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# A Transmissible Plasmid-Borne Pathogenicity Island Confers Piscibactin Biosynthesis in the Fish Pathogen *Photobacterium damselae* subsp. *piscicida*

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**The fish pathogen *Photobacterium damselae* subsp. *piscicida* produces the siderophore piscibactin. A gene cluster that resembles the *Yersinia* high-pathogenicity island (HPI) encodes piscibactin biosynthesis. Here, we report that this HPI-like cluster is part of a hitherto-uncharacterized 68-kb plasmid dubbed pPHDP70. This plasmid lacks homologs of genes that mediate conjugation, but we found that it could be transferred at low frequencies from *P. damselae* subsp. *piscicida* to a mollusk pathogenic *Vibrio alginolyticus* strain and to other Gram-negative bacteria, likely dependent on the conjugative functions of the coresident plasmid pPHDP60. Following its conjugative transfer, pPHDP70 restored the capacity of a vibrioferrin mutant of *V. alginolyticus* to grow under low-iron conditions, and piscibactin became detectable in its supernatant. Thus, pPHDP70 appears to harbor all the genes required for piscibactin biosynthesis and transport. *P. damselae* subsp. *piscicida* strains cured of pPHDP70 no longer produced piscibactin, had impaired growth under iron-limited conditions, and exhibited markedly decreased virulence in fish. Collectively, our findings highlight the importance of pPHDP70, with its capacity for piscibactin-mediated iron acquisition, in the virulence of *P. damselae* subsp. *piscicida*. Horizontal transmission of this plasmid-borne piscibactin synthesis gene cluster in the marine environment may facilitate the emergence of new pathogens.**

The emergence of bacterial pathogens in aquaculture systems has been a subject of great interest to microbiologists. Widespread horizontal gene transfer in marine environments and the close interactions among bacterial species in the cramped confines of aquaculture facilities make mobile-element acquisition a driving force in the evolutionary processes promoting the emergence of new virulence strategies (1–3). *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish photobacteriosis, is one of the most devastating pathogens in marine aquaculture (4). This bacterium, a member of the family *Vibrionaceae*, shares a species designation with *P. damselae* subsp. *damselae*, a bacterium that causes vibriosis in a variety of marine animals and is also the causative agent of infections in humans (5). The two *P. damselae* subspecies seem to cause disease through distinct mechanisms, and the differential presence of plasmids has contributed significantly to their divergence in virulence gene content (6, 7).

A major virulence factor in *P. damselae* subsp. *piscicida* is the metalloprotease A-B toxin AIP56, which induces apoptosis in fish macrophages and neutrophils. Interestingly, AIP56 is encoded in a 10-kb plasmid dubbed pPHDP10 (6). *P. damselae* subsp. *piscicida* spreads systemically following infection, suggesting that the pathogen faces an iron-limiting environment within the host. In this regard, it has been reported that *P. damselae* subsp. *piscicida* can obtain iron from human transferrin by a mechanism involving the production of a siderophore (8). Later, a gene cluster (the *irp* cluster) was found to be required for the biosynthesis of a phenolate-like siderophore in *P. damselae* subsp. *piscicida* (9). Initially, it was shown that insertional inactivation of the putative biosynthetic gene *irp1* abolished the biosynthesis of the *P. damselae* subsp. *piscicida* siderophore. Recently, the chemical structure of this siderophore, which was dubbed piscibactin, was determined and demonstrated to be similar but not identical to that of yersiniabactin (10). Indeed, the *irp* cluster was found to have a structure similar to that of the *Yersinia* high-pathogenicity island (HPI), a chromosome-borne high-pathogenicity island that encodes the biosynthesis and transport of the siderophore yersiniabactin in pathogenic *Yersinia* species (11). First identified in *Yersinia* spp., the HPI has been subsequently found in several *Enterobacteriaceae* genera (12, 13).

Mobile genetic elements appear to have played a major role in shaping the genome of *P. damselae* subsp. *piscicida*. In addition to pPHDP10, encoding AIP56 toxin, a conjugative plasmid, pPHDP60, which encodes a putative type II secretion system has been recently described (14), and an SXT/R391 family integrating conjugative element dubbed ICE*Pda*Spa1 was also identified in this subspecies (15). The structural features of the *irp* cluster and its similarities to the *Yersinia* HPI initially suggested that the pis-

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**TABLE 1** Strains and plasmids used in this study

| Strain or plasmid | Description or relevant characteristics[a] | Source or reference |
|---|---|---|
| Strains | | |
| *P. damselae* subsp. *piscicida* | | |
| DI21 | Highly virulent isolate, harbors pPHDP70, pPHDP60, pPHDP10 | Seabream, Spain |
| DI21-Rif | DI21 derivative, spontaneous Rif$^r$ mutant | 9 |
| CS31 | DI21 with pSJR52 inserted in pPHDP70; *irp1* disruptant | 9 |
| AR84 | CS31 cured of plasmid pPHDP70 | This study |
| *Vibrio alginolyticus* | | |
| TA15 | Isolate from carpet shell clams (*Ruditapes decussatus*) | 31 |
| AR13 | TA15 Δ*pvsA*, deficient in vibrioferrin siderophore production | This study |
| AR43 | AR13 transconjugant for pPHDP70::Km | This study |
| AR45 | AR13 transconjugant for pPHDP70::pSJR52 | This study |
| *Aeromonas salmonicida* RSP74.1 | Isolated from turbot (*Scophthalmus maximus*) | 51 |
| *Vibrio anguillarum* RV22 | Isolated from turbot (*Scophthalmus maximus*) | 52 |
| *Escherichia coli* | | |
| DH5α | Cloning strain | Laboratory stock |
| S17-1 λ pir | RP4-2(Km::Tn*7*, Tc::Mu-1) *pro-82* λ*pir recA1 endA1 thiE1 hsdR17 creC510* | Laboratory stock |
| XL1-blue MR | Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* | Stratagene |
| MG1655 | *E. coli* K-12, F$^-$ | 53 |
| BI533 | MG1655 Nal$^r$ | 54 |
| MC1061 | F$^-$ *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL hsdR2*($r_K^-$ $m_K^+$) *mcrA mcrB1* | 55 |
| Plasmids | | |
| pKD4 | Template for Km$^r$ gene | 21 |
| pNidKan | Suicide vector, derived from pCVD442, Km$^r$ | 19 |
| pSJR52 | 4,672-bp EcoRI-XbaI fragment including the 3′ end of *irp2* and the 5′ end of *irp1*, cloned into pNidKan | 9 |
| pPHDP70 | 68-kb native plasmid of strain DI21, contains piscibactin gene cluster | This study |
| pPHDP70::Km | pPHDP70 with a Km$^r$ gene from pKD4 inserted between *orf19* and *orf20* | This study |
| pPHDP70::pSJR52 | pPHDP70 with pSJR52 inserted: disrupts *irp1* gene | 9 |
| pPHDP60 | Conjugative 68-kb native plasmid of strain DI21 | 14 |
| pPHDP60::Cm | pPHDP60 with a Cm$^r$ gene inserted into *orf48* | 14 |

[a] Ap$^r$, ampicillin resistance; Km$^r$, kanamycin resistance; Rif$^r$, rifampin resistance; Cm$^r$, chloramphenicol resistance; Nal$^r$, nalidixic acid resistance.

cibactin biosynthesis genes might constitute a pathogenicity island (PAI) (9). In the present study, we report that the *irp* cluster is part of a novel mobilizable 68-kb plasmid here dubbed pPHDP70. pPHDP70 appears to be sufficient for piscibactin synthesis, and it proved to be required for *P. damselae* subsp. *piscicida* siderophore-mediated iron acquisition and virulence in fish. Notably, pPHDP70 provided piscibactin synthesis and utilization capabilities to a mollusk-pathogenic strain of *Vibrio alginolyticus* following its conjugative transfer aided by the coresident plasmid pPHDP60.

## MATERIALS AND METHODS

**Bacteria, plasmids, oligonucleotides, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. Marine bacteria were routinely grown at 25°C in either tryptic soy agar or tryptic soy broth (Difco) supplemented with 1% NaCl (TSA-1 and TSB-1, respectively). *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth or LB agar. Antibiotics were used at the following final concentrations: kanamycin (Km) at 25 μg ml$^{-1}$, ampicillin sodium salt (Ap) at 50 μg ml$^{-1}$, chloramphenicol (Cm) at 20 μg ml$^{-1}$, rifampin (Rif) at 50 μg ml$^{-1}$, and nalidixic acid (Nal) at 40 μg ml$^{-1}$. All stocks were filter sterilized and stored at −20°C. The iron chelator 2,2′-dipyridyl (Sigma) was prepared at 10 mM in water. The oligonucleotides used in this study are listed in Table 2.

**Cosmid library construction, DNA sequencing, and annotation.** Genomic DNA of *P. damselae* subsp. *piscicida* DI-21 was purified using the genome DNA kit from Q-BIOgene, partially digested with Sau3AI, and ligated into SuperCos 1 cosmid vector (Stratagene). The ligated products were packaged into bacteriophage lambda particles (Gigapack III Gold packaging extract; Stratagene, USA) and introduced into *E. coli* XL1-Blue MR cells. Nucleotide sequences were determined using the CEQ DTCS-Quick Start kit (Beckman Coulter) and a CEQ 8000 capillary DNA sequencer (Beckman Coulter). Plasmid annotation was carried out with RAST server (16), and a plasmid graphical map was generated with CGView Server (17). G+C content analyses were conducted with the Artemis Comparison Tool (18). Protein domains were searched using the Pfam database (http://pfam.sanger.ac.uk/).

**Construction of a *V. alginolyticus pvsA* mutant.** The *pvsA* gene of *V. alginolyticus* TA15, encoding a biosynthetic enzyme of the siderophore vibrioferrin, was deleted by allelic exchange with the Km$^r$ suicide vector pNidkan as previously described (19). A nonpolar deletion was constructed by using PCR amplification of the amino- and carboxy-terminal fragments of *pvsA*, which, when fused together, would result in an in-frame deletion. The plasmid construction containing the deleted allele was mated from *E. coli* S17-1λ*pir* into *Vibrio alginolyticus* TA15, selecting for Ap (TA15 is naturally Ap$^r$) and Km resistance for plasmid integration and subsequently for sucrose resistance (15% [wt/vol]) for a second recombination event.

**Construction of a kanamycin cassette-marked version of pPHDP70, conjugative assays, and plasmid curing.** We constructed a labeled pPHDP70 by inserting a Km$^r$ cassette through a double crossover between *orf19* and *orf20* (Fig. 1) by allelic exchange using the Ap$^r$ suicide vector pKEK229 (20). In brief, ca. 3,300-bp sequences upstream and downstream of the intergenic region that separates *orf19* and *orf20* were PCR amplified and ligated to a Km resistance gene amplified from plasmid

**TABLE 2** Oligonucleotides used in this study

| Purpose and oligonucleotide | Sequence | bp amplified |
|---|---|---|
| *entD-orf21* | | |
| HPI-103 | ATTGCGACGCATGTTGTATG | 2,134 |
| vibD-3′ | CATGCCTCCAAGGTTATCAC | |
| *parAB* | | |
| pCW046-5′-2 | ATTGCGACGCATGTTGTATG | 1,359 |
| pCW046-3′-1 | TATTCACGGTGATGACTAAC | |
| *orf28* (putative carbonic anhydrase) | | |
| mig5-5′-1 | ACGTTTGTTTTAGCAACAGC | 459 |
| mig5-3′-1 | TGTCACCTAATGATGTGGTG | |
| *orf48* (fimbrial usher) | | |
| pCW042-5′4 | CATTATGTCAGCATCTTCCG | 522 |
| usher-1 | TACTCGTGTAGTTATATCCG | |
| *repA* | | |
| RepA-F | GAAAGCTCAGATGCCATGCC | 2,407 |
| pCW044-5-1 | GAAAGCTCAGATGCCATGCC | |
| Amplification of pPHDP70 iterons | | |
| ITERON_F | GAAAGCTCAGATGCCATGCC | 1,429 |
| ITERON_R | GAAAGCTCAGATGCCATGCC | |
| Km$^r$ marker amplification from pKD4 | | |
| Kan-1 | GCGGATCCGGCTTACATGGCGATAGCTA | 1,115 |
| Kan-2 | GCGGATCCCCCCGCGCTGGAGGATCATC | |
| Flanking DNA between *orf19* and *orf20* for Km$^r$ marker insertion in pPHDP70 | | |
| HPmut1 | GCTCTAGACTATCTAACGCTTCGGGGAA | 3,314 |
| HPmut2 | GCGGATCCGCATTTCAGGACGATACACC | |
| HPmut3 | GCGGATCCGTTTACACATTGAGGACGAA | 3,314 |
| HPmut4 | GCGAATTCTGGCGCGGCATTTAAGGCCA | |
| Amplification of the Km$^r$ marker and flanking pPHDP70 DNA | | |
| HP-Sac-A | GCGAGCTCCTATCTAACGCTTCGGGGAA | 7,771 |
| HP-Sac-B | GCGAGCTCTGGCGCGGCATTTAAGGCCA | |
| *V. alginolyticus pvsA* deletion | | |
| TA15-pvsAMut1 | GCGGGCCCCGTTGACGACCACAGTACGA | 1,088 |
| TA15-pvsAMut2 | GCGAATTCATGGGTCAGCGCACGTGCAC | |
| TA15-pvsAMut3 | GCGAATTCCGGATTAAAGTGACTATCAC | 1,015 |
| TA15-pvsAMut4 | GCTCTAGAAATACGCCCGTTGACCTTAT | |

pKD4 (21). The three DNA fragments were ligated into the suicide vector pKEK229. The resulting plasmid, pAJR8, was mobilized from *E. coli* S17-1λ*pir* into *P. damselae* subsp. *piscicida* DI21-Rif. Suicide plasmid cointegration into pPHDP70 was selected on agar plates containing Ap, Km, and Rif. A second recombination event causing suicide plasmid loss and allelic exchange was selected by Km and sucrose (15% [wt/vol]) resistance. The resulting strain, DI21 pPHDP70::Km, was used as a donor in conjugation experiments carried out as previously described (15) using different bacterial species as recipients (Tables 1 and 3). In brief, exponentially growing cells of donor and recipient strains were mixed at a 1:1 ratio, concentrated 10 times by centrifugation, spotted on TSA plates prepared with seawater, and incubated at 25°C for 24 h. After incubation, mating spots were collected and resuspended to make serial dilutions. Transconjugants were selected at 25°C on TSA-1 plates containing kanamycin and the respective antibiotics to select for the recipient strain. The plasmid transfer frequency was expressed as the number of transconjugants per recipient cell. In order to obtain a strain cured of plasmid pPHDP70, we used a pAJR8 first-crossover strain and further selected for Km$^s$ and sucrose-resistant colonies that lost the pPHDP70-pAJR8 cointegrate. Plasmid DNA from transconjugants was extracted using the plasmid Midikit from Qiagen following the manufacturer's recommendations and subjected to restriction enzyme digestion with NotI.

**Siderophore production assay and growth under iron-limiting conditions.** To evaluate the ability of *P. damselae* subsp. *piscicida* and *V. alginolyticus* strains to grow under iron-restricted conditions, overnight cultures in TSB-1 of the parental and mutant strains were adjusted to an optical density at 600 nm ($OD_{600}$) of 0.5 and diluted 1:15 in CM9 minimal medium (19) in the presence of the iron chelator 2,2′-dipyridyl (Sigma) at concentrations of 60 μM (for *P. damselae* subsp. *piscicida*) and 16 μM (for *V. alginolyticus*). Growth under iron-sufficient conditions was measured

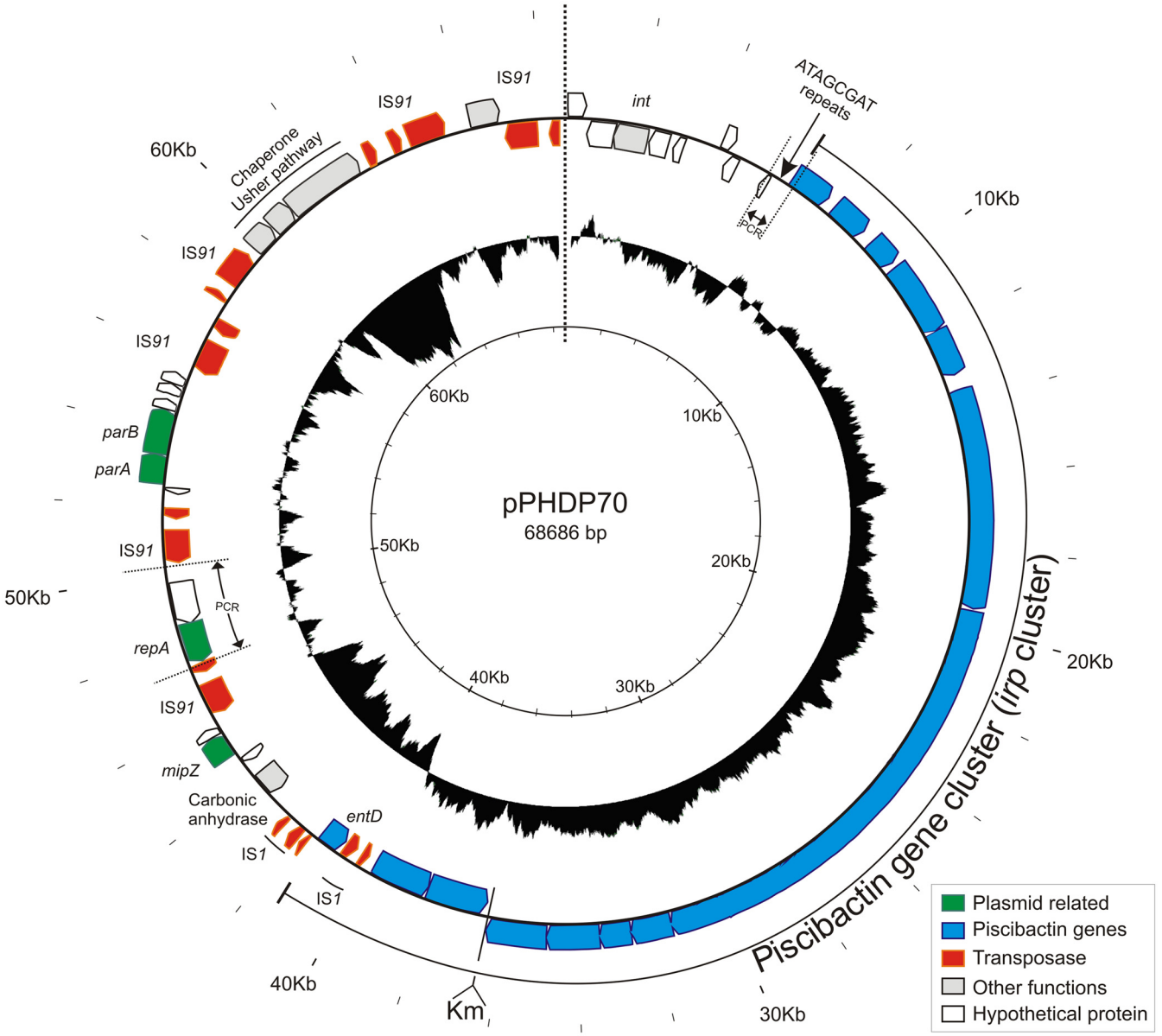



**FIG 1** Circular genome of pPHDP70. ORFs are shown as arrows in the outer track. The position of the putative iterons (ATAGCGAT repeats) is indicated. Km denotes the point where a Km$^r$ gene was inserted to mark pPHDP70. Iteron and *repA* regions are bracketed by discontinuous lines and labeled "PCR" to denote the PCR-amplified fragments that were used to assess potential replication determinants of the plasmid. The deviation of the G+C content from the mean (43.1%), calculated with a window of 500 bp, is indicated in the middle track. The inside track denotes positions in kilobases. Note that IS*91* is present in six copies and accounts for a 10% of the total plasmid sequence. The piscibactin siderophore gene cluster is highlighted.

by culturing the same strains in CM9 minimal medium supplemented with 10 μM ferric sulfate. Cultures were incubated at 25°C with shaking at 150 rpm, and growth ($OD_{600}$) was measured after 16 h. Siderophore production ($A_{630}$) was measured in 16-h culture supernatants using the chrome azurol-S (CAS) liquid assay (22). Assays were carried out in triplicate, and mean values with standard deviations are depicted.

**Analytical detection of piscibactin-Ga(III) complex.** A 1.36-liter batch of the centrifuged cell-free culture broth of *Vibrio alginolyticus* strain AR43 was concentrated under vacuum to 410 ml. A portion of 75 ml was transferred to a round-bottom flask, and 4.0 mg of $GaBr_3$ was added slowly over 5 min and gently stirred for another 10 min. After incubation at 4°C for 24 h, the solution was passed through one Oasis hydrophilic lipophilic balanced (HLB) cartridge (Waters) (35 cm$^3$, 6 g), which was previously conditioned and equilibrated with 60 ml of acetonitrile (solvent B) and 60 ml of water (solvent A). It was eluted with 30 ml of the following mixtures of solvent A and solvent B: 1:0, 7:3, 1:1, 7:3, and 0:1. The fraction eluted with A-B at 1:1, named VAPGL3 (3.0 mg), was subjected to high-pressure liquid chromatography (HPLC)-high-resolution electrospray ionization mass (HRESIMS) analysis using a Atlantis $dC_{18}$ column (100 by 4.6 mm, 5 μm) with a 35.0-min gradient from 10 to 100% $CH_3CN$-$H_2O$, then 5 min at 100% $CH_3CN$, and finally a 10.0-min gradient from 100 to 10% $H_2O$-$CH_3CN$, at a flow rate of 1 ml min$^{-1}$.

**Virulence assays.** The pathogenicity of *P. damselae* subsp. *piscicida* DI21-Rif (parental), CS31 (insertional mutant with disrupted *irp1*), and AR84 (pPHDP70-cured) strains for turbot (*Scophthalmus maximus*) was assayed using 10 fish (average weight, 15 g) per strain tested, as previously

**TABLE 3** Frequency of pPHDP70 transmissibility between strains

| Donor | Recipient | Frequency[a] |
|---|---|---|
| *P. damselae* subsp. *piscicida* DI21(pPHDP70::Km) | *E. coli* MC1061 | $1.5 \times 10^{-7}$ (pPHDP70) |
| | *V. alginolyticus* TA15 | $1.4 \times 10^{-7}$ (pPHDP70) |
| | *A. salmonicida* RSP74.1 | $1.2 \times 10^{-7}$ (pPHDP70) |
| | *V. anguillarum* RV22 | $1.1 \times 10^{-7}$ (pPHDP70) |
| *E. coli* MC1061(pPHDP70::Km) | *E. coli* BI533[b] | ND (pPHDP70) |
| *E. coli* MC1061(pPHDP70::Km, pPHDP60::Cm) | *E. coli* BI533 | $1.6 \times 10^{-7}$ (pPHDP70) |
| | *E. coli* BI533 | ND (pPHDP70 and pPHDP60) |
| | *E. coli* BI533 | $5 \times 10^{-5}$ (pPHDP60) |

[a] The frequency of conjugal transfer was calculated as the number of transconjugants per recipient cell. The results are the means of at least 3 independent assays; standard errors are ≤20%. The plasmid whose transmissibility was studied is indicated in parentheses (either pPHDP70 alone, pPHDP60 alone, or the simultaneous transmissibility of pPHDP70 and pPHDP60). ND, not detected.
[b] BI533 is Nal$^r$ *E. coli* MG1655.

described (23). Fish were maintained in 50-liter tanks with aeration and water recirculation and were kept under starvation during the experiment. Fish were inoculated intraperitoneally with 100 μl of a bacterial dose of $1 \times 10^6$ cells/fish, and mortalities were recorded daily for 7 days. A group of control fish was inoculated with 100 μl saline solution (0.85% NaCl). The statistical significance of differences in percent survival for *P. damselae* subsp. *piscicida* infection experiments was determined using the Kaplan-Meier method and a log rank test.

**Nucleotide sequence accession number.** The complete sequence of pPHDP70 has been deposited in the GenBank database with the accession number KP100338.

## RESULTS

**Identification of pPHDP70 in *P. damselae* subsp. *piscicida* DI21.** In previous work, we found that the siderophore produced by *P. damselae* subsp. *piscicida* DI21 was encoded by a gene cluster (*irp* cluster) whose structure resembled that of a pathogenicity island (9). To investigate the genetic context of this cluster, we constructed and screened a cosmid library of DI21. Two overlapping cosmids that contained *irp* cluster markers were shown to be contiguous, and additional sequencing revealed the complete sequence of a circular structure. Furthermore, during the last stages of this project, we finished sequencing the complete genome of *P. damselae* subsp. *piscicida* DI21 (GenBank accession no. GCA_000300355.3) (to be published elsewhere), which also revealed a plasmid molecule whose sequence and structure were identical to those inferred by the cosmid-based sequencing approach. We dubbed this circular molecule pPHDP70, a hitherto-uncharacterized plasmid from *P. damselae* subsp. *piscicida* strain DI21, isolated from a diseased seabream (*Sparus aurata*) on a fish farm in Galicia (northwestern Spain).

**Sequence analysis and general features of pPHDP70.** The nucleotide sequence of pPHDP70 is 68,686 bp and contains 53 potential open reading frames (ORFs). The gene organization and the G+C content of this plasmid are summarized in Fig. 1, and the similarities of the pPHDP70 gene products to proteins in *P. damselae* and other bacteria are listed in Table S1 in the supplemental material. The plasmid's average G+C content is 43.1%, similar to the mean G+C content of the *P. damselae* subsp. *piscicida* DI21 genome, which is 41.2%. There is a wide distribution of G+C content along pPHDP70, varying from 21% to 54%, suggesting that the plasmid contains genes from different sources. The lowest G+C content (21%) of the plasmid corresponds to a gene block that includes homologs (ORF47 and ORF48) of the *Edwardsiella ictaluri* proteins FimD and FimC, two components of a putative chaperone usher pathway (24).

The pPHDP70 backbone has been targeted by three distinct insertion sequence (IS) elements, IS*91*, IS*1*, and IS*3* (Fig. 1 and 2). IS*91* occurs in a multicopy fashion (six copies), accounting for 10% of the total plasmid sequence, whereas IS*1* (constituted by two ORFs, *tnpA* and *tnpB*) and IS*3* are found in double and single copy, respectively. Interestingly, pPHDP70 does not contain pseudogenes generated by insertion of transposases into coding sequences. Instead, multigenic modules are bracketed by IS*1* and IS*91* copies (Fig. 1). The IS*1* and IS*91* sequences are identical to those found in the previously described *P. damselae* subsp. *piscicida* plasmid pPHDP10 (accession no. DQ069059), which encodes the AIP56 toxin (Fig. 2). IS*1* is also found in other *Vibrio* species on plasmids and in chromosomal locations.

pPHDP70 shares four discrete sequence blocks with the pAQU1 backbone (Fig. 2), a plasmid recently sequenced from a Japanese isolate of *P. damselae* subsp. *damselae* (accession no. NC016983) (25). The shared blocks comprise 14 genes, with average similarity values between the two plasmids of 98% at the nucleotide level and 100% at the amino acid level, and putatively encode functions for replication and plasmid maintenance (*repA*, *parA*, *parB*, and *int*) as well as several hypothetical proteins. Interestingly, *repA* and *parAB* are flanked by copies of IS*91* in pPHDP70 (Fig. 2).

ORF33 showed 96% identity to the putative replication protein RepA of pAQU1; however, the putative iteron sequences described in pAQU1 are absent from pPHDP70. There is a region upstream of *orf9* (encoding a 3-deoxy-D-arabino-heptulosonate-7-phosphate [DAHP] synthase) containing 16 direct repeats (Fig. 1) of the octameric sequence ATAGCGAT that might constitute the iterons for RepA-mediated replication; however, we did not find sequences homologous to this octameric putative iteron in DNA databases. In an initial attempt to dissect the elements necessary for pPHDP70 replication, we ligated the iteron region to a kanamycin resistance (Km$^r$) cassette and assessed its ability to undergo replication in *E. coli* at 25°C and at 37°C. Regardless of the temperature, we were able to obtain Km$^r$ transformants only when iteron sequences coexisted with *repA* (*orf33*) cloned into a plasmid. However, these transformants, which were shown by PCR to contain the putative iterons and the Km$^r$ cassette, showed a microcolony phenotype. These results suggest that although the *cis*- and *trans*-acting elements tested (iterons and

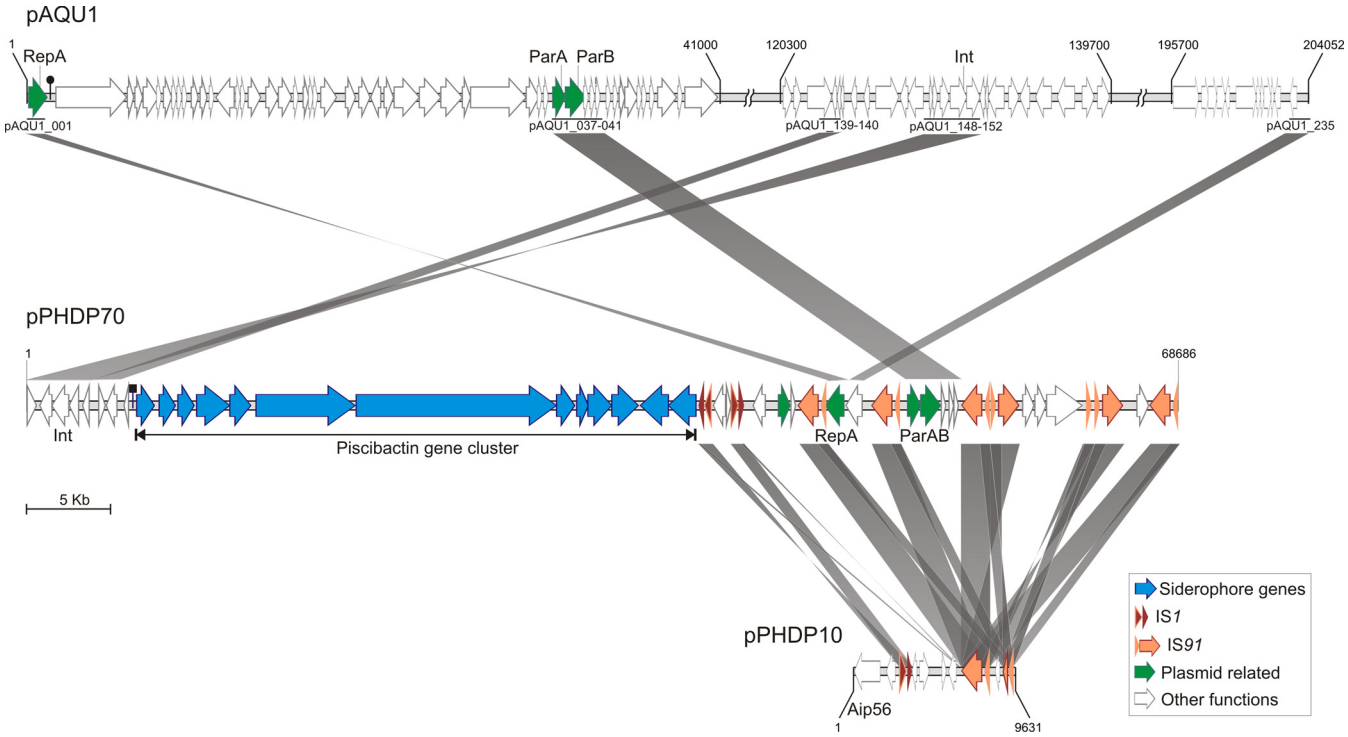



**FIG 2** Linear graphical representation and comparative analysis of the genomes of the two *P. damselae* subsp. *piscicida* plasmids pPHDP70 and pPHDP10 and the *P. damselae* subsp. *damselae* plasmid pAQU1. ORFs encoding transposases and plasmid-related functions are highlighted. Homologous ORFs shared between plasmids are denoted by diagonal gray bands. Note that pPHDP70 shares plasmid backbone genes with pAQU1 and insertion sequences with pPHDP10.

*repA*) appear to be sufficient to enable low levels of replication, efficient replication of pPHDP70 requires additional, unknown factors.

**The piscibactin cluster has similarity to chromosome-encoded HPI-like clusters in other species.** The hallmark of pPHDP70 is the presence of an HPI-like pathogenicity island that includes the 11 previously identified *irp* (iron-regulated proteins) genes (9). The complete sequence of pPHDP70 in the present study uncovered novel genes potentially involved in siderophore synthesis (*entD*) and transport (the ABC transporter genes *orf20* and *orf21*) (Fig. 3). Together with the previously reported putative outer membrane receptor gene *frpA*, *orf20* and *orf21* are candidates to constitute the dedicated transport system for the piscibactin siderophore.

Each siderophore-related pPHDP70 gene has a homologous counterpart in the *Yersinia* HPI, with the exceptions of *dahP*, *araC1*, *araC2*, *entD*, *orf20*, and *orf21*. The proteins shared between pPHDP70 and HPI showed similarity values at the amino acid level ranging from 44 to 62% (see Table S1 in the supplemental material), and the gene arrangement differs between the PAIs (Fig. 3). Although the two clusters are not exactly syntenic, both encode the determinants for biosynthesis, transport, and regulation of two distinct but similar siderophores, piscibactin (10) and yersiniabactin (26), respectively. Notably, by an *in silico* search in databases, we found gene clusters homologous to the pPHDP70-borne *irp* cluster in isolates of several *Vibrionaceae* species. These include human and marine animal pathogens such as *Vibrio cholerae*, *V. mimicus*, *V. coralliilyticus*, *V. ordalii*, and *V. anguillarum*. The closest clusters are found in *V. coralliilyticus* and *Photobacterium profundum* and show a high degree of synteny with the *irp* cluster (Fig. 3).

The pPHDP70 *irp* cluster includes two biosynthetic genes that are absent from the HPI and from many of the related *Vibrionaceae* clusters. One gene encodes a 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (ORF9) involved in chorismate synthesis and is necessary for production of salicilic acid, one of the piscibactin components (10). DAHP synthases specifically involved in providing chorismate for siderophore production have been reported in other *Vibrionaceae* (27). A second gene, *entD*, encodes the enzyme phosphopantheteinyl transferase, which is required to activate nonribosomal peptide synthesis (NRPS) domains (28). Homologs of *entD* have not been found in the other *irp*-like clusters (Fig. 3). In *Yersinia*, the phosphopantheteinyl transferase gene *ybtD*, which is homologous to the *P. damselae* subsp. *piscicida entD*, is located outside the HPI (29). It is tempting to speculate that *entD* may have been recruited by natural selection to be closely associated with the rest of the piscibactin synthesis-related genes. The recruitment hypothesis for *P. damselae* subsp. *piscicida entD* is reinforced by the observation that *entD* is bracketed between tandem copies of *tnpAB* transposase genes (IS*1*) (Fig. 3).

**pPHDP70 can be conjugally transferred to a variety of species of *Gammaproteobacteria*, helped by the coresident plasmid pPHDP60.** Although pPHDP70 appears to lack genes encoding a conjugation system, we investigated whether this plasmid could be conjugally transferred to different recipients. We found that pPHDP70::Km was transmissible from DI21-Rif to *Escherichia coli*, *Aeromonas salmonicida*, *Vibrio anguillarum*, and *V. alginolyti-*

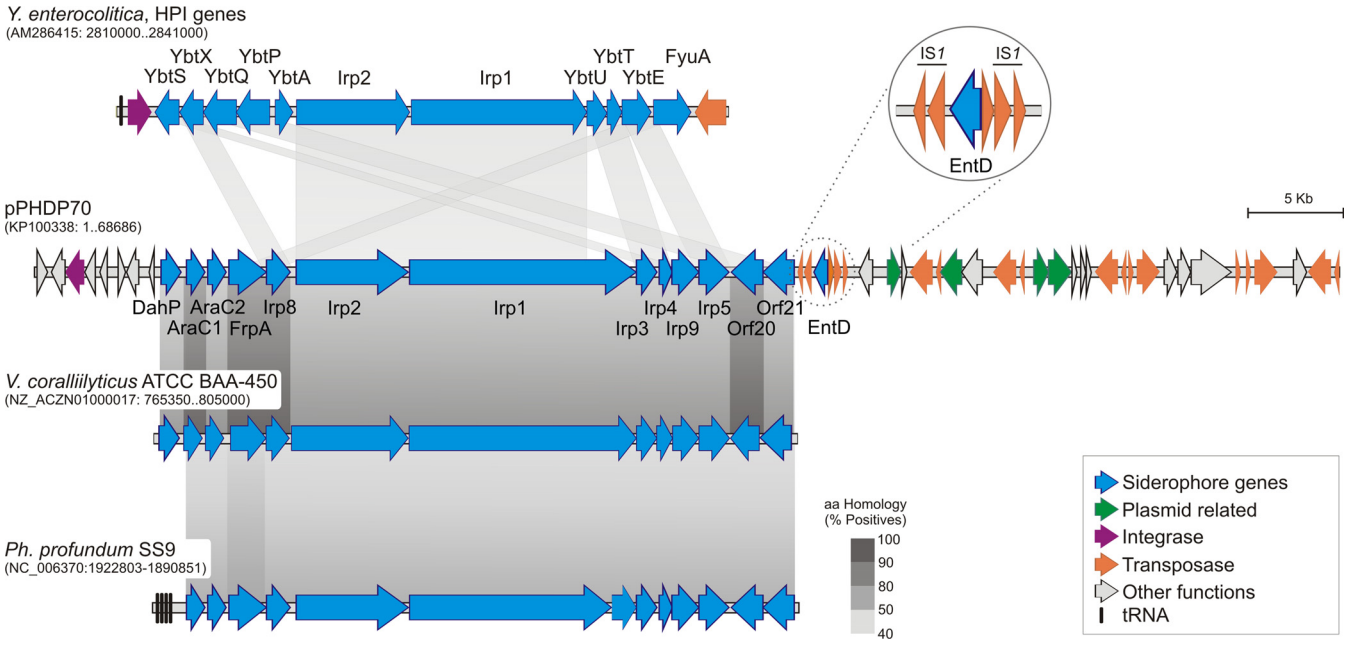



**FIG 3** Comparative analysis of *P. damselae* subsp. *piscicida* plasmid pPHDP70 with homologous gene clusters in *Yersinia enterocolitica* (high-pathogenicity island [HPI]), *Vibrio coralliilyticus*, and *Photobacterium profundum*, highlighting the region of siderophore-related genes (in blue). Homologous ORFs shared between pairs of sequences are connected by gray bands. The percent similarity refers to the amino acid sequences of the predicted proteins and is represented in similarity intervals in grayscale tones according to the scale shown at the bottom right. In parentheses below each species name, the GenBank accession number and the nucleotide position interval represented for each species are shown. Note that the cluster of pPHDP70 is plasmid borne and the homologous clusters are all chromosomal.

*cus* at low frequencies (Table 3). PCR assays targeting a number of different pPHDP70 markers and plasmid purification and restriction analyses revealed that the entire pPHDP70 plasmid was being transferred to the transconjugants (data not shown).

However, we were unable to detect transfer of pPHDP70::Km from the *E. coli* MC1061 (pPHDP70::Km) transconjugant (Table 3). This observation coupled with the apparent lack of pPHDP70-encoded conjugation machinery suggested that pPHDP70 mobilization relies on conjugative functions encoded elsewhere in the genome of *P. damselae* subsp. *piscicida* DI21. An *in silico* search in the genome sequence of this strain revealed a putative type IV secretion system in pPHDP60, a conjugative plasmid recently described (14). We hypothesized that pPHDP60 might enable the mobilization of pPHDP70 in *trans*. Consistent with this idea, we found that an *E. coli* MC1061 donor that harbored pPHDP70::Km and a Cm-labeled version of pPHDP60 (pPHDP60::Cm) could transfer pPHDP70::Km to *E. coli* BI533; both pPHDP70::Km and pPHDP60::Cm were independently mobilized to *E. coli* BI533 at frequencies of $1.6 \times 10^{-7}$ (pPHDP70::Km) and $5 \times 10^{-5}$ (pPHDP60::Cm) (Table 3). Of 40 pPHDP70::Km transconjugants tested from these conjugation assays, none was $Cm^r$, suggesting that transfer of pPHDP70 did not require concomitant transfer of pPHDP60 or cointegrate formation. Additionally, restriction analyses of plasmid DNA from transconjugants demonstrated that the pPHDP70 in the transconjugants did not contain additional DNA in its structure (data not shown). Since the two plasmids coexist in strain DI21, these findings suggest that pPHDP70 might be capable of conjugative transfer in the natural environment.

**pPHDP70 promotes piscibactin biosynthesis and utilization in a heterologous host upon conjugative transfer.** We recently determined the chemical structure of piscibactin and proposed a pathway for its biosynthesis based on an NRPS-mediated mechanism (10). The pPHDP70-borne *irp1* gene is necessary for piscibactin biosynthesis (9), but it is not clear whether pPHDP70 encodes all the biological functions required for piscibactin biosynthesis and its efficient utilization as an iron source. To explore this possibility, we used *V. alginolyticus*(pPHDP70) transconjugants derived as described above. The parental *V. alginolyticus* TA15 strain was originally isolated in an episode of mortality of carpet shell clam (*Ruditapes decussatus*) larvae (30) and harbors a gene cluster for production and utilization of the siderophore vibrioferrin. Vibrioferrin is a hydroxamate siderophore synthesized by an NRPS-independent mechanism (31) that does not share genes with the piscibactin NRPS-mediated mechanism. Therefore, we considered this a good model heterologous host to assess the sufficiency of pPHDP70 for piscibactin synthesis. TA15 vibrioferrin synthesis was abolished by inactivation of the biosynthetic gene *pvsA*. As expected, the *pvsA* mutant strain AR13 exhibited impaired growth under conditions of iron limitation and had reduced siderophore activity (Fig. 4A). In contrast, when the *pvsA* mutant *V. alginolyticus* harbored pPHDP70 (strain AR43), its ability to grow under iron-limiting conditions was restored and it had detectable siderophore activity (Fig. 4A). As a control, we introduced a pPHDP70 with an inactivated *irp1* gene (pPHDP70::pSJR52) by conjugation into AR13 (TA15 Δ*pvsA*). The resulting strain (AR45) exhibited impaired growth under iron limitation and no siderophore activity (Fig. 4A). These findings indicate that the conjugally transmitted pPHDP70 can bestow upon the heterologous *V. alginolyticus* host the capacity to produce a siderophore.

We used the methodology based on HLB cartridges and liquid chromatography-mass spectrometry (LC-MS) that we developed

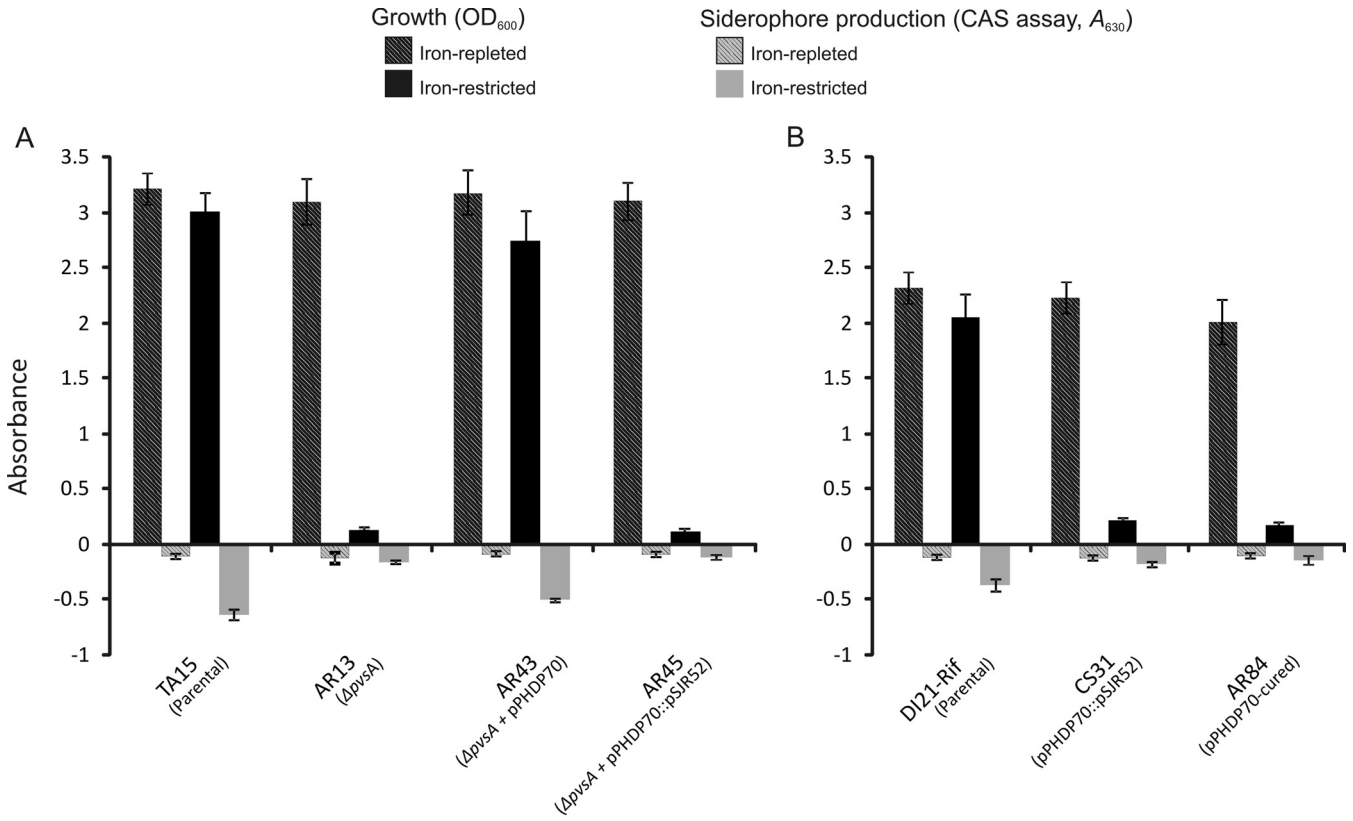


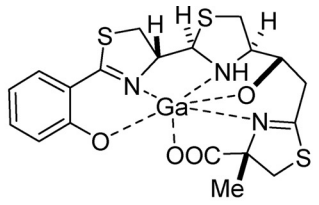

**FIG 4** Growth ($OD_{600}$) and siderophore production (CAS assay) ($A_{630}$) of *V. alginolyticus* (A) and *P. damselae* subsp. *piscicida* (B) parental strains and mutants in CM9 minimal medium after 16 h of cultivation under iron-replete conditions (10 μM ferric sulfate) and under iron-limited conditions (the iron chelator 2,2′-dipyridyl at 60 μM for *P. damselae* subsp. *piscicida*, and 16 μM for *V. alginolyticus*). The CAS assay yields more-negative values as the siderophore activity increases. The assays were carried out in triplicate, and mean values with standard deviations are shown.

for isolation of piscibactin (10) to test whether *V. alginolyticus* pPHDP70 transconjugants produced piscibactin. Cell-free culture supernatants of strain AR43 were incubated with an excess of $GaBr_3$, fractionated through HLB cartridges, and separated over a reverse-phase column by HPLC. In this way, we detected a compound with retention time ($t_R$) of 8.82 min, which, by HRESIMS analyses, showed an *m/z* of 519.9943/521.9927 and a distinctive gallium isotopic ratio (3:2) in concordance with those of piscibactin-Ga(III) complex (Fig. 5). Collectively, these observations provide strong evidence that pPHDP70 encodes the necessary biosynthetic, transport, and regulatory functions to enable synthesis and utilization of piscibactin following its conjugative transfer to a different species.

**Curing pPHDP70 from *P. damselae* subsp. *piscicida* impairs growth under iron-limiting conditions, abolishes piscibactin siderophore production, and diminishes virulence for fish.** We cured *P. damselae* subsp. *piscicida* DI21-Rif of pPHDP70 by inserting a suicide plasmid in the *irp1* gene and selecting for loss of the cointegrate to assess the role of pPHDP70 in iron utilization and virulence. PCR assays for plasmid markers as well as plasmid extraction and restriction analysis demonstrated the absence of pPHDP70 sequences from the cured strain, AR84 (data not shown). DI21-Rif and AR84 had similar growth in CM9 minimal medium supplemented with 10 μM $FeSO_4$ (iron-sufficient conditions) (Fig. 4B). However, when the same strains were grown in CM9 in the presence of the iron chelator 2,2′-dipyridyl, AR84 exhibited markedly impaired growth compared with the parental strain as well as reduced reactivity in the CAS siderophore assay, showing results similar to those for CS31, a DI21 derivative in which *irp1* is inactivated (Fig. 4B). In addition, chemical analysis did not detect the presence of piscibactin in the culture supernatants of AR84 (data not shown).

We inoculated turbot fingerlings with DI21-Rif, AR84, and CS31 to assess the contribution of pPHDP70 to the virulence of *P. damselae* subsp. *piscicida* for fish. The two non-piscibactin-producing strains (AR84 and CS31) caused much less mortality in the fish than DI21-Rif ($P < 0.001$ by the log rank test) (Fig. 6). Although the differences in the number of deaths caused by the cured strain versus insertional mutant did not reach statistical significance ($P = 0.152$), the cured strain caused fewer fish deaths (1 out of 10) than the single *irp* mutant (4 out of 10). Altogether, our results indicate that production of siderophores by the *irp* cluster in pPHDP70 contributes significantly to the virulence of *P. damselae* subsp. *piscicida* for fish.

**pPHDP70 shows geographical specificity within *P. damselae* subsp. *piscicida* isolates.** A variety of molecular evidence (32–36) indicates that there are two distinct *P. damselae* subsp. *piscicida* clonal lineages: a lineage that comprises all the European isolates and a lineage that includes the Japanese and U.S. isolates. We found that the distribution of the *irp* cluster in *P. damselae* subsp. *piscicida* isolates follows a geographical pattern and is restricted to European isolates (9). We wondered whether the modular pisci-



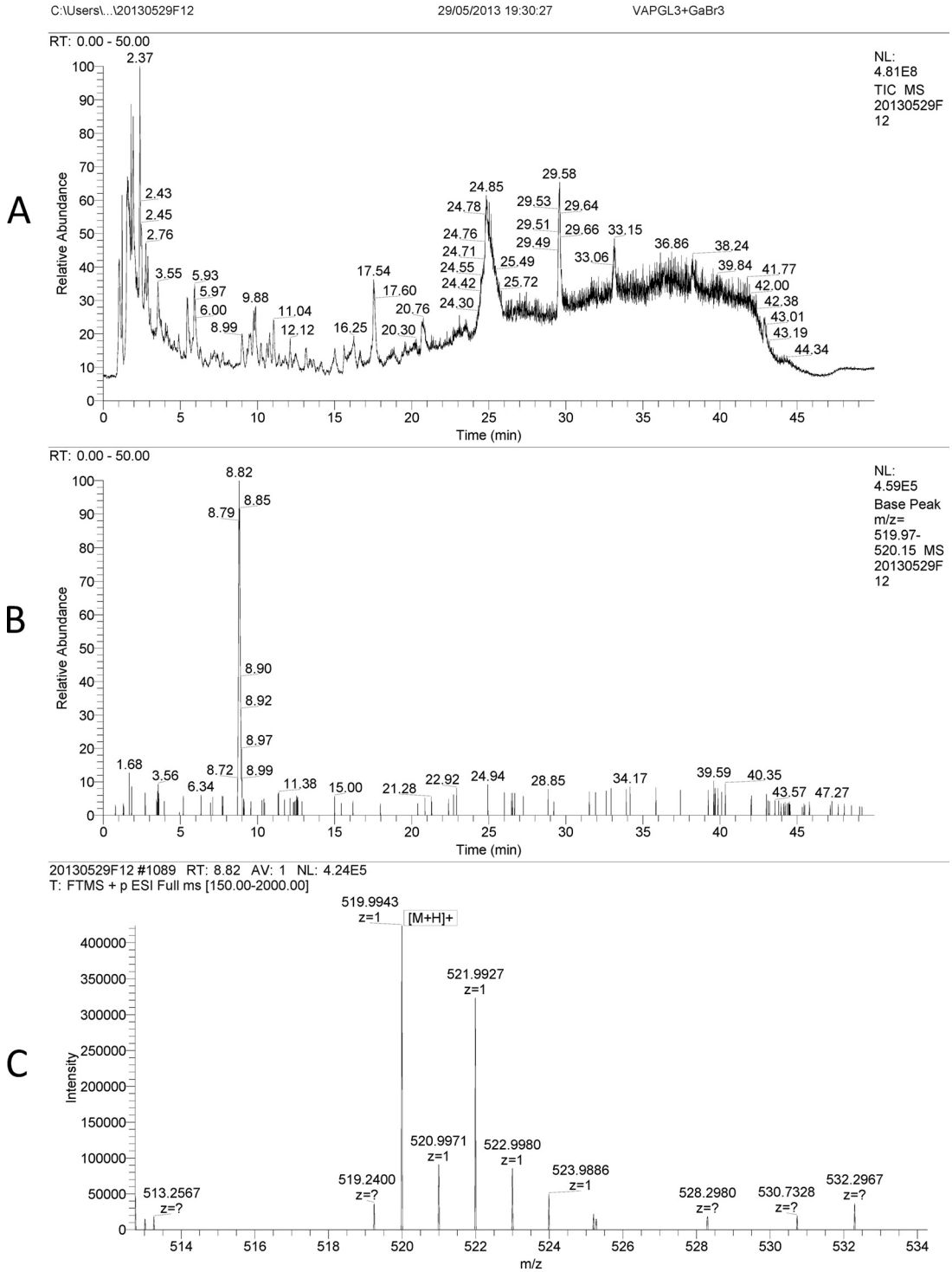




**FIG 5** LC-MS experiments for the detection of the piscibactin-Ga(III) complex. (A) Total ion chromatogram (TIC) of the fraction eluted with $H_2O$-$CH_3CN$ (1:1) from the Oasis HLB cartridge (VAPGL3) containing piscibactin-Ga(III) complex; (B) extracted mass chromatogram (*m/z* 519.97 to 520.15) showing the peak with retention time of 8.82 min; (C) (+)-HRESIMS of the peak at a $t_R$ of 8.82 min identified as piscibactin-Ga(III) complex: *m/z* 519.9943/521.9927 ($[M + H]^+$); calc. for $C_{19}H_{21}N_3O_4S_3Ga$, 519.9950/521.9941. Chromatographic conditions: Atlantis d$C_{18}$ column (100 by 4.6 mm, 5 μm) (Waters); 35-min gradient from 10 to 100% of $CH_3CN$ in $H_2O$, then 5 min at 100% $CH_3CN$, and finally 10 min from 100 to 10% $CH_3CN$ in $H_2O$; flow rate, 1 ml $min^{-1}$.

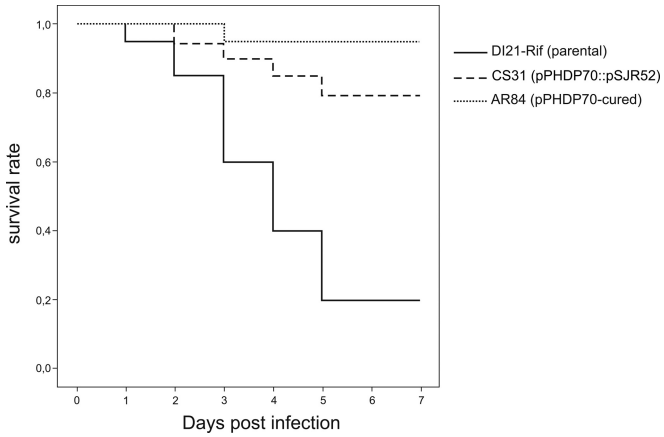



**FIG 6** Survival of turbot (*Scophthalmus maximus*) inoculated with *P. damselae* subsp. *piscicida* strains DI21-Rif (parental), CS31 (insertional mutation in *irp1* gene), and AR84 (pPHDP70 cured). The virulence of each strain was tested using 10 fish (average weight, 15 g), as previously described (23), inoculating $1 \times 10^6$ cells/fish. Mortalities were recorded daily for 7 days. The statistical significance of differences in percent survival for *P. damselae* subsp. *piscicida* strains was determined using the Kaplan-Meier method and a log rank test.

bactin-synthesizing plasmid pPHDP70 found in strain DI21 is conserved in *P. damselae* subsp. *piscicida* isolates. We used PCR assays to test for the presence of genes from different parts of pPHDP70 (*entD-orf21*, *parAB*, *orf28*, and *orf48*) in 14 *P. damselae* subsp. *piscicida* isolates from diverse regions of isolation (Table 4). The results were equivalent for all the genes assayed: all *P. damselae* subsp. *piscicida* strains isolated in Europe contained all the genes tested, whereas none of the American and Asian isolates tested contained any of these genes. Moreover, the primer pair combination used for *entD* allowed us to corroborate that this gene is invariably linked to the *irp* cluster (*orf21*) and separated from it by an IS*1* element. In addition, we also found that a total of 17 *P. damselae* subsp. *damselae* isolates from different origins (Table 4) tested negative in PCR tests for pPHDP70 markers.

## DISCUSSION

The *P. damselae* subsp. *piscicida irp* genes were known to be clustered in a pathogenicity island-like structure related to the *Yersinia* HPI (9) and were implicated in the synthesis of piscibactin, a phenolate siderophore (10). Here, we discovered that this HPI-like cluster is contained within a transmissible plasmid, pPHDP70. pPHDP70 is not self-transmissible; instead, its transmission from *P. damselae* subsp. *piscicida* to other organisms relied on

**TABLE 4** Results of PCR screening for presence of pPHDP70 gene markers in *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* strains

| Species and strain | Source | Presence of gene | | | |
|---|---|---|---|---|---|
| | | *entD-orf21* | *parAB* | *orf28* | *orf48* |
| *P. damselae* subsp. *piscicida* | | | | | |
| DI21 | Seabream, Spain | + | + | + | + |
| PC554.2 | Sole, Spain | + | + | + | + |
| PC435.1 | Sole, Spain | + | + | + | + |
| B51 | Seabream, Spain | + | + | + | + |
| 666.1 | Seabass, Portugal | + | + | + | + |
| IT-1 | Seabream, Italy | + | + | + | + |
| ATLIT 2 | *Morone* sp., Israel | + | + | + | + |
| ATCC 17911 | White perch, USA | − | − | − | − |
| ATCC 29690 | Yellowtail, Japan | − | − | − | − |
| EPOY 8803-II | Red grouper, Japan | − | − | − | − |
| MP-7801 | Yellowtail, Japan | − | − | − | − |
| MZS-8001 | Yellowtail, Japan | − | − | − | − |
| P3333 | Yellowtail, Japan | − | − | − | − |
| P3335 | Yellowtail, Japan | − | − | − | − |
| *P. damselae* subsp. *damselae* | | | | | |
| RM-71 | Turbot, Spain | − | − | − | − |
| RG-91 | Turbot, Spain | − | − | − | − |
| RG-153 | Turbot, Spain | − | − | − | − |
| RG-214 | Turbot, Spain | − | − | − | − |
| PC586.1 | Seabream, Spain | − | − | − | − |
| 309 | Mussel, Spain | − | − | − | − |
| LD-07 | Seabream, Spain | − | − | − | − |
| 340 | Seawater, Spain | − | − | − | − |
| 158 | Eel, Belgium | − | − | − | − |
| 162 | Eel, Belgium | − | − | − | − |
| PG-801 | Shrimp, Taiwan | − | − | − | − |
| J3G-801 | Shrimp, Taiwan | − | − | − | − |
| 192 | Dolphin, USA | − | − | − | − |
| ATCC 35083 | Brown shark, USA | − | − | − | − |
| 238 | Dolphin, USA | − | − | − | − |
| CDC-2227-81 | Human, USA | − | − | − | − |
| ATCC 33539 | Damselfish, USA | − | − | − | − |

pPHDP60, a coresident plasmid that encodes a type IV secretion system. Conjugal transfer of pPHDP70 to a *V. alginolyticus* strain deficient in siderophore synthesis enabled the transconjugant to grow under low-iron conditions and to synthesize piscibactin, providing strong evidence that pPHDP70 encodes all the information for synthesis and transport of this siderophore. A strain cured of pPHDP70 showed impaired growth under low-iron conditions, no piscibactin synthesis, and markedly reduced virulence in fish. Thus, pPHDP70 is an unusual virulence plasmid that contains a transmissible form of a pathogenicity island that is ordinarily locked into the chromosome.

In general, the *irp* cluster has a higher G+C content than the remainder of the plasmid, consistent with the idea that during pPHDP70 evolution, the *irp* cluster, which was derived from a different source than the rest of the plasmid, fused with an ancestral version of the plasmid. The heterogeneous origin of pPHDP70 genes is also suggested by the diverse taxonomy of the species showing the closest homologs to pPHDP70 genes. Thus, pPHDP70 can be considered a mosaic, whose evolution appears to have depended on recombination of genetic information from a variety of sources, including a likely chromosomal *irp* cluster.

We have shown that gene clusters homologous to pPHDP70 *irp* cluster are present in several species of vibrios. The high similarity at the amino acid level among homologs and the fact that they share the same functional domains in the biosynthetic proteins (data not shown) strongly suggest that these different *Vibrio* species produce piscibactin-related siderophores. Indeed, a recent study revealed that closely related *Vibrio* species in the marine environment can produce the same or similar siderophore molecules, suggesting that evolutionary forces have promoted the use of the siderophore-mediated mechanism as a "public good" to be used by species of the same microbial community (37).

It is important to note that none of the homologous clusters found in other *Vibrionaceae* is plasmid borne; they are all chromosomal. Indeed, one of the distinctive features of the *P. damselae* subsp. *piscicida irp* cluster is that it is a plasmid-borne PAI-like element. Although PAIs were first identified as chromosomal regions (38), parts of some virulence plasmids are also considered PAIs: the invasion regions of *Shigella* plasmids (39) and the *Yersinia* plasmid cluster encoding Yop proteins (40). *Pantoea agglomerans*, a plant pathogen, is thought to have recently evolved into a host-specific tumorigenic pathogen by acquisition of a plasmid-borne PAI (41). However, in contrast to the pPHDP70 cluster, these other examples of plasmid-borne PAIs do not have chromosomal counterparts. Chromosomal gene clusters similar to the pPHDP70 *irp* cluster include the *Yersinia* HPI and homologous clusters described in yersiniabactin-producing strains of *Escherichia*, *Citrobacter*, *Klebsiella*, and *Serratia* (12, 13, 42). In addition, an HPI was found as part of an integrative and conjugative element (ICE) in *E. coli* (HPI-ICE*Ec1*) (43), in *K. pneumoniae* (HPI-ICE*Kp1*) (44), and in *Enterobacter hormaechei* (HPI-ICE*Eh1*) (45). Recently, an ICE in *Streptococcus equi* that putatively encodes the synthesis of a yersiniabactin and piscibactin-like siderophore was reported, whose biosynthetic proteins bear similarity to HPI- and *irp*-encoded proteins (46). Similarly, the mobile element ICE*Pm1*, common to *Proteus mirabilis*, *Providencia stuartii*, and *Morganella morganii*, contains the *Yersinia* HPI within its structure (47). These observations, together with the similarity of some *Vibrionaceae* clusters to the *Yersinia* HPI and the remarkable chemical similarities of piscibactin and yersiniabactin, suggest that HPI/*irp*-like clusters have evolved as potential horizontally transmissible elements in diverse bacterial species. Thus, piscibactin- and yersiniabactin-synthetic genes appear to have evolved on and likely exchanged between a variety of mobile elements, including plasmids, ICE, and PAIs, which enables their transmission among diverse bacteria in both in terrestrial and aquatic ecosystems.

Horizontal gene transfer is thought to be extensive among the *Vibrionaceae* (48). The transmissibility of pPHDP70 is another example of the key role that mobile elements have had in underlying the emergence of disease-causing vibrios. Notably, we demonstrated that a virulence plasmid can be transferred from a fish pathogen (*P. damselae* subsp. *piscicida*) to a mollusk pathogen (*Vibrio alginolyticus*) that inhabits the same environment. This suggests that pPHDP70 might also be capable of undergoing horizontal transfer under natural conditions, perhaps at frequencies higher than those we found in our study, which was conducted under laboratory conditions.

*P. damselae* subsp. *piscicida* may be an obligate pathogen, as no free-living state has been described to date. This pathogen is predicted to face an iron-limiting environment in fish, and therefore the acquisition and spread of pPHDP70 might have constituted a driving force in the evolution of the European *P. damselae* subsp. *piscicida* lineage. The restriction of pPHDP70 to European isolates is consistent with the existence of two lineages and suggests that pPHDP70 might be useful as a genetic marker for epidemiological typing of *P. damselae* subsp. *piscicida* strains. Our findings also leave open the question of which siderophore is produced by Asian and American *P. damselae* subsp. *piscicida* isolates as well as by *P. damselae* subsp. *damselae* strains, which do not harbor pPHDP70.

When *P. damselae* subsp. *piscicida* was cured of pPHDP70, the strain's virulence was markedly diminished, indicating that pPHDP70 is a bona fide virulence plasmid. The importance of siderophore production in virulence has been reported for other fish pathogens (49), and in some cases this biological function has been found to be plasmid encoded, as is the case for the anguibactin siderophore system of *Vibrio anguillarum* encoded in plasmid pJM1 (50). The possibility that pPHDP70 carries additional genes with a role in virulence (for example, the chaperone usher pathway genes) cannot be ruled out, since the cured strain caused fewer fish deaths (1 out of 10) than the single *irp* mutant (4 out of 10), although these differences did not reach statistical significance ($P = 0.152$). Additional studies will be needed to ascertain whether pPHDP70 encodes properties in addition to piscibactin-mediated iron uptake that also contribute to *P. damselae* subsp. *piscicida* virulence.

Our results highlight the emerging similarities between terrestrial and marine animal pathogens, in terms of both the presence of similar siderophore-mediated iron-sequestering systems and their location on mobile elements. The recent discovery of a fish-pathogenic *Edwardsiella tarda* strain that acquired the locus of enterocyte effacement by horizontal transfer (2) indicates that lateral gene flow can surmount barriers between aquatic and terrestrial ecosystems and has a powerful influence on the generation of new virulence strategies. Our findings represent the first description of an HPI-like cluster borne on a mobilizable plasmid and demonstrate that the piscibactin cluster can be efficiently expressed in a recipient species following conjugative transfer. Transmission of this plasmid among different bacterial species in

the marine environment enables recipients to synthesize a novel siderophore and thereby to potentially occupy new niches.

## ACKNOWLEDGMENTS

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