, curation of pPHDD1 could only be selected under circumstances that likely involved the loss of other genomic sequences or the occurrence of spontaneous mutations elsewhere on the genome.

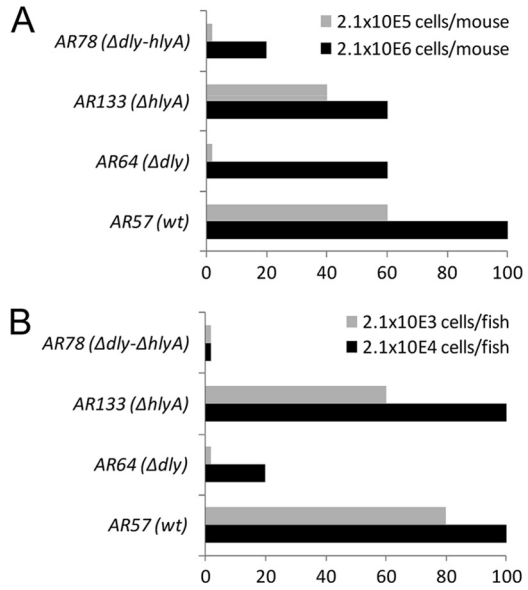

FIG. 8. Mouse (A) and fish (B) virulence assays with *P. damselae* subsp. *damselae* strains, using two different doses. The results are expressed as the percent mortality.

Contribution of *dly* and *hlyA* to *P. damselae* subsp. *damselae* virulence for mice and fish. Dly has been recognized as a cytolytic toxin with lethal activity for mice. We assayed the roles of *dly* and *hlyA* on the ability of *P. damselae* subsp. *damselae* to cause death in mice after tail vein inoculation, using two doses of 2.1×10^6 and 2.1×10^5 CFU per mouse. Mice died within 12 to 48 h postinfection, and *P. damselae* subsp. *damselae* was recovered from the spleen and liver as pure cultures. The parental strain caused death in 100% of mice inoculated with 2.1×10^6 cells and in 60% of mice inoculated with 2.1×10^5 cells (Fig. 8A). However, we found that all of the mutants showed some degree of reduction in their virulence. Interestingly, although the single Δ*hlyA* and *dly* mutants still maintained the ability to kill 60% of mice with the high dose, we found that the *dly* mutant was unable to cause death at the lower dose. Since the hemolytic activity results on blood agar suggested the existence of both an additive and synergistic effect between Dly and HlyA, we wanted to test the effect that mutation of the two hemolysin genes had on virulence. We found that the *hlyA dly* double mutant caused the death of only one animal at the high dose, suggesting that a synergistic effect between Dly and HlyA is necessary for maximal virulence in mice.

Since *P. damselae* subsp. *damselae* RM-71 was isolated from diseased turbot, we also assayed the effect of *dly* and *hlyA* mutations on the virulence for fish by intraperitoneal inoculation, using two doses of 2.1×10^4 and 2.1×10^3 CFU per fish. The fish died within 24 to 72 h postinfection, and *P. damselae* subsp. *damselae* was recovered from the kidney as pure cultures. The parental strain caused death in 100% of fish inoculated with 2.1×10^4 cells and in 80% of fish inoculated with 2.1×10^3 cells (Fig. 8B). The Δ*hlyA* mutant only showed a slight reduction in virulence at the lower dose but maintained the same virulence values of the parental strain at the high dose. However, the Δ*dly* mutant only killed 20% of fish at the high dose and no fish at the low dose. No fish deaths were recorded with the *hlyA dly* double mutant. These results suggest not only that Dly and HlyA play a synergistic effect in virulence for fish but also that Dly is the main contributor of the two hemolysins in *P. damselae* subsp. *damselae* virulence for fish. Based on the results obtained with the single and double mutants, it is clear that plasmid pPHDD1 is necessary for maximal virulence in mice and fish infected with *P. damselae* subsp. *damselae*.

DISCUSSION

Strains of *P. damselae* subsp. *damselae* have been isolated from aquatic environments and as causative agents of disease in a variety of aquatic animals and humans (41). Despite recent reports of fatal human cases due to this bacterium (2, 53), knowledge of the genetic basis of virulence of this bacterium remained quite limited to early studies on Dly toxin. The virulence of *P. damselae* subsp. *damselae* in mice has been previously correlated with the ability to produce Dly (28, 29). Similarly, the symptoms caused by this bacterium in fish have been related to the ability to produce extracellular products, which included phospholipase and hemolysin activities (16). The genomic location of Dly gene remained elusive until now. We found that *dly* is encoded on pPHDD1 in strain RM-71, a novel 153-kb plasmid of *P. damselae* subsp. *damselae*. In accordance with our findings, previous studies reported that all of the strongly hemolytic isolates contained a ca. 150- to 170-kb plasmid, but not the weakly hemolytic ones, with the exception of ATCC 33539 that contained a plasmid but was moderately hemolytic (18, 47). Like many large plasmids, pPHDD1 appears to have a mosaic-like structure due to modular evolution processes in which DNA sequence stretches are acquired by horizontal gene transfer and reorganized by general recombination, transposition, and site-specific recombination (14, 15). In this sense, it is noticeable that different pPHDD1 modules show similarity to plasmid-borne genes from different bacterial taxa. Although the *tra* and *tad* genes bear similarity to *Vibrio* plasmids, the *par* genes are highly similar to the sequences of *Enterobacteriaceae* plasmids.

Tad clusters are involved in the pathogenesis of several bacteria (8, 49). Interestingly, this is the first report of a plasmid-borne Tad cluster in a member of the *Vibrionaceae*, which suggests that conjugative transfer is one of the mechanisms for Tad cluster genes spread in the marine environment. We found that pPHDD1 can be mobilized to *P. damselae* subsp. *piscicida* but at a very low frequency. The reasons for this low transfer rate are unknown. Possible explanations include plasmid incompatibility or entry exclusion mechanisms due to residing plasmids or the necessity for SOS response induction or any environmental stress signal in order to trigger conjugative transfer (4). Although no evident addiction systems were found in pPHDD1 by protein homology searches, curation of this plasmid was only achieved as a rare event that likely involved the occurrence of additional mutations or DNA loss elsewhere in the genome. The reasons for this resistance to curation, as well as the mutations that likely allow pPHDD1 to be lost from cells, are currently unknown.

The existence of two main hemolytic categories among *P. damselae* subsp. *damselae* strains has been largely reported,

with a clear distinction between strongly hemolytic and weakly hemolytic strains on blood agar plates (7, 30, 42). Southern blot analysis revealed that the *dly* gene was found only in highly hemolytic strains (9). In accordance with these previous observations, in the present study we found a direct correlation between pPHDD1 and the *dly* gene and the production of large hemolytic halos on sheep blood agar.

A variety of erythrocyte sources from homeotherms have been reported to be sensitive to *P. damselae* subsp. *damselae* cells and extracellular products (28). In addition, it was known that turbot erythrocytes were sensitive to the extracellular products of *P. damselae* subsp. *damselae* cells (16). To date, hemolysis caused by *P. damselae* subsp. *damselae* was explained exclusively in terms of Dly, although previous studies had suggested the possibility that other hemolysins might be produced (27). Our study has brought into play a hitherto-unknown factor, the pore-forming toxin HlyA. Our experiments with single and double mutants demonstrated that Dly is not only responsible for the strong hemolytic phenotype in *P. damselae* subsp. *damselae*. Rather, we found that both Dly and HlyA contribute to hemolysis. Interestingly, since it can be concluded from the results with the Δ*dly* mutant, Dly makes a differential contribution to hemolysis according to the erythrocyte source, being the major contributor in rats, turbot, and humans. This selectivity might be explained by the different lipid compositions of erythrocyte membranes of each species (25). The lipid composition of mammal erythrocyte membranes is almost identical, with the exception of sphingomyelin being partially replaced equimolarly by phosphatidylcholine in various species and having a direct effect on membrane thermostability (25). That Dly toxin shows phospholipase D activity against sphingomyelin (29) might explain the different degrees of hemolysis observed in the Δ*hlyA* and Δ*dly* mutants depending on the blood source tested. In addition, a possible connection between sphingomyelin content, Dly-mediated hemolysis, and temperature cannot be ruled out. Since sphingomyelin plays a role in membrane thermostability, the removal of sphingomyelin choline head groups by Dly might explain the wider turbid hemolytic halo observed at 37°C in parental and Δ*hlyA* strains (the two producing Dly) with respect to the observed halos at 25°C.

Although the hypothesis of an additive effect between Dly and HlyA might explain most of the hemolytic halos observed in the different mutant combinations and blood sources, there is also evidence that Dly and HlyA may act in a synergistic manner. The experiments carried out in *E. coli* suggest that the effect of the two hemolysins being produced at the same time in the same cell is stronger than the mere addition of their individual contributions. Synergistic effects between hemolysins have been well documented (5, 33, 46). An explanation of how Dly and HlyA interact synergistically can be drawn from the data available for *V. cholerae* cytolysin VCC. This hemolysin has specificity for cholesterol, which is essential for oligomerization and pore formation (56). It has been proposed that the choline head group of sphingomyelin has an inhibitory effect on VCC pore formation since it shields the cholesterol ring from VCC, constituting the so-called umbrella model (55, 56). This model might be applied to the synergistic effect between Dly and HlyA in *P. damselae* subsp. *damselae*, where Dly's removal of the choline head group from sphingomyelin would allow HlyA to enhance its hemolytic activity.

The finding that pPHDD1-negative strains produce weak hemolytic halos, together with the observation that the double mutant still produces small halos, clearly suggests the existence of additional hemolysins non-pPHDD1 encoded in *P. damselae* subsp. *damselae* strains. In this regard, previous studies had reported the existence of one major and two minor components with hemolytic activity in *P. damselae* subsp. *damselae* supernatants by thin-layer isoelectric focusing assays (27). An *in silico* analysis of the *P. damselae* subsp. *damselae* ATCC 33539 genome reveals the existence of two genes annotated as encoding putative hemolysin-like proteins (GenBank accession no. ADBS00000000; ORFs VDA_003208 and VDA_002420). These putative hemolysins might be responsible for the basal small hemolytic halos observed both in the double mutant and in the pPHDD1-negative strains, and further studies are necessary to ascertain the role of these proteins in hemolysis. Similarly, the observation that a transconjugant *P. damselae* subsp. *piscicida* that received pPHDD1 produced smaller hemolytic halos than did the subsp. *damselae* parental strain might also be partly explained by the existence of non-pPHDD1-encoded hemolysins that contribute to hemolysis.

Our results demonstrate that pPHDD1 is necessary for full virulence of *P. damselae* subsp. *damselae* for mice and fish. The double mutant, lacking pPHDD1-encoded *hlyA* and *dly* genes, caused death in only 20% mice at the high dose and 0% of fish at the two doses assayed, whereas the two single mutants proved to be more virulent than the double mutant. We found that mutation of *hlyA* had less effect on virulence than mutation of *dly* in the two animal models. This observation was consistent with the results obtained in the hemolysis assays, in which the *hlyA* mutant produced larger hemolysis halos than the *dly* mutant. The contribution to virulence of these two genes together was higher than the sum of their individual contributions, which suggests that a synergy between Dly and HlyA toxin is necessary for maximal virulence.

We have found that pPHDD1 is widespread in *P. damselae* subsp. *damselae* strains isolated from diseased marine fish. Thus, strains of this pathogen inhabiting the aquatic environments and infecting poikilotherm animals contain virulence factors that might be of potential concern for human health. Future comparative studies of the genomes of *dly*-positive and plasmidless *P. damselae* subsp. *damselae* strains isolated from mammals and poikilotherms will help to elucidate the key features that allow this marine bacterium to cause disease and explain how pPHDD1 constituted a driving force for the emergence of the highly hemolytic lineage of *P. damselae* subsp. *damselae*.

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