Synergistic and Additive Effects of Chromosomal and Plasmid-Encoded Hemolysins Contribute to Hemolysis and Virulence in Photobacterium damsela subsp. damsela

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Photobacterium damsela subsp. damsela causes infections and fatal disease in marine animals and in humans. Highly hemolytic strains produce damselfin (Dly) and plasmid-encoded HlyA (HlyA\(_{pl}\)). These hemolysins are encoded by plasmid pPHDD1 and contribute to hemolysis and virulence for fish and mice. In this study, we report that all the hemolytic strains produce a hitherto uncharacterized chromosome-encoded HlyA (HlyA\(_{ch}\)). Hemolysis was completely abolished in a single hlyA\(_{ch}\) mutant of a plasmidless strain and in a dly hlyA\(_{pl}\) hlyA\(_{ch}\) triple mutant. We found that Dly, HlyA\(_{pl}\), and HlyA\(_{ch}\) are needed for full hemolytic values in strains harboring pPHDD1, and these values are the result of the additive effects between HlyA\(_{pl}\) and HlyA\(_{ch}\) on the one hand, and of the synergistic effect of Dly with HlyA\(_{pl}\) and HlyA\(_{ch}\) on the other hand. Interestingly, Dly-producing strains produced synergistic effects with strains lacking Dly production but secreting HlyA, constituting a case of the CAMP (Christie, Atkins, and Munch-Petersen) reaction. Environmental factors such as iron starvation and salt concentration were found to regulate the expression of the three hemolysins. We found that the contributions, in terms of the individual and combined effects, of the three hemolysins to hemolysis and virulence varied depending on the animal species tested. While Dly and HlyA\(_{pl}\) were found to be main contributors in the virulence for mice, we observed that the contribution of hemolysins to virulence for fish was mainly based on the synergistic effects between Dly and either of the two HlyA hemolysins rather than on their individual effects.

The marine bacterium Photobacterium damsela subsp. damsela (formerly Vibrio damsela) is a primary pathogen for a variety of marine animals, such as sharks, dolphins, crustaceans, mollusks, and fish (1–3). In addition, this bacterium can cause a highly severe necrotizing fasciitis in humans that may lead to a fatal outcome (4–7).

Early studies described that P. damsela subsp. damsela isolates from marine animals and from human clinical cases exhibited hemolytic activity (8, 9). It was reported that a correlation between the ability of P. damsela subsp. damsela to cause disease in mice and the production of large amounts of a cytolytic toxin with hemolytic activity that was later named damselfin (Dly) existed (8). Dly was characterized as a cytolsin with phospholipase D activity against sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine, and its main molecular activity consists of the removal of the polar choline groups of choline-containing membrane lipids (10, 11). The removal of the polar choline phosphate head group of sphingomyelin by Dly was found to enhance the hemolytic effect of staphylococcal delta-toxin, an observation that constituted the first evidence that Dly can act synergistically with hemolysins produced by other cells (11). The term “synergy” defines a process in which one or more factors in a system has intrinsic lytic activity and the combined factors cause erythrocyte lysis at a rate greater than the sum of the individual rates (12). One well-described synergistic effect is the so-called CAMP (Christie, Atkins, and Munch-Petersen) reaction, originally defined to be a synergistic hemolytic process produced by the interaction of a hemolysin (CAMP factor) of group B streptococci (Streptococcus agalactiae) with beta-toxin (a sphingomyelinase) of Staphylococcus aureus (13).

Recently, we found that Dly toxin is encoded by pPHDD1, a 153-kb plasmid that also encodes a homologue of HlyA (HlyA\(_{pl}\)), and demonstrated that synergistic effects between Dly and HlyA\(_{pl}\) contribute to hemolysis and virulence in pPHDD1-harboring P. damsela subsp. damsela strains (14). HlyA\(_{pl}\) shows a 50% identity to Vibrio cholerae VCC, a hemolysin of the pore-forming toxin (PFT) family. PFTs are released as water-soluble monomeric proteins that diffuse toward the target membrane, forming amphipathic noncovalently associated oligomers that render a ring-like structure called a prepore. After contact with the cell membrane, a subsequent conformational change leads to its insertion as a transmembrane channel and to pore formation (15, 16).

We observed that Dly ΔhlyA\(_{pl}\) mutants retained hemolytic activity at levels similar to those for naturally occurring pPHDD1-negative strains, which suggested the presence of yet uncharacterized chromosome-encoded hemolysins. In the present study, we report the identification and characterization of a chromosome-encoded HlyA (HlyA\(_{ch}\)) in both pPHDD1-harboring and plasmidless strains that was highly similar (92% identity at the amino acid level) to pPHDD1-encoded HlyA\(_{pl}\). We demonstrate here that hemolysis in strains containing pPHDD1 is due to the additive effect between HlyA\(_{pl}\) and HlyA\(_{ch}\) on the one hand, and to the synergistic activity of Dly with HlyA\(_{pl}\) and HlyA\(_{ch}\) on the other hand. Additional data on the interaction among these three he-
molsyins and with hemolysins from other species, their role in virulence, and their transcriptional regulation by environmental factors are discussed.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used here and those derived from this study are listed in Table 1. Additional *P. damselae* subsp. *damselae* isolates used in the PCR-based screening for the presence of *hlyA* are described elsewhere (14). *P. damselae* subsp. *damselae* cells were routinely grown at 25°C on tryptic soy agar (TSA) supplemented with 1% NaCl (TSA-1) or tryptic soy broth supplemented with 1% NaCl (TSB-1). Sheep blood agar plates (Oxoid) were used for hemolysis assays and for conjugative matings. Mouse and turbot blood was aseptically collected and added to TSA-1 at a final concentration of 5% (vol/vol) to obtain mouse and turbot blood agar, respectively. For hemolysis assays on agar plates, a single colony of each strain was transferred from the plate and resuspended in TSB-1, and 100-μl aliquots of serial decimal dilutions were spread on TSA-1 plates, selecting for kanamycin resistance. The single *hly* insertion mutation in strain LD-07 was generated by amplifying an internal fragment of 1,000 bp of *hlyA* ligated into vector pWKS30 to generate pAJR37. The pAJR37 plasmid was mobilized from *E. coli* S17-1-λpir into the rifampin-resistant derivative of *P. damselae* strains.

**Mutant construction and gene complementation.** 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 of more than 90% of the coding sequence. Amplification was carried out using Hi-Fidelity Kapa *Taq* (Kapa). Allelic exchange was performed as previously described using the Km′ suicide vector pNidKan containing the *sacB* gene, which confers sucrose sensitivity, and R6K ori, which requires the *pir* gene product for replication (17). The plasmid constructions containing the deleted alleles were transferred from *E. coli* S17-1-λpir into a rifampin-resistant derivative (AR57) of *P. damselae* subsp. *damselae* RM-71. For conjugative matings, exponentially growing cells of donor and recipient strains were mixed, a drop (100 μl) was placed directly onto a sheep blood agar plate, and the plate was incubated 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 on TSA-1 plates, selecting for kanamycin resistance for plasmid integration and subsequently for sucrose resistance (15% [wt/vol]) for a second recombination event. This led to four *P. damselae* subsp. *damselae* strains (Table 1). The presence of the correct alleles was confirmed by PCR. The single *hlyA* insertion mutation in strain LD-07 was generated by amplifying an internal fragment of 1,000 bp of *hlyA* ligated into vector pWKS30 to generate pAJR37. The pAJR37 plasmid was mobilized from *E. coli* S17-1-λpir into the rifampin-resistant derivative of *P. damselae* strains.

### Table 1. Strains and plasmids used and constructed in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td><strong>P. damselae subsp. damselae</strong></td>
<td>Isolated from turbot (<em>Psetta maxima</em>), strongly hemolytic, carrying pHDD1</td>
<td>30</td>
</tr>
<tr>
<td>RM-71</td>
<td>RM-71 derivative, spontaneous rifampin-resistant mutant</td>
<td>14</td>
</tr>
<tr>
<td>AR57</td>
<td>AR57 with in-frame deletion of <em>hlyA</em> gene</td>
<td>14</td>
</tr>
<tr>
<td>AR64</td>
<td>AR57 with in-frame deletion of <em>dly</em> gene</td>
<td>14</td>
</tr>
<tr>
<td>AR133</td>
<td>AR57 with in-frame deletion of <em>hlyA</em> and <em>dly</em> genes</td>
<td>14</td>
</tr>
<tr>
<td>AR78</td>
<td>AR57 with in-frame deletion of <em>hlyA</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td>AR129</td>
<td>AR57 with in-frame deletion of <em>hlyA</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td>AR119</td>
<td>AR57 with in-frame deletion of <em>hlyA</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td>AR158</td>
<td>AR57 with in-frame deletion of <em>hlyA</em> and <em>hlyA</em> genes</td>
<td>This study</td>
</tr>
<tr>
<td>AR89</td>
<td>AR57 with in-frame deletion of <em>hlyA</em> and <em>hlyA</em> genes</td>
<td>This study</td>
</tr>
<tr>
<td>LD-07</td>
<td>Isolated from seabream (<em>Sparus aurata</em>), weakly hemolytic</td>
<td>30</td>
</tr>
<tr>
<td>AR111</td>
<td>LD-07 derivative, spontaneous rifampin-resistant mutant</td>
<td>This study</td>
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<tr>
<td>AR112</td>
<td>AR111 with <em>hlyA</em> gene disrupted by insertion mutation, Km′</td>
<td>This study</td>
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<td><strong>E. coli</strong></td>
<td>Cloning strain, recA</td>
<td>Laboratory stock</td>
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<tr>
<td>DH5α</td>
<td>Δ(mcrA)183 Δ(mcrCR-hsdS2888-oriT173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Y1hfpF15-oriT173 recA thi pro ΔhsdR-hsdM RP4-2 Tc:Mm Km::Tn7 Δpir Tp′ Sm′</td>
<td>Stratagene</td>
</tr>
<tr>
<td>XL1-Blue MR</td>
<td>Laboratory isolate from rat skin</td>
<td>This study</td>
</tr>
<tr>
<td>S17-1-λpir</td>
<td>Laboratory isolate, sea bass (<em>Dicentrarchus labrax</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>Streptococcus agalactiae AR170</td>
<td>Cloning strain, Ap′</td>
<td>Spanish Type Culture Collection (CECT)</td>
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<td>Vibrio cholerae H9004</td>
<td>Cloning strain, Ap′</td>
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<td>This study</td>
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<td>pWK30</td>
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<td>Suicide vector derived from PCVD442, Km′</td>
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<tr>
<td>pHPR309</td>
<td>lacZ reporter plasmid, mob Gm′</td>
<td>20</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Low-copy-no. cloning vector, Cm′</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pAJR27</td>
<td>pWK30 with <em>hlyA</em> gene from RM-71</td>
<td>14</td>
</tr>
<tr>
<td>pAJR29</td>
<td>pACYC184 with <em>dly</em> gene from RM-71</td>
<td>14</td>
</tr>
<tr>
<td>pAJR55</td>
<td>pHPR309 with <em>hlyA</em> gene from RM-71</td>
<td>This study</td>
</tr>
<tr>
<td>pAJR45</td>
<td><em>hlyA</em>; lacZ fusion in pHPR309</td>
<td>This study</td>
</tr>
<tr>
<td>pAJR51</td>
<td><em>dly</em>: lacZ fusion in pHPR309</td>
<td>This study</td>
</tr>
<tr>
<td>pAJR53</td>
<td><em>hlyA</em>; lacZ fusion in pHPR309</td>
<td>This study</td>
</tr>
</tbody>
</table>
per fish. The mortalities were recorded daily for 3 days (mice) and 4 days (turbot), and the results from the three independent groups of five animals were pooled and expressed as mortality percentages. All the protocols of animal experimentation used in this study have been reviewed and approved by the Animal Ethic Committee of the University of Santiago de Compostela.

**Statistical analysis.** The statistical analysis of the hemolytic activity data to assess the contributions of the three hemolysins was carried out using one-way and multifactor analysis of variance (ANOVA; full model, including interaction). For statistical analysis of differences between two groups, we used the Student t test. The β-galactosidase data (promoter expression levels) under changing concentrations of either NaCl or 2,2’-dipyridyl were subjected to a regression analysis. For the statistical analysis of the results of the fish and mouse virulence experiments, differences among data for live/dead animals for each pooled group of 15 animals were compared using a chi-square test. The SPSS statistical software package (version 20; IBM SPSS, Inc., Chicago, IL) was used for all statistical analyses. Adjusted P values of <0.05 were considered statistically significant.

**RESULTS**

A novel HlyA hemolysin is encoded in the chromosome of plasmidless and pHDD1-harboring *P. damselae* subsp. *damselae* strains. We searched the complete genome sequence of *P. damselae* subsp. *damselae* ATCC 33539 (GenBank accession no. ADBS00000000) for candidate hemolysin genes and found that the translated product of open reading frame VDA_002420 in chromosome I showed 92% identity to the previously described pHDD1-encoded hemolysin HlyA. Here we refer to these two hemolysins as HlyA<sub>pl</sub> (plasmid encoded) and HlyA<sub>ch</sub> (chromosome encoded). Interestingly, while the similarity between the coding sequences of the two genes at the nucleotide sequence level was 91%, the 250-bp sequences upstream of the translational start codon containing the putative promoters showed only 72% identity (i.e., 28% divergence) between them, with three insertions of 14, 30, and 20 bp in hlyA<sub>pl</sub> promoter which were absent in the hlyA<sub>ch</sub> promoter sequence (data not shown). Nucleotide sequence substitutions between hlyA<sub>pl</sub> and hlyA<sub>ch</sub>-coding sequences allowed the design of allele-specific PCR primers, and a PCR-based screening for specific hlyA<sub>ch</sub> sequences demonstrated that all of a total of 16 hemolytic *P. damselae* subsp. *damselae* isolates (14) tested contained the hlyA<sub>ch</sub> gene, including those harboring plasmid pHDD1 (6 strains) and those lacking pHDD1 (10 strains) (data not shown). We found that the genomes of several species of *Vibrio* whose genomes have been completely sequenced encode homologues of HlyA in chromosome II, with identity values at the amino acid level ranging from 47% to 50%. These homologues of HlyA in chromosome II, with identity values ranging from 47% to 50%. These

**Construction of lacZ transcriptional fusions and β-galactosidase assays.** DNA fragments corresponding to *P. damselae* subsp. *damselae* dly, hlyA<sub>ch</sub>, and hlyA<sub>pl</sub> presumptive promoter regions were obtained by PCR. The PCR-amplified regions, extending from about 1 kb upstream of the start codon to about 50 to 100 bp downstream of the start codon for each tested gene, were fused to a promoterless *lacZ* gene as previously described (18). After 48 h of incubation at 25°C, cells were washed off the cellophane with a minimum volume of saline solution (0.85% [wt/vol] NaCl) and reattached to an optical density at 600 nm of 1. The cell suspensions were centrifuged at 13,000 rpm for 5 min, and the supernatants were filtered through 0.22-µm-pore-size membranes and stored at −30°C until needed. For hemolytic liquid assays, a modification of the method of Bernheimer was used (19). Briefly, sheep erythrocytes (Oxoid) were washed with phosphate-buffered saline (PBS) and centrifuged at 3,000 rpm for 5 min at 4°C. Washes were repeated until the supernatant was visibly clear of hemoglobin. Different volumes of crude erythrocytes were washed and tested to assess the minimal volume necessary to get the maximal absorbance (the absorbance obtained by lysis of the erythrocytes with distilled water). Two hundred microliters of crude erythrocytes were enough to get the maximal absorbance, and consequently, the erythrocytes were adjusted with PBS to 0.5 ml per dilution tube assayed and 0.5 ml of serially diluted ECP samples was added. After 2 h of incubation at 25°C, the mixtures were centrifuged at 3,000 rpm for 5 min at 4°C to remove undamaged erythrocytes. This assay is based on the measure of the released hemoglobin, whose concentration is estimated by reading the absorbance at 545 nm in a spectrophotometer and adjusted using nonlysed washed cells as a control. One unit of hemolytic activity was expressed as the amount of ECP which caused the release of 50% of the hemoglobin in the standardized erythrocyte suspension. All hemolytic assays were carried out in triplicate, and mean values with standard deviations are depicted.

**Mouse and fish virulence assays.** Virulence assays were carried out with BALB/c mice (age, 6 to 8 weeks; weight, 26 to 30 g), as well as with turbot (*Psetta maxima*) (average weight, 15 g), in three groups of five animals each per strain tested. 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 in 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 (22). Mice were inoculated intraperitoneally in the tail vein, and turbot were inoculated intraperitoneally with 0.1 ml of 10-fold serial dilutions of the bacterial suspensions. The actual number of injected CFU was determined by plate count on TSA-1. The final doses assayed corresponded to 2.1 × 10<sup>6</sup> bacterial cells per mouse and 2.1 × 10<sup>6</sup> bacterial cells

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Three Hemolysins in *P. damselae*
results altogether demonstrate that HlyAch is a hemolytic factor in pPHDD1-harboring and also in plasmidless \textit{P. damselae} subsp. \textit{damselae} strains.

To better understand the contribution of HlyAch to hemolysis, we generated \textit{hlyAch} mutants under different genetic contexts (Fig. 2). On sheep blood, the \( \Delta hlyA_{ch} \Delta hlyA_{pl} \) double mutant (AR158), which only secretes Dly, produced an almost unperceivable and turbid hemolytic halo. This observation, together with the previously reported weak halo produced by the \( \Delta hlyA_{pl} \) mutant (AR78) (14), which produces only HlyAch, clearly suggests that the strong hemolytic halo of AR133 (\( \Delta hlyA_{pl} \)) on sheep blood agar is the result of a synergistic effect between Dly and HlyAch rather than an additive sum of the individual effects of these two hemolysins. Interestingly, we observed on sheep blood agar that the single \( \Delta hlyA_{ch} \) mutant (AR129) lacked the hemolytic halo that would be expected by the action of HlyAch. Most surprisingly, deletion of \textit{dly} in AR129 (yielding AR119) caused a recovery of hemolytic activity on sheep blood (Fig. 2), which suggests that repression or inhibition of HlyApl activity is mediated, directly or indirectly, by Dly on a \( \Delta hlyA_{ch} \) background (see below). Altogether, the results using sheep blood agar indicate that Dly is unable by itself to produce complete lysis of sheep erythrocytes but is necessary to produce synergistic effects with either of the two HlyA hemolysins in this erythrocyte source.

On mouse and turbot blood, the \( \Delta hlyA_{ch} \Delta hlyA_{pl} \) double mutant (AR158) showed only a slight reduction of hemolytic activity compared to AR57. However, deletion of \textit{dly} in strain AR129, resulting in the \( \Delta dly \Delta hlyA_{ch} \) double mutant (AR119), caused a reduction of the hemolytic halo of >80%. The results using mouse and turbot blood agar confirm the main role of Dly in hemolysis of these two erythrocyte species.

Hemolysis in strains harboring pPHDD1 is the result of a synergistic effect between Dly and either of the two HlyA hemolysins (HlyApl and HlyAch) and of additive effects between HlyApl and HlyAch. In order to obtain a quantitative measure of the contribution of each of the three hemolysins to the hemolytic phenotype, we carried out assays with sheep erythrocyte suspensions and bacterial extracellular products (ECPs) obtained under standard TSA growth conditions (Fig. 3A). We found significant differences in the hemolytic activities obtained for each assayed strain (\( P = 6.6 \times 10^{-19} \) by ANOVA). The results showed that HlyApl was the most active (75 hemolytic units [HU]), followed by HlyAch (33 HU), while Dly alone did not produce hemolysis (0 HU), demonstrating that the presence of at least one of the two HlyA hemolysins is necessary and sufficient to produce detectable hemolysis of sheep erythrocytes (\( P < 0.001 \) by Student’s \( t \) test). No significant differences (\( P = 0.0841 \) by Student’s \( t \) test) between the sum of the individual values obtained for the HlyApl- and the HlyAch-producing mutants (75 and 33 units, respectively) and the value obtained with the mutant that produced the two HlyA (96 units) were detected, suggesting that the contribution of HlyApl and HlyAch to hemolysis constitutes an additive effect (the multifactor ANOVA analysis [\( P = 0.15 \]) did not detect an interaction between HlyApl and HlyAch).

In addition, we found that the full hemolytic activity of the parental strain did not result from a mere sum of the individual

FIG 1 HlyAch is necessary for hemolysis of sheep erythrocytes in plasmidless strain AR111. The respective insertional mutant derivative for the \textit{hlyAch} gene (strain AR112) lacks detectable hemolytic activity.

FIG 2 Hemolytic activity of the \textit{P. damselae} subsp. \textit{damselae} parental strain and mutants in sheep, mouse, and turbot blood agar plates. The genotype of the strain (either parental or deletion mutant) is given in parentheses. The hemolysins being produced are indicated below the strain name.
activities of each hemolysin. Mutation of dly strongly prevented the values obtained with the parental strain from being achieved. The Δdly mutant producing the two HlyA hemolysins achieved 96 units, whereas in the presence of Dly (i.e., in the parental strain), this value increased to 316 units. In the multifactor ANOVA analysis, this fact was demonstrated by a significant strong interaction between Dly and either of the two HlyA hemolysins (the interaction of Dly, HlyAch, and HlyApl yielded a P value of $1.22 \times 10^{-11}$ by ANOVA). This observation, together with the data obtained with the Δdly ΔhlyApl (33 units) and ΔhlyAch ΔhlyApl (196 units) strains (the interaction between Dly and HlyAch yielded a P value of $8.70 \times 10^{-13}$ by ANOVA), clearly demonstrates that Dly interacts synergistically with either of the two HlyA hemolysins.

The synergistic and additive effects among hemolysins are reproduced in an *Escherichia coli* background. In order to gain additional evidence of the synergistic and additive effects between hemolysins, we assayed the hemolytic phenotype conferred by each gene individually and by combinations of two or three genes to *Escherichia coli* (Fig. 4). Cells of *E. coli* harboring the dly gene showed an almost undetectable hemolytic halo compared to the halo observed in *E. coli* cells harboring either hlyApl or hlyAch, confirming again the low activity of Dly against sheep erythrocytes and confirming that either of the two HlyA hemolysins is necessary and sufficient to cause hemolysis of sheep erythrocytes. The halo shown by *E. coli* harboring the hlyApl gene was wider than that shown by *E. coli* harboring the hlyAch gene, consistent with the 2-fold higher values obtained with HlyApl than HlyAch in the liquid hemolytic assays (see above). This differential hemolytic activity of the two HlyA toxins was also visible when each was combined with Dly. In addition, the existence of synergistic effects between Dly and each HlyA was demonstrated by the much stronger haloes observed in their different combinations compared with the haloes produced by the individual hemolysins. The widest and most translucent halo was observed in *E. coli* harboring the three hemolysin genes. Introduction of the two distinct hlyA genes together produced a halo which did not differ substantially from

![FIG 3](image-url) Synergistic and additive effects among the Dly, HlyApl, and HlyAch hemolysins are demonstrated by calculation of the hemolytic activity of the ECPs of *P. damselae* subsp. *damselae* on sheep erythrocytes. (A) ECP of individual strains; (B) combinations of ECPs from different pairs of strains mixed in a 1:1 ratio. The release of hemoglobin in the supernatant was measured at A540. One hemolytic unit is defined as the amount of hemolysin which lyses 50% of sheep erythrocytes. All assays were carried out in triplicate, and mean values with standard deviations are shown. The results for bars with different letters are significantly different from each other (one-way ANOVA).

![FIG 4](image-url) Transformation of *Escherichia coli* DH5α with different combinations of the *P. damselae* subsp. *damselae* hemolysin genes *dly*, *hlyApl*, and *hlyAch* demonstrates the synergistic and additive effects of hemolysins against sheep erythrocytes on sheep blood agar plates.
the halo seen with introduction of \( hlyA_{ch} \) alone, indicating an additive rather than a synergistic effect between \( hlyA_{ch} \) and \( hlyA_{pl} \). Altogether, these results support the hypothesis that in strains harboring plasmid pPHDD1, hemolysis is due to the synergistic activity of Dly with \( hlyA_{ch} \) and \( hlyA_{pl} \), on one hand, and to the additive effect between \( hlyA_{ch} \) and \( hlyA_{pl} \), on the other hand.

The synergistic effect between Dly and HlyA constitutes a case of the CAMP reaction. We saw, as indicated above, that hemolysis in the parental strain is the result of synergistic effects between Dly and HlyA. This phenomenon might constitute a CAMP reaction. This is a two-step process where a first-step agent modifies the erythrocyte membrane in a nonlytic or sublytic fashion, making it vulnerable to the action of the second-step agent (the so-called CAMP factor). The CAMP reaction was described to be a synergistic hemolytic process produced by the interaction of the beta-toxin sphingomyelinase of \( Staphylococcus aureus \) with a hemolysin (CAMP factor) of \( Streptococcus agalactiae \), causing increased hemolysis where the two toxins converge (13).

To assess whether synergy in \( P. damselae \) subsp. \( damselae \) hemolysis constitutes a case of the CAMP reaction, we cultured Dly-producing strains (the parental strain and the double mutant for the two \( hlyA \) genes, AR158) surrounded by non-Dly-producing strains on a sheep blood agar plate. The hemolytic halo of two non-Dly-producing strains was increased and acquired a half-moon shape at the area that intersects with the halo of partial hemolysis produced by Dly both in the parental strain (Fig. 5A) and in the AR158 mutant (Fig. 5B). Mutation of the \( dly \) gene prevented us from observing the CAMP reaction (Fig. 5C), demonstrating that Dly acts as the first-step agent. CAMP reactions between two parental strains were not observed (Fig. 5D) because the factor limiting the size of the hemolytic halo is the extent of the HlyA diffusion zone rather than that of the Dly diffusion zone. Hence, the translucent hemolytic halo produced by each parental strain is not susceptible to further improvement when additional Dly is supplied. This phenomenon can also be observed when the Dly halo produced by AR158 overlaps the hemolytic halo produced by AR64 (Fig. 5E).

CAMP reactions can therefore be observed in plasmidless \( AR111 \) and in \( \Delta dly \) \( P. damselae \) subsp. \( damselae \) strains. HlyA (in either of the two forms, \( hlyA_{ch} \) or \( hlyA_{pl} \)) is present in the two types of strains, making this toxin a good candidate to act as a CAMP factor. To prove this hypothesis, different \( hlyA \) mutants were tested for CAMP reactions in the presence of the Dly-producing strain AR158 (Fig. 5E). A strain producing only \( hlyA_{ch} \) (AR78) showed a strong CAMP reaction at the intersection with the zone of Dly diffusion. However, as expected, the triple mutant AR89 as well as the \( hlyA_{ch} \) mutant (AR112) of a pPHDD1-negative strain yielded no CAMP reactions (Fig. 5E). We therefore propose that Dly acts as a first-step agent and the pore-forming toxin HlyA acts as a CAMP factor in the CAMP reactions observed in \( Photobacterium damselae \) subsp. \( damselae \) strains.

Since previous studies reported the existence of CAMP reactions in \( Streptococcus agalactiae \) and \( Rhodococcus equi \) (13, 28), we also tested whether the hemolytic activity of these two species is enhanced by the interaction with \( P. damselae \) subsp. \( damselae \) Dly. As a result, we observed the existence of synergistic effects between Dly and these two species (Fig. 6). We also found that Dly produces CAMP reactions with \( Vibrio cholerae \) and with \( Aeromonas schubertii \) (Fig. 6). Taken together, these results indicate that Dly is capable of producing CAMP reactions with a variety of species that produce CAMP factors.

Dly exerts an inhibitory effect on \( hlyA_{ch} \) activity against sheep erythrocytes in a \( \Delta hlyA_{ch} \) background. We observed, as
indicated above, that the single hlyAch mutant (AR129) undergoes a drastic decrease in its hemolytic activity compared with that of the parental strain and that the dly mutation in this strain to yield AR119 Δdly ΔhlyAch causes a significant recovery of the hemolytic activity (P = 4.5 × 10^{-7} by Student’s t test) (Fig. 3A). This suggests that upon deletion of hlyAch from the genome, Dly plays a role in the repression or inhibition of HlyAch activity. In order to assess at which level this inhibition occurs, we tested two different hypotheses: (i) whether it occurs in a bacterial cell-independent manner or (ii) whether it occurs in a bacterial cell-dependent manner.

To test whether inhibition occurred in a cell-independent fashion by means of protein-protein interactions in the extracellular medium, we mixed ECPs from strain AR158 ΔhlyAch ΔhlyAch, producing only Dly, with ECPs of strains that produce either both of the HlyA hemolysins (the Δdly strain) or only one of the two HlyA hemolysins (the Δdly ΔhlyAps and Δdly ΔhlyAch strains) and conducted liquid hemolytic assays. Since, as said above, the ECPs of strain AR158 alone yield no hemolytic values (Fig. 3A), we can attribute the hemolytic values of the combinations containing AR158 ECPs to the synergistic effects between extracellular products. As a result, we found that all the combinations of mixed ECPs from different strains yielded values of hemolytic units similar to those yielded by a single strain producing the same combination of hemolysins (Fig. 3A and B), with the notable exception of ECPs from strains AR158 and AR119. The definite demonstration that the inhibition of HlyAch does not occur via extracellular interactions between hemolysins comes from the comparative study of the hemolytic values of the ΔhlyAch mutant (1.8 units) (Fig. 3A) and of the combination of ECPs from AR158 and AR119 (223 units) (Fig. 3B) (P = 4.29 × 10^{-6} by Student’s t test). In the two cases, the hemolysins that are present are the same. However, the ECP mix behaves as if all the hemolysins are being normally produced and functional, whereas in the ΔhlyAch mutant, there is a phenomenon of inhibition/repression. Additional confirmation that repression/inhibition of HlyAch activity is not due to protein-protein interactions between hemolysins at the extracellular level can be drawn from the results obtained with E. coli strains harboring different gene combinations (see above and Fig. 4).

The next hypothesis that we tested was whether Dly-mediated inhibition of HlyAch occurs at cellular levels, i.e., whether the presence of the P. damselae subsp. damselae cells rather than their ECPs is necessary to produce the repression/inhibition phenomenon. We therefore investigated whether mutation of the hlyAch gene had any effect on the transcriptional activity of hlyAch expression. We could not find significant differences (P = 0.0791 by Student’s t test) in the β-galactosidase activities of the hlyAch promoter in the parental and the ΔhlyAch genetic backgrounds tested (17,450 versus 16,183 units), clearly indicating that the inhibition of HlyAch in a ΔhlyAch background does not occur at the transcriptional level.

We know that inhibition of HlyAch activity is evidenced when Dly is being produced in the same cell that undergoes the inhibition (Fig. 2 and 3A). We therefore wanted to investigate whether Dly-mediated inhibition of HlyAch can also be observed in a cell-dependent manner in cells that are exposed to exogenous Dly supplied in the extracellular environment, even in those cells that do not produce Dly. For this purpose, we cultured (i) parental strain AR57, (ii) the AR158 ΔhlyAch ΔhlyAch double mutant (which produces only Dly), and (iii) mutant AR64 Δdly on the center of sheep blood agar plates. After 3 days of growth (enabling Dly to be produced and diffused through the agar plate), we streaked five different mutants radially from the plate edge toward the center of the plate (Fig. 7). As a result, we found that the radially streaked double Δdly ΔhlyAch mutant and the single Δdly mutant underwent an enhancement of their hemolytic activity by synergistic effects in the presence of Dly (Fig. 7A and B) which was not observed if Dly was absent (Fig. 7C). The slight hemolysis produced by the ΔhlyAch mutant was totally inhibited in the presence of exogenous Dly (Fig. 7A and B). Most interestingly, Dly-mediated inhibition was clearly visible in the Δdly ΔhlyAch double mutant, where hemolysis was produced only in the absence of exogenous Dly, whereas cells growing on the Dly diffusion area were unable to produce hemolysis. Interestingly, a synergistic effect was observed at the edge of the Dly diffusion area in the Δdly ΔhlyAch mutant, with this synergistic effect likely being derived from the interaction of Dly with HlyAch molecules which were secreted before the repression/inhibition started. As expected, no HlyAch inhibition could be observed in any of the strains assayed in experiments with the dly mutant growing on the plate center (Fig. 7C).

From these results and considering the results presented above, we propose that inhibition of HlyAch activity does not occur under bacterial cell-free conditions. Rather, this inhibition is evidenced at cellular levels in a ΔhlyAch background, and it occurs not only when Dly is produced inside the cell but also when Dly is provided in the extracellular environment.

Expression of the three hemolysins is regulated at the transcriptional level by iron and sodium chloride concentrations. Many Vibrio spp. can live both in a free-living form and in a pathogenic lifestyle, and P. damselae subsp. damselae is not an exception, since it is known to survive in seawater microcosms in a culturable stage for at least 1 year, maintaining its infective potential for fish (29). It would be expected that expression of the P.
**P. damselae** subsp. **damselae** hemolysins does not occur while the bacterium is in a free-living style in the ocean but, rather, occurs when the bacterium has established contact with a host. We examined the changes in promoter activity for the three hemolysin genes when the cell faced two situations that mimic entry into a host: (i) low iron availability and (ii) a decrease in the sodium chloride concentration. As a result, we found a positive correlation between the $/H9252$-galactosidase activity values of the three promoters and the concentrations of the iron chelator 2,2'-dipyridyl (which causes a decrease in iron availability) (Fig. 8A).

To test whether salt concentration acts as an environmental regulatory signal, we measured promoter expression in the presence of three increasing concentrations of NaCl in LB medium. We found that the $/H9252$-galactosidase activity of the three promoters decreased as the salt concentration was increased (Fig. 8B), denoting a negative correlation between the transcriptional activity of the three hemolysin genes and salt concentration. Interestingly, we found that in an *E. coli* XL1-Blue MR background, there was no transcriptional response of the three promoters to changes in NaCl concentration (data not shown), suggesting that this regulation is mediated by a *P. damselae* subsp. **damselae** mechanism which is absent in *E. coli*. Moreover, we found a transcriptional response to salinity when the three promoters were introduced in plasmidless strain AR111 (data not shown), which suggests that the genetic determinants that mediate the salinity-dependent regulation are not encoded by pPHDD1 but, rather, are encoded by the chromosomes of *P. damselae* subsp. **damselae**.

** Contribution of *P. damselae* subsp. **damselae** hemolysins to virulence for mice and fish.** In a previous work, we found evidence that Dly and HlyApl contribute to the virulence of *P. damselae* subsp. **damselae** for mice and fish (14). Following the discovery of the hlyAch gene in the present study, we wanted to evaluate the contribution of the three hemolysins to *P. damselae* subsp. **damselae** virulence. For this purpose, we conducted virulence tests in mice, inoculating the parental strain as well as the seven possible combinations of hemolysin gene mutants. Statistically significant differences were observed among the assayed strains ($P = 1.444 \times 10^{-8}$ by chi-square test) (Fig. 9A). The parental strain caused death in the 15 animals inoculated, whereas no deaths were recorded among animals inoculated with the triple mutant ($P = 4.32 \times 10^{-8}$), which indicates that at least one of the hemolysins is required for virulence. As a general observation, we found that...
strains producing any combination of two hemolysins caused higher mortality ratios than a strain producing only one of those two hemolysins, with the exception of the hlyApl mutant, which yielded a slightly lower mortality value than the hlyApl hlyAch double mutant. By comparing the three different double mutants (AR158, AR119, and AR78, strains producing only one of the three individual hemolysins) with the triple mutant (AR89), we observed that although each of the three hemolysins individually is sufficient to cause death in mice, each one contributes to the virulence to a different degree. The mutant AR158, which produces only Dly, killed 12 animals \( (P = 7.744 \times 10^{-6}) \), whereas the mutants producing HlyApl (AR119) and HlyAch (AR78) killed 9 \( (P = 3.362 \times 10^{-4}) \) and 3 \( (P = 0.06789) \) animals, respectively. These results suggest that the contribution of HlyAch to virulence is the lowest among the three toxins. In this regard, we found that although the strain with deletion of hlyAch (AR129) \( (P = 0.1432) \) alone or in combination with deletion of hlyApl (AR158) \( (P = 0.06789) \) caused a lower percentage of dead mice than the parental strain, the differences were found not to be significant (Fig. 9A).

The virulence data altogether suggest that albeit the highest values of mortality for mice (death of all the animals tested) are achieved only when the three hemolysins are being produced, Dly and HlyApl are the main contributors to the virulence of this bacterium for mice.

Because P. damselae subsp. damselae is a primary pathogen for fish, we judged it to be of maximum interest to assess the role of each of the three hemolysins in virulence for turbot (Fig. 9B). Statistically significant differences were evidenced among the strains tested \( (P = 6.587 \times 10^{-14}) \). Most interestingly, of the three single hemolysin-producing mutants, only the Dly-producing strain (AR158) was able to cause death in fish \( (P = 0.01431) \). The observation that no fish deaths were recorded with either the \( \Delta \)hlyApl \( \Delta \)dly (AR119) or the \( \Delta \)hlyAch \( \Delta \)dly (AR78) double mutants demonstrates that either of the two HlyA hemolysins alone does not cause death in turbot but, rather, that one HlyA hemolysin needs the presence of either Dly or the other HlyA hemolysin to cause death. The production of Dly in combination with either of the two HlyA hemolysins causes an increase in the number of animals killed with respect to the number of animals killed by strains that produce Dly alone, and this increase is particularly significant \( (P = 0.0006501) \) when Dly is combined with HlyAch. This clearly suggests that, unlike what is observed in mice, the contribution of hemolysins to virulence for fish is not so much based on the individual effects of each hemolysin but, rather, on the synergistic effects between Dly and HlyA.

**DISCUSSION**

*P. damselae* subsp. *damselae* is a hemolytic bacterium that causes disease and death in a variety of marine animals and also in humans. Tissue damage and hemorrhagic areas are characteristics of *P. damselae* subsp. *damselae* infections (6, 30), suggesting that membrane-damaging factors and particularly hemolysins contribute to pathogenesis. We previously reported that the phospholipase D Dly and the pore-forming toxin HlyApl encoded by plasmid pPHDD1 are two main contributors to the hemolytic activity and virulence in this species (14). Strains lacking pPHDD1 as well as a \( \Delta \)dly \( \Delta \)hlyApl mutant are hemolytic, pointing out the production of other hemolysins. After searching the complete genome sequence of *P. damselae* subsp. *damselae* ATCC 33539, we identified HlyAch, a novel hemolysin encoded by chromosome I of this bacterium. Its 92% amino acid sequence identity to pPHDD1-encoded HlyApl and its 50% identity to *V. cholerae* VCC suggest that HlyAch belongs to the family of pore-forming toxins. Early studies had demonstrated the existence of one major and two minor components with hemolytic activity in the supernatants of *P. damselae* subsp. *damselae* strains (31), and we propose that they correspond to Dly, HlyApl, and HlyAch.

We have demonstrated here that HlyAch is responsible for hemolysis in *P. damselae* subsp. *damselae* strains that lack pPHDD1. Since hlyAch is located on chromosome I and is also present in plasmidless strains, it is likely that hlyAch has its evolutionary origin in an hlyApl-like gene. In line with this, many species of *Vibrio* harbor hlyAch homologues in their chromosomes. The two HlyA hemolysins showed differential activity against sheep erythrocytes. Transcriptional lacZ fusions revealed that the two genes have similar expression levels, which suggests that their differen-
tial hemolytic activity is due to punctual amino acid substitutions. We demonstrated that HlyApl and HlyAch contribute to hemolysis of sheep erythrocytes in an additive manner, and their high sequence similarities suggest that their additive effect in hemolysis is due to a dosage effect.

It was previously reported that Dly is strongly active against mouse and fish erythrocytes but weakly active against sheep erythrocytes (31), and this is consistent with the results obtained with the ΔhlyAch ΔhlyApl double mutant. The differential sphingomyelin content among erythrocyte membranes of mammal species (32) might explain the different susceptibilities of sheep and mouse erythrocytes to the action of Dly.

Albeit Dly alone is weakly active against sheep erythrocytes, Dly is essential to yield the highest hemolytic values due to a synergistic effect with either of the two HlyA hemolysins. This synergistic reaction can be considered a typical CAMP reaction (13). Previous work demonstrated that Dly shows sphingomyelinase activity and can act synergistically with staphylococcal delta-toxin on sheep erythrocytes (11). Sphingomyelinases are capable of interacting with outer leaflet membrane phospholipids and are responsible for the incomplete hemolytic areas observed in sheep blood agar plates (33,34). The group of second-step agents, the so-called CAMP factors, is more heterogeneous and includes non-enzymatic bacterial exoproteins as pore-forming toxins, as well as phospholipases, lipases, and cholesterol oxidases (35). The umbrella model (36, 37) provides an explanation of how Dly and HlyA might cooperate synergistically to produce cell lysis. It has been demonstrated that cholesterol is required for different pore-forming toxins to induce cytolysis, as is the case for V. vulnificus cytolysin (16, 38), staphylococcal alpha-toxin (39), and V. cholerae VCC (36, 40). Hydrolysis of choline head groups in membranes by sphingomyelinases has been demonstrated to facilitate access to cholesterol and elicit destruction of the membrane by second-step agents (37,41). We demonstrated in our study that

![Graph A](image)

![Graph B](image)

**FIG 9** Results of mouse (A) and turbot (B) virulence assays with *P. damselae* subsp. *damselae* parental strain and mutants. The results are expressed as percent mortality (number of dead animals/number of animals assayed). Numbers inside the bars denote the number of dead animals. Results from three independent groups of 5 animals each per strain tested were pooled and compared using a chi-square test. Asterisks denote statistically significant differences between strains: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.
the action of Dly as a first-step agent allows the second-step agents HlyA<sub>pl</sub> and HlyA<sub>ch</sub> to enhance their hemolytic activity and to produce CAMP reaction. In addition, we observed CAMP reactions between Dly and <i>V. cholerae</i>, <i>S. agalactiae</i>, <i>R. equi</i>, and <i>Aeromonas schuberti</i> CAMP factors. In <i>S. agalactiae</i> the second-step agent has been reported to be a ceramide-binding protein (42), whereas in <i>R. equi</i> it is a cholesterol oxidase (41). Since it has been demonstrated that deletion of the <i>V. cholerae</i> VCC gene leads to a total suppression of hemolysis (43), we suggest that the unknown CAMP factor produced by <i>V. cholerae</i> is indeed VCC. Albeit <i>A. schuberti</i> has been reported to be hemolytic (44), the molecular basis of hemolysis in this species remains uncharacterized.

The drastic hemolytic reduction observed in the ΔhlyA<sub>ch</sub> mutant in sheep erythrocytes is likely due to a repression/inhibition of HlyA<sub>ch</sub> hemolytic activity mediated by Dly. It is interesting to note that the predominant role of Dly in the lysis of mouse and turbot erythrocytes prevents us from observing whether this repression also occurs in these two blood types. Recently, it was reported that <i>V. cholerae</i> HlyA hemolysis is negatively regulated via the quorum-sensing-regulated transcription factor HapR, and this negative regulation is exerted at the transcriptional and posttranslational levels (45). We demonstrated, using transcriptional lacZ fusions, that inhibition of HlyA<sub>ch</sub> in <i>P. damselae</i> subsp. <i>damselae</i> does not occur at the transcriptional level, and liquid hemolytic assays demonstrated that it is not due to interactions among the hemolysins and does not take place under bacterial cell-free conditions. Rather, we propose that HlyA<sub>ch</sub> inhibition occurs in a bacterial cell-dependent manner in the presence of intra- or extracellular Dly. The exact mechanism by which this inhibition takes place remains to be elucidated and might be related to inhibition of maturation and/or secretion of HlyA<sub>ch</sub> or to HlyA<sub>ch</sub> degradation in a ΔhlyA<sub>ch</sub> mutant background in the presence of Dly molecules.

We have demonstrated that iron-deficient conditions enhance expression of the <i>dly</i>, <i>hlyA<sub>pl</sub></i>, and <i>hlyA<sub>ch</sub></i> genes. Since the ability to obtain iron from host tissues is crucial for most pathogenic bacteria in order to establish an infection (46), hemolysin production is expected to facilitate hemoglobin release as a source of iron. Indeed, hemoglobin availability and the susceptibility to bacterial infections are directly related (47), and a functional heme uptake system has been described in <i>P. damselae</i> subsp. <i>damselae</i> (48). Previous studies reported that <i>P. damselae</i> subsp. <i>damselae</i> cultures containing Na<sup>+</sup> ion concentrations of 0.8% underwent a considerable reduction in Dly toxin levels (8). Our results show that increasing sodium chloride concentrations induced a decrease in expression of the three hemolysin promoters. It is unlikely that <i>P. damselae</i> subsp. <i>damselae</i> cells secrete the three hemolysins while in a free-living stage in seawater. Interestingly, the environmental conditions in which higher transcriptional expression values were obtained (iron deficiency and low salt concentration) simulate the conditions that the bacterium encounters upon entry into a host. Therefore, low iron and low salinity might act as molecular signals that trigger expression of Dly and HlyA hemolysins. The fact that the influence of salinity in the transcription of the hemolysin genes was not reproduced in an <i>E. coli</i> background suggests that this regulation might be controlled by a mechanism which is specific to marine bacteria or even specific to <i>P. damselae</i> subsp. <i>damselae</i>. Studies to assess the molecular basis of this salinity-mediated regulation are under way.

In a previous work, we demonstrated that plasmid pHDD1 is necessary for the full virulence of <i>P. damselae</i> subsp. <i>damselae</i> for mice and fish (14). Here we demonstrate that virulence for mice resides mainly in pHDD1-encoded Dly and HlyA<sub>pl</sub> since deletion of <i>hlyA<sub>ch</sub></i> did not produce a statistically significant decrease in virulence for mice. The virulence data for mice clearly indicated that Dly is highly toxic for this species, in agreement with the findings of previous studies (11). In addition, we found that the two double mutants AR119 and AR78, which produce only HlyA<sub>pl</sub> and HlyA<sub>ch</sub>, respectively, are avirulent for fish. Thus, we can conclude that either of the two HlyA hemolysins alone does not cause death in turbot, with the presence of either Dly (synergistic effect) or the other HlyA hemolysin (additive effect) being necessary to cause death. It was somehow surprising that HlyA<sub>ch</sub> with homologues in several <i>Vibrio</i> species, some of them potential fish pathogens, was not toxic for fish in our virulence model unless it was accompanied by Dly. However, Dly alone was able to cause death in turbot, and its virulence was enhanced by the presence of either HlyA<sub>ch</sub> or HlyA<sub>pl</sub>. Thus, we can conclude that while Dly and HlyA<sub>ch</sub> are the main contributors to the virulence for mice, the contribution of hemolysins to virulence for fish is very much based on the synergistic effects between Dly and either of the two HlyA hemolysins rather than on their individual effects. The reason for the differential behavior of HlyA toxins for mice and for fish is currently unknown.

Altogether, our results demonstrate that, depending on the erythrocyte and animal source, the outcomes achieved with the individual hemolysins and of the interactions among the three hemolysins are very different. We found a good correlation between hemolysis and virulence data in mice and fish. As an example, the parental strain and the mutants showing wide hemolytic haloes on either mouse or turbot blood agar plates (Fig. 2) broadly corresponded to the ones that caused higher percentages of mortality for the cognate species (Fig. 9). An exception to this was the observation that HlyA<sub>ch</sub> alone was weakly hemolytic in mouse blood agar, while a strain producing only HlyA<sub>ch</sub> caused death in 9 of 15 mice inoculated. However, we can guess from the results of previous studies that the role of the <i>P. damselae</i> subsp. <i>damselae</i> hemolysins is not necessarily reduced to lysis of erythrocytes (9). Rather, these three proteins could be exerting effects on other cell types in the animal host that would pass unadvertised in the blood agar plates.

To date, the great majority of synergistic effects reported between hemolysins are between toxins produced by two different bacterial species, with the only exception being <i>Bacillus cereus</i>, which produces synergistic effects with hemolysins produced simultaneously by the same cell (12). Therefore, to our knowledge, <i>P. damselae</i> subsp. <i>damselae</i> represents the first report in a Gram-negative bacterium of synergistic hemolysis occurring within the same species. This synergistic process between Dly and either of the two HlyA forms might be of special relevance in the severity of <i>P. damselae</i> subsp. <i>damselae</i> pathogenesis and highlights the importance of synergistic interactions among virulence factors in pathogenic bacteria.

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**I. Oligomerization of Vibrio cholerae cytolsins yields a pentameric pore and has a dual specificity for cholesterol and sphingolipids in the target membrane. J. Biol. Chem. 276:1375–1380.**

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