

## NOTE

## Electron microscopic evidence that expression of capsular polysaccharide by *Photobacterium damsela* subsp. *piscicida* is dependent on iron availability and growth phase

Ana do Vale<sup>1,\*</sup>, Anthony E. Ellis<sup>2</sup>, Manuel T. Silva<sup>1</sup>

<sup>1</sup>Institute for Molecular and Cell Biology, University of Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal

<sup>2</sup>Marine Laboratory, Fisheries Research Services, PO Box 101, Victoria Rd, Aberdeen AB11 9DB, Scotland, UK

**ABSTRACT:** The expression of capsular polysaccharide (CPS) by the fish pathogen *Photobacterium damsela* subsp. *piscicida* was analysed in the virulent strain DI 21 in relation to the growth phase and presence or absence of available iron in the culture medium. Bacterial cells were processed for electron microscopy by a procedure that improves visualisation of the capsule through stabilisation with polycationic ferritin, and electron micrographs of ultrathin sections were scanned with an acquired computerised image analyser to measure capsular area. Cells grown under iron-limited conditions always had a significantly lower amount of capsular material on their surfaces than iron-supplemented cells, even when cells from different culture phases were compared. Irrespective of the presence or absence of iron in the culture medium the amount of CPS decreased with the age of the culture, i.e., from early log phase to late log phase to stationary phase. The *in vivo* significance of this regulatory role of iron remains to be investigated.

**KEY WORDS:** *Photobacterium damsela* · Bacterial capsule · Capsule regulation · Iron and capsular expression

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*Photobacterium damsela* subsp. *piscicida* (previously *Pasteurella piscicida*) is the causative agent of fish pasteurellosis, a serious bacterial disease affecting different economically important marine fish species such as yellowtail, gilthead seabream, striped jack, and sea bass (Thune et al. 1993, Magariños et al. 1996a). Different studies have identified several factors/mechanisms important in the virulence of *P. damsela* subsp. *piscicida* (see Romalde & Magariños 1997). Among them, the existence of efficient iron uptake systems was found to correlate with *P. damsela* subsp. *piscicida* pathogenicity. It has been demonstrated that this bacterium possesses a high affinity iron uptake system involving the production of both a siderophore and several iron-regulated outer membrane proteins, as well as a sidero-

phore-independent mechanism responsible for the uptake of iron from haem-containing compounds (Magariños et al. 1994). Iron was also found to play a regulatory role in the synthesis of some proteolytic enzymes by *P. damsela* subsp. *piscicida* (Magariños et al. 1994). The presence of capsular polysaccharide (CPS) material also seems to have an important role in virulence as the presence of a capsule on *P. damsela* subsp. *piscicida* results in decreased levels of phagocytosis by gilt-head seabream macrophages *in vitro* (Arijo et al. 1998). Furthermore, an increased resistance to the bactericidal action of seabream serum *in vitro* (Magariños et al. 1996b), as well as a reduction, by 2 to 3 log units, in the LD<sub>50</sub> values in challenge experiments have been reported (Magariños et al. 1996b).

The expression of *Photobacterium damsela* subsp. *piscicida* CPS can be modulated by culturing the bacteria in a glucose-enriched medium (Bonet et al. 1994). Although the effect of iron as a regulator of the expression of certain virulence factors by *P. damsela* subsp. *piscicida* has been reported (Magariños et al. 1994), the influence of iron availability and growth phase on the CPS expression by this pathogen has not previously been addressed.

In this study, the CPS expression by a virulent strain of *Photobacterium damsela* subsp. *piscicida*, grown either under iron-limited or iron-supplemented conditions is compared. The influence of growth phase on the capsule expression by this fish pathogen was also investigated.

**Materials and methods.** *Photobacterium damsela* subsp. *piscicida* strain DI 21 (a virulent strain, kindly provided by Prof. Alicia Toranzo, University of Santiago de Compostela, Spain) was maintained at –70°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) supplemented with NaCl to a final concentration of 1% (TSB-1) and with 15% (v/v) glycerol. For the experiments, the stock bacteria were routinely cul-

\*E-mail: avale@ibmc.up.pt

tured overnight on brain heart infusion agar (Oxoid Ltd, Basingstoke, UK) and sub-cultured in TSB-1. Iron-limited conditions were obtained by adding the iron chelator ethylenediamine-di-(O-hydroxyphenyl-acetic acid) (EDDHA; Sigma, St. Louis, MO, USA) to the TSB-1 medium at a final concentration of  $100 \mu\text{g ml}^{-1}$ . Iron-supplemented conditions were obtained by addition of  $\text{FeCl}_3$  (Fe) to the TSB-1 medium at a final concentration of  $10 \mu\text{M}$ .

Cell surface fine structure of *Photobacterium damsela* subsp. *piscicida* was analysed by transmission electron microscopy of ultrathin sections by a capsule stabilisation procedure (Jacques et al. 1994), using cationised ferritin, to improve capsule visualisation. This technique has been used to visualise the capsule of *P. damsela* subsp. *piscicida* (Magariños et al. 1996b). Improved capsule visualisation in this bacterium by cationised ferritin probably results from the interaction between ferritin and capsular sialic acid, which is known to be present on the surface of this fish pathogen (Jung et al. 2000). The technique using cationised ferritin was applied to bacteria grown in TSB-1+EDDHA or TSB-1+Fe at  $25^\circ\text{C}$  in a rotary shaker at 100 rpm and collected at the beginning (Fig. 1, a) or end (Fig. 1, b) of exponential phase and at stationary phase (Fig. 1, c). Bacteria fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M; pH 7.2) were allowed to react with polycationic ferritin (Sigma) at a final concentration of  $1 \text{ mg ml}^{-1}$  in the above cacodylate buffer, for 30 min at  $25^\circ\text{C}$  and were post-fixed in 1% osmium tetroxide in the cacodylate buffer for 3 to 4 h. Samples were embedded in Epon 812 and thin sections were cut with an LKB Ultratome III, stained with uranyl acetate and lead citrate (Silva et al. 1987) and examined under a Zeiss EM 10C electron microscope. The experiment was repeated twice. From electron micro-

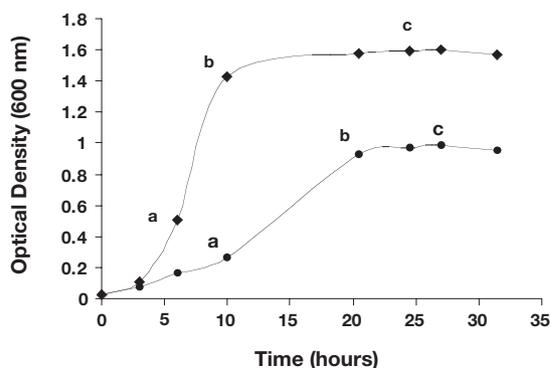
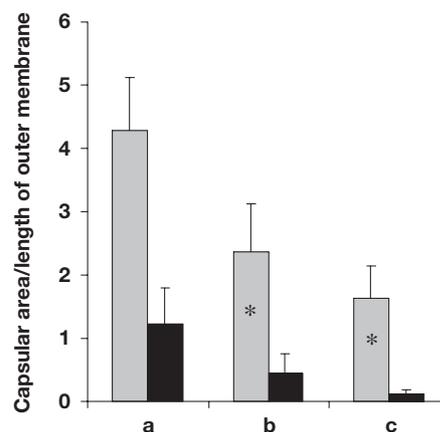


Fig. 1. *Photobacterium damsela* subsp. *piscicida*. Growth of the virulent strain DI 21 under iron-supplemented (◆) or iron-limited conditions (●). Samples were collected at the beginning (a) and end (b) of exponential phase and at the stationary phase (c) and were subsequently analysed by transmission electron microscopy as described in the text

scope pictures with a magnification of  $36\,500\times$ , representative surface bacterial profiles were analysed using an acquired computerised image analyser (Leica Quantimet 500MW, Leica, Cambridge, UK). This software allowed the quantification of capsular area per length of bacterial outer membrane. Only free surface profiles in perpendicular sections were used for analysis. The results of the quantitative study described in Fig. 2 represent averages and standard deviations of 9 to 15 measurements per sample. Statistical analysis was done using Student's *t*-test.

**Results and discussion.** In preliminary experiments we found that a clearly visible capsule cannot be seen in *Photobacterium damsela* subsp. *piscicida* cells processed for electron microscopy using either negative staining or the conventional glutaraldehyde/osmium fixation technique for sectioned samples (not shown). It is known that, in some species, the bacterial capsule is a difficult structure to examine using conventional electron microscopic techniques. Characteristic biophysical features of bacterial capsules are their high water content and their degree of negative charge (Bayer 1990). Because the capsule is highly hydrated, the dehydration for electron microscopy often causes the capsule to collapse (Bayer et al. 1985), which is unavoidable if protective measures are not taken. Several preservation procedures have been developed to prevent structural collapse during fixation and dehydration, therefore allowing the electron microscopic visualisation of bacterial capsules (Bayer 1990). One involves the stabilisation of the capsular material by cationised ferritin, which has been used as a marker for anionic charges, and binds both to CPS and lipopolysaccharides (LPS) (Bayer 1990, Anderson 1998). Our results obtained with samples processed for ultrastructural analysis by the capsule stabilisation procedure with ferritin (Jacques et al. 1994) show that the amount of capsule on the surface of *P. damsela* subsp. *piscicida* cells varied, depending on both the culture conditions and growth phase (Figs. 2 & 3). The amount of capsule covering cells was maximal on iron-supplemented bacteria collected at the beginning of the exponential phase and minimal on iron-limited cells harvested at the stationary phase. Cells grown under iron-limited conditions always had a significantly lower amount of capsular material on their surfaces than iron-supplemented cells, even when cells from different culture phases were compared. Irrespective of the presence or absence of iron in the culture medium the amount of CPS decreased with the age of the culture, i.e., from early log phase to late log phase to stationary phase. This observation indicates that studies on virulence mechanisms of bacterial pathogens should take into consideration the growth phase of the cultures.

Fig. 2. *Photobacterium damsela* subsp. *piscicida*. Capsular polysaccharide expression by DI 21 cells grown under iron-supplemented (grey bars) or iron-limited (black bars) conditions and collected at the early exponential phase (a), late exponential phase (b) or stationary phase (c). Capsular polysaccharide expression was determined by measuring the areas of ferritin deposition around bacterial cells as described in the text. Statistical differences (Student's *t*-test) for the comparison between capsular areas were significant ( $p < 0.05$ ) except for the comparison between the pair of values labelled with \* ( $p = 0.06$ )



The regulation of bacterial capsule expression is complex, involving many different factors (Costerton et al. 1981, Whitfield & Roberts 1999). Metal availability has been reported to affect the production of some bacterial capsular exopolymers (Geesey & Jang 1989). Iron-limited growth conditions obtained by the addition of the iron chelator 2,2-dipyridyl, deferoxamine

mesylate or apotransferrin to culture media have been shown to markedly reduce the amount of capsular material covering the cells of *Pasteurella multocida* (Jacques et al. 1994). On the other hand, the capsule of *Azotobacter chroococcum* appeared larger and more diffuse under iron-restricted conditions and it was suggested that such a capsule might serve to enhance iron

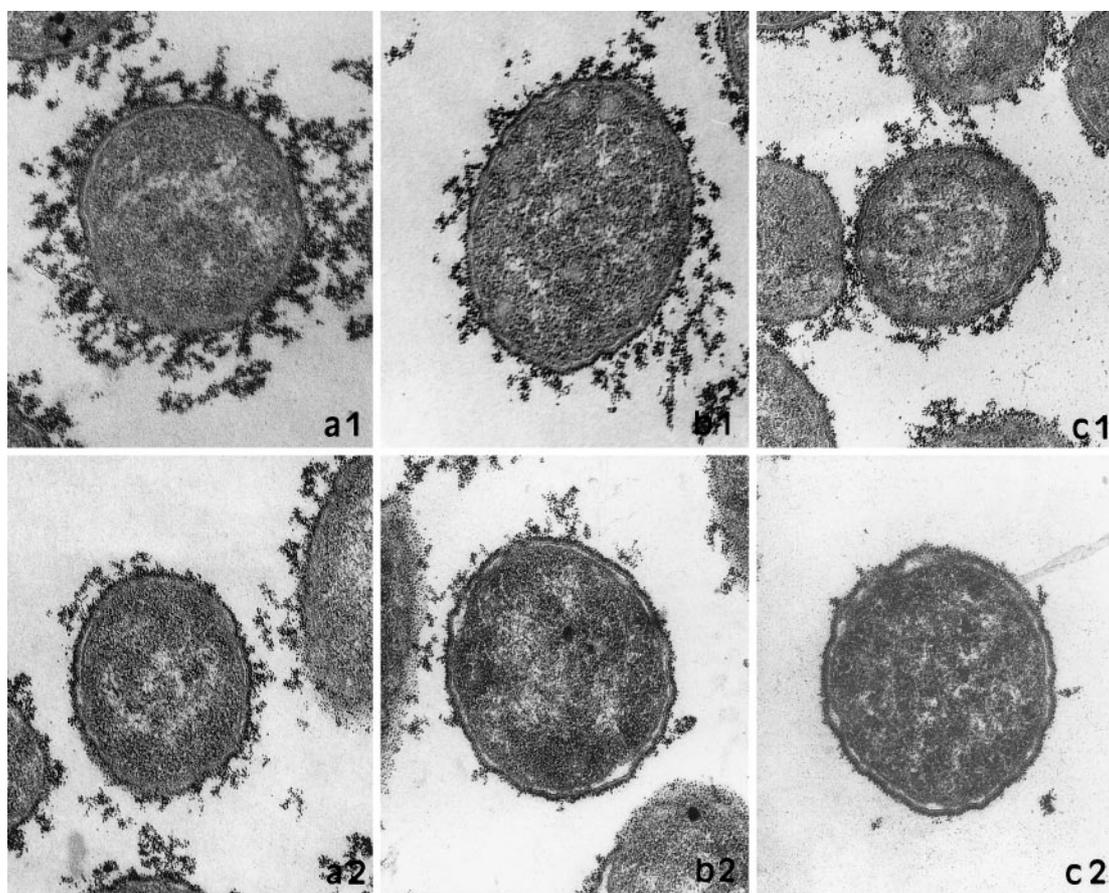


Fig. 3. *Photobacterium damsela* subsp. *piscicida*. Transmission electron micrographs of thin sections of DI 21 cells labelled with cationized ferritin. Cells were grown under iron-supplemented (a1, b1, c1) or iron-limited (a2, b2, c2) conditions and collected at the early exponential phase (a1, a2), late exponential phase (b1, b2) or stationary phase (c1, c2). Magnification 30000 $\times$

sequestration under these conditions (Ferrala et al. 1986). Factors such as growth phase and growth rate were also found to influence CPS production. Wright et al. (1999) demonstrated that *Vibrio vulnificus* expression of CPS varied with the growth phase, being higher in the logarithmic phase than in the stationary phase. It was also documented that capsule production by *P. multocida* in broth culture was maximal during the early log phase of growth (Jacques & Foiry 1987). Similarly, it was also proposed that cell growth rate could be a mechanism by which *Streptococcus* Type III CPS expression is regulated (Paoletti et al. 1996).

In conclusion, our results demonstrate that the amount of capsule in *Photobacterium damsela* subsp. *piscicida* virulent strain DI 21 varies with the age of the bacterial cultures and that iron plays a regulatory role in the CPS expression by this fish pathogen. The *in vivo* significance of this iron-dependent mechanism remains to be investigated.

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