Phobalysin, a Small β-Pore-Forming Toxin of *Photobacterium damselae* subsp. *damselae*

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*Photobacterium damselae* subsp. *damselae*, an important pathogen of marine animals, may also cause septicemia or hyperaggressive necrotizing fasciitis in humans. We previously showed that hemolysin genes are critical for virulence of this organism in mice and fish. In the present study, we characterized the *hlyA* gene product, a putative small β-pore-forming toxin, and termed it phobalysin P (PhlyP). PhlyP formed stable oligomers and small membrane pores, causing efflux of K+, with no significant leakage of lactate dehydrogenase but entry of vital dyes. The latter feature distinguished PhlyP from the related *Vibrio cholerae* cytolsin. Attack by PhlyP provoked a loss of cellular ATP, attenuated translation, and caused profound morphological changes in epithelial cells. In coculture experiments with epithelial cells, *Photobacterium damselae* subsp. *damselae* led to rapid hemolysin-dependent membrane permeabilization. Unexpectedly, hemolysins also promoted the association of *P. damselae* subsp. *damselae* with epithelial cells. The collective observations of this study suggest that membrane-damaging toxins commonly enhance bacterial adherence.

**Materials and Methods**

**Bacterial strains and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Culture conditions and conjugative matings used in this study have been described previously (9, 14, 15). The *Staphylococcus aureus* strains have been described previously (24). Adherence assays with *Escherichia coli* were performed with DH5α.

**Mutant construction and gene complementation.** For construction of a cheA mutant, a cheA internal fragment of 1,734 bp was amplified by PCR from *P. damselae* subsp. *damselae* strain AR57 by using Kapa Taq DNA polymerase (Kapa) and the primers cheA-Forward (CTTTATTGC AAGTTGCAGCGC) and cheA.Reverse (GGTAGGAGCCACTTTCAT GA). The resulting DNA product was ligated into the TA vector pSPark (Canvax). Subsequently, the DNA insert was cut out by digestion and cloned into the BamHI site of the suicide vector pNidKan (Table 1), resulting in pAJR80. As a derivative of pCVD442, pNidKan contains the R6K ori, requiring the pir gene product for replication. Insertion of the suicide vector into the chromosome by a single crossover results in a Km² phenotype. After conjugational matings, *P. damselae* subsp. *damselae* Km² ex-
TABLE 1 Strains and plasmids used in this study

| Strain or plasmid      | Description                                                                 | Reference or source |
|------------------------|-----------------------------------------------------------------------------|---------------------|
| Strains               |                                                                             |                     |
| *P. damsela* subsp. *damsela* strains |
| AR57                   | RM-71 derivative, spontaneously rifampin-resistant mutant                   | 9                   |
| AR78                   | AR57 with in-frame deletion of *dly* and *hlyA* genes                       | 9                   |
| AR158                  | AR57 with in-frame deletion of *hlyA* and *hlyA* genes                      | 14                  |
| AR119                  | AR57 with in-frame deletion of *hlyA* and *hlyA* genes                      | 14                  |
| AR89                   | AR57 with in-frame deletion of *pidD* gene                                  | 14                  |
| AR239                  | AR57 with in-frame deletion of *pidD* and *tadV* genes                      | 15                  |
| AR252                  | AR57 with suicide plasmid pAIR80 inserted into the cheA gene                | This study          |
| AR264                  | AR89 with suicide plasmid pAIR80 inserted into the cheA gene                | This study          |
| AR272                  | AR89 complemented with pAIR38                                              | This study          |
| E. coli strains        |                                                                             |                     |
| DH50                   | Cloning strain; recA                                                        | Laboratory stock    |
| S17-1 *pitr*           | recA thi pro Δhsl(R− M−) RP42-Tc-Mu-Km-Tn7 *pitr* Tp' Sm'                 | 70                  |
| Plasmids               |                                                                             |                     |
| pSpark                 | PCR cloning vector; Amp′                                                    | Canvax              |
| pHRP309                | lacZ reporter plasmid; mob Gm′                                             | 71                  |
| pTrcHisA               | His<sub>6</sub>-tag fusion expression vector; Amp′                          | Invitrogen          |
| pTrcHisA-PPhlyP        | pTrcHisA with pro-PhlyP                                                     | This study          |
| pNidKan                | Suicide vector, derived from pCV342; Km′                                    | 72                  |
| pAIR80                 | pNidKan containing an internal fragment of the cheA gene                    | This study          |
| pAIR38                 | pHRP309 with the *hlyA* gene from strain RM-71                             | 9                   |
| pAIR76                 | pHRP309 with the *pidD* gene from strain RM-71                             | 15                  |

Conjugants were isolated. Disruption of the cheA gene was confirmed by PCR. For *hlyA* complementation, pAIR38 was transferred by conjugation to *P. damsela* subsp. *damsela* strain AR89 (triple mutant [TM]).

Cloning, expression, and purification of toxins. Extracellular products (ECPs) of the various *P. damsela* subsp. *damsela* strains were obtained by growing the bacteria on cellophane membranes placed on LB agar in petri dishes (14-cm diameter). In our hands, this procedure achieved as follows. First, ECPs of *P. damsela* subsp. *damsela* were collected by rinsing each cellophane membrane with 2 ml 0.85% NaCl. Purification of PhlyP was described previously (25). In brief, Origami B cells carrying pQE30-pVCC were grown, induced with IPTG (isopropyl-β-d-thiogalactoside), and harvested by centrifugation. Pellets were resuspended in binding buffer (50 mM NaPO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0, 1 mg/ml lysozyme, 4 mM Pefabloc), and bacteria were lysed and disrupted by ultrasonication. The supernatant was filtered using 0.2-μm-pore-size filters and applied to Ni-NTA-agarose (Qiagen); further purification steps were performed according to the Qiagen protocol. Fractions with the highest protein concentrations were pooled and dialyzed in 20 mM Tris-HCl, pH 6.8, 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 2% [wt/vol] SDS, and bromophenol blue) and harvested by centrifugation. Fractions with the highest protein concentrations were dialyzed in 20 mM Tris-HCl, pH 6.8, and concentrated by using the Bio-Rad protein assay. For the production of recombinant pPhlyP (pVCC), the gene encoding pPhlyP was amplified by PCR from *P. damsela* subsp. *damsela* AR57 by using high-fidelity Kapa Taq DNA polymerase (Kapa) and primers PhlyP-Forward (5’-AACATT TCTTACACCTGCGA) and PhlyP-Reverse (5’-TTACACCAATGCTAAT). The amplified DNA was purified, digested, and cloned into the BamHI site of pTrcHisA (Invitrogen Life Technologies), resulting in pTrcHisA-pPhlyP, which was transformed into Shuffle Express competent E. coli cells (New England BioLabs [NEB]). Induction was performed according to the NEB protocol. N-terminally His<sub>6</sub>-tagged pPhlyP was...
affinity purified from E. coli cell lysates by using Ni-NTA agarose (Qiagen) following Qiagen protocols. The purity of the material was estimated to be at least 90%, as estimated by densitometric analysis of Coomassie-stained SDS-PAGE gels, and the concentration was estimated to be ~20 μg/ml as assessed by an anti-His 
Western blot with His 
-tagged pVCC serving as a standard. S. aureus alpha-toxin and the single-amino-acid-exchange D152C mutant were prepared as published elsewhere (see references 26 and 27 and references therein).

Hemolysis. Hemolysis assays were performed with rabbit erythrocytes (RRCs). The release of hemoglobin was measured in the supernatant by determining the A 
. Assays were carried out by mixing serial dilutions of filtered ECPs (stocks contained 3 mg/ml total protein) and washed RRCs. One hemolytic unit is defined as the amount of hemolysis which lyses 50% of RRCs, calculated with a hemolytic standard of fully lysed RRCs (deionized water). ECPs from different mutants were diluted 2-fold in phosphate-buffered saline (PBS) in microtiter plates. To 50 μl of diluted toxin in each well, 50 μl of 5% rabbit erythrocytes was added and subsequently incubated at 37°C for 1 h. To remove cholesterol, RRCs were incubated for 30 min at 37°C with methyl-β-cyclodextrin (MβCD; Sigma-Aldrich), at 0.5, 1.0, or 2.0 mM. To restore cholesterol in membranes, MβCD-treated RRCs were washed with PBS and supplemented with 400 μg/ml water-soluble cholesterol (Sigma-Aldrich). The absorbance of hemoglobin in supernatants was measured at 405 nm by use of an enzyme-linked immunosorbent assay (ELISA) reader.

Osmoprotection. Assays were performed by exposing RRCs to toxins in the presence or absence of various osmolytes. Purified pVCC or ECPs from strain AR119 (ΔHy/ΔHy/ΔHy), which produces only PhlyP/PhlyA 
, were serially diluted in PBS and mixed with RRCs in the absence of osmoprotectants or in the presence of 80 mM sucrose, 80 mM maltotriose, or 10% dextran 4. Samples were incubated at 37°C for 1 h. Subsequently, hemolysis was quantified by measuring the absorbance of supernatants at 405 nm. In some experiments, cells were subsequently pelleted and resuspended in fresh PBS to wash out osmoprotectants; samples were then tumbled at the time points indicated in the relevant figure.

Transmission electron microscopy (TEM) of PhlyP-treated erythrocyte ghosts. For preparation of PhlyP-treated erythrocyte ghost membranes, 5 × 10
 washed RRCs/ml in osmoprotection buffer (20 mM Tris-HCl, pH 7.0; 0.1% bovine serum albumin (BSA); 30 mM dextran 4) (28) were incubated with 3.75 μg/ml of PhlyP for 1 h at RT. Subsequently, RRCs were washed in osmoprotection buffer and lysed in 5 mM sodium phosphate, pH 8. RRC membranes were washed twice and then resuspended in 100 μl of PBS. Droplets of the PhlyP-loaded ghost preparation were applied to carbon-coated Formvar films mounted on electron microscope nickel grids and exposed to glow discharge immediately before analysis. Absorbed samples were negatively stained with ammonium molybdate or uranyl acetate, and specimens were examined with a Zeiss EM 902 instrument.

Cells and culture conditions. HaCaT cells (non-virally transformed human keratinocytes) (29) were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 GlutaMAX-I medium with 10% fetal calf serum, 1% HEPES buffer, 1% penicillin-streptomycin in a humidified incubator with 5% CO2 at 28°C. Human keratinocytes (29) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 1% HEPES buffer, 1% penicillin-streptomycin in a humidified incubator with 5% CO2 at 28°C. Human keratinocytes are grown to confluence in six-well plates, with or without coverslips, for microscopy- or culture-based assays, respectively. The next day, bacteria (100 μl/well of a suspension in exponential growth phase, i.e., OD 600nm = 0.4) were recovered by centrifugation, and the pellet was resuspended in medium containing ECPs from different P. damselae subsp. damselae strains. S. aureus alpha-toxin, mutant (D152C) alpha-toxin, or pVCC. Subsequently, these suspensions were added to cells, and the cocultures were incubated for 15 min at 37°C. Next, cells were washed twice before they were harvested and resuspended in 500 μl PBS. Dilutions of each sample were prepared, plated onto LB agar plates, and incubated overnight at 25°C. Finally, colony counts were assessed by visual inspection.

Motility measurements. For motility determinations, bacteria were stabbed into semisolid LB plates containing 0.22% agar. Growth diame-

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Staining with trypan blue. ECPs (final dilution, 1:100) of the strains indicated in the figures were added to cell suspensions containing 5 × 10
 HaCaT cells/ml. One milliliter of cell suspension was mixed with 10 μl of each of the ECPs to be tested. The mixture was incubated at 37°C for 1 h. Subsequently, cells were centrifuged and resuspended in 100 μl of PBS; 50 μl of treated cells was mixed with 50 μl of 0.4% trypan blue. Stained and unstained cells were counted in a Neubauer chamber.

PI influx assay and flow cytometry. To assess membrane perforation, toxin-treated cells were incubated with propidium iodide (PI; 50 μg/ml) for 1 min and then fixed. Nuclei were stained with Hoechst 33342 (Cell Signaling Technology), and cells were analyzed by wide-field fluorescence microscopy. The number of sub-G1 events in toxin–treated cell populations or controls was determined by staining ethanol-fixed cells with PI and subsequent analysis by flow cytometry using a FACScan instrument (BD) as described previously (32). Ethanol treatment was omitted for measuring toxin-dependent PI influx into cells.

Flame photometry for measuring K+ ions. Cellular K+ was quantified by flame photometry as described previously (33). In brief, cells were washed three times with ice-cold K+-free choline buffer. Cells were subsequently lysed by incubation for 30 min in choline buffer—0.5% Triton X-100 at RT on a shaker. Lysates were analyzed for K+ K+ with an M401 flame photometer (Sherwood, United Kingdom) using propane gas.

Western blots. Cells were lysed directly in loading buffer (65 mM Tris, 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 2% [wt/vol] SDS, and bromophenol blue) and heated for 5 min at 95°C. Proteins were separated by SDS-PAGE (10%) and electroblotted onto a nitrocellulose membrane. After blocking for 1 h at RT in skim milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% [vol/vol] Tween 20), the membrane was incubated with a primary antibody, washed three times in TBST, and incubated with an HRP-conjugated secondary antibody for 1 h at RT. After three washing steps, bound antibody was detected by an enhanced chemiluminescence (ECL) assay (Roche Applied Science).

Fluorescence microscopy and DIC microscopy. Cells grown on glass coverslips were incubated or not with ECPs or bacteria as detailed in the figure legends. After incubation, cells were washed in PBS and subsequently fixed with 2% paraformaldehyde in PBS for 10 min at RT. For staining of mitochondria or F-actin in cells, we used the MitoTracker mitochondrion-selective probe (Molecular Probes, Invitrogen) or Alexa 488 Fluor-conjugated phalloidin, respectively; in the case of F-actin staining, cells were permeabilized with 0.1% Triton X-100 for 10 min prior to application of the probe. Coverslips were mounted on slides with Fluoro
cell prep (bioMérieux SA), and samples were examined in a Zeiss Axiosvert 200M epifluorescence microscope equipped with a Plan Apochromat 100X/1.4-numerical-aperture oil-immersion differential interference contrast (DIC) objective. For DIC microscopy, a Zeiss POL filter set was used. Digital images were acquired with a Zeiss AxioCam camera. Image processing was done using Zeiss AxioVision software rel. 4.8 and Adobe Photoshop.

Adherence assays. HaCaT cells were seeded at a density of 4 × 10
 well in six-well plates, with or without coverslips, for microscopy- or culture-based assays, respectively. The next day, bacteria (100 μl/well of a suspension in exponential growth phase, i.e., OD 600nm = 0.4) were recovered by centrifugation, and the pellet was resuspended in medium containing ECPs from different P. damselae subsp. damselae strains. S. aureus alpha-toxin, mutant (D152C) alpha-toxin, or pVCC. Subsequently, these suspensions were added to cells, and the cocultures were incubated for 15 min at 37°C. Next, cells were washed twice before they were harvested and resuspended in 500 μl PBS. Dilutions of each sample were prepared, plated onto LB agar plates, and incubated overnight at 25°C. Finally, colony counts were assessed by visual inspection.

Mobility measurements. For motility determinations, bacteria were stabbed into semisolid LB plates containing 0.22% agar. Growth diame-

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FIG 1 PhlyP, a new β-pore-forming toxin. (A) Alignment of amino acid sequences of pro-PhlyP, pro-VCC, and VVC (the prodomain of VVC is not contiguously expressed with the hemolysin domain and the lectin domain). Sequences were aligned by use of ClustalW 2.0 (68) and displayed with version 3.21 of BOXSHADE, written by K. Hofmann and M. Baron (http://www.ch.embnet.org/software/BOX_form.html). The prodomain of PhlyP is shaded in green, and...
tors around the puncture site were measured. Ten plates were evaluated for each condition.

**Statistics.** Data displayed are derived from ≥3 independent experiments; in the figures, columns show mean values, and error bars indicate standard errors (SE). The statistical significance of differences between two mean values was assessed using two-sided, unpaired Student’s t test. Analysis of variance (ANOVA) and Tukey’s post hoc analysis were employed for multiple comparisons. In the figures, degrees of significance are indicated as follows: *, *P ≤ 0.05; **, *P ≤ 0.01; and ***, *P ≤ 0.001 (P values of ≤0.05 were assumed to indicate significance).

**RESULTS**

**PhlyP forms small membrane pores.** Previously, we noted that highly hemolytic strains of *P. damselae* subsp. *damselae* are particularly virulent (9). Further, the data suggested that *hlyA*ep plays major roles in both virulence and hemolytic activity (14). Sequence comparison suggested that *hlyA*ep encodes a hemolisyn related to *Vibrio cholerae* cytolsin (VCC), leaving unmatched, however, an additional lecmin domain present in pro-VCC (pVCC) (9) (Fig. 1A). Also, homology-based structure prediction with the aid of I-TASSER (34) inferred a structure similar to that of pVCC (Fig. 1B). To obtain experimental evidence that PhlyP is in fact a small β-PFT, we incubated ECPs from *P. damselae* subsp. *damselae* strain AR119 (*ΔhlyA*epΔhly*A*ch) produces only HlyA*ep* with erythrocyte ghosts and separated firmly bound proteins by SDS-PAGE. ECPs from strain AR119 yielded a 50-kDa species and one with a high apparent molecular mass (250 kDa), both of which were absent from samples treated with ECPs of the triple mutant AR89 (*ΔhlyA*epΔhly*A*chΔhly*P*). At RT, the high-molecular-mass band resisted SDS and trypsin digestion (Fig. 1C), and it was lost only after heating to 95°C in SDS. Thus, PhlyP, similar to other small β-PFTs, appears to form stable oligomers (28, 35).

Transmission electron microscopy of pore complexes formed by PhlyP on red cell membranes (“ghosts”) revealed typical circular structures associated with membranes (Fig. 1D).

To approximate pore size, we next performed osmoprotection experiments (36). PFTs induce an uncontrolled influx of water into red cells, which ultimately leads to lysis. However, when osmolytes with hydrodynamic diameters (HDs) above the effective pore size are present, cells are protected by retention of water in the extracellular space. The differential ability of various osmolytes to suppress lysis allows an approximation of pore size, as previously shown for pVCC (37). Osmoprotection assays were carried out with ECPs from the PhlyP-producing strain AR119; pVCC served as a control. Although pVCC-dependent hemolysis was significantly reduced by maltotriose (molecular mass, 504.4 g mol⁻¹; HD, ~1.2 nm) (Fig. 2A, left panel), inhibition of PhlyP-dependent hemolysis was inefficient (Fig. 2B, left panel). In contrast, dextran 4, which has an HD of ~3 nm, protected cells equally well against either of these toxins. When RRCs which had been treated with PFTs in the presence of an active osmoprotectant (and thus were protected from lysis) were pelleted and resuspended in PBS, hemolysis occurred after incubation for 1 h at 37°C, indicating that osmoprotectants had not prevented binding of toxins to RRCs (Fig. 2A and B, right panels). The results suggested that PhlyP creates pores with an effective diameter of >1.2 and <3.0 nm, which are thus wider than those formed by VCC.

**Cholesterol is required for PhlyP-dependent hemolysis.** As exemplified by VCC, cholesterol-dependent cytolysins (CDCs) (38) are not the only PFTs that require cholesterol in target cell membranes to function properly (39). When cholesterol was extracted from RRCs by use of methyl-β-cyclodextrin (MβCD), we observed that PhlyP failed to lyse cells (Fig. 2C, left panel), suggesting that PhlyP requires cholesterol in order to cause hemolysis. The prediction that the effect of MβCD was actually a consequence of cholesterol depletion was confirmed by adding back cholesterol to depleted RRCs (Fig. 2C, right panel), as this fully restored the ability of PhlyP to cause hemolysis.

**Extracellular products of Photobacterium damselae subsp. damselae contain active PhlyP.** Proteases in the extracellular environment (40) or located in the host cell membrane (41) process pVCC to yield mature, active VCC, which oligomerizes and forms heptameric transmembrane pores (25). Sequence analysis indicated that *hlyA*ep encodes a small β-PFT related to VCC (9) that is also produced as a protoxin, but cleavage sites of pVCC (25) are not conserved in pPhlyP. However, secretion products of strain AR119 contained hemolytic activity which was not fully blocked by low temperature or inhibitor of tumor necrosis factor alpha processing 2 (TAPI-2), a metalloproteinase inhibitor that inhibits processing of pVCC to VCC by cellular metalloproteinases (41) (see Fig. S1A and B in the supplemental material). Therefore, it appeared likely that these ECPs contained mature PhlyP. Indeed, preparative isoelectric focusing (IEF), which has been employed to purify VCC (28), followed by ion-exchange chromatography allowed us to isolate the hemolytic activity from ECPs of strain AR119. It focused at a pI of ~5.5, eluted as a single peak from a Mono S column (Fig. 3A, left panel), and had an apparent molecular mass of ~50 kDa as determined by SDS-PAGE (Fig. 3A). The band was cut out from gels and subjected to Edman degradation, which revealed the sequence N terminus-valine-alanine-serine-aspartic acid-glutamine-C terminus, matching positions 155 to 159 of the amino acid sequence predicted from *hlyA*ep. The corresponding protein is expected to comprise 447 amino acids and to have a molecular mass of 50.47 kDa, in line with its electrophoretic migration velocity (Fig. 3A).

**Purified PhlyP is cytotoxic to nucleated cells.** Cytotoxic activity of *P. damselae* subsp. *damselae* has been described previously (42), but the roles of individual *P. damselae* subsp. *damselae* hemolysins in this context have not been investigated. Purified PhlyP provoked a marked, time- and dose-dependent drop of intracellular ATP in HaCaT cells (Fig. 3B and C). In contrast to the large pore-forming protein streptolysin O, no significant leakage of lactate dehydrogenase (LDH) (140 kDa) was observed with PhlyP (Fig. 3D). Notably, however, PhlyP, but not VCC, rapidly caused a
significant influx of PI (Fig. 3E), in line with the differential behavior of these toxins in osmoprotection assays.

Loss of potassium ions is a hallmark of PFT action and is held responsible for many of their downstream effects (27, 33, 43, 44). PhlyP caused a loss of intracellular K⁺ in HaCaT cells and also in AB.9 cells from zebrafish (Fig. 3F). Phosphorylation of eIF2α and inactivation of mTORC1 are important consequences of the PFT-dependent loss of intracellular K⁺ from cells (27), both leading to attenuation of translation (33, 45, 46). The Western blot in Fig. 3G documents that PhlyP leads to dephosphorylation of p70S6K, a substrate of TORC1, and to hyperphosphorylation of eIF2α. Consistently, incorporation of puromycin in growing polypeptide chains was markedly reduced, indicating that PhlyP inhibits protein synthesis (Fig. 3H).

**Recombinant pro-PhlyP recapitulates cytotoxic effects.** In order to provide evidence that the cytotoxicity of PhlyP did not

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**FIG 2** PhlyP forms small membrane pores, and its hemolytic activity requires cholesterol in cell membranes. (A and B) Osmoprotection experiments were performed with pVCC (A) and PhlyP (ECPs of AR119) (B). The left graphs show hemolysis in the presence of osmoprotectants, and the right graphs show hemolysis after washout of osmoprotectants. Data show mean values ± SE (n = 3). (C) RRCs were depleted of cholesterol by using methyl-B-cyclodextrin (MβCD) and were challenged with ECPs of strain AR119 (left). Replenishment of cholesterol was conducted by adding soluble cholesterol to cholesterol-depleted RRCs (right). Data are mean values ± SE (n = 3).
**FIG 3** *P. damselae* subsp. *damselae* extracellular products contain mature PhlyP. (A) The left panel shows the elution profile from the Mono S column; note the single peak in the right half of the profile, which contained the hemolytic activity. The right panel shows an SDS-PAGE gel with purified PhlyP (two peak fractions from the elution profile), indicated by an open triangle. (B) ATP was measured after incubation of HaCaT cells with various concentrations of purified PhlyP for 2 h. Data are mean values ± SE (*n* = 3). ***, *P* ≤ 0.001 in two-sided, unpaired Student’s *t* test. (C) Results of ATP assays after incubation of HaCaT cells with purified PhlyP (100 ng/ml) for various times. Data shown are percentages of the level in untreated controls and are mean values ± SE (*n* = 3). (D) HaCaT cells were treated for 10 min with the indicated toxins, and lactate dehydrogenase (LDH) was measured in supernatants. Control, saline without toxin (HBSS). Data are mean values ± SE (*n* = 3). **, *P* ≤ 0.01 in two-sided, unpaired Student’s *t* test. (E) HaCaT cells in suspension were incubated with PhlyP (100 ng/ml) for 4 min, PI (50 µg/ml) was added, and samples were analyzed after 1 min at 37°C by flow cytometry. FL3H, fluorescence intensity (log) for PI; counts, number of events per channel. Red line, PhlyP; green line, VCC; filled area, untreated cells. (F) Cellular K⁺ levels were determined by flame photometry in HaCaT or AB.9 cell lysates after exposure of cells to PhlyP. Cells were incubated with purified PhlyP (100 ng/ml) (HaCaT cells) or ECPs from strain AR119 (produces only PhlyP) (AB.9 cells). Intracellular levels of potassium were determined after 10 min. Data are percentages of the levels in untreated controls and are mean values ± SE (*n* = 3). ***, *P* ≤ 0.001 in two-sided, unpaired Student’s *t* tests. (G) HaCaT cells were treated with purified PhlyP (100 ng/ml) for the indicated times at 37°C and were analyzed by Western blotting for phosphorylation of p70S6K or eIF2α. (H) HaCaT cells were incubated with purified PhlyP (100 ng/ml) or VCC (10 ng/ml) for 10 min at 37°C. Subsequently, cells were incubated with 10 µg/ml puromycin (1 h at 37°C), which is incorporated into nascent proteins (69). Finally, cells were analyzed by Western blotting using antibodies directed against puromycin.
depend on factors potentially copurified from *P. damselae* subsp. *damselae* ECPs, we sought to perform experiments with a heterologously expressed toxin. To this end, we cloned and expressed the protoxin (pPhlyP) as a His$_6$/H$_{11003}$ fusion protein in *E. coli* (Fig. 4A and B) to analyze the dose and time dependency of various effects of heterologously expressed, affinity-purified material on HaCaT cells. The earliest change that was consistently observed was an influx of PI (50% positive cells after 7.5 min of incubation with 100 ng/ml pPhlyP) (Fig. 4C), followed by a drop of ATP (Fig. 4D), depolymerization/redistribution of F-actin, morphological alterations of mitochondria (Fig. 4G) similar to what had been observed for listeriolysin (47), and (de)phosphorylation of S6K and eIF2$_\alpha$ (Fig. 4E), all commencing at ~15 min. By 1 h, at a toxin concentration of 100 ng/ml, incorporation of puromycin—an indicator of ongoing protein synthesis—was largely inhibited (Fig. 4F). After 2 h, a significant increase in sub-G$_1$ events was seen, indicating irreversible DNA damage (see Fig. S1C in the supplemental material).

*Photobacterium damselae* subsp. *damselae* causes rapid hemolysin-dependent damage. To evaluate the cytotoxicity of PhlyP and bacteria in context, we infected cultures of epithelial cells with *P. damselae* subsp. *damselae* strains expressing all or none of the three known hemolysins, or one hemolysin only. First, HaCaT cells infected with *P. damselae* subsp. *damselae* were stained with PI, which is excluded from intact cells. Infection with the wild-type (WT) strain, encoding the three known hemolysins and expressed pPhlyP, reproducibly resulted in ~50% PI-positive cells after 7.5 min of incubation with 100 ng/ml pPhlyP (Fig. 4C). Subsequently, cells were incubated or not for 1 h at 37°C with 10 μg/ml puromycin, which is incorporated into nascent proteins (69). Cells were analyzed by Western blotting for puromycin and α-tubulin. (G) HaCaT cells were incubated for the indicated times with 100 ng/ml His$_{11003}$-tagged pPhlyP and then either washed, fixed, and treated with 0.1% Triton X-100 prior to staining with phalloidin-Alexa 488 (upper panels) or washed and treated with MitoTracker Green FM (200 nM) for 30 min at 37°C (lower panels). bars = 20 μm.
led to permeabilization of the majority of cells within 30 min (Fig. 5A, upper panels); considerable permeabilization was already noticed even after 15 min (data not shown). In contrast, keratinocytes infected with the TM, in which all three hemolysin genes are disrupted (14), did not stain with PI (Fig. 5A, lower panels).

Incubation of HaCaT cells with ECPs of the WT strain, but not the TM strain, recapitulated the effect of infection on membrane permeability (Fig. 5B) and caused a loss of cellular ATP (Fig. 5C); complementation of the TM strain with pAJR38 (yielding AR272) restored the ability to reduce ATP levels (see Fig. S1D in the supplemental material). Moreover, the frequency of sub-G1 events increased (Fig. 5D). Next, we extended coculture experi-

**FIG 5** *P. damselae* subsp. *damselae* toxins cause fulminant membrane damage. (A) HaCaT cells were infected with washed wild-type (WT) or triple mutant (TM) *P. damselae* subsp. *damselae* at a multiplicity of infection (MOI) of 1:30, incubated for 30 min at 37°C, stained for 1 min with PI (50 μg/ml), and subsequently fixed and stained with Hoechst 33342. Bars = 50 μm. Representative images are shown. (B) HaCaT cells were incubated for 8 min with ECPs (6 μg/ml total protein) from the WT or TM strain and stained as described for panel A, and PI-positive cells in digital microscopic images were enumerated. The graph shows mean values ± SE for 4 independent experiments. ***, P ≤ 0.001 in two-sided, unpaired Student’s t test. (C) HaCaT cells were incubated with ECPs of the wild-type or triple mutant strain. After 1 h, the cellular ATP level was determined. Data are mean values ± SE (n = 4). ***, P ≤ 0.001 in two-sided, unpaired Student’s t test. (D) Cells were incubated with normal medium or ECPs for 48 h and then processed for flow cytometric analysis of DNA content. Columns show numbers of sub-G1 events. Data shown are mean values ± SE (n = 3). *, P ≤ 0.05 in two-sided, unpaired Student’s t test. (E) HaCaT cells were incubated for 1 h at 37°C with ECPs containing single toxins, as indicated. Control, medium only. Cells were stained with trypan blue (TB), and TB-positive and -negative cells were counted in a Neubauer chamber. Data are mean values ± SE (n = 3). ***, P ≤ 0.001 in two-sided, unpaired Student’s t test. HaCaT (F) and AB.9 (G) cells were treated as described for panel E prior to measurement of ATP. Graphs show mean values ± SE (n = 3). ***, P ≤ 0.001 in two-sided, unpaired Student’s t test.
ments to measure the effects of ECPs from double mutant strains encoding only one of the three known hemolysins each (Table 1). Among these strains, AR119, which expresses only PhlyP, yielded the strongest effects on membrane integrity (Fig. 5E) and cellular ATP levels (Fig. 5F). Notably, ECPs of AR119 also induced a loss of ATP in AB.9 cells from zebra fish (Fig. 5G).

Hemolysins promote bacterial adherence. When analyzing mammalian cells infected with *P. damselae* subsp. *damselae* by differential interference contrast microscopy, we made the fortuitous observation that *P. damselae* subsp. *damselae* WT bacteria decorated the surfaces of HaCaT cells, whereas comparably few bacteria of the TM strain adhered to cells (Fig. 6A).

To verify this unexpected result by using an independent approach and to quantify the effect, we enumerated bacteria adhering to cells by a culture-based assay. Several-fold larger numbers of bacteria (CFU) were obtained with WT bacteria than with TM bacteria, suggesting that hemolysins conferred adherence to cells (Fig. 6B). Addition of ECPs of the WT strain to TM bacteria reconstituted the adherent phenotype. Using double mutant bacteria, we found that each of the three known hemolysins was capable of enhancing bacterial adherence to cells (Fig. 6B).

In order to clarify whether hemolysins of *P. damselae* subsp. *damselae* also promoted adherence of other bacteria to HaCaT cells, we tested the effect of WT ECPs on adherence of *E. coli*. The results obtained with this combination suggested that the activity of ECPs did not depend on some *P. damselae* subsp. *damselae*-specific, nonsecreted factor(s) (see Fig. S2A, left panel, in the supplemental material). Next, we asked whether purified pVCC could also enhance adherence of *E. coli* to HaCaT cells; the confirmatory results are shown in Fig. S2A, right panel.

Up to this point, all adherence assays had been performed with *E. coli* or *P. damselae* subsp. *damselae*, i.e., Gram-negative, motile bacteria. Flagellated bacteria may move along concentration gradients of nutrients or ions (48), which might form when the nutrients or ions leak from target cells of PFTs. To investigate whether the apparent hemolysin-dependent attachment of bacteria to cells depended on bacterial chemotaxis, we extended our experiments to include *S. aureus*, a Gram-positive, nonmotile species. As shown in Fig. S2B in the supplemental material, ECPs of *P. damselae* subsp. *damselae*, purified pVCC, and alpha-toxin all enhanced the adherence of an alpha-toxin-negative *S. aureus* strain, suggesting that hemolysin-dependent adherence did not depend on bacterial chemotaxis but not excluding a role of chemotaxis in the case of hemolysin-dependent association of motile bacteria with target cells. Therefore, we deleted the cheA gene from the *P. damselae* subsp. *damselae* WT and TM strains. CheA is a histidine kinase that senses changes in the concentration of nutrients through transmembrane chemoreceptors and leads to flagellar motor switching (49). Among the three cheA genes carried on the *Vibrio cholerae* chromosome, only cheA2 is directly related to chemotaxis (50). Interestingly, only one cheA homologue was found to be carried on the *P. damselae* subsp. *damselae* chromosome. Although deletion of cheA decreased the spread of both the *P. damselae* subsp. *damselae* WT and TM strains on semisolid agar plates (see Fig. S2C), as expected, no significant effect on the association of *P. damselae* subsp. *damselae* with HaCaT cells was observed (Fig. 6C). This indicated that the enhanced association of bacteria and target cells was independent of chemotaxis. Hence, it likely reflected increased adherence. Bacterial adherence commonly involves interactions between pili and host cells. The role of type IV pili in adherence to host cells has been demonstrated for many Gram-negative bacteria (51–53). Deletion of the *P. damselae* subsp. *damselae* prepli peptidase gene pilD severely affects hemolysin secretion and prevents pilus formation (15). In addition to pilD, the gene encoding TadV, another prepli peptidase, which belongs to the tight adherence (tad) cluster carried on pPHDD1, is involved in the production of type IV pili (54). TadV partially compensates for PilD and is responsible for the residual release of hemolytic activity observed with ΔpilD mutants (15). Therefore, we investigated whether exogenous hemolysins are able to promote the association of target cells with *P. damselae* subsp. *damselae* strains lacking either pilD or both pilD and tadV. As expected, ΔpilD bacteria showed a marked (~10-fold) decrease of adherence, and complementation of the ΔpilD mutant with the pilD gene restored the ability to produce pili and large hemolytic halos (15). Although the number of pilus-negative bacteria adhering to target cells was much reduced, the addition of WT ECPs increased adherence of both the ΔpilD single mutant and the ΔpilD ΔtadV double mutant (Fig. 6D). The fold increase was similar to that for pilus-expressing wild-type bacteria, indicating that the adherence-enhancing effect of hemolysins did not depend on pilI.

Next, we wished to address the question of whether the effect of membrane-damaging toxins on the increased adherence of bacteria depended on active responses of target cells. The observation that an alpha-toxin with a single amino acid mutation, with a far lower hemolytic activity than that of wild-type toxin, exerted a weaker effect on adherence than that of the wild-type toxin (see Fig. S2B, right panel, in the supplemental material) indicated that membrane perforation is involved. This would be expected, for instance, if signaling pathways triggered by membrane perforation played a role. By phosphorylating phosphatidylinositolos at the 3'-OH position of the inositol ring, phosphatidylinositol 3-kinases (PI3Ks) modulate the composition and function of biological membranes of eukaryotic cells in response to many types of stress. Therefore, we investigated the effect of low-molecular-weight inhibitors of PI3Ks on toxin-dependent bacterial adherence. Three functional classes of PI3Ks are distinguished based on their preferred substrates. ZSTK474, a class I/II-Selective PI3K inhibitor, did not reduce hemolysin-dependent adherence (data not shown), but LY294002, which also inhibits class III PI3K, led to a significant inhibition of adherence (Fig. 6E). Therefore, hemolysin-dependent increases of bacterial adherence to target cells appear to depend on class III PI3K-mediated cellular responses.

**DISCUSSION**

We report here the primary characterization of PhlyP, a major contributor to the virulence of *P. damselae* subsp. *damselae* (14). PhlyP is encoded on a plasmid that is found in ~20% of environmental isolates of *P. damselae* subsp. *damselae* (13). It is conceivable that the plasmid spreads to other bacterial species (9). Because *P. damselae* subsp. *damselae* has the potential to cause serious infections, it is relevant to elucidate the basis of its virulence. The characterization of PhlyP represents an important step toward this goal. We showed that PhlyP is a small B-PFT, with distinct properties, which exerts multiple toxic effects on nucleated target cells. Further, the results led to the conclusion that hemolysins are general promoters of bacterial adherence to target cells.

The observation that PhlyP is a small pore-forming protein was
FIG 6  Hemolysins promote bacterial adherence to target cells. (A) HaCaT cells were infected with the *P. damselae* subsp. *damselae* WT (AR57) strain or TM (AR89) strain. The distribution of bacteria was analyzed by differential interference contrast microscopy. Bars = 10 μm. (B) Results of adherence assays based on CFU counts. (Left) HaCaT cells infected with *P. damselae* subsp. *damselae* TM or WT. (Middle) HaCaT cells infected with *P. damselae* subsp. *damselae* TM and treated (white columns) or not (black column) with ECPs obtained from the WT strain (6 μg/ml total protein). (Right) HaCaT cells infected with the TM (black column) or double mutant strains, each expressing one of the known hemolysins only (white columns). Data shown are mean values ± SE (*n* = 3). * (left and middle panels), *P* ≤ 0.05 as determined by two-sided, unpaired Student’s *t* test; * and ** (right panel), *P* ≤ 0.05 and *P* ≤ 0.01, respectively, as determined by ANOVA. (C) Results of adherence assays based on CFU counts. The bars show mean numbers of CFU (± SE [*n* = 3]) determined for samples of HaCaT cells infected with *P. damselae* subsp. *damselae* WT or TM (black) and their respective *cheA* mutants. ns, nonsignificant, i.e., *P* > 0.05, in two-sided, unpaired Student’s *t* tests. (D) HaCaT cells were infected with *P. damselae* subsp. *damselae* AR239 (∆*pilD*) or AR252 (∆*pilD ∆tafV*) in the presence of ECPs from the WT strain. For determination of the numbers of adherent AR239 and AR252 bacteria, a 10-fold larger volume of the harvested coculture than that of all other strains analyzed here was plated for CFU counts. Data shown are mean values ± SE ([*n* = 3]). ** and ***, *P* ≤ 0.01 and *P* ≤ 0.001, respectively, in two-sided, unpaired Student’s *t* tests. (E) HaCaT cells were infected with the TM and WT strains in the presence of Ly294002 (1 μM). Data are mean values ± SE (*n* = 3). ns, nonsignificant; *, *P* ≤ 0.05 in two-sided, unpaired Student’s *t* test.
predicted from nucleic acid sequences. Several lines of experimental evidence, including functional (osmoprotection), morphological (circular structures in electron micrographs), and biochemical (SDS-stable complexes) evidence, support this contention. Also, in line with previous observations with \textit{V. cholerae} cytolysin (VCC), cholesterol depletion in red cells reduced the lytic activity of PhlyP. Although PhlyP thus shares typical characteristics with other members of the small \beta-PFT family, there are substantial differences even between PhlyP and VCC, the closest known ortholog, with 50% identity at the amino acid level. First, the PhlyP transmembrane channel is wider than the VCC channel, in line with the fact that the narrow point in the VCC \beta-barrel is formed by a heptad of tryptophan residues (W318) (55), whereas the corresponding position in PhlyP is a serine (S341), which is less bulky. A second distinguishing feature of PhlyP that deserves mentioning is the absence of a second lectin domain. VCC contains two contiguous lectin domains: the so-called \beta-trefoil domain and the more C-terminal \beta-prism domain (55). Deletion of the \beta-prism domain virtually eliminated hemolytic activity (56), which led to the conclusion that the \beta-trefoil domain in VCC is inactive (57). Although devoid of the \beta-prism domain, PhlyP applied at nanomolar concentrations kills mammalian cells, suggesting that its trefoil domain is active. In support of this, an amino acid substitution in the \beta-trefoil domain renders PhlyP substantially less hemolytic, but the activity is fully recovered by replacing the defective domain with the wild-type sequence (13). Notably, PhlyP’s hemolytic, but the activity is fully recovered by replacing the de-

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