The two subspecies of *Photobacterium damselae* express virulence factors that are encoded by mobile DNA elements

C.R. Osorio, A.J. Rivas, M. Balado, A. Rodríguez-Blanco, B. Puentes, and M.L. Lemos*

Department of Microbiology and Parasitology, Institute of Aquaculture, University of Santiago de Compostela, Santiago de Compostela 15782, Spain. (manuel.lemos@usc.es)

Introduction

Photobacterium damselae is a marine gram-negative bacterium of the family *Vibrionaceae,* widespread in marine environments, that includes strains classified into two distinct subspecies, namely subsp. *piscicida* and subsp. *damselae*. *P. damselae* subsp. *piscicida* is the causative agent of pasteurellosis, a disease affecting a number of fish species in marine aquaculture systems worldwide, but not affecting homeotherms. *P. damselae* subsp. *damselae* affects a variety of marine animals but has also been reported as the causative agent of fatal cases in humans. It is considered as a primary pathogen causing wound infections and hemorrhagic septicemia in different species of wild fish, as well as in reared fish of economical importance in aquaculture. Several important virulence factors have been reported in both subspecies (Osorio and Lemos, in press). In this report we show that some of these virulence factors like toxins, hemolysins or siderophore biosynthesis are encoded by mobile DNA elements and that some of them can be horizontally transmitted.

Results and Discussion

A pathogenicity island encoding a siderophore biosynthesis and transport gene cluster of P. damselae *subsp.* piscicida *is part of a 70 kb mobilizable plasmid*

We have previously reported that siderophore biosynthesis in subsp. *piscicida* is encoded by a gene cluster which is part of a pathogenicity island (PAI) closely similar to the *Yersinia enterocolitica* HPI island. This cluster encodes the biosynthesis, transport and utilization of a phenolate siderophore not yet characterized, that has a relevant role in the pathogenicity of this bacterium (Osorio *et al.*, 2006). This pathogenicity island that spans about 45 kb has been now completely sequenced and characterized. It contains features typical of PAIs, as presence of an integrase gene and genes encoding transposases, although its excision and mobility have not been proved so far. This PAI has been demonstrated to be part of a 70 kb plasmid not reported previously in this bacterium, which was dubbed pPHDP70. This plasmid harbours numerous transposase genes, as well as genes encoding replication and partitioning functions but lacks genes for a conjugative apparatus. Nevertheless, it was found to undergo conjugative transfer from *P. damselae* susbp. *piscicida* to *Vibrio* species although at a very low rate, suggesting that pPHDP70 can take advantage of the conjugative machinery encoded elsewhere in the cell. pPHDP70 is present in all *P. damselae* subsp. *piscicida* strains isolated from Europe, but it is absent from strains isolated from Japan. It is also absent from all subsp. *damselae* strains tested. It is noteworthy that homologous gene clusters exist in the published genomes of *P. profundum* SS9 and *V. cholerae* RC385, although their role in siderophore biosynthesis remains untested.

An ICE element of P. damselae *subsp.* piscicida *can mobilize virulence plasmids*

Integrating and Conjugative Elements (ICEs) of the SXT family, first described in *V. cholerae*, have been described in a small number of bacteria (Burrus *et al*., 2006). One of these elements (ICE*Pda*Spa1) has been previously reported and characterized in a strain of *P. damselae* subsp. *piscicida* isolated from diseased fish in Spain (Juiz-Rio *et al.*, 2005; Osorio *et al.*, 2008), constituting the first complete description of an ICE element from a fish pathogen. This ICE proved to be an ~100 kb element which shared ~97-% sequence identity

to the *V. cholerae* SXT element in genes that encode essential ICE functions including integration/excision, conjugal transfer and regulation. However, ICE*Pda*Spa1 contains approximately 25 kb of DNA sequence not found in SXT, including a tetracycline resistance locus, and 10 of these 25 kb are ICE*Pda*Spa1-specific DNA not found in any other ICE of the SXT family described to date. This variable DNA is inserted at five points which are considered as *hotspots* for acquisition of new DNA. We found that ICE*Pda*Spa1 can be excised from the chromosome and be transmitted to other *Photobacterium* strains as well as to *E. coli*. Interestingly, ICE*Pda*Spa1 can mobilize the *P. damselae* subsp. *piscicida* virulence plasmid pPHDP10 (encoding the apoptoptic toxin AIP-56, Do Vale *et al*., 2005) via formation of cointegrates, suggesting that SXT-reated ICEs can acquire new DNA and promote the horizontal flux of virulence genes (Osorio *et al.*, 2008). In addition, we have found evidences that ICE*Pda*Spa1 can promote the conjugative transfer of pPHDP70 (harboring the HPI-like pathogenicity island) when the two elements coexist into an *E. coli* strain that does not harbour a conjugation machinery by itself. Our findings not only reveal the plasticity of the genome of this fish pathogen, but also demonstrate that different mobile DNA elements can interact to spread antibiotic resistance and virulence determinants. Furthermore, we recently found that ICE elements containing tetracycline-resistance determinants, are also present in other vibrios isolated from fish-rearing facilities in Spain. This suggests that environmental factors, like presence of antibiotics coming from fish treatments, could facilitate the spread among marine bacteria of ICE elements and hence of other mobile DNA elements.

A megaplasmid from P. damselae *subsp.* damselae *encodes two hemolysins*

The major virulence factor known to date in *P. damselae* subsp. *damselae* is damselysin, a potent cytolysin with hemolytic activity, encoded by the *dly* gene. This is an exotoxin with phospholipase D activity against sphingomyelin, and is able by itself to cause death in mice. However, a direct relationship between hemolysis and the presence of *dly* gene was never demonstrated to date. In addition, some studies suggest that this gene may be located in an unstable genetic element.

To identify the genes responsible for hemolysis in *P. damselae* subsp. *damselae* and their genetic background, we constructed a cosmid gene library in *E. coli* from the RM71 strain isolated from turbot. The hemolytic activity of cosmid clones was evaluated by plating on blood agar. Among approximately 300 clones analyzed, one of them showed a halo of hemolysis similar to the *P. damselae* parental strain. This clone was subcloned and subjected to partial sequencing. It was found that the hemolytic activity was encoded by an approximately 5 kb fragment containing two opposing ORFs: one encoding the damselysin, and another one that encoded a protein belonging to the pore forming toxins family, HlyA. By sequencing overlapping cosmids we demonstrated that *dly* and *hlyA* genes are part of a hitherto undescribed plasmid of approximately 150 kb, which was named pPHDD1. The sequence analysis of this plasmid revealed the existence of *tra* genes, suggesting that it could be a conjugative plasmid. These data were confirmed by the recent completion of the genome sequencing of the type strain of this bacterium (GenBank accesion number ADBS00000000). However, pPHDD1 is not exactly identical to the plasmid reported in the type strain, since pPHDD1 contains extra DNA sequences related to bacteriophages and plasmid replication functions. This suggests that *Photobacterium damselae* plasmids are continuously evolving by events of gene gain or loss.

When analyzing the presence of pPHDD1 in a collection of strains of *P. damselae* subsp. *damselae* from different origins, it was found that the strains with stronger hemolytic activity were positive for different markers located in the plasmid, including *dly* and *hlyA*, indicating the existence of a direct relationship between the presence of pPHDD1 and the hemolytic capacity. The analysis of mutants with deletions in *hlyA* and *dly*, obtained by allelic exchange, showed the following results: *dly* gene mutant produced in blood agar a hemolytic halo much smaller than the wild type, although it was a translucent halo which suggests a complete hemolysis. The Δ*hlyA* mutant produced a large hemolytic turbid halo, suggesting that this mutant does not produce complete hemolysis of erythrocytes. A double mutant for both genes

presented only basal hemolytic activity, probably due to a low activity hemolysin encoded by the chromosome. Strains cured of pPHDD1 showed a hemolytic phenotype identical to that of double mutants, indicating that this plasmid actually contains the genetic determinants of hemolysis and that it does not harbor additional genes encoding hemolysins other than HlyA and damselysin. Furthermore, when these two genes were individually cloned into *E. coli* HlyA was found to produce a translucent halo, while damselysin generated a turbid halo. The two ORFs cloned together in the same cell caused a much larger halo. These data suggest a possible synergistic interaction between these two hemolysins.
Overall, the results indicate that the molecular basis of hemolysis in *P. damselae* subsp. *damselae* lies mainly in two proteins: damselysin toxin and HlyA, and both are encoded by plasmid pPHDD1, present only in the hemolytic strains.

Conclusions

In summary, both subspecies of *P. damselae* express a number of different virulence factors that are encoded by mobile DNA elements. We have demonstrated that some of these elements can be horizontally transmitted to other bacteria by conjugation. Our results indicate that both bacteria have genomes with a great plasticity and suggest that some of their more relevant pathogenic characteristics could have being acquired by horizontal gene transfer.

Acknowledgements

This work was partially supported by project CSD2007-00002 (Consolider Aquagenomics), and by grant AGL2009-12266-C02-01 (cofunded by the FEDER Programme from the European Union), from the Ministry of Science and Innovation of Spain to M.L.L., and by grant INCITE08PXIB235028PR from Xunta de Galicia, Spain to C.R.O.

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