NEW ASPECTS IN THE BIOLOGY OF FISH PATHOGEN *Photobacterium damselae* subsp. *piscicida*: PILI, MOTILITY AND ADHERENCE TO SOLID SURFACES

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INTRODUCTION

Photobacterium damselae subsp. *piscicida* (*Phdp*, previously *Pasteurella piscicida*) is one of the most important halophilic bacterial pathogens, and the causative agent of fish pasteurellosis, a serious disease affecting several economically important marine fish species (Romalde, 2002).

Pasteurellosis was first observed in natural populations of white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) in 1963 in Chesapeake Bay. Until recently, Europe was considered to be free of fish pasteurellosis, but from 1990 to the present, several outbreaks occurred in cultured fish populations in different European countries (Romalde, 2002). Based on an exhaustive characterization, a complete description of the pathogen was obtained. *Phdp* is a Gram-negative, nonmotile, pleomorphic bacterium. The virulence of *Phdp* is reported to correlate with the presence of a polysaccharide capsular layer (Acosta et al., 2006) and a plasmid-encoded virulence factors. The presence of bacterial pili (or fimbriae) and their relation to agglutination of red blood cells were demonstrated early by Duguid and coworkers in 1955, but in *Phdp*, not any strain described in the literature was able to hemagglutinate sheep, rat, rabbit, yellowtail, horse, trout or chicken erythrocytes. Moreover, as far as we are aware, the movement of *Phdp* bacteria on semi-solid or solid surfaces and biofilm formation has not previously been described.

Epifluorescence, light, transmission, scanning and confocal microscopy studies performed until now did not reveal pili on different *Phdp* strains. In contrast with several studies, the results reported in this work revealed the presence of long appendages (similar in appearance to type 4 bacterial pili) in several *Phdp* strains, and that new features of this pathogen (motility, attachment to solid surfaces and biofilm formation) could be mediated by these structures.

MATERIAL AND METHODS

Five virulent *Phdp* strains were used in this study. We studied the hemagglutinating activity of these strains with human group A erythrocytes. *Phdp* strains were examined by TEM and SEM to confirm the presence of pili. For bacterial microscopic examinations, bacteria were stained using a polyclonal antibody against *Phdp* strain 94/99 as we described previously (Acosta et al., 2009). Motility assays were performed as previously described with some modifications. Quantitative estimation of biofilm formation was performed by the method of O'Toole and Kolter (1998) with some modifications. Samples for CLSM were prepared as for standard immunofluorescence studies without fixation, and bacterial viability within biofilms was determined by using the BacLight LIVE/DEAD bacterial viability kit (Mol. Probes Inc.).

RESULTS

We have shown that *Phdp* expresses pilus-like surface structures which resemble in several aspects the surface appendages of other bacteria (Fig.1 and Fig.2). To our knowledge, such appendages have not previously been described in this species.

Although, historically *Phdp* is described as non-motile, which is related to the lack of flagella and therefore its inability to swim, the strains used in this study migrated in the medium-plastic interface of solid media, referred to as twitching motility (Fig. 3).

Furthermore, we report here that *Phdp* forms biofilms on submerged surfaces and not at the air-liquid interface on microtiter plates. Biofilm production was found to be strongly dependent on incubation time, culture medium, as well as the strain used. Microscopic examination of biofilms by SEM and CLSM revealed that Phdp display extensive cellular chaining and cell elongation during biofilm formation in vitro (Fig. 4). Cellular chaining may facilitate an increased flow of nutrients through the interior of the biofilm and could also afford an increased structural coherence during stress conditions.

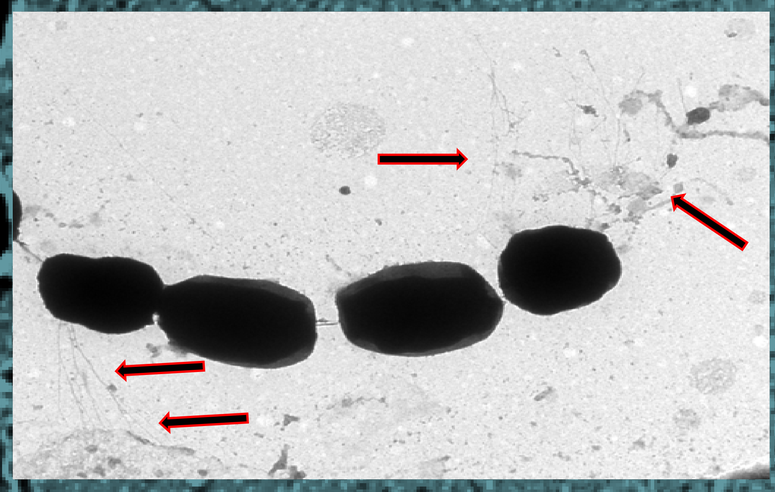

Figure 1. TEM microphotograph obtained from *Phdp* bacteria attached to the interface between the agar medium and the Petri dish. Arrows indicate pili-like structures. *Magnif:* ×*12.000*

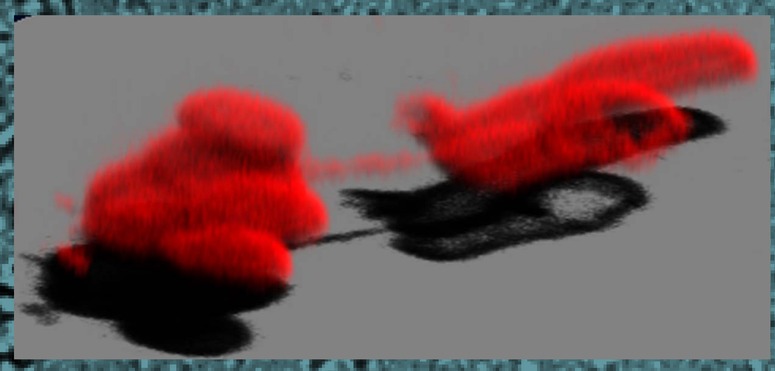

Figure 2. CLSM image of *Phdp*. It shows a pili-like structure between two bacteria. 3D reconstruction.

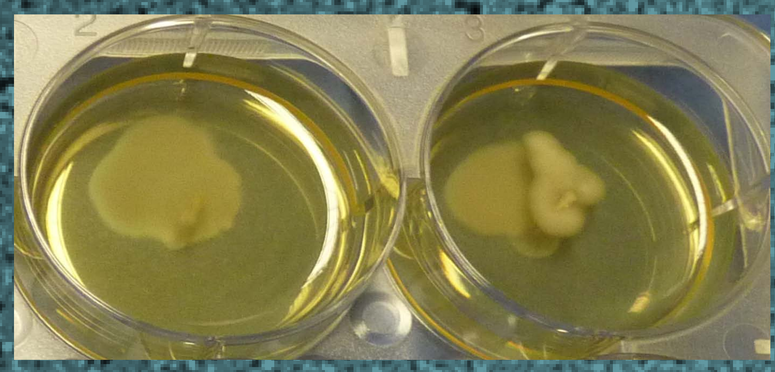

Figure 3. Motility assays of *Phdp* strains. Bacteria were inoculated with a toothpick into the bottom of BHIA medium in Petri dishes, and incubated at 25ºC for 48h.

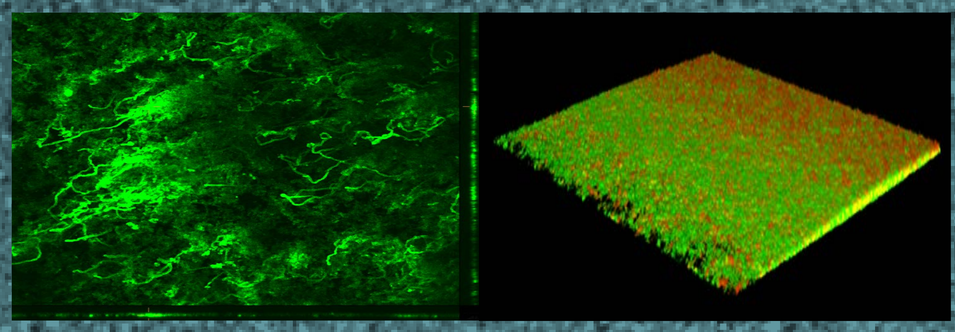

Figure 4a. Maximum intensity projection of a fixed 4 days old biofilm formed on a glass slide. Bacteria were stained with the *anti-Phdp* antibody and prepared as for standard immunofluorescence studies.
Figure 4b. CLSM image of unfixed 6 day old biofilm formed by *Phdp* strains and stained with LIVE/DEAD. Live bacteria are in green and dead ones in red.

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DISCUSSION AND CONCLUSIONS

We have shown that *Phdp*, a facultative intracellular fish pathogen, expresses pilus-like surface structures resembling in several aspects other bacteria surface appendages. To our knowledge, such appendages have not been described previously in this species. Although historically *Phpd* is described as non motile, the strains used in this study migrated in the medium-plastic interface, referred to as twitching motility.

We report here that *Phdp* forms biofilms on submerged surfaces and not at the air-liquid interface on microtiter plates. Biofilm production was found to be strongly dependent on incubation time and culture medium, as well as on the strain used. Microscopic examination of biofilms by SEM and CLSM revealed that *Phdp* displays extensive cellular chaining and cell elongation during biofilm formation *in vitro*.

Based on our results, standardized analyses of *Phdp* surface appendages, biofilms, motility and their impact on *Phdp* survival, ecology and pathobiology are now more feasible, and will help to open up new research areas in several fields of the ecology and the pathogenesis of this important fish pathogen.