Invasion of fish epithelial cells by *Photobacterium damsela* subsp. *piscicida*: evidence for receptor specificity, and effect of capsule and serum

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***Photobacterium damsela* subsp. *piscicida* is a fish pathogen which causes serious disease in commercial warmwater fish species. Because information on the initial stages of the infection is scarce, an investigation of the invasion ability of this pathogen was undertaken utilizing a fish epithelial cell line (epithelioma papillosum carpio, EPC), a virulent capsulated strain of *P. damsela* (MT1415), an avirulent non-capsulated strain of *P. damsela* (EPOY-8803-II) and *Escherichia coli* HB101 as a non-invasive control. *P. damsela* was found to be able to adhere to and invade fish epithelial cells and remain inside them for 6–9 h. There were no significant differences in invasiveness between the capsulated and non-capsulated strains. A kinetics study demonstrated that *P. damsela* invasiveness was more efficient at low m.o.i., reaching saturation at higher m.o.i., suggesting internalization may be receptor-mediated. Invasion efficiency (IE) was significantly higher than in the control *E. coli* HB101. Engulfment of bacteria was possibly by an endocytic process and was unaffected by killing the bacteria with UV light. However, heat-killed bacteria had significantly reduced invasion capability. Ultrastructural studies showed that inside the epithelial cells, the bacteria remained within large vacuoles for a few hours and no evidence of intracellular replication was found, by either fluorescence or electron microscopic studies. Normal sea bass serum slightly reduced the invasion capability of the MT1415 strain, but heat-inactivated normal serum had no effect. On the other hand, heat-inactivated fish antiserum raised against the same strain reduced the percentage of invaded epithelial cells by 50%. As for other pathogens, an intracellular phase of *P. damsela* may be a mechanism to delay or avoid phagocytosis and host immune responses, favouring the spread of infection.**

Keywords: *Photobacterium damsela* subsp. *piscicida*, fish epithelial cells, invasion efficiency

INTRODUCTION

Pasteurellosis is a serious bacterial disease caused by *Photobacterium damsela* subsp. *piscicida* (previously *Pasteurella piscicida*) which affects commercially important warmwater fish species, such as sea bass, white bass, yellowtail, striped bass and gilthead seabream (Thune *et al.*, 1993). The pathology of pasteurellosis has

been widely reported (Kubota *et al.*, 1970; Wolke, 1975; Hawke *et al.*, 1987; Toranzo *et al.*, 1991; Noya *et al.*, 1995b), and acute and chronic infections have been described.

The pathogenesis of *P. damsela* is poorly understood. It has been demonstrated that the polysaccharide capsular layer has an important role in the virulence of the pathogen (Magariños *et al.*, 1996b), as also have the extracellular products and iron availability (Magariños *et al.*, 1992, 1994). Regarding the interaction of *P.*

Abbreviations: EPC, epithelioma papillosum carpio; IE, invasion efficiency.

damselae with phagocytes, the results have been contradictory. Whilst morphologically intact bacteria within macrophages have been found *in vivo* (Kubota *et al.*, 1970; Nelson *et al.*, 1981; Kusuda & Salati, 1993; Noya *et al.*, 1995a, b), suggesting that *P. damsela* can survive inside macrophages, *in vitro* studies (Skarmeta *et al.*, 1995; Arijó *et al.*, 1998) have indicated that macrophages from three different fish species were able to kill the bacteria. More recently, Barnes *et al.* (1999) have confirmed that this species is unable to respond to oxidative attack such as that experienced during the macrophage respiratory burst. On the other hand, it has been suggested that *P. damsela* could avoid host defence mechanisms and antimicrobial agents by intracellular survival in non-phagocytic cells (Magariños *et al.*, 1996a) since it has been demonstrated that this bacterium is capable of invading different fish cell lines (Magariños *et al.*, 1996a; Yoshida *et al.*, 1997).

The ability to invade epithelial cells is a key determinant of virulence for several human pathogenic bacteria such as *Escherichia coli* and *Yersinia*, *Salmonella* and *Shigella* species (Galán, 1994; Zierler & Galán, 1995). Amongst fish pathogens, this capacity has been demonstrated for *Aeromonas hydrophila* (Leung *et al.*, 1996; Tan *et al.*, 1998) and *Vibrio anguillarum* (Wang *et al.*, 1998). *P. damsela* is considered weakly or moderately adherent and invasive to various fish cell lines (Romalde & Magariños, 1997). Moreover, it showed a high binding capacity to fish intestines (Magariños *et al.*, 1996a). The adherence seemed to be mediated by a protein or glycoprotein receptor of the bacterial cell surface, and the internalization of the bacteria was an actin-microfilament-dependent mechanism (Magariños *et al.*, 1996a). Although these authors have indicated that *P. damsela* is able to remain viable inside the cells for at least 2 d, and to spread from cell to cell, it has not been demonstrated that internalization in fish cell lines is a specific process.

This study was carried out to analyse and obtain a better understanding of *P. damsela* internalization, by studying invasion efficiency (IE), the kinetics of invasion, the role of the capsule, and the effects of heating and exposure to UV light, serum and antiserum on the invasiveness of this species.

METHODS

Bacteria. A virulent capsulated strain (MT1415) and an avirulent non-capsulated strain (EPOY-8803-II) (Magariños *et al.*, 1996b) of *P. damsela* subsp. *piscicida* were used in the present study. The capsulated strain was originally isolated from an outbreak of pasteurellosis in sea bass (*Dicentrarchus labrax*) in Italy and was obtained from the Marine Laboratory Collection, Aberdeen; the non-capsulated strain was isolated from red grouper (*Epinephelus akaara*) in Japan and was obtained from the Microbiology Department, Málaga University, Spain (Arijó *et al.*, 1998). Strains were cultured on tryptic soy agar (TSA) supplemented with 2% NaCl (TSA-2) at 22 °C for 48 h, or in tryptone soya broth (TSB) supplemented with 2% NaCl (TSB-2) overnight at 22 °C with shaking.

Tissue culture. Epithelioma papillosum carpio (EPC) cells were grown in 75 cm² flasks containing Glasgow modification of Eagle's minimal essential medium (G-MEM) (ICN) with 10% fetal bovine serum (FBS, Sigma). In all the experiments, EPC cells were grown to a confluence of 80–90% (between 2×10^6 and 1×10^7 cells per 25 cm²).

Bacterial hydrophobicity. The cell surface hydrophobicity of the *P. damsela* strains used was studied using the salt aggregation test (SAT). The assay was performed essentially as described by Lindhal *et al.* (1981). Briefly, serial doubling dilutions of ammonium sulphate were made ranging between 4 M and 0.003 M in 0.002 M sodium phosphate buffer (pH 6.8). To 50 µl of each dilution, 50 µl of bacterial suspension ($\approx 10^{10}$ c.f.u. ml⁻¹) was added. The lowest concentration at which agglutination occurred was recorded for each strain.

Adherence assay. Bacteria were grown as indicated above and 25 µl added to each well of a 24-well tissue culture plate containing a sterile glass coverslip seeded with EPC cells. The plates were centrifuged (150 g, 5 min) to achieve contact between bacteria and cells. The infected cultures were incubated at 22 °C from 15 min to 24 h. After incubation, coverslips were washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.7 mM KH₂PO₄, pH 7.2) to remove non-adherent bacteria, fixed with methanol, stained with Giemsa and mounted with 90% glycerol in PBS.

Quantitative invasive assay. Invasiveness of *P. damsela* MT1415 was tested and *E. coli* HB101 (Sigma) a non-invasive strain, was used as a negative control. From an overnight TSB culture, *E. coli* was inoculated into TSB and grown at 37 °C for 3 h to obtain an exponential-phase culture.

EPC cells were seeded in 75 cm² flasks and grown until 80–90% confluent. New medium was added to the cells, which were then suspended using a scraper. Cells were counted in a counting chamber and distributed in microtubes (1×10^5 EPC cells per tube) containing 1 ml of G-MEM supplemented with 10% FBS. Serial dilutions of freshly grown, exponential-phase bacteria suspended in PBS were prepared so as to produce m.o.i.s ranging from 0.1 to 10^6 bacteria per epithelial cell. One hundred microlitres of the bacterial suspensions was added to each tube. Each dilution was prepared in duplicate. After adding the bacteria, the microtubes were centrifuged for 5 min at 150 g to initiate contact between bacteria and epithelial cells. The infected EPC cells were incubated for 2 h, centrifuged and resuspended for 1 h in 1 ml medium containing 100 µg gentamicin (Gibco) ml⁻¹ to kill extracellular bacteria. The infected cells were washed with medium and then lysed with 1 ml 1% Triton X-100 for 10 min. One hundred microlitres of this suspension was serially diluted in PBS and the bacteria were quantified by viable count after growth for 48 h at 22 °C on TSA-2 for *P. damsela* and 24 h at 37 °C on TSA for *E. coli*.

To confirm that the conditions used in the antibiotic assay described were sufficient to eliminate the extracellular bacteria, the bactericidal activity of gentamicin was determined essentially as described by Barnes *et al.* (1991), with the following modifications. A single antibiotic concentration (100 µg ml⁻¹) was used in G-MEM. Inocula were prepared by serial tenfold dilution from a stock suspension of 10^{11} bacteria ml⁻¹ of *P. damsela* MT1415 to give similar concentrations to those employed in the antibiotic protection assay, ranging from 5×10^{10} to 5×10^4 bacteria ml⁻¹. The suspensions and antibiotic were incubated for 1 h at room temperature. Following incubation, residual antibiotic was diluted out in

fresh TSB-2 prior to determining bacterial viability by plating onto TSA-2.

Study of invasion of EPC cells by *P. damsela* using fluorescence microscopy. The technique described by Devrets & Campbell (1991) and Bandín *et al.* (1995) was used to differentiate adherent from intracellular bacteria. Bacteria were harvested, washed (2000 *g* at 4 °C for 15 min), resuspended in PBS and the OD₅₄₀ adjusted to 1.5–2.0. The bacteria were then labelled by incubation with 0.1 mg FITC (isomer I, Sigma) ml⁻¹, 0.1 M NaHCO₃, pH 9.0 at 22 °C for 60 min. Bacteria were pelleted at 1500 *g* for 5 min and washed free of unbound FITC with PBS (5–10 times). After washing, the bacterial concentration was determined in a counting chamber and adjusted to 10⁸–10⁹ cells ml⁻¹. EPC cells were seeded in 25 cm² flasks and incubated at 22 °C until 80–90% confluent. Prior to infection, the cells were scraped from the flasks and distributed equally into tubes (1 ml per tube); 25 µl labelled bacteria was then added to each tube. After centrifugation (150 *g* for 5 min) and gentle resuspension, the infected EPC cells were incubated at 22 °C in a rotary mixer (20 r.p.m.) for various times. At the end of the incubation period, the infected cells were washed three times with PBS (150 *g* for 5 min, 4 °C) to remove free bacteria, centrifuged and resuspended in 100 µl PBS. Ethidium bromide (Sigma) was then added (500 µg ml⁻¹, final concentration), and after mixing, a 10 µl drop was placed on a glass slide and overlaid with a coverslip.

Preparations were observed with a fluorescence microscope (Leica DM LB). Intracellular bacteria fluoresced green and extracellular bacteria fluoresced orange. The percentage of EPC cells with at least one intracellular bacterium was calculated as the mean of random counts of 100 cells, repeated at least three times. All the assays were carried out in triplicate.

Heat and UV light treatment. To investigate if invasiveness depended on the viability of *P. damsela* and/or a heat-labile receptor, bacteria were killed by either heat or UV light. Bacteria grown for 24 h on a TSA-2 plate were treated with UV light for 2 h. For heat treatment, the bacteria were suspended in PBS and heated at 80 °C for 10 min. Three plates of TSA-2 for each treatment were inoculated to confirm the non-viability of the bacteria. After inactivation, bacteria were labelled with FITC and incubated with EPC cells (m.o.i. ≈ 25) as indicated above.

Production of sea bass anti-*P. damsela* antiserum. Sea bass, 1–2 kg, were kept in seawater aquaria at 20 °C. Fish were immunized with a formalin-inactivated bacterin made from a 48 h culture of *P. damsela* MT1415 grown in TSB-2 at 22 °C. The bacterin was emulsified 1:1 in Freund's complete adjuvant. Fish were intramuscularly injected (100 µl), followed by an identical immunization 30 d after initial injection. Sera were collected 14 d after the second immunization and stored at –20 °C until required. Normal serum was collected from non-immunized fish. The agglutinating titres were determined as described by Roberson (1990). The titre of the antiserum against strain MT1415 was 1:16. No agglutination was detected after incubation of MT1415 with normal serum.

Treatment with normal serum and antiserum. Heat-inactivation of the sera was carried out at 45 °C for 15 min in order to inactivate complement (Sakai, 1981).

Invasion assays were conducted as described above. After FITC-labelling and washing, the bacteria were suspended in PBS to about 3 × 10⁸ ml⁻¹. Aliquots (100 µl) were placed into five microcentrifuge tubes (in triplicate) and incubated with 500 µl PBS as control, sea bass normal serum, heat-inactivated

normal serum or heat-inactivated antiserum for 10 min at room temperature. After centrifugation (1500 *g*, 5 min), bacteria were washed and resuspended in PBS. Bacteria were added to EPC (m.o.i. ≈ 15–19) and incubated for 2 h. Visualization of intracellular bacteria was carried out as above by the fluorescence method.

Transmission electron microscopy. The techniques described by Watanabe *et al.* (1988) for rabbit platelets and by Gutenberger *et al.* (1997) for trout leucocytes were used. EPC cells obtained as described above were infected with bacteria. After centrifugation (150 *g* for 5 min) and gentle resuspension, the infected EPC cells were incubated for 1–9 h at 22 °C in a rotary mixer (20 r.p.m.), washed three times with PBS (900 *g* for 5 min) to remove non-attached bacteria and pelleted. The pellet was resuspended in 2% glutaraldehyde buffered with 0.05 M PIPES (Hayat, 1986) at pH 7.2 and fixed for 1 h at room temperature. After washing twice with 0.025 M PIPES and centrifugation at 230 *g* for 10 min, two drops of 2% agarose (agarose type VII, low gelling temperature <30 °C) maintained at 37 °C were added. The cells were centrifuged at 230 *g* for 5 min at room temperature and then left on ice until the agarose gelled. The gel was cut into small pieces (1 mm³), postfixed with 1% aqueous osmium tetroxide for 1 h, and left overnight at room temperature in 2% aqueous uranyl acetate. Dehydration in an acetone series was followed by embedding in araldite (Durcupam ACM) using propylene oxide as an intermediate solvent. Ultrathin sections (70–80 nm) were obtained with an LKB Ultratome III, stained on the grid with Fahmy's lead citrate (Lewis & Knight, 1977) and examined with a Hitachi H-300 electron microscope.

Statistical analysis. All the results were analysed by applying a unifactorial ANOVA test and probabilities of <0.05 were considered significant. The data are presented as means ± SD.

RESULTS

Adherence

Light microscope examination of infected EPC cells demonstrated extracellular *P. damsela* MT1415 in close association with the plasma membrane of the epithelial cells within 15 min of infection (data not shown).

Kinetics of bacterial invasion

The bactericidal test confirmed that the gentamicin concentration and incubation time used for the invasion assay were sufficient to kill the extracellular bacteria. Bacterial IE was calculated as: (number internalized c.f.u. at the end of the assay/starting inoculum) × 100. Invasion efficiency of *P. damsela* MT1415 was tested at different m.o.i.s, from 0.16 to 1.6 × 10⁶ bacteria per EPC cell (Fig. 1a). Maximum IE (2.77%) was observed at an m.o.i. of 0.162 and was reduced to about 1% at m.o.i.s of 1.6 and 16.0. At higher m.o.i.s, IE values decreased gradually. The non-invasive control *E. coli* HB101 was tested over a similar range of m.o.i.s and the maximum IE was 0.0038% (data not shown).

When bacterial invasion ability was expressed as the total number of intracellular c.f.u. recovered from the infected EPC cells (Fig. 1b), a progressive increase was found from the lowest m.o.i., reaching a value of ~1.3 × 10⁶ at m.o.i. 1.6 × 10⁴. Thereafter, the number

Fig. 1. Characteristics of invasion of EPC cells by *P. damsela* subsp. *piscicida* MT1415 after 2 h incubation and 1 h gentamicin treatment. All assays were conducted in duplicate. Results are presented as means $\pm$ SD. (a) IE of the bacteria at different m.o.i.s. (b) Total number of bacteria internalized at different m.o.i.s. The number of internalized bacteria increased progressively and significantly ($P < 0.05$), and reached saturation at m.o.i. 1.6×10^4 . Thereafter, there were no significant ($P > 0.05$) differences when the m.o.i. was increased. (c) Mean number of bacteria internalized per EPC cell at different m.o.i.s. As in (b), the number of bacteria per cell increased up to m.o.i. 1.6×10^4 and thereafter no significant increase was recorded.

increased slightly but not significantly, suggesting a limitation on entry at m.o.i.s. $\geq 1.6 \times 10^4$.

The number of internalized c.f.u. per EPC cell resulting from varying the m.o.i. (Fig. 1c) increased slowly for the first four m.o.i.s and then there was a sharp increase up to 6.5 bacteria per EPC cell at m.o.i. 1620. This value doubled at m.o.i. 16000 and thereafter remained constant at m.o.i.s. $\geq 1.6 \times 10^4$.

Quantification of *P. damsela* internalization by the fluorescence method

The fluorescence staining assays confirmed the presence of intracellular micro-organisms (Fig. 2). By using this method, it was possible to quantify the percentage of EPC cells infected over time, as well as the intensity of infection (i.e. number of bacteria per infected EPC cell) over time.

Uptake of *P. damsela* strain MT1415 by EPC cells was concentration- and time-dependent (Fig. 3a). Depending on bacterial concentration, between 33 and 63% of EPC cells had one or more internalized bacteria within 1 h of *P. damsela* inoculation. The percentage of infected EPC cells reached a plateau by 3 h. Significant differences were always found between 1 and 3 h of incubation, but not between 3 h and longer incubation times (5 or 6 h).

The number of intracellular bacteria per epithelial cell was studied over time. In this case, EPC cells were infected as above and incubated for 1, 3 and 6 h. Two 6 h incubations were carried out (6 and 6#). In 6#, the cells were washed and new medium was added 3 h after infection in order to remove extracellular bacteria from the medium. After incubation, 100 EPC cells were randomly examined and the number of bacterial cells (1,

Fig. 2. EPC cell infected by *P. damsela* MT1415 as observed by fluorescence staining. Green bacteria, intracellular; orange bacteria, extracellular. Bar, 100 μ m.

2, 3, 4, 5, 6 or more) in each of the infected cells was counted (Fig. 3b). The number of intracellular bacteria per EPC cell was time-dependent. After 1 h incubation, 44% of infected EPC cells had only 1 intracellular bacterium and 9% had six or more. After 6 h, 17% had one intracellular bacterium and 41% had six or more. The number of EPC cells with one bacterium was significantly ($P < 0.05$) reduced from 1 to 3 and 6 h, whereas the number of epithelial cells with six or more

Fig. 3. Infection of EPC cells by *P. damsela* (MT1415) over time. (a) Cells were infected with 2.28×10^8 (◆), 4.64×10^8 (●), or 9.92×10^8 (■) bacteria (m.o.i. ≈ 5 , 30 and 60, respectively). Results are expressed as means $\pm$ SD from three replicates for each experiment. As the bacterial concentration increased, so did the percentage of EPC cells infected. There was a significant ($P < 0.05$) increase between 1 and 3 h of incubation, but not between 3 h and longer incubation times. (b) Bars show number of intracellular bacteria per EPC cell. Time 6# represents a 6 h incubation period in which the bacteria were eliminated from the medium after 3 h. Results are expressed as means $\pm$ SD from a representative experiment carried out in quadruplicate. Bacterial concentration was 9.92×10^8 (m.o.i. ≈ 60).

bacteria was significantly ($P < 0.05$) increased from 1 to 3 and 6 h. However, when the bacteria were removed from the medium after 3 h and the cells incubated for a further 3 h (6#), there was no significant difference between the values at 3 and 6# h of incubation suggesting that the increase in the number of intracellular bacteria per EPC cell between 3 and 6 h resulted from uptake of further bacteria from the medium, rather than division of bacteria within the cells.

Hydrophobicity and invasion of the virulent and avirulent strains

Non-capsulated *P. damsela* strain EPOY-8803-II aggregated with a lower (0.25 M) concentration of ammonium sulphate than the capsulated strain MT1415 (> 4 M). For invasion assays, EPC cells were infected with equivalent bacterial concentrations ($\approx 10^8$ bacteria ml^{-1} , m.o.i. ≈ 15) of the virulent MT1415 and non-virulent EPOY-8803-II strains, incubated for 1 and 3 h and observed by fluorescence microscopy. There were no significant differences between the percentage of EPC cells invaded by the two strains, either after 1 h (MT1415, $37.8 \pm 1.8\%$; EPOY-8803-II, $38.83 \pm 1.04\%$) or 3 h (MT1415, $49.3 \pm 5.1\%$; EPOY-8803-II, $49.0 \pm 0.9\%$) incubation.

Effect of heat, UV light, serum and antiserum on bacterial invasiveness

After heat and UV light treatments, no viable bacteria could be detected on TSA-2 plates, confirming complete inactivation of the bacteria. Invasiveness of heat-inactivated bacteria was greatly reduced ($9.3 \pm 1.5\%$ EPC cells invaded) compared with the non-inactivated bacteria ($52.2 \pm 4.0\%$ EPC cells invaded). However, bacteria inactivated by UV light were still able to invade up to

$44.8 \pm 1.5\%$ of EPC cells, which was not significantly ($P > 0.05$) different from the non-treated viable bacteria.

Capsulated bacteria incubated with heat-inactivated normal serum invaded a similar percentage of EPC cells ($47.5 \pm 4.4\%$) as the control (bacteria incubated with PBS) ($49.2 \pm 2.0\%$). However, when bacteria were incubated with fresh normal serum, the percentage of EPC cells infected was significantly ($P < 0.05$), but not greatly, reduced ($36.0 \pm 1.5\%$). Incubation of the bacteria with heat-inactivated sea-bass antiserum provoked a significant ($P < 0.05$) decrease in the number of epithelial cells with intracellular bacteria ($24.5 \pm 1.0\%$).

Ultrastructure of *P. damsela* infection

Ultrastructural studies of EPC cells infected with *P. damsela* provided information on the alterations which occurred both in host cells and bacteria. EPC cells infected with bacteria were incubated for 1, 3, 5, 7 and 9 h; non-infected EPC cells were used as control. No changes in cell morphology occurred in the control cell cultures over the experimental period (data not shown).

After 1 h infection, both extra- and intracellular bacteria could be seen. Extracellular bacteria were in close association with the plasma membrane (Fig. 4a). EPC cells showed cytoplasmic extensions around the bacteria which were interpreted as the first steps in the engulfment process (Fig. 4b). The intracellular bacteria were always situated within membrane-bound vacuoles (Fig. 4b, c) and no evidence of membrane disintegration was found. Vacuoles with multiple bacteria were observed (Fig. 4c) which may have been the result of several bacteria being engulfed at the same time or of vacuole fusion. Five hours after infection, most bacteria were located within large membrane-bound vacuoles and no evidence of bacteria free in the cytoplasm was seen.

Fig. 4. Transmission electron micrographs of EPC cells infected with *P. damsela* MT1415 after 1–3 h incubation. (a) After 1 h incubation. Both extracellular (arrow) and intracellular (arrowhead) bacteria can be seen. N, nucleus of epithelial cell. (b) After 1 h incubation. A cytoplasmic extension (arrow) is visible around one extracellular bacterium close to a vacuole that already contains an internalized bacterium (arrowhead). (c) After 3 h incubation. Once internalized, bacteria are always located inside membrane-bound vacuoles. Large vacuoles may contain two or more bacteria. Bars, 1 µm.

Fig. 5. Transmission electron micrographs of EPC cells infected with *P. damsela* MT1415 after 7–9 h incubation. (a) After 7 h incubation. Bacteria-containing vacuole close to the EPC cell surface. The cytoplasm of the EPC cell shows degenerative characteristics (asterisk). (b) After 7 h incubation. Exit of *P. damsela* from an EPC cell. The bacteria are still associated with the cellular membrane (arrows). (c) After 9 h incubation. Epithelial cells are lysed and bacteria are extracellular. Some instances of extracellular division can be seen (arrow). N, nucleus of epithelial cell. Bars, 1 µm.

By 7 h post-infection, EPC cells showed degenerative changes (Fig. 5a, b). There was a decrease in the number of intracellular bacteria and the bacteria-containing vacuoles were now located closer to the EPC cell surface, separated from the medium by a thin edge of cytoplasm. Furthermore, although many extracellular bacteria were found, most of them being associated with cellular membranes, no evidence of rupture of the plasma membrane of EPC cells was observed. At 9 h post-infection, epithelial cells appeared to be lysed and most

bacteria were extracellular. Some instances of extracellular bacterial division were observed (Fig. 5c). Despite many observations, at no time was intracellular replication or bacterial destruction observed.

DISCUSSION

P. damsela subsp. *piscicida* has the ability to adhere to and invade fish epithelial cells. This invasion ability has been previously demonstrated by Magariños *et al.*

(1996a) and Yoshida *et al.* (1997). However, this is the first study of the kinetics and specificity of this process. Visual evidence of the intracellular phase of *P. damsela* inside fish epithelial cells was provided by electron microscopic observations and the light microscopic fluorescence technique. Moreover, the invasion of fish epithelial cells by *P. damsela* was confirmed by the gentamicin assays, since this antibiotic selectively kills extracellular bacteria.

Magariños *et al.* (1996a) indicated this species was weakly or moderately invasive. These authors tested various fish cell lines at an m.o.i. of 100 and found that up to 10^3 bacteria were recovered from EPC cells after the antibiotic assay. Unfortunately, the concentration of neither the bacteria or the fish cells was stated in this report so it is not possible to compare the results directly with the present data. The invasive properties of a species depend greatly on the bacterial strain and cell lines used, as has been demonstrated for *Salmonella*, where the IE varied between ≈ 6 and 38% depending on the cell lines and the bacterial strain tested (Mills & Finlay, 1994). On the other hand, we have demonstrated that, for this species, m.o.i. has a clear effect on the number of bacteria that invade the cells. IE of *P. damsela* was maximal at the lowest m.o.i. (0.16), whereas the number of internalized bacteria and the number of bacteria per epithelial cell reached saturation at higher m.o.i.s ($\geq 1.6 \times 10^4$). This relation between m.o.i. and invasion should be borne in mind in future studies on *P. damsela* invasiveness since the value of m.o.i. chosen represents a compromise between number of internalized bacteria and IE.

The maximum IE observed for *P. damsela* in the present study represents 2% of the initial inoculum, a percentage significantly lower than those indicated for some human invasive bacterial pathogens, such as *Salmonella typhimurium* (Huang *et al.*, 1998) or *Yersinia enterocolitica* (Small *et al.*, 1987), which showed IEs of 48.9% and 21%, respectively. However, the level found here for *P. damsela* is higher than those reported for some other invasive species, such as *Campylobacter jejuni* (0.1–0.2%) (Konkel *et al.*, 1993), and similar to the enteroinvasive *E. coli* 11 (2.9%) (Small *et al.*, 1987). The present study also showed that the induced uptake of *P. damsela* by the EPC cells was not a non-specific property of the cell line, since the cells were not invaded by the non-invasive *E. coli* HB101.

More intracellular organisms and higher numbers of bacteria per epithelial cell were recorded as the m.o.i. increased, until a saturation level was reached at high m.o.i.s ($\geq 1.6 \times 10^4$). These results suggest that internalization of *P. damsela* by EPC cells is a process mediated by receptor–ligand interactions, since this type of process is characterized by saturability (García-Peñarrubia *et al.*, 1992). Both epithelial cells and bacteria seem to be involved in the internalization process. The host cell plays an active role since the internalization of the bacteria is inhibited by cytochalasin D (Magariños *et al.*, 1996a), indicating that it is dependent upon host actin microfilaments. Moreover, the ultrastructural

analysis by transmission electron microscopy described here provides evidence that *P. damsela* is ingested into vacuoles, possibly by an endocytic process, in a similar way to pathogens such as *Salmonella*, *Shigella* spp. and *Yersinia* (Finlay & Falkow, 1988). On the other hand, heat-killed bacteria were unable to invade the epithelial cells, whilst the UV-light-inactivated bacteria retained their invasive capabilities. This suggests that engulfment does not require *P. damsela* to be viable, but integrity of its surface components is necessary to permit interactions between the bacteria and the surface of the host cells. These results agree with those of Magariños *et al.* (1996a), who found that adhesive capacities are affected by heat and sugars, and suggested that adherence may be mediated by a glycoprotein of the bacterial or host cell surface.

Once internalized by non-phagocytic cells, micro-organisms have different strategies to survive within the host cells. Some bacteria, such as *Shigella* spp. (Sansone *et al.*, 1986) and *Rickettsia* (Heinzen *et al.*, 1993), escape from the vacuole to multiply in the cytoplasm and later infect adjacent cells. However, there are numerous bacterial species that remain inside the vacuoles of the epithelial cells throughout infection. Species such as *Salmonella* and *Yersinia* (Finlay & Falkow, 1988; Janda *et al.*, 1991), *Haemophilus influenzae* (Virji *et al.*, 1991) or group B streptococci (Rubens *et al.*, 1992) are included in this group. *P. damsela* did not seem to escape from the vacuoles. At no time during infection were bacteria observed free in the cytoplasm. Instead, they were found within large cytoplasmic vacuoles where they remained viable for several hours. The intracellular time period seems to be dependent on the cell line used and assay conditions since Magariños *et al.* (1996a) found that *P. damsela* remained inside CHSE-214 cells for at least 2 d, whilst in the present study the bacteria were liberated from the EPC cells 7–9 h after infection.

Some invasive bacteria replicate inside the host cells (Finlay & Falkow, 1988). However, during the present study, examination of a large number of electron micrographs failed to show evidence of intracellular multiplication of *P. damsela*. Data from the fluorescence assay indicated an increasing number of intracellular bacteria per cell at longer incubation times, which could be due either to continuous invasion or to replication of the organisms inside the EPC cells. However, when the extracellular bacteria were eliminated from the medium there was no further increase in the number of intracellular bacteria, which supports the electron microscopic observations that *P. damsela* does not seem to replicate inside the EPC cells. Bacterial replication seemed to occur once the bacterium had left the host cell and was observed extracellularly 9 h after infection.

The virulence factors that contribute to IE of epithelial cells by *P. damsela* are unknown. Significant differences in IE between non-capsulated and capsulated *P. damsela* strains were not observed, although the surface hydrophobicity of the avirulent non-capsulated EPOY-

8803-II strain is strong according to the criteria proposed by Santos *et al.* (1990), whilst the virulent capsulated strain MT1415 is not hydrophobic. Similar results were found by Magariños *et al.* (1996a) who studied the adherence to tissues of capsulated (virulent) and non-capsulated (avirulent) strains of *P. damsela*. The lack of effect of the bacterial capsule on adherence or invasion has been reported in other Gram-negative bacteria, such as *H. influenzae* (Lipuma & Gilsdorf, 1987; Roberts *et al.*, 1984) and enterotoxigenic *E. coli* (Guerina *et al.*, 1983). However, studies on the role of bacterial capsules in adherence or invasion to host cells are contradictory. It has been postulated that a capsule may reduce adhesiveness in several species, such as *E. coli* (Runnels & Moon, 1984), *H. influenzae* (St Geme & Falkow, 1991; Virji *et al.*, 1991), *Actinobacillus pleuropneumoniae* (Jacques *et al.*, 1991) and *Pasteurella multocida* (Jacques *et al.*, 1993). On the other hand, it has been found that the *E. coli* capsule may promote adherence (Davis *et al.*, 1981; Chan *et al.*, 1982). Recent studies with *H. influenzae* (St Geme & Cutter, 1996) have suggested that the level of encapsulation, and not the presence of capsule by itself, influences the adhesive interactions of the organism with its host cells. In *P. damsela*, the level of encapsulation seems also to be important, since although the constitutive capsule of virulent strains does not interfere with adhesion, a thicker capsular material decreases adherence to host tissues (Magariños *et al.*, 1996b). Since both capsulated and non-capsulated strains of *P. damsela* appear to be able to invade fish epithelial cells, the virulence of the capsulated bacteria cannot be explained solely on the basis of their ability to invade host cells.

Incubation of the bacteria prior to invasion with fresh normal sea bass serum slightly, but not significantly, reduced the percentage of EPC cells invaded by the encapsulated strain, whilst incubation with heat-inactivated normal serum had no effect on invasiveness. Although this could suggest a certain degree of inhibition by the complement of fish serum on capsulated bacteria, the percentage of infected epithelial cells was still high. It has been demonstrated that bacterial capsules confer resistance to bactericidal activity of serum (Amaro *et al.*, 1994; Daly *et al.*, 1996), including that of *P. damsela* (Arijo *et al.*, 1998; Magariños *et al.*, 1996b). This slight reduction in invasiveness with fresh normal fish serum may be a consequence of some bacteria with a thinner capsule being killed by the action of complement. Incubation with heat-inactivated antiserum against strain MT1415 had a significant effect on invasion, indicating that antibodies reduce the invasiveness of the bacteria, presumably by blocking the surface receptors involved in adhesion and/or invasion. The effect of antiserum is certainly specific as heat-inactivated normal serum did not decrease invasiveness and did not agglutinate the bacteria as did the antiserum. Thus, nonspecific interactions with fish serum proteins have no effect on bacterial invasiveness.

The presence of intracellular bacteria in host cells has been interpreted as a mechanism to avoid phagocytosis

and host immune responses, reducing their effectiveness and favouring the spread of infection (Virji *et al.*, 1991; Magariños *et al.*, 1996a; Galán, 1994; Amaro *et al.*, 1994; Daly *et al.*, 1996). As Virji *et al.* (1991) pointed out, bacteria do not need intracellular replication, only the ability to enter and survive inside the host cells and to exit. The present data show that *P. damsela* can invade fish epithelial cells, remain intracellular for a few hours, leave the host cells by lysing them and replicate extracellularly. As the capsule confers resistance to complement-dependent killing (Magariños *et al.*, 1996b; Arijo *et al.*, 1998), the intracellular environment may be more important to escape contact with phagocytes. Although some studies have indicated that *P. damsela* survives inside macrophages (Kubota *et al.*, 1970; Nelson *et al.*, 1981; Kusuda & Salati, 1993; Noya *et al.*, 1995a, b), Skarmeta *et al.* (1995) and Arijo *et al.* (1998) found that fish macrophages have the ability to kill the bacteria, and Barnes *et al.* (1999) have recently confirmed that *P. damsela* does not have adaptive defences against the bactericidal activities of macrophages. Since macrophages are able to kill *P. damsela*, the invasion of epithelial host cells may avoid, or at least delay, the contact between bacteria and macrophages, so contributing to the advance of disease.

Currently, there are no commercially available vaccines showing any degree of protection against pasteurellosis (Magariños & Romalde, 1997). Furthermore, chemotherapeutic failure is widely reported. This may partially reflect the growing body of evidence suggesting an intracellular phase to the life-cycle of this organism (Yoshida *et al.*, 1997; Magariños *et al.*, 1996a; Magariños & Romalde, 1997). The present data lend further support to this contention, demonstrating that internalization is a specific process and not passive uptake by the cell line. This intracellular phase, coupled with other factors such as the capsule (Arijo *et al.*, 1998), may aid the pathogen in avoiding the host's cellular and humoral defences. Identification of the components and mechanisms involved in this internalization process may provide alternative targets for vaccination. There is evidence for activation of the host cell's actin system and a role for glycoproteins (Magariños *et al.*, 1996a). It may be that a type III secretion system or similar is involved. Work towards characterizing these components is continuing in our laboratory.

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