Short communication

Survival against exogenous hydrogen peroxide of Photobacterium damselae subsp. piscicida under different culture conditions

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Photobacterium damselae subsp. piscicida is a fish pathogen responsible for important losses in aquaculture world-wide. Several studies on its virulence mechanisms have been carried out and outer membrane proteins involved in the acquisition of iron or production of extracellular products have been suggested as the main determinants of its virulence for fish (Magarinós, Santos, Romalde, Rivas, Barja & Toranzo 1992; Magarinós, Romalde, Lemos, Barja & Toranzo 1994). However, the actual methods of invasion and survival inside the host are still unknown and while some authors have reported the presence of intact bacteria inside fish cells, suggesting the ability of the bacterium to survive as an intracellular pathogen (Noya, Magarinós, Toranzo & Lamas 1995; López-Dóriga, Barnes, dos Santos & Ellis 2000), others have observed that this pathogen is highly susceptible to oxidative radicals generated during the macrophage respiratory burst (Skarmeta, Bandín, Santos & Toranzo 1995; Barnes, Balebona, Horne & Ellis 1999a).

Reactive oxygen species (ROS) such as hydrogen peroxide and superoxide are generated during the macrophage respiratory burst in response to microbial infection. Bacterial pathogens must overcome the toxic effects of ROS to establish infections. Production of superoxide dismutase and catalase enzymes, which decompose superoxide and peroxide radicals, respectively, have been reported to contribute to the virulence of a number of pathogens (Franzon, Arondel & Sansonetti 1990; Lefebre & Valvano 2001; Uzzau, Bossi & Figueroa-Bossi 2002). Thus, the ability of catalase to decompose peroxide radicals increases survival of bacteria in the presence of peroxide.

In addition, increased levels of catalase activity when bacteria are cultured under certain conditions, such as the presence of peroxide radicals or until the stationary phase, have been reported (Stortz, Tartaglia & Ames 1990; Loewen 1997). Moreover, the fact that most catalases are iron-cofactored suggests that growth under different iron concentrations may have some effect on this enzyme activity.

Catalase activity has been reported in P. damselae subsp. piscicida (Barnes et al. 1999a), however, the role of this enzyme in the protection against peroxide has not yet been determined. For this reason, the resistance to peroxide radicals of P. damselae subsp. piscicida cells grown under iron limited and replete conditions, and pulsed with hydrogen peroxide, has been evaluated in this study.

Two strains of P. damselae subsp. piscicida have been included in this study. The virulent strain (Lg41/01) (LD$_{90}$ = 2.2 × 10$^4$ CFU g$^{-1}$) was isolated from diseased sole, Solea senegalensis Kaup, showing typical signs of pseudotuberculosis, and the non-virulent strain (Epo) (LD$_{90}$ > 1.0 × 10$^8$ CFU g$^{-1}$; Magariños, Bonet, Romalde, Martinez, Congregado & Toranzo 1996) kindly supplied by Dr K. Muroga (Faculty of Applied
Biological Science, Hiroshima University, Japan). Isolates were cultured in 250-mL flasks containing 100 mL of tryptic soya broth supplemented with 2% NaCl (TSBS) at 22°C until the early stationary phase (O.D. 600 nm = 1.0). The effect of iron concentration on the cultures was evaluated in cells grown in TSBS supplemented with 2,2-dipyridyl (100 μM) or ferric chloride (100 μM) according to the methodology described by Barnes et al. (1999a). Bacterial survival against peroxide after a potential induction of catalase by hydrogen peroxide was tested according to Barnes, Bowden, Horne & Ellis (1999b) by adding 20 μM hydrogen peroxide to mid-exponential phase cultures and 2 mM hydrogen peroxide to early stationary phase cultures.

Cells were harvested, washed and resuspended in phosphate-buffered saline (PBS) to a density of 10⁹ CFU mL⁻¹ (O.D. 600 nm = 1.00). Aliquots of 100 μL were used to inoculate 9.9 mL PBS containing hydrogen peroxide at concentrations of 0, 0.05, 0.1, 0.5, 1 and 10 mM. Samples were incubated for 1 h at 22°C and surviving bacteria were enumerated by viable counts on tryptic soya agar with 2% NaCl (TSAS) plates. The survival of H₂O₂-treated bacteria was expressed as the percentage of colony forming units recovered compared with untreated samples. An ANOVA test was performed to compare the results of the experiments.

Previous studies with *P. damselae* subsp. *piscicida* exposed to photochemically generated superoxide radicals show that bacterial inactivation is overcome when catalase is added to the medium (Barnes et al. 1999b), thus indicating the important effect of hydrogen peroxide on the inactivation of this bacterium. Results obtained in this study indicate that *P. damselae* subsp. *piscicida* shows increased survival when exposed to peroxy radicals when cells have previously been in contact with hydrogen peroxide. Both the virulent and non-virulent strains were inactivated after 1 h incubation with 10 mM H₂O₂, however, when decreasing concentrations of peroxide were used, a higher degree of resistance to peroxide was observed in the virulent strain compared with the non-virulent strain (Fig. 1).

Figure 1 Survival of *Photobacterium damselae* subsp. *piscicida*, strains Epoy (a) and Lg41/01 (b) to exogenous peroxide. (▲) Stationary phase cultures; (■) cultures treated at the mid-exponential phase with 20 μM peroxide followed by 2 mM peroxide in the early stationary phase; (■) cells grown in TSBS with 100 μM 2,2-dipyridyl; (■) cells grown in TSBS with 100 μM ferric chloride.
A significant ($P < 0.05$) increase in the survival rates of the non-virulent strain was observed when cultures were pulsed with hydrogen peroxide compared with cells cultured until the stationary phase. In contrast, this increase has not been observed for the virulent strain, which always showed higher survival regardless of the growth phase or the pulse with hydrogen peroxide. Peroxide induction of catalase and increased cell survival have been reported for several bacterial pathogens (Loewen, Switala & Triggs-Raine 1985; Barnes et al. 1999b; Vattanaviboon & Mongkol suk 2001). Results obtained in this study suggest that peroxide-decomposing enzymes induced in the strain Epoxy only by peroxide treatment could protect these cells from oxidation, whilst decreasing survival rates observed in cells grown in other conditions could be attributable to lower levels of catalase and peroxidase activities. In contrast, the high survival rates observed in the virulent strain in stationary phase cultures, and in cells cultured in the presence of iron or pulsed with hydrogen peroxide suggest the presence of higher levels of catalase activity in the cells grown under these conditions, although a possible relationship with virulence remains to be demonstrated. Furthermore, the presence of a capsule in the virulent strain may have an important role in the protection of $P.\ damselae$ subsp. $piscicida$ cells against peroxide. This capsule would partially contribute to the increased survival of the virulent strain compared with strain Epoxy, a non-capsulated strain (Magariños et al. 1996).

When bacteria were cultured under iron limited conditions, a significant decrease ($P < 0.05$) in survival was observed for both strains compared with cells grown under iron replete conditions or pulsed with peroxide. The decrease in bacterial survival in cultures grown under iron limited conditions suggests the presence of an iron-cofactored catalase in $P.\ damselae$ subsp. $piscicida$. In this way, the ability to obtain iron from the host would determine the ability to cope with the radicals generated during the respiratory burst. It should also be noted that decomposition of superoxide anions primarily generated during the phagocytic respiratory burst depends on the activity of a ferric superoxide dismutase in $P.\ damselae$ subsp. $piscicida$ (Barnes et al. 1999a). Additional studies to demonstrate the presence of iron as a cofactor in the catalase, and the sensitivity of $P.\ damselae$ subsp. $piscicida$ to the radicals generated during the macrophage respiratory burst, are in progress.

References


SodCI and SodCIII in intracellular bacteria: correlation with their relative contribution to pathogenicity. *Molecular Microbiology* 46, 147–156.


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