Response of *Pseudomonas aeruginosa* PAO following exposure to hydrogen peroxide

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Abstract

Hydrogen peroxide (H_2O_2) is used in various applications to prevent, control, or decrease bacterial activity in e.g. cooling water, hospitals, recreational waters and the food industry. The aim of the work reported here was to investigate the response of *P. aeruginosa* following exposure to H_2O_2 during both logarithmic and stationary phases of growth. The catalase levels were determined following exposure to H_2O_2, and the general cellular response was investigated by pulse-labelling using ^{35}S methionine. Stationary phase cells did not demonstrate a stress response to H_2O_2. Where *de novo* protein synthesis was inhibited, cells were less susceptible to growth inhibition, indicating an inverse stress response to H_2O_2 in *P. aeruginosa*. The addition of H_2O_2 to cultures in logarithmic growth phase resulted in the induction of a short lag phase. The growth rate following a return to logarithmic growth phase was lower than before addition of H_2O_2, and was inversely related to the concentration of H_2O_2 added. Oxidising stress elicited *de novo* synthesis of four proteins within 5 min following exposure to stress. Cellular catalase levels doubled from 16 U·mg^{-1} protein to over 30 U·mg^{-1} protein within 10 min following exposure to oxidising stress but no new catalase isozymes were induced. H_2O_2 was demonstrated to interrupt cell division as well as to decrease the ensuing rate of division in *P. aeruginosa*, and the culture did not exhibit an effective stress response to H_2O_2.

Introduction

Hydrogen peroxide (H_2O_2) is used in various applications to prevent, control, or decrease bacterial activity. It is used amongst others in industrial water systems to control biofouling, in swimming pools and for the disinfection of surfaces and pipelines in food and other industries (Baldry and Fraser, 1988; Characklis, 1990; Cloete et al., 1992). Surfaces exposed to water are often colonised by bacteria which grow to form biofilms (Characklis, 1990; Cloete et al., 1992). These biofilms are composed largely of gram-negative aerobic rods. *Pseudomonas aeruginosa* is one of the dominant members of biofilm communities and is often used as a model organism in the investigation of biofouling processes and control strategies (Characklis, 1990; Cloete et al., 1992).

Hydrogen peroxide is formed as a by-product during aerobic metabolism along with superoxide (Fridovich, 1978), and reacts with a wide array of biological macromolecules such as DNA, proteins and membrane lipids (Tao et al., 1989). For example, H_2O_2 penetrates cells, causing site-directed damage especially of DNA due to metal-dependant OH formation (Storz et al., 1990).

Many bacteria have been reported to respond to a wide range of environmental stresses including heat, cold, salt, UV radiation and oxidising stresses (e.g. exposure to a sub-inhibitory concentration of H_2O_2). Such stress responses result, generally, in the synthesis of small sets of stress proteins which lead to tolerance of the cells to further exposure to otherwise lethal levels of the same stress (Völker et al., 1992; Watson, 1990). A variety of bacteria, all facultative anaerobes, exhibit oxidising stress response by producing oxidant-degrading as well as damage-repair enzymes, provided that the initial concentration of H_2O_2 is not lethal (Storz et al., 1990). These include *Escherichia coli, Salmonella typhimurium* (Storz et al., 1990), and *Bacillus subtilis* (Hartford and Dowds, 1992). A variety of defence genes to naturally occurring oxidising agents have been characterised in *Escherichia coli*. These defence genes encode various superoxide dismutases, catalases, alkyl hydroperoxide reductases and glutathione reductases, as well as DNA repair enzymes (Ahern, 1993; Storz et al., 1990). Few reports have focused on oxidising stress response in strict aerobes, and none have addressed the response of *P. aeruginosa* upon exposure to H_2O_2.

The aim of the work reported here was to investigate the possible inhibitory activity of H_2O_2 towards *P. aeruginosa* in various stages of growth, as well as the response of *P. aeruginosa* following exposure to H_2O_2 in order to shed more light on the suitability of H_2O_2 as a biofouling control agent.

Materials and methods

Bacterial strains and chemicals

Pseudomonas aeruginosa PAO1 was obtained from the Deutsche Sammlung von Mikroorganismen. The culture was maintained on R2A agar slants (Reasoner and Geldreich, 1985) with 1% glycerol, and was subcultured monthly. Casamino acids (Difco), soluble starch and glucose (BDH chemicals), peptone, yeast extract and bacteriological grade agar (Biolab), Na pyruvate, K_2HPO_4 and $MgSO_4$ (Merck) and chloramphenicol (Sigma) were used throughout this study. H_2O_2 (8.8 mol·ℓ^{-1}) was obtained from Saarchem.

Reaction during 24 h following exposure to sub-inhibitory oxidising stress

Pseudomonas aeruginosa PAO1 was cultured for 24 h in R2A broth (Reasoner and Geldreich, 1985), but omitting sodium pyruvate,

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at 28°C while shaking at 120 r·min^{-1}. The minimum inhibitory concentration (MIC) of H_2O_2 was determined as described previously (Brözel et al., 1993). Briefly, 10µℓ volumes of a washed cell suspension were inoculated into tubes containing R2A broth (pH 6.8) with increasing concentrations of H_2O_2. These tubes were incubated at 30°C for 24 h. The lowest concentration of H_2O_2 inhibiting growth was taken as the MIC. After 24 h the cultures were exposed to sub-inhibitory oxidising stress by adding H_2O_2 to one fourth of the MIC (0.75 mmol·ℓ$^{-1}$ H_2O_2). The MICs and culturable counts were determined at 5, 10, 20, 30 and 60 min, and 2, 3, 4, 6, 8 and 12 h after exposure to stress. The culturable counts were determined by plating serial dilutions onto triplicate plates of R2A agar (Reasoner and Geldreich, 1985) and incubating these at 28°C for 48 h.

To determine the effect of *de novo* protein synthesis upon tolerance of cells to H_2O_2, 24 h-old cultures of *P. aeruginosa* were exposed to 5 µg·mℓ$^{-1}$ chloramphenicol. Cells were exposed to H_2O_2 stress 2 min after exposure to chloramphenicol as described above. The MICs were determined at the times as given above.

Effect of oxidising stress on survival of oxidant treatment

Twenty-four hour-old cultures of *P. aeruginosa* PAO1 (cultured in R2A broth for 24 h at 28°C with shaking at 120 r·min^{-1}) were exposed to 8.8 mmol·ℓ$^{-1}$ and 17.6 mmol·ℓ$^{-1}$ H_2O_2 for 60 min. The numbers of surviving cells (culturable count) were determined after 60 min by plating serial dilutions onto R2A agar plates (Reasoner and Geldreich, 1985) in duplicate. Plates were incubated at 30°C for 72 h. The effect of pre-exposure to oxidant was studied by treating cultures with 0.35 mmol·ℓ$^{-1}$ H_2O_2 for 60 min. These cultures were then exposed to 8.8 and 17.6 mmol·ℓ$^{-1}$ H_2O_2 as described above. The percentage surviving cells was calculated by dividing the number of surviving cells by the initial number of cells and multiplying by 100.

Determination of the effect of H_2O_2 on the growth rate

The growth rate of *P. aeruginosa* PAO in R2A broth at 28°C with shaking at 120 r·min^{-1} was determined by absorbance at 550 nm (A_{550}). Broths were inoculated with 1 mℓ of a culture in the late log phase. To determine the effect of H_2O_2 on growth rate, a series of concentrations of H_2O_2 (0.22, 0.88, 2.20, 3.50 and 4.40 mmol·ℓ$^{-1}$) was added to various cultures during the early logarithmic phase of growth. Readings were taken at 15 min intervals. The extent of the bacteriostatic effect of H_2O_2 was determined by neutralisation of H_2O_2 at various times after addition by the addition of bovine catalase (Sigma) (200 U·mℓ$^{-1}$ final concentration). One unit (U) is the amount of enzyme catalyzing decomposition of 1 µmol·min^{-1}.

To determine whether *P. aeruginosa* exhibited an oxidising stress response during logarithmic growth phase, logarithmically growing cultures were exposed to 0.22 mmol·ℓ$^{-1}$ H_2O_2 prior to inoculation into fresh R2A broth. Cultures were then challenged with 0, 0.44 and 1.76 mmol·ℓ$^{-1}$ H_2O_2, and the A_{550} was determined every 15 min.

Investigation of *de novo* protein synthesis following exposure to H_2O_2

In order to determine whether exposure to H_2O_2 resulted in *de novo* synthesis of certain proteins in *P. aeruginosa* PAO, 1 mℓ volumes of H_2O_2 - stressed cultures were pulse - labelled with 1 µℓ (15 µCi) of ^{35}S methionine (Amersham, UK) at 0, 5, 15, 25 and 55 min and at 2, 3, 4 and 6 h following stress. Incorporation of radioactive methionine was stopped 5 min after each interval by the addition of 160 µℓ of cold methionine (200 mmol·ℓ$^{-1}$). The radiolabelled proteins were indicative of proteins synthesised during the 5 min following sampling time.

Total cell proteins of the respective samples were prepared for SDS PAGE as follows: Labelled cells were washed twice in phosphate buffer and resuspended in 1 mℓ of sample treatment buffer (50 mℓ·ℓ$^{-1}$ mercaptoethanol, 100 g·ℓ$^{-1}$ glycerol, 82 mmol·ℓ$^{-1}$ Tris and 69 mmol·ℓ$^{-1}$ SDS) and 20µℓ PMSF (phenylmethylsulfonyl fluoride) (2.5 mg·mℓ$^{-1}$ in 96% ethanol) (PMSF inhibits protein degradation by inhibition of protease activity (Colby and Chen, 1992)). Samples were exposed to ultrasonication for 4 x 15 s bursts and were then heat-treated for 10 min at 96°C. Total cell proteins were separated at 60 mA in 125 g·ℓ$^{-1}$ acrylamide gels using the discontinuous buffer system of Laemmli and Favre (1973). Labelled proteins were recorded by exposure of vacuum - dried gels to autoradiography films (Amersham Hyperfilm - ßmax; Amersham, UK) for 24 h. Autoradiography films were developed by the procedures as specified by the manufacturer.

Characterisation of catalase production

The levels of cellular catalase following exposure to oxidising stress were determined as follows: Logarithmically growing cultures were exposed to 88 and 176 µmol·ℓ$^{-1}$ H_2O_2 and samples were taken at 10, 20, 30, 60 and 90 min following exposure to stress. Cellular catalase was assayed as follows: Cells were harvested by centrifugation at 10 000 x g, washed in 0.5 mol·ℓ$^{-1}$ phosphate buffer (pH 7.0) (PB) and suspended in 500µℓ PB. Cells were lysed in suspension by ultrasonication for 4 x 15 s. Catalase was quantified by monitoring the disappearance of H_2O_2 spectrophotometrically at 240 nm according to the method of Beer and Sizer (1952). Cell lysates were supplemented with 1 500 µℓ PB, and 1 000 µℓ H_2O_2 (8.8 mmol·ℓ$^{-1}$) was added at t_0. One unit of catalase decomposed 1 µmol·min^{-1} of H_2O_2 at pH 7.0 (Beer and Sizer, 1952).

Different forms of catalase were distinguished using discontinuous native polyacrylamide slab gel electrophoresis. Crude cell lysates were prepared as described above, and 10 µℓ volumes were loaded onto native polyacrylamide gels prepared as described by Katsuwon and Anderson (1989). Electrophoresis was performed at 10 mA. Catalases were localised as follows: Gels were soaked in 30 mmol·ℓ$^{-1}$ H_2O_2 for 5 min. Gels were then washed with deionised water and transferred into a solution of $FeCl_3$ (20 g·ℓ$^{-1}$) and $K_3Fe(CN_6)$ (10 g·ℓ$^{-1}$). As soon as a blue colour developed, gels were rinsed carefully with deionised water and photographed. In this procedure catalase appears as a yellow area, indicative of decomposition of H_2O_2, whereas the background stains blue, showing ferric iron oxidised by H_2O_2 (Woodbury et al., 1971).

Results

Reaction during 24 h following exposure to sub-inhibitory oxidising stress

The minimum inhibitory concentration (MIC) of H_2O_2 over the 12 h period following stress did not increase above the initial value (Fig. 1). On the contrary, exposure to a sub-inhibitory concentration of H_2O_2 sensitised cells to further treatment with the oxidant (Fig. 1). Although *P. aeruginosa* cultures were temporarily more resistant to H_2O_2 between 30 and 60 min after exposure to stress than after 5, 10 and 20 min, this level was still

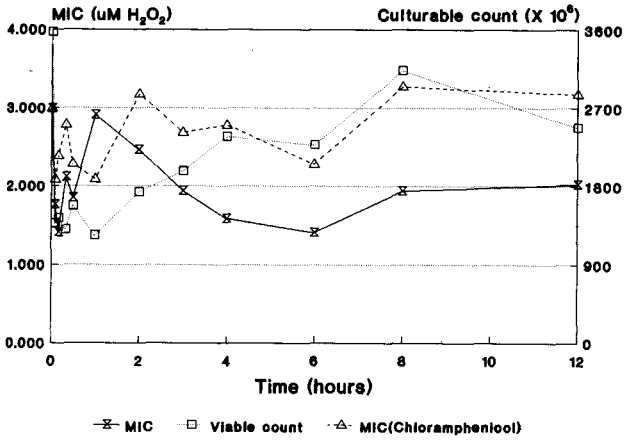

Figure 1

Minimum inhibitory concentration (MIC) of H_2O_2 as well as culturable count of P. aeruginosa *cultures during 12 h following exposure to a sub-inhibitory concentration of oxidant. The MIC was determined for cultures stressed with and without an intact protein synthesising system (5 mg·mℓ^{-1} chloramphenicol).*

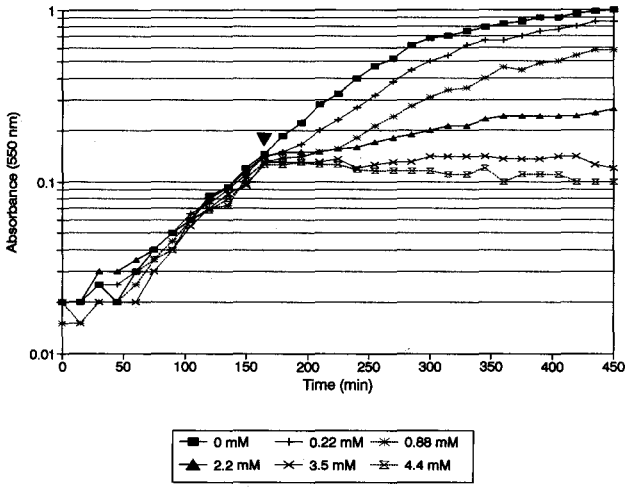

Figure 2

Response of P. aeruginosa *PAO following exposure to various concentrations of H_2O_2. The growth rate was determined following exposure of logarithmically growing cultures to various concentrations of H_2O_2. The arrow indicates the time of addition of H_2O_2.*

less than the initial resistance of 3 mmol·ℓ^{-1}. The decline in resistance after 60 min following exposure to stress indicated that the mechanism of increased resistance could not be upheld. However, the increased resistance at 60 min after exposure to stress was also demonstrated by the increase in the culturable count after 60 min (Fig. 1). This showed that cells either started to divide again, or at least recovered to the culturable state. Yet these cells were again more susceptible to further H_2O_2 treatment (Fig. 1).

Exposure to sub-inhibitory H_2O_2 stress during inhibition of *de novo* protein synthesis by chloramphenicol caused *P. aeruginosa*

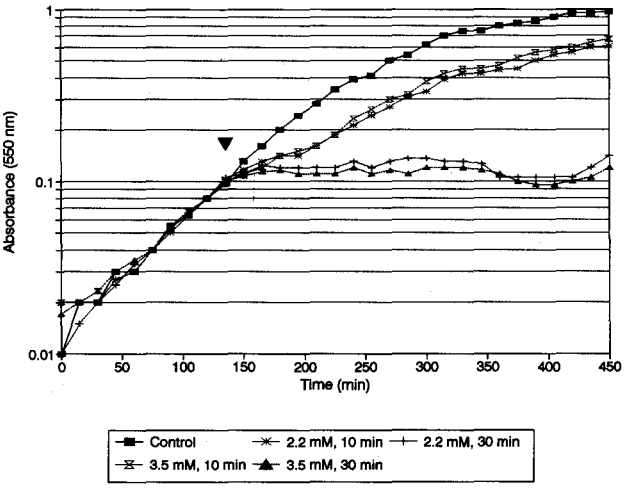

Figure 3

The recovery of growth of P. aeruginosa *PAO following exposure to 0, 2.2 and 3.5 mmol·ℓ^{-1} H_2O_2 for various time periods was determined by removal of H_2O_2 at 10 and 30 min by the addition of an excess of bovine catalase. The growth rate was determined as described in materials and methods. The arrow indicates the time of addition of H_2O_2*

to be more resistant than when disposing over an intact protein synthesis mechanism (Fig. 1). The MIC of unstressed cultures in the presence of chloramphenicol was not found to be meaningfully lower than in its absence (data not shown), so that 5 µg·mℓ^{-1} chloramphenicol itself did not affect the MIC of H_2O_2 on *P. aeruginosa*. This implies that the exposure to H_2O_2 stress elicited a response which rendered cells more susceptible, and that this response was linked to *de novo* protein synthesis. Even at 8 h after H_2O_2 stress, cells defective in protein synthesis during exposure to stress, were more resistant than before stress, whereas cells with an intact protein synthesis mechanism were not (Fig. 1).

Treatment of stationary phase cells with H_2O_2 made the cells more susceptible to lethal concentrations of H_2O_2. Pre-stressed cultures exposed to 8.8 mmol·ℓ^{-1} H_2O_2 for 60 min exhibited an 82.5 % survival compared to 112 % for unstressed cultures. Pre-stressed cultures exposed to 17.6 mmol·ℓ^{-1} H_2O_2 for 60 min exhibited a 79.2 % survival compared to 96.6 % for unstressed cultures.

The effect of H_2O_2 on the growth rate

The addition of H_2O_2 to cultures in logarithmic growth phase resulted in the induction of a short lag phase (Fig. 2). The growth rate following a return to logarithmic growth phase was lower than before addition of H_2O_2, and was inversely related to the concentration of H_2O_2 added (Fig. 2). Colorimetric quantification of H_2O_2 was attempted, but proved unsuccessful due to interference by the green pigment produced by *P. aeruginosa*. We therefore added bovine catalase (Sigma) in excess (200 U·mℓ^{-1} final concentration) at certain times following addition of H_2O_2 in order to remove all residual H_2O_2 from the medium. This served to indicate how long after depletion of H_2O_2, growth would recur. When the available H_2O_2 was degraded 10 min following addition, the cultures recovered from the induced lag phase ca. 30 min later and returned to the initial growth rate (Fig. 3). When the available

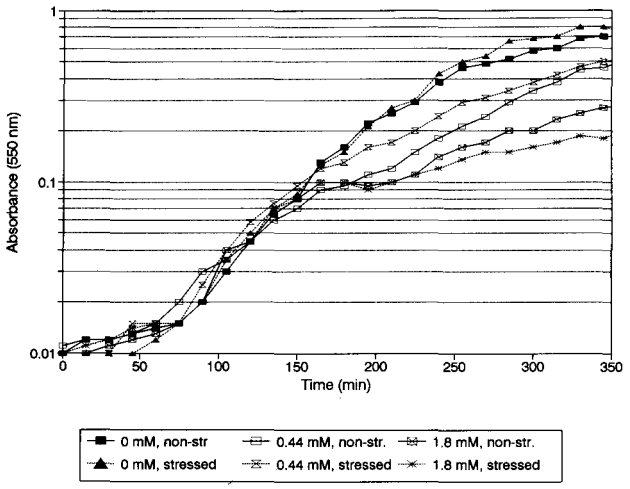

Figure 4

Growth rate of stressed (0.22 mmol·ℓ⁻¹ H_2O_2) (▲) and non-stressed (■) P. aeruginosa PAO following addition of various concentrations of H_2O_2. The arrow indicates the time of addition of H_2O_2.

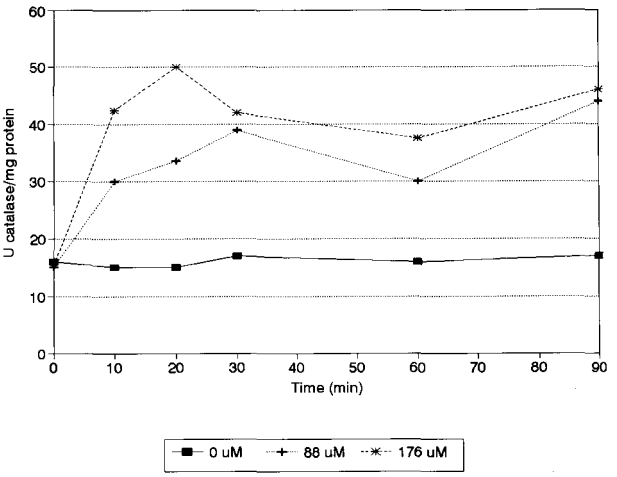

Figure 6

Effect of H_2O_2 stress on cellular catalase levels in P. aeruginosa *PAO. Logarithmically growing cultures were exposed to 0, 88 and 176 mmol·ℓ⁻¹ H_2O_2. Cell extracts were prepared at various times following addition, and catalase was assayed as described in* ***Materials and methods****.*

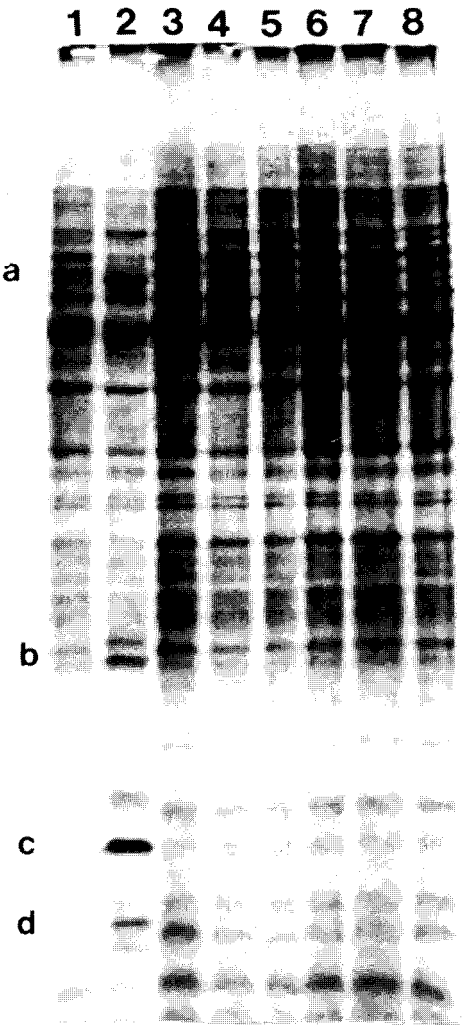

Figure 5

Autoradiograph of ^{35}S methionine pulse-labelled proteins of P. aeruginosa *separated by SDS PAGE. Logarithmic cultures were exposed to 0.176 mmol·l⁻¹ H_2O_2 and 1 ml samples were pulse labelled before (lane 1) and at 5 min (lane 2), 30 min (lane 3), 60 min (lane 4), 2 h (lane 5), 3h (lane 6), 4 h (lane 7) and 6 h (lane 8) following exposure to H_2O_2 stress. Letters a to d indicate the positions of* de novo *synthesized proteins (lane 2).*

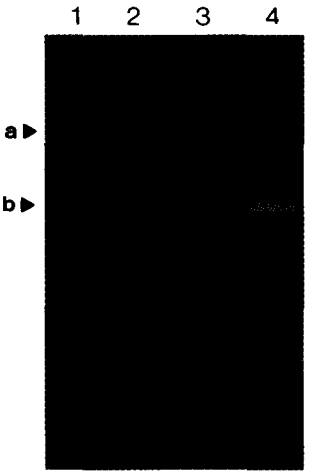

Figure 7

Native polyacrylamide gel of catalase in extracts from P. aeruginosa *PAO. Logarithmically growing cultures were exposed to 0 (lane 2), 88 (lane 3) and 176 mmol·ℓ⁻¹ H_2O_2 (lane 4) for 60 min and extracts were prepared as described in* ***Materials and methods****. Bovine catalase was included as control (lane 1, a).*

H_2O_2 was degraded 30 min following addition, cultures did not recover from the induced lag phase (Fig. 3).

Cultures pre-stressed during the logarithmic growth phase were protected from the additional bacteriostatic effect of a low concentration of H_2O_2 (0.44 mmol·ℓ^{-1}) as no lag phase was apparent following addition (Fig. 4). The specific growth rate was, however, decreased. Where pre-stressed cultures were exposed to a higher concentration of H_2O_2 (1.76 mmol·ℓ^{-1}), a lag phase was induced, and the specific growth rate following return to logarithmic phase was lower than in unstressed cultures (Fig. 4).

De novo protein synthesis following exposure to H_2O_2

Oxidising stress elicited *de novo* synthesis of four proteins within 5 min following exposure to stress (Fig. 5). Synthesis of all four was, however, terminated again and these were not detectable in samples taken 30 min after exposure to stress. No further changes in the *de novo* protein synthesis profile were detected in stressed cultures.

Catalase in cultures following exposure to H_2O_2

Cellular catalase levels doubled from 16 U·mg^{-1} protein to over 30 U·mg^{-1} protein within 10 min following exposure to oxidising stress (Fig. 6). A higher concentration of catalase was present in cultures exposed to 176 µmol·ℓ^{-1} H_2O_2 than in those exposed to 88 µmol·ℓ^{-1} H_2O_2. These increased levels of catalase were maintained for at least 90 min following stress. Only one catalase isozyme was detected in cultures of *P. aeruginosa*, also following exposure to oxidising stress so that the increased level of catalase was not due to induction of further catalases, but to increased production of the constitutive catalase (Fig. 7).

Discussion

Effect of H_2O_2 on the growth rate of *P. aeruginosa*

The arrest of cell division upon addition of H_2O_2 indicated the interruption of one or more processes involved in macromolecular synthesis. Protein synthesis did not appear to be interrupted as indicated by the whole cell protein profile of *de novo* synthesised proteins. It is possible that the cell membranes were damaged in some way. It is well established that H_2O_2 causes DNA strand breaks as well as hydroxylation of bases in *E. coli*, resulting in temporary termination in replication (Storz et al., 1990). However, bacterial cells possess various repair enzymes which repair the damaged DNA (Ahern, 1993). The time required for the recovery of cell division was proportional to the quantity of H_2O_2 added, indicating that more time was required for repair where the concentration of H_2O_2 was greatest, and that the degree of damage to macromolecules was greater. However, the decreased growth rate following return to logarithmic growth indicated a more permanent damage. This damage appeared to be separate from the damage leading to interruption of cell division.

Stress response of *P. aeruginosa*

Pseudomonas aeruginosa in the stationary phase did not exhibit a protective oxidising-stress response to H_2O_2. Rather, it appeared to have an inverse stress response, becoming more susceptible after exposure to sub-inhibitory stress. The increased susceptibility was not due to cell damage caused by the sub-inhibitory stress as the culturable count decreased only slightly. The inoculum was also standardised spectrophotometrically, so that the tubes used to determine MIC were not inoculated with fewer cells. *Pseudomonas aeruginosa* PAO1 did exhibit some form of recovery, 30 min after exposure to H_2O_2-induced stress. This indicated that some degree of protective mechanism was induced or repaired within 20 min following stress exposure, albeit at a lower level than in the unstressed culture. This mechanism could not be maintained despite the fact that cells were growing as the MIC decreased again after 60 min.

Pseudomonas aeruginosa in the logarithmic growth phase exhibited a degree of oxidising stress response. The level of cellular catalase was doubled where cultures were exposed to 0.176 mmol·ℓ^{-1} H_2O_2. Where such cultures were subsequently challenged with 0.44 mmol·ℓ^{-1} H_2O_2, they proved more resistant than unstressed cultures, probably due to the higher catalase levels. Because pre-stressed cultures exposed to a higher concentration of H_2O_2 proved more susceptible, despite the enhanced level of catalase, than were unstressed cultures, the preliminary stress must have rendered cells more susceptible.

Pseudomonas aeruginosa did exhibit a response to H_2O_2 stress, although this was limited. Because the inhibition of *de novo* protein synthesis in stationary phase cells decreased the susceptibility to H_2O_2, it appeared that the response of *P. aeruginosa* to oxidising stress had a net deleterious effect on the cell. Certain specific responses must have exerted a negative effect on the physiological state of the cells. Two possibilities should be considered. Firstly, inactivation and repair enzymes could be absent or poorly expressed. *P. aeruginosa* has at least three UV-inducible stress genes (Warner-Bartnicki and Miller, 1992). However, none of these genes lead to increased protection to damage by UV light (Warner-Bartnicki and Miller, 1992). Although it encodes for a UV-inducible RecA protein, and this controls at least three *din* (damage-inducible) genes, the response does not lead to any degree of detectable protection to UV damage (Katsuwon and Anderson, 1989). This is probably due to lack or poor expression of one or more of the UV-damage repair enzymes (Warner-Bartnicki and Miller, 1992). Similarly, it is possible that enzymes for the repair of H_2O_2 - induced damage are either poorly expressed or lacking, so that response to oxidising stress does not lead to increased protection.

Alternatively, certain enzymes expressed upon H_2O_2 stress could have rendered cells more susceptible. It is possible that stress response systems in *P. aeruginosa* have either reverted to non-functioning forms, or are the primitive predecessors of functioning stress response systems of the other pseudomonads. Root surface peroxidases of many plants produce superoxide anions and H_2O_2 (Katsuwon and Anderson, 1989). Pseudomonads colonising the rhizosphere, such as *P. fluorescens* and *P. putida*, express high levels of catalase and superoxide dismutase and have further oxidising stress-inducible catalases, affording them protection from the root defence system (Katsuwon and Anderson, 1989). *Pseudomonas aeruginosa*, although being an autochthonous soil and water organism, is not a rhizosphere coloniser (Korsten and Lubbe, 1993). The reason for this could be that *P. aeruginosa* does not possess an oxidising stress-inducible response, so that it cannot survive in the rhizosphere.

Implications for treatment of water systems with H_2O_2

The aim of treatment of industrial water systems with antimicrobial agents is to inhibit microbial growth, partly by killing of cells, and partly by a decrease in the growth rate of surviving cells (Characklis, 1990). The timing of addition of antimicrobial agents to industrial water systems is disputable. Whereas some

authors support continuous addition, others advocate intermittent, batch or "slug" dosing at certain intervals (Characklis, 1990; Cloete et al., 1992). The disadvantage of continuous addition is the volume of product consumed. The disadvantage of intermittent addition is that surviving bacteria recover at a set time following addition, so that a high concentration of antimicrobial agent is required to challenge the large number of cells.

H_2O_2 was demonstrated as being able to interrupt cell division as well as to decrease the ensuing rate of division in *P. aeruginosa*. Furthermore *P. aeruginosa* did not exhibit an effective stress response to H_2O_2. Intermittent addition of H_2O_2 would effectively serve to inhibit growth of *P. aeruginosa* because it interrupts cell division. Where cells would recover, the growth rate would be lower than normal. Furthermore, the cells in the system would not adapt to H_2O_2 treatment as would facultative anaerobes, but rather be sensitised to further exposure to H_2O_2. *Pseudomonas aeruginosa* does not develop resistance to other oxidising bactericides such as hypochlorous acid and 3-bromo-5-chlorodimethylhydantoin following long-term exposure (Pietersen et al., 1995). In the light of the data presented, intermittent addition of 2 mmol·ℓ^{-1} H_2O_2 every 4 h is advised. This would serve to control bacterial growth with the added advantage that no toxic residues would remain in any effluent.

Acknowledgements

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