The adaptation of *Rhizobium leguminosarum* bv. *phaseoli* to oxidative stress and its overlap with other environmental stress responses

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This paper reports the adaptation of *Rhizobium leguminosarum* bv. *phaseoli* to oxidative stress and the investigation of its overlap with other environmental stress responses. Treatment of *R. leguminosarum* bv. *phaseoli* cells with low concentrations of either menadione (MD, a superoxide generating agent) or 1-chloro-2,4-dinitrobenzene (CDNB, which depletes GSH levels) induced an adaptive response which resulted in cells becoming resistant to subsequent treatment with high concentrations of these oxidative stress compounds. There was overlap between the adaptive response to MD-generated superoxide stress and the response previously demonstrated in this organism to H₂O₂ (A. J. Crockford, G. A. Davis & H. D. Williams, 1995, *Microbiology* 141, 843–851); pretreatment with H₂O₂ was protective against cell killing by MD and vice versa. In contrast, similar experiments indicated only a limited overlap between the responses to H₂O₂ and CDNB-mediated GSH depletion. It was also found that H₂O₂, but not MD or CDNB, adaptation protected cells against subsequent osmotic challenge and heat shock. Carbon-starved cells were more resistant to H₂O₂ and MD killing than exponentially growing cultures, but were more sensitive to CDNB-mediated GSH depletion. Therefore, this work shows that there is a substantial, but incomplete overlap between the responses of *R. leguminosarum* to different forms of oxidative and other environmental stresses.

Keywords: oxidative stress, starvation, osmotic stress, heat-shock, superoxide, glutathione, hydrogen peroxide

INTRODUCTION

Aerobic micro-organisms have to deal with reactive oxygen species such as the superoxide anion radical, H₂O₂ and the hydroxyl radical which are formed as an unavoidable consequence of an aerobic lifestyle (Fridovich, 1978; Halliwell & Gutteridge, 1989). These oxidants can cause wide ranging damage to the living cell through their detrimental effects on key macromolecules (Brot *et al.*, 1981; Demple & Linn, 1982; Levin *et al.*, 1982). Consequently, living organisms have evolved ways of protecting themselves against reactive oxygen species by the involvement of enzymic defence mechanisms such as catalase and superoxide dismutase, and through the synthesis of antioxidant molecules such as GSH (Halliwell & Gutteridge, 1989).

The adaptation of bacteria to oxidative stress is best studied in enteric bacteria, where OxyR positively regulates the genes encoding the proteins involved in the adaptive response to H₂O₂, and the SoxR and SoxS proteins are essential regulators of the superoxide adaptive response (Christman *et al.*, 1989; Demple, 1991). There is a degree of overlap between the two oxidative stress responses and they overlap with the SOS and heat-shock adaptations (Demple, 1991; Farr & Kogoma, 1991). H₂O₂-adapted enteric bacteria show increased resistance to heat and 2-D PAGE shows that there are some common proteins synthesized during oxidative stress and nutrient starvation (Nystrom, 1993; Christman *et al.*, 1985; Siegle & Kolter, 1992).

We are currently investigating the response of the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum* bv. *phaseoli* to environmental stress in an attempt to understand its ability to adapt to and survive in adverse

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; MD, menadione.

soil conditions and how stress might affect its ability to form nitrogen-fixing symbioses with leguminous plants (Graham, 1992). Little is known about the physiological responses of *Rhizobium* spp. to the kinds of stress they might encounter in the soil, during nodulation or when differentiated into bacteroids in the mature, nitrogen-fixing root nodule. However, a number of stress conditions are known to interfere with the development and function of the *Rhizobium*-legume symbiosis, including temperature, acid and osmotic stresses (La Favre *et al.*, 1986; Michiels *et al.*, 1994; Zahran & Sprent 1986; Evans *et al.*, 1980). Adaptation to acid stress is probably the best studied stress response in *Rhizobium* spp. and a positive correlation has been found between acid tolerance, exopolysaccharide production and the ability to generate a pH gradient under acid conditions (Tiwari *et al.*, 1992; O'Hara *et al.*, 1989; Chen *et al.*, 1993). In *R. leguminosarum* bv. *trifolii* there is evidence that acid tolerance is linked to a megaplasmid which also carries certain symbiotic nitrogen-fixing functions (Chen *et al.*, 1993).

We have previously reported that *R. leguminosarum* can mount an adaptive response to H_2O_2 -mediated oxidative stress and this response involved a modest induction of catalase activities (Crockford *et al.*, 1995). Interestingly, catalase showed cell-density-dependent regulation controlled by the accumulation of extracellular components in the growth medium. This paper extends our previous work to study the response of these bacteria to other forms of oxidative stress and investigates the extent of cross-protection between adaptation to different oxidative stress agents. We also investigate whether oxidatively stressed *R. leguminosarum* cells acquire resistance to other environmental stress conditions such as osmotic, heat-shock and starvation stresses.

METHODS

Bacteria and growth conditions. *Rhizobium leguminosarum* bv. *phaseoli* strain 4292 used in this study was kindly provided by Dr Allan Downie. It is a rifampicin-resistant strain which carries the bv. *phaseoli* Sym plasmid pRP2JI (Lamb *et al.*, 1982). It was grown in YEM medium at pH 7.5 (Vincent, 1970) comprising (g l⁻¹): yeast extract (0.4, Oxoid), K₂HPO₄ (0.5), NaCl (0.1), MgSO₄·7H₂O (0.2), mannitol (10), or TY medium comprising (g l⁻¹): bactotryptone (5), yeast extract (3), CaCl₂·6H₂O (1.3). For solid medium, agar was added at 15 g l⁻¹. For routine culture maintenance, growth of starter cultures and for plating for viable counting, rifampicin was added at 25 µg ml⁻¹, but it was not added to cultures used in stress experiments.

Culture methods for stress treatments and adaptation experiments. Strain 4292 was inoculated into YEM medium and grown with shaking (200 r.p.m.) at 30 °C to an OD₆₀₀ of 0.2, the pH of the culture at this stage being about 7.45. Aliquots (5 ml) of the culture were transferred into sterile tubes and H_2O_2 , menadione (MD), 1-chloro-2,4-dinitrobenzene (CDNB) or NaCl added to the desired final concentration. The cultures were then incubated as before. For heat-shock experiments, cultures were shifted from 30 °C to 45 °C. Samples were taken immediately prior to and periodically after the stress addition, diluted in YEM minus mannitol and plated onto TY agar containing rifampicin (25 µg ml⁻¹) to monitor cell viability. Colonies were counted after 3–4 d incubation at 30 °C. For

adaptation experiments, overnight cultures were used to inoculate 50 ml YEM to an initial OD₆₀₀ of 0.01. When the culture had grown to reach an OD₆₀₀ of 0.2, H_2O_2 , MD or CDNB was added to a final concentration of 200 µM. After the pretreated cells had been incubated for 2 h, a sample was taken to determine culture viability and then H_2O_2 , MD or CDNB was added to a final concentration of 3, 50 or 2 mM, respectively, or for osmotic stress NaCl was added to 2.5 M and the culture sampled at intervals for viable counting. Heat-shock was achieved by shifting the culture to 45 °C. *R. leguminosarum* 4292 cultures were carbon-starved by growing in YEM containing 0.01 % mannitol to stationary phase (OD₆₀₀ 0.5).

RESULTS AND DISCUSSION

Adaptation of *R. leguminosarum* bv. *phaseoli* to MD and CDNB

R. leguminosarum can adapt to H_2O_2 -induced oxidative stress (Crockford *et al.*, 1995) and we were interested to determine if it could also adapt to other forms of oxidative stress, in particular the superoxide anion radical and depletion of the antioxidant molecule GSH. MD is a quinone that generates superoxide *in vivo* by redox cycling reactions (Halliwell & Gutteridge, 1989). To determine the degree of sensitivity of *R. leguminosarum* to MD, exponentially growing cultures were harvested and exposed to MD concentrations from 0.1 to 50 mM and their viability followed. Concentrations of MD as high as 10 mM had little or no effect on viability, but at 50 mM MD viability was quickly lost (data not shown). To look at the ability of *R. leguminosarum* to adapt to MD, exponentially growing cultures were pretreated with a sub-lethal dose of MD (200 µM) for 2 h and subsequently challenged with a 50 mM dose of MD and cell viability followed. The results in Fig. 1(a) show that pretreating cultures with 200 µM MD led to considerably increased survival compared to untreated cultures. For example, 6 h after addition of the lethal MD concentration, 73 % survival was found in pretreated cultures compared to just 21 % survival in the untreated cultures. Cultures pretreated with MD in the presence of chloramphenicol (100 µg ml⁻¹) did not acquire any resistance to 50 mM MD (Fig. 1a), indicating that *de novo* protein synthesis is required for adaptation of *R. leguminosarum* to MD-generated superoxide stress. Therefore, in its ability to adapt to H_2O_2 (Crockford *et al.*, 1995) and superoxide stress, *R. leguminosarum* shows a similar response to *Escherichia coli* (Dempsey & Halbrook, 1983), *Salmonella typhimurium* (Christman *et al.*, 1985), *Bacillus subtilis* (Dowds *et al.*, 1987) and yeast (Flattery-O'Brian *et al.*, 1993; Collinson & Dawes, 1992; Jamieson, 1992).

CDNB causes oxidative stress by depleting the intracellular levels of the antioxidant molecule GSH by forming conjugates with GSH, either spontaneously or by enzyme-catalysed reactions with GSH-transferases (Plummer *et al.*, 1981; Habig & Jakoby, 1981; Christman *et al.*, 1985). GSH is a ubiquitous tripeptide that has a protective effect in both mammalian and bacterial cells against oxidative damage by removing hydroperoxides. It is present at high levels (5–10 mM) in some bacteria, such as *E. coli* (Apontowiel & Berenda, 1975) and the presence

Fig. 1. Adaptation of *R. leguminosarum* to MD and CDNB. Cells were grown exponentially in YEM medium to an OD₆₀₀ of 0.2 (4×10^8 c.f.u. ml⁻¹), which represents 100% survival, and then pretreated with 200 μ M MD (a) or 200 μ M CDNB (b) for 2 h in the presence and absence of chloramphenicol (100 μ g ml⁻¹). After 2 h, MD (a) or CDNB (b) were added to cultures at final concentrations of 50 mM and 2 mM, respectively, and samples were taken to determine cell viability. Symbols: no pretreatment (■), pretreated in the presence (○) and absence (●) of chloramphenicol. Data points are mean values of duplicates from a representative experiment.

of GSH-reductase in the plant fraction of extracts from nitrogen-fixing root nodules is evidence that GSH may play a role in the root nodule tissue as a defence against H₂O₂ (Dalton *et al.*, 1986). The effect of concentrations of CDNB from 0.1–5 mM on the viability of growing *R. leguminosarum* cultures was investigated. CDNB had no effect at 0.1 mM, but following addition of 2 mM CDNB, viability was rapidly lost. The data in Fig. 1(b) show that cultures pretreated with a non-lethal dose of CDNB were considerably more resistant to killing following addition of a lethal concentration, demonstrating that *R. leguminosarum* can mount an adaptive response to CDNB-mediated GSH depletion. For example, 6 h after addition of 2 mM CDNB there was 7.7% and 0.023% survival in the pretreated and untreated cultures, respectively. Cultures

pretreated in the presence of chloramphenicol did not acquire CDNB resistance, indicating that *de novo* protein synthesis is required for adaptation of *R. leguminosarum* to GSH depletion by CDNB (Fig. 1b). The observation that *R. leguminosarum* can adapt to CDNB implies a role for GSH in oxidative stress protection in this bacterium.

Overlap between *R. leguminosarum* adaptive responses to oxidative stress agents

E. coli possesses a regulatory response to superoxide anions that is distinct from that involved in protecting cells against H₂O₂ (Farr & Kogoma, 1991), while in *S. typhimurium* pre-adaptation to H₂O₂ leads to greater resistance to MD (Christman *et al.*, 1985). The proteins induced by these individual stresses comprise largely separate regulons controlled by OxyR and the SoxR–SoxS proteins, respectively (Dempse, 1991). Therefore, we investigated whether the *R. leguminosarum* adaptive response to H₂O₂ (Crockford *et al.*, 1995) was distinct from those demonstrated above to MD and CDNB. *R. leguminosarum* cultures were exposed to doses of H₂O₂, MD or CDNB, capable of inducing adaptive responses (Fig. 1; Crockford *et al.*, 1995), and after a 2 h adaptation period, cultures were challenged with lethal doses of the various oxidative stress agents. The results of these experiments (Fig. 2) indicated that cross-protection against H₂O₂ killing was obtained by pretreating cells with MD or CDNB (Fig. 2a). Similarly, pretreatment of cultures with a low concentration of H₂O₂ gave modest, but reproducible, cross-protection against MD (Fig. 2b). In contrast, pretreatment with a low concentration of CDNB resulted in a small sensitizing effect to subsequent MD treatment as shown by the reduced survival of pretreated compared to untreated cultures (Fig. 2b). Similarly, adaptation of cultures to H₂O₂ or MD led to a reduction in survival, very marked with MD, following CDNB treatment (Fig. 2c). This may be due to the H₂O₂ and particularly the MD treatment depleting intracellular GSH reserves prior to CDNB treatment. The data imply that there is significant overlap between the adaptive responses of *R. leguminosarum* to H₂O₂ and superoxide-mediated stress. In contrast, there is only limited overlap between the responses to H₂O₂ and CDNB, as pretreatment with CDNB afforded cross-protection against H₂O₂ but not vice versa, and no overlap in the responses to MD and CDNB. Therefore, the adaptive response to GSH depletion, mediated by CDNB, is distinct from those to H₂O₂ and superoxide.

The apparent sensitization effect on cells of MD treatment to CDNB killing may reflect the presence of acutely superoxide-sensitive proteins. In *E. coli* the enzymes 6-phosphogluconate dehydrogenase, α , β -dihydroxyisovalerate dehydratase and aconitase are particularly sensitive to superoxide (Gardner & Fridovich, 1991a, b; Kuo *et al.*, 1987), but can be reactivated by GSH. A plausible explanation of our data is that non-lethal doses of MD may inactivate superoxide-sensitive enzymes in *R. leguminosarum*. Consequently, GSH levels become depleted during reactivation processes making

Fig. 2. Overlap between *R. leguminosarum* adaptive responses to oxidative stress agents. *R. leguminosarum* cultures were grown as described in the legend to Fig. 1 and then pretreated with H₂O₂ (□), MD (●) or CDNB (▲) at concentrations of 200 µM or left untreated (■). After 2 h, H₂O₂ (a), MD (b) or CDNB (c) were added to all cultures to final concentrations of 3, 50 and 2 mM, respectively, and samples were taken periodically to determine cell viability. Data points are mean values of duplicates from a representative experiment.

the cells acutely sensitive to subsequent CDNB treatment. A similar explanation could account for the sensitizing effect of H₂O₂ pretreatment prior to CDNB challenge. An additional factor might be the depletion of intracellular NAD(P)H levels via redox cycling reactions of MD compromising the ability of the NADPH-requiring enzyme GSH-reductase to regenerate GSH from GSSG, prior to CDNB addition.

Investigation of the overlap between oxidative stress and other stresses

Considerable overlap between different stress responses, including oxidative stress has been demonstrated in enteric bacteria (Farr & Kogoma, 1991). In their soil environment rhizobia would frequently encounter a range of environmental stress conditions (Graham, 1992). Therefore, we looked at the survival responses of *R. leguminosarum* to various environmental stresses including osmotic stress, heat-shock and carbon starvation and their overlap with the oxidative stress responses. Preliminary experiments showed that exponentially growing cultures were killed fairly rapidly when osmotically stressed by addition of NaCl to a final concentration of 2.5 M. To determine if there was cross-protection between oxidative stress and osmotic stress, exponentially growing cultures were exposed to non-lethal doses of H₂O₂, MD or CDNB (all at 200 µM) for 2 h prior to challenge with 2.5 M NaCl. The data in Fig. 3(a) show that prior H₂O₂ adaptation was very effective in protecting against osmotic challenge. After 5 h, H₂O₂-adapted cultures showed 1.5% survival compared to 0.14% in the non-adapted culture. Osmotic stress survival was not enhanced by pretreatment with MD or CDNB (Fig. 3a). Similarly, to determine whether there was cross-protection between oxidative stress and thermotolerance, cultures preadapted to H₂O₂, MD or CDNB at 30 °C were shifted to 45 °C and their viability followed. H₂O₂-adapted cultures showed modestly increased survival compared to the non-adapted culture, while both MD and CDNB adaptation led to a small decline in survival relative to the control (Fig. 3b).

In response to osmotic stress, *R. meliloti* synthesizes trehalose as a compatible solute (Breedveld *et al.*, 1990, 1993) and also accumulates the disaccharide under micro-oxic conditions (Hoelzle & Streeter, 1990). It has recently been proposed that trehalose might function as a general stress protectant in microbes rather than simply a reserve of carbon and energy (Strom & Kaasen, 1993). So a possible explanation for cross-protection against different stress conditions may be the induction of a general stress protectant such as trehalose. Some proteins, identified originally as heat-shock proteins, may act as general anti-stress proteins; for example, the heat-shock proteins GroES and GroEL are induced by peroxide and superoxide-mediated oxidative stress as well as by starvation and DNA damage in enteric bacteria (Farr & Kogoma, 1991).

Stationary phase cultures of *R. leguminosarum* are more resistant to H₂O₂ than exponential cultures and resistance is not linked to increased catalase activity (Crockford *et*

Fig. 3. Overlap between adaptive responses of *R. leguminosarum* to oxidative stress agents and osmotic stress and heat-shock. *R. leguminosarum* cultures were grown as described in the legend to Fig. 1 and then pretreated with H₂O₂ (□), MD (●) or CDNB (▲) at concentrations of 200 µM or left untreated (■). After 2 h, cultures were either subjected to osmotic stress by addition of NaCl to a final concentration of 2.5 M (a) or heat-shocked by shifting the temperature from 30 °C to 45 °C (b). Samples were taken periodically to determine cell viability. Data points are mean values of duplicates from a representative experiment.

al., 1995). Stationary phase cultures of a number of different bacteria are very resistant to a variety of stresses, including oxidative stress, as indeed are cultures starved of specific nutrients (Siegle & Kolter, 1992; Nystrom, 1993). To establish whether *R. leguminosarum* has a similar response, the effects of oxidative stress on carbon-starved *R. leguminosarum* were compared with those of exponentially growing cultures. Growth of *R. leguminosarum* in YEM containing 0.01% (w/v) mannitol, in contrast to the usual concentration of 1%, led to the culture entering stationary phase at an OD₆₀₀ of 0.53 rather than 1.5. We concluded that the premature entry into stationary phase resulted from carbon-limitation of growth. The YEM medium has a low buffering capacity which would be

Fig. 4. The effect of carbon starvation on the ability of *R. leguminosarum* to survive oxidative stress. Exponentially growing cultures (open symbols) or cultures which had been in a carbon-starvation-induced stationary phase for 4 h (closed symbols) were treated with (final concentration) H₂O₂ (5 mM, □, ■), MD (50 mM, ○, ●) or CDNB (2 mM, △, ▲) and samples were taken periodically to determine cell viability. Data points are mean values of duplicates from a representative experiment.

further reduced in the carbon starvation medium. However, it seems unlikely that the responses of exponentially growing and starved cultures to oxidative stress were unduly complicated by differences in the pH of the two cultures as the growing culture was at pH 7.45 (OD₆₀₀ 0.2) and the carbon-starved, stationary phase culture was at pH 7.7. Fig. 4 shows that carbon-starved *R. leguminosarum* are significantly more resistant to H₂O₂ than growing cultures. After a 5 mM H₂O₂ challenge of 1.5 h, there was 2.5% viability in the starved culture but only 0.007% in the growing culture. Carbon-starved cells were also more resistant to MD, with 60% viability in starved cultures after 7 h, compared to just 16% in the growing cultures. In contrast, exponentially growing cells were significantly more resistant to CDNB than starved cells. A possible explanation for this is that starved cells carefully control the regeneration of GSH from GSSG as it could only be achieved in carbon-starved cultures at the expense of reducing power derived from vital endogenous reserves. Consequently, starved cells may be sensitized to CDNB-mediated GSH depletion. These data indicate overlap in the adaptation of *R. leguminosarum* to carbon starvation and oxidative stress, but also show again a difference in the response to GSH depletion.

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