Superoxide dismutase and catalase in *Photobacterium damsela* subsp. *piscicida* and their roles in resistance to reactive oxygen species

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***Photobacterium damsela* subsp. *piscicida* (formerly *Pasteurella piscicida*) is the causative agent of pasteurellosis or pseudotuberculosis in warm water marine fish. Enzymes which neutralize reactive oxygen species, produced during aerobic metabolism or during respiratory burst in fish macrophages, are important virulence factors in many pathogens. This study characterizes a periplasmic superoxide dismutase (SOD) and a cytoplasmic catalase in *P. damsela*. Purification and partial amino-terminal sequencing confirmed the SOD to be iron-cofactored, with a high degree of homology to other bacterial FeSODs. The SOD was common to all strains analysed in terms of type, location and activity, whilst the catalase varied in activity between strains. The catalase was constitutively expressed, but the SOD appeared to be repressed under low oxygen conditions. In spite of the presence of a periplasmic SOD, *P. damsela* was susceptible to killing by exogenous superoxide anion generated in a cell-free system. Addition of exogenous SOD to this system did not abolish the bactericidal effect; however, addition of catalase was protective. These results suggest that lack of periplasmic catalase may be implicated in susceptibility to killing by reactive oxygen species.**

Keywords: *Photobacterium damsela* subsp. *piscicida*, superoxide dismutase, catalase, antioxidant defences, periplasmic FeSOD

INTRODUCTION

Photobacterium damsela subsp. *piscicida* (formerly *Pasteurella piscicida*, Gauthier *et al.*, 1995), the causative agent of pseudotuberculosis or pasteurellosis, is responsible for extensive losses in aquaculture. Pasteurellosis has been reported in wild and cultured marine fish species in Japan (Kubota *et al.*, 1970; Ueki *et al.*, 1990), USA (Snieszko *et al.*, 1964; Hawke *et al.*, 1987) and more recently in Europe, mainly affecting sea bass and gilthead sea bream (Toranzo *et al.*, 1991; Balebona *et al.*, 1992).

Studies on the phenotypic and serological characteristics

of this pathogen have demonstrated that *P. damsela* constitutes a highly homogeneous taxon (Magariños *et al.*, 1992). Moreover, extracellular products and iron have been implicated in the pathogenesis of this disease in fish (Magariños *et al.*, 1992, 1994). However, despite these studies, information on the pathogenesis of the infection and interaction with phagocytes are still scarce and in some cases contradictory results have been reported (Noya *et al.*, 1995a; Skarmeta *et al.*, 1995).

Cell metabolism of oxygen produces reactive species toxic for many micro-organisms (Welch *et al.*, 1979; Bandín *et al.*, 1993; Barnes *et al.*, 1996; Hardie *et al.*, 1996). This toxicity is mediated by products resulting from the univalent reduction of molecular oxygen, including the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\cdot$) (Fridovich, 1995; Keyer *et al.*, 1995; Hampton *et al.*, 1996). Additionally, infectious organisms may encounter re-

Abbreviations: GS, glutamine synthetase; MDH, malate dehydrogenase; SOD, superoxide dismutase.

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active oxygen species from respiratory burst activity of phagocytic cells. Consequently, enzymes involved in antioxidant defences in pathogens have been associated with microbial virulence. Among these enzymes, superoxide dismutases (SODs), a family of three metallo-enzymes, are capable of disproportionating O_2^- , the first reactive oxygen species formed in the metabolic reduction of oxygen, into H_2O_2 . Subsequently, the presence of catalase activity decomposes H_2O_2 into oxygen and water.

Both SOD and catalase have been reported to protect certain pathogenic bacteria during the respiratory burst following phagocytosis. Thus, SODs of *Shigella flexneri* (Franzon *et al.*, 1990), *Listeria monocytogenes* (Welch *et al.*, 1979) and the fish pathogen *Aeromonas salmonicida* (Barnes *et al.*, 1996) have been reported to reduce the killing efficacy of the oxygen radicals, whereas catalase activity is thought to protect *Staphylococcus aureus* (Mandell, 1975). However, the protective role of these enzyme activities is variable and catalase appears to make a limited contribution to virulence in *Shigella flexneri* (Franzon *et al.*, 1990) and *Salmonella typhimurium* (Tsolis *et al.*, 1995). Thus, the contribution of a particular detoxifying enzyme for reactive oxygen intermediates in protection against phagocytic killing mechanisms may vary with the organism and the model system studied. Studies on survival of *Nocardia* spp. in polymorphonuclear leukocytes have demonstrated that bactericidal activity at early time points is due to oxidative metabolism, whereas killing after 3 h was achieved by both oxidative and non-oxidative mechanisms (Beaman & Beaman, 1984). Resistance to early killing in phagocytes is, therefore, more likely to be mediated by enzymes which provide protection against oxygen-dependent microbicidal mechanisms.

Intracellular location of these enzymes has been reported to have an important role in the ability of bacteria to survive oxidative stress (Gregory *et al.*, 1973). Thus, a periplasmic location could contribute to the elimination of reactive oxygen species from external sources such as phagocytes, while a cytoplasmic location may be more relevant in dealing with radicals generated during cellular metabolism.

Contradictory results have been reported on the survival of *P. damsela* in fish macrophages. *In vitro* studies have shown that this pathogen is killed by fish macrophages (Skarmeta *et al.*, 1995). However, histological studies of infected fish have observed intact bacteria inside macrophages, suggesting that they could survive the phagocyte respiratory burst (Noya *et al.*, 1995a). Information on the role of SOD and catalase activity in this fish pathogen could contribute to the understanding of the interactions between phagocytes and *P. damsela* cells.

To our knowledge, no previous studies on the presence, type and location of SOD and catalase in *P. damsela* have been carried out. To provide such information and to determine the role of these enzymes in the possible protection of the bacterium against oxidative stress are the aims of this communication.

METHODS

Bacterial strains. Six strains of *P. damsela* (MT1376, MT1377, MT1378, MT1379, MT1380 and MT1415), originally isolated from clinical outbreaks of pasteurellosis affecting gilthead sea bream in Greece and Italy, or yellowtail in Japan, were obtained from the Marine Laboratory, Aberdeen, collection. Another two strains of *P. damsela* from the American Type Culture Collection, Manassas, VA, USA (ATCC 17911 and ATCC 29690) were also included in this study. Bacteria were stored at -80°C in tryptic soy broth (Oxoid) supplemented with 2% NaCl (TSB-2) and 20% glycerol. Bacteria were cultured on tryptic soy agar (Oxoid) containing 2% NaCl (TSA-2) and incubated at 22°C for 48 h. One colony was used to inoculate 10 ml broths which were incubated with shaking overnight at 22°C . Aliquots (25 μl) of these cultures were used to inoculate 250 ml flasks containing 100 ml TSB-2 and they were incubated at 22°C in a rotary shaker (150 r.p.m.).

Preparation of crude extracts. Cells grown as described above were harvested by centrifugation at 3500 g for 15 min at 4°C and washed twice in 25 mM potassium phosphate buffer, 1 mM disodium EDTA, pH 7.2, 0.5 mM PMSF (Sigma) and finally resuspended in 2 ml of the same solution. Suspensions were sonicated on ice at 8 μm for 90 s (6×15 s with 30 s cooling between bursts) and finally centrifuged at 16000 g for 45 min at 4°C . Supernatants were assayed for the presence of SOD and catalase on acrylamide gels. Protein content was determined by using the BCA assay (Pierce & Warriner).

Partial purification of SOD. Frozen cell paste (1 g wet wt) was resuspended in 2 ml 25 mM potassium phosphate buffer, 1 mM disodium EDTA, pH 7.2, containing 0.5 mM PMSF. Cell suspensions were sonicated as described above and centrifuged at 100000 g for 1 h at 4°C .

Supernatant was filtered (0.22 μm) and 200 μl aliquots were applied to a Superose 6 HR10/30 column connected to an FPLC system (Pharmacia). Chromatography was performed in 50 mM Tris/HCl, pH 7.7, containing 50 mM NaCl and 0.05% sodium azide at a flow rate of 0.5 ml min^{-1} . Fractions (1 ml) containing SOD activity from three replicate runs were pooled and applied to a Mono-Q HR5/5 anion exchange column and eluted at 1 ml min^{-1} on a linear gradient of 50–400 mM NaCl in 50 mM Tris/HCl, pH 7.7, over 40 ml. SOD activity was detected in three fractions; however, one fraction (F26) contained most of the SOD activity and minor contamination (one major and three minor bands detected in SDS-PAGE by Aurodyne; Amersham). This fraction was desalted using a fast desalt column HR10/10 (Pharmacia) and reappplied to the Mono-Q column, this time eluting over a more gradual gradient (150–300 mM NaCl over 40 ml). Fraction 24 contained only 1 band in addition to SOD. An aliquot (200 μl) of this fraction was applied to Superose 12 HR10/30 and eluted in 50 mM Tris/HCl, pH 7.7, containing 50 mM NaCl at 0.5 ml min^{-1} . SOD was eluted in one peak over two 1 ml fractions. Fraction 9 (the leading edge of the peak) contained essentially pure SOD as analysed by native and SDS-PAGE, stained with Coomassie brilliant blue, and Western blotting onto PVDF (Immobilon P, Millipore), stained with Aurodyne (Amersham).

Amino-terminal sequencing. Purified SOD protein was excised from an SDS-PAGE gel and subjected to automated Edman sequencing on an Applied Biosystems model 477A pulsed-liquid sequencer, equipped with on-line PTH analysis, at the Protein Facility, Department of Molecular and Cell Biology, University of Aberdeen.

Electrophoresis and *in situ* staining. Electrophoresis was performed on non-denaturing acrylamide gels using the Bio-Rad Mini Protean II System with Tris/glycine buffer. Samples were applied to 10% bisacrylamide gels prepared with 1.5 M Tris/HCl, pH 8. Electrophoresis was carried out at 200 V.

SOD activity was visualized on gels following the methodology described by Beauchamp & Fridovich (1971). Briefly, gels were washed in distilled water, then soaked in a solution of 2.45 mM nitro blue tetrazolium (Sigma) for 20 min, followed by a 10 min incubation in a solution containing 28 mM tetramethylethylenediamine (TEMED, Sigma), 0.028 mM riboflavin (Sigma) and 36 mM potassium phosphate buffer, pH 7.8. Gels were illuminated on a light box to obtain a dark background with achromatic bands corresponding to SOD activity.

Catalase activity was visualized on non-denaturing acrylamide gels following the methodology specified by Woodbury *et al.* (1971). After electrophoresis, gels were washed three times in distilled water for 20 min and soaked in a solution of 0.015% H_2O_2 . Activity was then visualized by transferring the gels to a solution of 1% ferric chloride, 1% potassium ferricyanide. Regions corresponding to catalase activity were identified as yellow bands on a green background.

Intracellular location of SOD and catalase activities. Several methods were compared to obtain periplasmic extracts with low cytoplasmic contamination.

Cell suspensions. Bacterial cultures (100 ml broth in 250 ml flasks) were incubated at 22 °C until late exponential phase (33 h) in a rotary shaker (150 r.p.m.). Cells were harvested by centrifugation at 3500 *g* for 10 min at 4 °C and standardized turbidimetrically ($OD_{540} = 1.0$) to 1.0×10^9 cells ml^{-1} . Aliquots containing 50 ml of this cell suspension were used for spheroplast extractions.

CHAPS-lysozyme extraction. This method was developed according to a modification by Barnes *et al.* (1996) of the method described by Stabel *et al.* (1994). After centrifugation of the bacterial suspensions (50 ml) with 10^9 cells ml^{-1} , cells were resuspended to their original concentration in 0.2 M Tris/HCl, pH 8, and an equal volume of 0.2 M Tris/HCl, pH 8, supplemented with 1.0 M sucrose and 0.5% CHAPS was added. Lysozyme (Sigma) was added to achieve a final concentration of 100 $\mu g\ ml^{-1}$ and cells were exposed to mild osmotic shock by addition of 20 ml cold distilled water. After incubation with gentle shaking at 22 °C for 2 h, cell suspensions were centrifuged at 3500 *g* for 10 min at 4 °C. Filter-sterilized supernatants were assayed for SOD, malate dehydrogenase (MDH) and glutamine synthetase (GS) (see below).

Osmotic shock. Cell suspensions (50 ml) with 10^9 cells ml^{-1} were exposed to osmotic shock as previously described by Stabel *et al.* (1994). Cell suspensions were washed twice with an equal volume of cold 10 mM Tris/HCl, pH 8, 30 mM NaCl. After centrifugation at 3500 *g* for 10 min at 4 °C, washed cells were resuspended in 2.5 ml 30 mM Tris/HCl, pH 8, 20% sucrose to which 10 ml 250 mM EDTA was added. The suspension was shaken at 22 °C for 10 min and cells harvested by centrifugation at 3500 *g* for 10 min at 4 °C. Pelleted cells were resuspended in 2.5 ml cold water, shaken for 10 min at 4 °C and centrifuged at 3500 *g* for 10 min at 4 °C. Supernatants were filter-sterilized and assayed for MDH and GS. SOD activity was visualized on acrylamide gels.

Chloroform extraction. This was performed essentially according to Ames *et al.* (1984). Bacterial suspension (50 ml) was

centrifuged at 3500 *g* for 10 min at 4 °C and washed in saline solution (0.145 M NaCl). Supernatants were discarded, pellets resuspended in residual saline solution and chloroform (100 μl) was added. After incubation at 22 °C for 15 min, 0.5 ml 10 mM Tris/HCl, pH 8, was added and whole cells were pelleted by centrifugation at 3500 *g* for 10 min at 4 °C. The upper fraction of the supernatants was removed, filter-sterilized and assayed for MDH and GS. SOD was visualized on acrylamide gels. A modification of the method described by Klotz & Hutcheson (1992) was also employed. Bacterial suspensions were washed with 10 mM Tris/HCl, 30 mM $MgCl_2$, pH 7.3, and resuspended in 1 ml of the same buffer before addition of 15 μl chloroform. After 15 min at 4 °C, an additional volume of 1 ml ice-cold buffer was added and the suspension was centrifuged at 3500 *g* for 10 min at 4 °C. Supernatants were carefully collected, filtered and assayed for SOD, MDH and GS.

Whole-cell extracts. Bacterial suspensions (50 ml) were washed twice with 25 mM sodium phosphate buffer containing 1 mM disodium EDTA, pH 7.2, 0.5 mM PMSF and finally resuspended in 2 ml buffer. Suspensions were sonicated on ice at 8 μm for 90 s (six 15 s bursts with 15 s cooling periods) and then centrifuged at 16000 *g* for 45 min at 4 °C. Supernatants were filtered and assayed for MDH, GS and SOD.

Enzyme assays. MDH, a cytoplasmic marker enzyme (Smith & Winkler, 1979), was assayed as described in Worthington Enzyme Manual (1993). Enzyme activity was calculated based on the decrease of A_{340} at 25 °C due to oxidation of NADH by the enzyme. Freshly prepared 6 mM oxaloacetic acid (0.1 ml), 3.75 mM NADH (0.2 ml) (both prepared in 0.1 M potassium phosphate buffer, pH 7.4) and 0.1 M potassium phosphate buffer pH 7.4 (2.6 ml) were placed in a 3 ml cuvette, path length 1.0 cm, at 25 °C. After incubating the cuvette in the spectrophotometer for 3–4 min to achieve temperature equilibration and establish blank rate, if any, 0.1 ml of the sample was added to the cuvette and the decrease in A_{340} was recorded for 10 min. The rate of decrease in absorbance was calculated from the linear portion of the curve.

GS, also described as a cytoplasmic marker (Ames *et al.*, 1984), was assayed according to Cánovas *et al.* (1991). GS activity was calculated based on the concentration of γ -glutamyl hydroxamate produced. The reaction was performed in vials containing 85 μl 0.8 M imidazole/HCl, pH 7, 20 μl 2.25 M hydroxylamine, 20 μl 0.75 M sodium arsenate, 20 μl 0.11 M manganese chloride, 20 μl 15 mM ADP, 500 μl 0.18 M glutamine, 65 μl distilled water and 20 μl of the sample. Blank tubes were prepared for each sample, replacing glutamine by an equal volume of distilled water. After incubation at 37 °C for 15 min, 250 μl GS reagent (10% ferric chloride in 0.2 M HCl, 24% TCA, 18.5% HCl; 1:1:1) was added. Vial contents were shaken and placed in 1 ml cuvettes with a 1.0 cm light path. A_{540} was determined for each sample.

SOD was quantified using xanthine and xanthine oxidase as a source of superoxide and reduction of cytochrome *c* as a detector of the presence of superoxide anions as described by McCord & Fridovich (1969).

Enzyme stability. MDH, GS and SOD stability to the treatments used for spheroplasting was determined. Bacterial suspensions (50 ml) with 10^9 cells ml^{-1} were centrifuged and resuspended in 2 ml of the solutions described for spheroplasting above and sonicated at 8 μm for 90 s (six 15 s bursts with 15 s cooling periods). After sonication, the spheroplast extraction protocols described above were applied to the crude extracts. MDH, GS and SOD were quantified as described above.

Localization of SOD with diazo-NDS. Diazo-NDS was prepared from 7-aminonaphthalene-1,3-disulphonic acid (Fluka) and sodium nitrite, as described by Pardee & Watanabe (1968). Localization of SOD was determined as described by Britton & Fridovich (1977). Briefly, a mid-exponential culture of *P. damsela* MT1415 (50 ml TSB-2) was washed in 50 mM Tris/HCl, 5 mM MgCl₂, pH 8.0, and resuspended to the original density in the same buffer. The culture was split into two and one half was treated with the diazo-NDS compound (final concentration 5 mM), whilst the other was kept as control. The test and control were incubated at 25 °C for 1 h and the reaction was quenched by addition of imidazole to 100 mM. Cells were washed extensively in 50 mM Tris/HCl, 5 mM MgCl₂, pH 8.0, and resuspended in 5 ml of the same solution. Cells were sonicated on ice as described above. Control sonicate was split into two; half was treated with 5 mM diazo-NDS to determine the effect of the compound on SOD. Equal quantities of protein were analysed by native PAGE (12%) and stained for SOD activity. Test, control and control lysates subsequently treated with diazo-NDS following lysis were assayed for GS as described above.

Determination of the type of SOD. The metal present in the active site of the SOD molecule was determined by using inhibition studies according to Dunlap & Steinman (1986). Briefly, zones of activity attributable to Cu/ZnSODs were identified by their elimination when 1.9 mM KCN was added to the staining mixture. Zones of FeSODs were identified by their resistance to cyanide and their elimination when 3.7 mM H₂O₂ was present. Resistance to both cyanide and H₂O₂ is characteristic of MnSODs.

Estimation of native molecular mass. The native molecular mass of SOD from *P. damsela* strains was determined following the methodology described by Hedrick & Smith (1968) based on the dependence of mobility of proteins on the acrylamide concentration. Cell extracts were electrophoresed on separate non-denaturing gels containing 8, 9, 10, 11, 12 and 13% acrylamide. The following proteins were used as molecular mass standards: α -lactalbumin (14200 Da), carbonic anhydrase (29000), chicken egg albumin (45000) and bovine serum albumin (monomer 66000; dimer 132000) (all from Sigma). SOD was visualized as described above and molecular mass markers were stained with Coomassie brilliant blue.

Effect of the presence of oxygen on SOD expression. Bacteria were cultured in aerobic and mild anaerobic conditions. For aerobic growth bacteria were cultured in 500 ml flasks with a 5:1 ratio of flask volume to medium volume. Flasks were incubated at 22 °C in a rotary shaker at 200 r.p.m. Anaerobic cultures were carried out in 100 ml screw-capped Duran bottles filled to the top with medium and incubated at 22 °C with very gentle agitation to avoid cell sedimentation. Samples were taken hourly from both cultures and OD₅₄₀ was determined. The number of culturable cells was determined at different time intervals by viable cell counting on TSA-2.

Bacteria were cultured as described above under aerobic and mild anaerobic conditions until late exponential and stationary phase. Sonicates were prepared as described above and used to detect and quantify SOD as described above.

Effect of iron concentration on SOD production. Bacteria were cultured in TSB-2, TSB-2 containing 200 μ M ferric chloride (FeCl₃) or TSB-2 + 100 μ M dipyrrolyl (Sigma). Bacteria were twice passaged on the relevant medium prior to culture to deplete/augment iron stores. Cultures (250 ml broth in 500 ml flasks) were incubated at 22 °C at 150 r.p.m. for 48 h

and used for preparation of sonicates. SOD quantity and type were determined under the various conditions.

Bacterial susceptibility to killing by cell-free-generated superoxide anion. Susceptibility of *P. damsela* to oxygen radical species was evaluated using a cell-free photochemical radical generating system described by Karczewski *et al.* (1991). Briefly, a 6.25 mM methionine solution was prepared in PBS, pH 7.2, containing 4% glucose. Assay solutions containing 0.2, 0.1 and 0.05 mM riboflavin were prepared in the methionine solution. Bacterial suspensions (10⁸ c.f.u. ml⁻¹) in PBS were diluted in the various riboflavin concentrations and applied to 96-well microplates. O₂⁻ was generated by photo-reduction of riboflavin, methionine being used as hydrogen donor. Microplates were exposed to light from a light box for 10 min to initiate superoxide anion production and subsequently incubated in the dark for 5 h at 22 °C. After incubation, plates were centrifuged at 400 g for 10 min and the supernatants removed. TSB-2 was added to support bacterial growth and the plates were incubated at 22 °C for 18 h.

Bacterial viability was quantified by adding 10 μ l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg ml⁻¹; Sigma) per well, according to Graham *et al.* (1988). After 15 min incubation at room temperature, the OD₆₃₀ was determined using a microplate reader (System 7000 running Revelations Software, Dynatech). The suitability of the assay for determining viable numbers of *P. damsela* was verified prior to the experiment by correlating absorption of MTT at 630 nm with viable cell count determined on TSA-2. A linear range of cell numbers within which reduction of MTT accurately reflected viable counts was determined and the subsequent experiments were designed to fit within this range.

The effect of methionine or riboflavin separately on the viability of *P. damsela* was determined by performing the assay described in the presence of PBS, 4% glucose and 6.25 mM methionine, or PBS, 4% glucose and 0.2, 0.1 or 0.05 mM riboflavin, respectively.

The effect of the addition of catalase and SOD on the bactericidal activity of the radicals was determined by addition of catalase (2400 U ml⁻¹), SOD (1200 U ml⁻¹) or both to the wells. The effect of the addition of the inhibitor of SOD activity, sodium nitroprusside (100 μ M), on bacterial viability was also determined.

RESULTS

Detection of SOD and catalase activity on acrylamide gels

All strains included in this study showed a similar clear band of SOD activity on the gels (Fig. 1a). All bands migrated to the same point in 10% acrylamide gels, suggesting a similar enzyme was expressed by all strains. Identical protein concentrations were loaded for all the strains, thus the similar intensity of the SOD bands indicates that the *P. damsela* strains assayed contained similar levels of SOD activity.

The mobility of the bands of catalase activity in native gels was similar for all of the strains examined. Although this technique is not accurately quantitative, the differences in intensity of the bands suggests different levels of activity in the extracts of different strains (Fig. 1b).

Fig. 1. SOD and catalase activities detected in sonicated extracts of *P. damselae* subsp. *piscicida*. Gel stained for (a) SOD activity and (b) catalase activity.

Table 1. Stability of MDH, GS and SOD to the treatments used for spheroplasting

Values are relative to those obtained in sonicated samples.

Treatment	MDH (%)	GS (%)	SOD (%)
CHAPS-lysozyme	145.0	169.0	73.4
Osmotic shock	105.0	91.0	95.8
Chloroform	48.2	90.2	94.5

Intracellular location of SOD and catalase activities

Assayed enzymes showed different degrees of stability to the treatments used for spheroplasting. When CHAPS-lysozyme extraction or osmotic shock were applied to previously sonicated cells, higher levels of MDH activity were detected compared to sonicated extracts (Table 1). However, treatment with chloroform after sonication significantly inhibited MDH activity. Indeed, only 48.2% of the initial activity could be detected (Table 1). In contrast, GS was not significantly affected by any of the spheroplasting protocols. Thus, this enzyme was

Table 2. Protein, GS and SOD content of periplasmic extracts prepared by different methods

Values are relative to those obtained in sonicated samples.

Treatment	Protein (%)	GS (%)	SOD (%)
CHAPS-lysozyme	98.0	56.8	81.0
Osmotic shock	28.5	9.7	24.2
Chloroform (22 °C)	20.0	<1.0	62.6
Chloroform (4 °C)	2.6	<1.0	1.7

selected in favour of MDH as a cytoplasmic marker in this study.

SOD showed stability to the treatments used for osmotic shock and chloroform extractions. However, the use of the detergent CHAPS with lysozyme resulted in the loss of more than 25% activity.

Four methods to selectively release periplasmic contents of *P. damselae* cells without cytoplasmic contamination were compared. Screening of periplasmic extracts for GS activity recorded the highest levels of the enzyme in sonicated fractions. The lowest activity was detected in chloroform-treated samples, representing less than 1% of the total GS observed in sonicated extracts (Table 2). Due to the low level of cytoplasmic contamination, chloroform was considered the most suitable extraction protocol for release of periplasmic proteins.

Different levels of SOD activity from the different methods were observed on polyacrylamide gels, the maximum activity corresponding to sonicated samples. Bands corresponding to SOD activity were less intense in samples from CHAPS-lysozyme, osmotic shock and chloroform extraction at 4 °C than in periplasmic fractions obtained by extraction with chloroform at 22 °C. The different methods used in this study resulted in variable volumes of periplasmic extracts. Thus although some treatments like CHAPS-lysozyme released a high percentage of the protein content of the cells (Table 2), the high level of dilution of the samples required by the protocol did not make it possible to obtain extracts with high protein concentrations. When extracts from sonicated and chloroform-treated samples containing equal amounts of protein were loaded on the gels, a more intense band of SOD activity was found in chloroform extracts than in sonicated samples, showing that SOD represents a higher proportion of the total protein content in periplasmic extracts obtained with chloroform than in sonicated samples. In sonicated samples, both periplasmic and cytoplasmic proteins were released during the treatment.

Quantification of SOD activity obtained in periplasmic extracts and sonicated cells showed that CHAPS-lysozyme extraction released the highest percentage of activity. However, the high level of cytoplasmic contamination shown by GS contents indicates that this method cannot conclusively locate SOD activity in the periplasmic space. In contrast, the high percentage of

Fig. 2. Protein and catalase activity in sonicated and periplasmic fractions of *P. damsela* subsp. *piscicida* MT1415. (a) Gel stained with Coomassie brilliant blue. Lanes: 1, 2, sonicates; 3, 4, osmotic shock extract; 5, 6, chloroform extraction according to Ames *et al.* (1984). (b) Gel stained for catalase activity. Lanes: 1, 2, sonicates; 3, osmotic shock; 4, chloroform extraction according to Ames *et al.* (1984).

SOD activity coupled with very low cytoplasmic contamination indicates that chloroform extraction at 22 °C is the most appropriate method for isolating SOD from the periplasmic space in *P. damsela*. The periplasmic location of SOD was confirmed using diazo-NDS. This reagent does not penetrate the cytoplasmic membrane, but can easily enter the periplasmic space where it may inactivate certain enzyme activities by coupling with histidine and tyrosine residues (Pardee & Watanabe, 1968). It has previously been used to disprove the proposed periplasmic location of FeSOD in *Escherichia coli* (Britton & Fridovich, 1977). In this study, diazo-NDS was shown to inactivate SOD and GS in sonicates of *P. damsela* but, in contrast to the observations made in *E. coli* (Britton & Fridovich, 1977), it inhibited SOD activity when whole cells were treated with the compound, suggesting periplasmic location of the enzyme. GS, a cytoplasmic enzyme, was not inhibited when whole cells were treated with diazo-NDS, confirming that the compound was unable to permeate the cytoplasmic membrane.

Chloroform extraction according to Ames *et al.* (1984) was selected to determine the intracellular location of catalase in *P. damsela* cells. Treatment of sonicated extracts with chloroform following the protocol described for spheroplasting did not result in different intensity bands of catalase activity in acrylamide gels, indicating that catalase is stable to this process. No catalase activity was observed when periplasmic extracts were applied to the gels, thus indicating that catalase is not located in the periplasmic space of *P. damsela* (Fig. 2).

Determination of type and approximate native molecular mass of SOD

In all cases, clear bands corresponding to SOD activity persisted after the addition of 1.9 mM KCN in the staining mixture (Fig. 3). However, this activity was

sensitive to H_2O_2 , indicating that the SOD present in these strains is probably a ferric enzyme. The native molecular mass of SOD from *P. damsela* was estimated to be 39.8 kDa.

Purification and amino-terminal sequencing of SOD

SOD was purified from sonicates by a combination of gel filtration and anion exchange chromatography. Purified SOD had a specific activity of approximately 1000 U (mg protein)⁻¹. Partially purified SOD, following Superose 6 and the first passage on a Mono-Q column, resulted in a specific activity of 910 U (mg protein)⁻¹, suggesting >90% purity was achieved after these two steps. SDS-PAGE of purified SOD in reducing sample buffer, followed by Western blotting and staining with Aurodyne, resulted in a single band with an approximate molecular mass of 25 kDa (Fig. 4). Amino-terminal sequencing of the first 20 residues, coupled with a database search using OWL (on the Leeds University database browser) revealed that SOD from *P. damsela* showed a high degree of sequence homology to other bacterial FeSODs (Table 3).

Effect of the presence of oxygen on SOD expression

P. damsela strains grew at different rates when cultured under aerobic or mild anaerobic conditions. Fig. 5 shows that cells cultured in highly aerated flasks reached stationary phase after 38 h, whilst cells grown under mild anaerobic conditions experienced a shorter exponential phase reaching stationary phase after 20 h with a lower OD.

Extracts from cells grown under aerobic conditions contained higher specific SOD activity [28.8 ± 0.5 U (mg protein)⁻¹] compared to those from low oxygen cultures [8.1 ± 0.8 U (mg protein)⁻¹] when assayed in late exponential phase. Inhibitory studies with KCN and H_2O_2 showed that *P. damsela* cells grown to exponential or

Fig. 3. Inhibitory effect of KCN and H_2O_2 on SOD activity from cells grown under differing iron concentrations. Gel stained (a) for catalase with no inhibitor, (b) for SOD in the presence of 1.9 mM KCN, (c) for SOD in the presence of 3.7 mM H_2O_2 and (d) with Coomassie brilliant blue. Lanes: 1, sonicate from bacteria cultured in TSB-2; 2, sonicate from bacteria cultured in TSB-2 with 100 μ M dipyridyl; 3, sonicate from bacteria cultured in TSB-2 with 200 μ M $FeCl_3$.

stationary phase produce FeSOD under both aerobic and mild anaerobic conditions.

Effect of iron concentration on SOD production

Growth under normal, iron-supplemented and iron-depleted conditions did not result in a different SOD activity band on acrylamide gels. In all cases it was possible to observe a similar band on the gels that was inhibited by H_2O_2 but was resistant to KCN. However, specific SOD activity was affected by iron-depleted conditions. Cells grown in the presence of 100 μ M

Fig. 4. SDS-PAGE of purified SOD, blotted onto immobilon P and stained with Aurodyne. Lanes: 1, 3, Promega mid-range markers (phosphorylase B, 97400 Da; bovine serum albumin, 66200; glutamate dehydrogenase, 55000; ovalbumin, 42700; aldolase, 40000; carbonic anhydrase, 31000; soybean trypsin inhibitor doublet 21500/19700; lysozyme, 14400); 2, purified SOD.

dipyridyl contained only 7.1% SOD activity [1.6 ± 0.2 U (mg protein) $^{-1}$] detected in cells grown in normal TSB-2 [22.0 ± 0.2 U (mg protein) $^{-1}$] or iron-supplemented TSB-2 [22.5 ± 0.4 U (mg protein) $^{-1}$].

Bacterial susceptibility to killing by superoxide anion

Susceptibility to oxygen radical species generated *in vitro* was determined for two strains selected according to their virulence: MT1415 from sea bream affected with pasteurellosis and ATCC 29690, a strain avirulent for sea bream (Magariños *et al.*, 1992). Superoxide anions were generated photochemically from a range of concentrations of riboflavin, with methionine as electron donor.

Both strains showed high susceptibility to the radicals generated in this cell-free system and no differences were observed between the strains. Thus the decrease in cell viability in terms of OD₆₃₀ due to reduction of MTT has

Table 3. Amino-terminal amino acid sequence of *P. damsela* and closely related bacterial SODs

Sequences for bacteria other than *P. damsela* obtained from OWL. Underlined residues differ from the *P. damsela* sequence.

Species	SOD type	1				5				10				15				20			
<i>Photobacterium damsela</i>	Fe	ala	phe	glu	leu	pro	ala	leu	pro	tyr	ala	lys	asp	ala	leu	glu	pro	his	ile	ser	ala
<i>Pseudomonas aeruginosa</i>	Fe	ala	phe	glu	leu	pro	<u>pro</u>	leu	pro	tyr	<u>glu</u>	lys	<u>asn</u>	ala	leu	glu	pro	his	ile	ser	ala
<i>Escherichia coli</i>	Fe	<u>ser</u>	phe	glu	leu	pro	ala	leu	pro	tyr	ala	lys	asp	ala	leu	<u>ala</u>	pro	his	ile	ser	ala
<i>Salmonella typhimurium</i>	Fe	<u>ser</u>	phe	glu	leu	pro	ala	leu	pro	tyr	ala	lys	asp	ala	leu	<u>ala</u>	pro	his	ile	ser	ala
<i>Pseudomonas putida</i>	Fe	ala	phe	glu	leu	pro	<u>pro</u>	leu	pro	tyr	ala	<u>his</u>	asp	ala	leu	<u>glu</u>	pro	his	ile	ser	ala
<i>Photobacterium leiognathi</i>	Fe	ala	phe	glu	leu	pro	ala	leu	pro	<u>phe</u>	ala	<u>met</u>	<u>asn</u>	ala	leu	glu	pro	his	ile	ser	<u>glu</u>
<i>Escherichia coli</i>	Mn	<u>ser</u>	<u>tyr</u>	<u>thr</u>	leu	pro	<u>ser</u>	leu	pro	tyr	ala	<u>tyr</u>	asp	ala	leu	glu	pro	his	<u>phe</u>	<u>asp</u>	<u>lys</u>
Bovine erythrocyte	Cu/Zn	<u>ala</u>	<u>thr</u>	<u>lys</u>	<u>ala</u>	<u>val</u>	<u>cys</u>	<u>val</u>	<u>leu</u>	<u>lys</u>	<u>gly</u>	<u>asp</u>	<u>gly</u>	<u>pro</u>	<u>val</u>	<u>glu</u>	<u>gly</u>	<u>thr</u>	<u>ile</u>	<u>his</u>	<u>phe</u>

Fig. 5. Growth curves for *P. damsela* subsp. *piscicida* MT1415 cultured under aerobic (■) and mildly anaerobic (▲) conditions.

been shown only for one strain (Fig. 6). This killing was not reduced by addition of SOD to the system, suggesting the involvement of killing mechanisms other than O_2^- . However, addition of catalase or both SOD and catalase to the medium greatly reduced bacterial killing. Addition of 100 μ M sodium nitroprusside, a SOD inhibitor, did not make any difference to bacterial killing compared to controls (data not shown).

It was observed that increasing concentrations of riboflavin overcame the protective effect of catalase, and SOD and catalase. This inactivation was not due to toxicity of riboflavin or methionine, since cell viability in the presence of each of these compounds separately was not affected. Some inactivation was detected even with the lowest riboflavin concentration used in the presence of the enzymes. These results show the high susceptibility of *P. damsela* to the oxygen radical species, or metabolites thereof, generated in this system.

DISCUSSION

SODs, which catalyse the dismutation of O_2^- , may be the first line of defence against oxygen toxicity. We have found that all the strains of *P. damsela* included in this

study contain SOD activity. A single band of SOD activity was observed on acrylamide gels, indicating the presence of one type of SOD with an estimated native molecular mass of 39.8 kDa. Inhibitory studies with KCN and H_2O_2 indicated this SOD is a ferric enzyme. Amino-terminal sequencing corroborated this finding, with *P. damsela* SOD having >90% homology with numerous other bacterial FeSODs. Purification followed by SDS-PAGE of the purified enzyme revealed a single band with a molecular mass of approximately 25 kDa after Coomassie staining. Most bacterial SODs comprise two subunits of equal size, with the metal cofactor at the active site (Yost & Fridovich, 1973; Keele *et al.*, 1970). The molecular mass determined under reducing conditions suggests two subunits of 25 kDa each, giving a total native molecular mass of 50 kDa.

Several types of SOD have been found in different bacterial species such as *E. coli* (Gregory *et al.*, 1973), *Legionella pneumophila* (St John & Steinman, 1996) and *Aeromonas salmonicida* (Barnes *et al.*, 1996). In many cases, one type of SOD is located in the periplasmic space of the cell, possibly contributing to the elimination of O_2^- generated extracellularly or at the membrane, while the other types may be found in the cytoplasm. In this study, only one SOD enzyme, the FeSOD located in the periplasm, was identified in *P. damsela*.

Pathogens must compete with the host for iron and the ability of pathogenic bacteria to acquire iron is essential for their growth *in vivo*. Magariños *et al.* (1994) observed that iron overload had an enhancing effect on the virulence of *P. damsela* for fish. When fish were treated with ferric ammonium citrate, haemin or haemoglobin, prior to bacterial inoculation, LD₅₀ values were reduced by as much as 4 log units. Results obtained by these authors showed that *P. damsela* has the ability to obtain iron from transferrin and haem compounds. In this study, a retardation in the growth of *P. damsela* was observed when cultured under iron-restricted conditions. Assuming that SOD contributes to the pathogenicity of *P. damsela*, the reliance on an iron-containing SOD could partially explain the enhancing effect of iron on the virulence of this pathogen and the reduced growth rate when iron is limiting. Furthermore, free iron contributes to oxidative stress in bacteria through its involvement in Fenton reactions, catalysing

Fig. 6. Effect of 5 h exposure to photochemically generated oxygen radicals on viability of *P. damsela* subsp. *piscicida* MT1415. Assays contained (a) 0.2, (b) 0.1 and (c) 0.05 mM riboflavin; controls (d) were performed in the absence of riboflavin. Enzymes added: none ($\square$); SOD ($\triangle$, 1200 U ml⁻¹); catalase ($\circ$, 2400 U ml⁻¹); both SOD and catalase ($\bullet$). Viability was determined by measurement of MTT reduction at 630 nm. Data are means $\pm$ SD of triplicate values.

the formation of hydroxyl radicals from peroxide (Halliwell & Gutteridge, 1984). This reduction in overall iron load may result in a reduced requirement for SOD as the potential for oxidative damage is reduced.

SODs have been reported to be regulated by oxygen-sensitive systems in several organisms (Tardat & Touati, 1991; Fridovich, 1995). In *P. damsela*, SOD levels detected in cells grown under highly aerated and non-aerated conditions suggest that SOD may be repressed under low aeration. A significant increase in specific SOD activity per cell was observed in cells grown in aerated conditions for 30 h. This activity decreased when cells were old and the culture was in stationary phase (48 h incubation), possibly due to bacterial death. Lower levels of SOD present in cells cultured under mild anaerobic conditions could be sufficient to cope with O_2^- generated from the lower levels of oxygen present in the medium. Moreover, as Fridovich (1995) reported, the presence of a certain minimal level of SOD helps the cell to survive if it is suddenly exposed to an aerobic environment. Interestingly, cells cultured under anaerobic conditions grew more quickly and reached stationary phase earlier than those grown under aerobic

conditions, albeit with a lower final OD. This may result from the low amount/absence of SOD in the cytoplasm, making aerobic growth stressful on the bacterium.

While some species such as *Enterococcus faecalis* lack catalase activity, different isozymes have been identified in other species such as *Pseudomonas syringae* (Klotz & Hutcheson, 1992). In this study only one type of catalase was detected in all the strains of *P. damsela* analysed, according to migration in acrylamide gels. However, the intensity of the bands differed among the strains.

Cell fractionation was used to determine the intracellular location of SOD and catalase. Attempts to form spheroplasts by different methods yielded different results. Production of spheroplasts by chloroform at 22 °C confirmed the presence of periplasmic SOD in *P. damsela*. The low levels of GS activity in the extracts obtained by treatment with chloroform, both at 22 °C and 4 °C, indicate that both methods successfully prevent cytoplasmic contamination during spheroplasting. However, the incubation at 4 °C in the presence of 10 mM Tris/HCl, 30 mM MgCl₂ and 150 μ l chloroform did not allow the visualization of SOD activity on

polyacrylamide gels and low SOD activity was detected in the extracts. This method probably resulted in a much lower degree of spheroplasting as the low total protein content of the extracts confirmed.

Some proteins are inactivated by exposure to chloroform (Ames *et al.*, 1984). This was the case for MDH, commonly used as a cytoplasmic marker. Although this enzyme does not seem to be inactivated after osmotic shock, a different cytoplasmic marker had to be selected when chloroform was used for preparation of spheroplasts. GS was not significantly inactivated by any of the methods used in this study and thus was selected as the enzyme of choice for detecting cytoplasmic contamination. In this study, the use of chloroform to obtain spheroplasts, coupled with assay of GS to estimate cytoplasmic contamination, was found most appropriate in establishing the periplasmic location of SOD and the absence of catalase in this region in *P. damselae*.

The location of FeSOD in the periplasm is unusual, with the early reports of periplasmic FeSOD in *E. coli* (Gregory *et al.*, 1973) having been subsequently disproven (Britton & Fridovich, 1977). The only precedent for periplasmic FeSOD is that of *Aquaspirillum magnetotacticum* which possesses FeSOD and MnSOD, both of which are located periplasmically (Short & Blakemore, 1989). However, the identity of the SOD in this study was supported both by inhibitor studies and by amino-terminal sequencing. The evidence locating the enzyme in the periplasm is also compelling, with the absence of GS and catalase activity in periplasmic extracts confirming the suitability of the extraction procedure. Further corroboration is presented by inactivation of SOD in whole cells after treatment with diazo-NDS, the same technique used to disprove the periplasmic location of *E. coli* FeSOD (Britton & Fridovich, 1977). Any explanation for the opposing locations of SOD and catalase in *P. damselae* would be complex as a result of the numerous theories relating to the mechanisms of oxygen radical toxicity presented in the literature. In aqueous environments the reactivity and oxidizing capacity of O_2^- are very low (Halliwell & Gutteridge, 1984). However, in non-polar environments O_2^- is a powerful proton acceptor, nucleophile and reducing agent (Frimer, 1982; Halliwell & Gutteridge, 1984). Biological membranes, including the bacterial cell envelope and outer membrane, are known to be hydrophobic and O_2^- produced in this environment could be extremely damaging, destroying phospholipids. Halliwell (1981) reported that much of the O_2^- formed within cells is generated by membrane-bound systems. Furthermore, oxygen is more soluble in non-polar solvents than in aqueous solutions. One may speculate, therefore, that *P. damselae* has greater need of a superoxide detoxifying enzyme at the membrane where the radical can inflict the greatest damage.

However, using this model, the question of protection against oxidative DNA damage in the cytoplasm remains unanswered. Halliwell & Gutteridge (1984) proposed a model for O_2^- -mediated generation of hydroxyl radicals based on a Haber-Weiss-type Fenton

reaction in which O_2^- reduces iron salts to produce Fe(II). Oxidation of Fe(II) to Fe(III) by simultaneous reduction of peroxide then generates the hydroxyl radical. This model suggests a role for superoxide of merely transferring electrons to free iron. However, biological reductants such as thiols and NADH are far more abundant in the cell (Imlay & Fridovich 1991) and are equally capable of performing this task (Winterbourn, 1979; Imlay & Linn, 1988). Thus Keyer *et al.* (1995) presented data consistent with oxidative excision of iron by O_2^- from [4Fe-4S] clusters in dehydratases, increasing the pool of free iron for catalysis of oxidative DNA damage. The means by which *P. damselae* counters this form of oxidative damage cannot be answered conclusively from data presented in this paper. Certainly, cytoplasmic catalase is important in protection against such damage by removing cytoplasmic H_2O_2 , the precursor of hydroxyl radicals. However, as no accurate quantification was made of the percentage, if any, of SOD present in the cytoplasm, it is not possible to comment on the fate of any free O_2^- generated in the cytoplasm. Current models for protein secretion do not preclude the FeSOD from being predominant in the periplasm with small quantities remaining in the cytoplasm (Randall & Hardy, 1989). Following transient interaction with the leader peptide, protein folding is modulated such that interaction with export apparatus is kinetically favoured over folding to produce the mature protein. As a result, the bulk of leader-associated proteins are exported, but this does not rule out a small proportion folding into an active export-incompetent state within the matrix (Randall & Hardy, 1989).

The present results demonstrate that *P. damselae* is susceptible to reactive oxygen species. Exposure of bacteria to photoreduced riboflavin in the presence of methionine resulted in a significant reduction in bacterial survival compared to the controls. The bactericidal effect was abolished by addition of catalase, but not exogenous SOD to the medium. The periplasmic location of SOD suggests that *P. damselae* cells should be able to protect against exogenous superoxide anions and this may, in fact, be the case. However, in the strains examined, catalase was absent from the periplasm. In addition, there is no induction of catalase in response to sublethal concentrations of peroxide (unpublished data). This suggests that the weak link in the oxidative defence strategies of *P. damselae* lies in dealing with H_2O_2 generated by SOD in the periplasm. The protection afforded by addition of catalase to the killing assays corroborates this hypothesis. Furthermore, the addition of both SOD and catalase simultaneously to the bactericidal assays did not make a significant difference to protection against O_2^- compared with the addition of catalase alone. This suggests that *P. damselae* contains sufficient SOD activity to disproportionate exogenous O_2^- but that the susceptibility to killing results from accumulation of H_2O_2 . Although all the strains assayed here produced a catalase, the level of activity detected in gels appeared to vary between strains. It would be of

interest to know if strains producing higher levels of catalase are more resistant to killing by reactive oxygen species.

Several authors have postulated that *P. damsela* can survive within fish macrophages: histopathological studies carried out by Kubota *et al.* (1970) and Nelson *et al.* (1981) showed the presence of yellowtail macrophages with engulfed intact bacteria. Noya *et al.* (1995b) also reported the existence of unaffected *P. damsela* cells within macrophages of gilthead sea bream weighing 0.5 g. However, the same authors indicated that macrophages from larger sea bream (20–30 g) killed the pathogen. Furthermore, Skarmeta *et al.* (1995) reported that macrophages from sea bass and sea bream killed the bacterium *in vitro* and observed that superoxide anions generated *in vitro* are implicated in the killing process of *P. damsela*.

The present data indicate that *P. damsela* is ill-equipped to protect itself from the bactericidal products of phagocyte respiratory burst and hence corroborate the findings of Skarmeta *et al.* (1995). Future work may be fruitfully directed towards studies of how the bacterium might avoid phagocytosis to survive in the host. Some recent work is of interest in this context; virulent strains of *P. damsela* possess a polysaccharide capsule which confers resistance to normal serum killing (Magariños *et al.*, 1996). Also, while non-capsulated strains are opsonized by normal serum, capsulated strains are not (Arijo *et al.*, 1998). Thus the presence of a capsule may reduce the opportunities of fish macrophages to phagocytose the bacterium *in vivo*. Furthermore, *P. damsela* is highly invasive in fish epithelial tissue culture cell lines with evidence of actin polymerization and cell to cell migration (Magariños *et al.*, 1996). Unpublished observations in our laboratory have confirmed these results in EM studies and the ability of *P. damsela* to reside within EPC and Sea Bass Larval cell lines has been observed. Thus, *P. damsela*, while lacking defences against the bactericidal activities of macrophages, may avoid phagocytosis by growing in non-phagocytic host cells and producing an anti-opsonizing capsule when in the extracellular milieu. One may speculate that the increased growth rate observed during anaerobic culture reflects a preference for survival in non-phagocytic host cells, where oxygen will be in short supply.

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