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Oxidative stress in the moderately halophilic bacterium *Deleya halophila*: Effect of NaCl concentration

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Abstract. The sensitivity of *Deleya halophila* to oxidative stress caused by hydrogen peroxide (H_2O_2) was found to vary, depending on the NaCl concentration of the growth medium. Pretreatment of the bacteria at a low concentration of H_2O_2 (50 μM) protected the cells against the lethal effects of higher levels (1–2 mM) of H_2O_2. Exposure of *D. halophila* cells to 50 μM H_2O_2 resulted in the induction of several proteins (hydrogen peroxide-inducible proteins, hips). However, the kinetics of induction, the extent of induction and the number of hips appear to be influenced by the salt concentration of the growth medium. Five of the hips exhibited apparent molecular masses identical to those of five heat shock proteins (hsps).
Key words. Deleya halophila; oxidative stress; viability; protein synthesis; heat shock.

In bacteria, in response to non-lethal doses of oxidative agents, there is a stimulation of the production of enzymes that scavenge superoxide radicals, hydroxyl radicals and hydrogen peroxide, as well as of other proteins that alleviate the toxic effect. These induced proteins represent adaptive responses to oxidative stress since they prepare cells against subsequent challenge with otherwise lethal levels of these oxidants[1–3, 6, 18].

In the enteric bacteria *Escherichia coli* and *Salmonella typhimurium*, an H_2O_2-adaptive response stimulates the synthesis of about thirty proteins[2, 13, 17]. Synthesis of several of these polypeptides, including the enzymes catalase and alkylhydroperoxide reductase, is positively controlled by the product of the *oxy R* gene[2, 9, 18]. It has also been shown that *oxy R* mutants are hypersensitive to oxidative stresses and highly mutable under aerobic growth conditions[2, 7, 18]. In the gram-positive bacterium *Bacillus subtilis*, eight proteins were induced by treatment with a low concentration of H_2O_2[14]. In general, many of the proteins induced by H_2O_2 are also induced by a variety of other stresses, including heat shock[2, 13, 17].

Recently, we have demonstrated that the kinetics of induction of the heat shock proteins (hsps) in the moderately halophilic bacterium *Deleya halophila* are influenced by the NaCl concentration of the growth medium. Moreover, it has been shown that growth in high salt (2.5 M NaCl) conditions conferred on these bacteria the ability to maintain a high growth rate at elevated temperatures[10]. In addition, the effects of sudden changes in external NaCl concentration on the patterns of protein synthesis of *D. halophila* have also been investigated[4].

In this study, we report the effect of oxidative stress caused by H_2O_2 on the survival of *D. halophila*. Furthermore, the alteration of protein patterns during the adaptive response to non-lethal levels of H_2O_2 is also investi-

gated. The results show that the responses of the *D. halophila* cells to oxidative stress were dependent on the NaCl concentration.

Materials and methods

Deleya halophila CCM 3662 (kindly provided by Prof. A. Ramos-Cormenzana, Spain) was maintained and cultured as previously described [10]. The NaCl concentration requirement for optimal growth of *D. halophila* at 30 °C is 1 M NaCl [4].

The survival of *D. halophila* cells challenged with various concentrations of H_2O_2 was carried out as follows. Aliquots (2 ml) of cells growing exponentially in minimal medium containing 1 M or 2.5 M NaCl at 30 °C were taken, and H_2O_2 was added to give a final concentration of 50 µM, 0.5 mM, 1 mM, 5 mM and 10 mM, respectively. Treated cells were incubated at 30 °C for 45 min and the H_2O_2-challenge was terminated by dilution in minimal medium. Appropriate dilutions of cells were propagated on plates containing full medium and colonies were counted after 24 h at 30 °C.

For pretreatment experiments, H_2O_2 was added (final concentration, 50 µM) to exponentially growing cells and incubation was continued for 45 min at 30 °C before the second challenge with increased concentrations of H_2O_2. The effect of 50 µM H_2O_2 on growth was determined as follows: exponentially growing cells were challenged with 50 µM H_2O_2, and aliquots of cells were taken at successive time intervals, diluted and plated. Colonies were counted after 30 h of incubation at 30 °C.

Cells growing in minimal medium containing either 1 M or 2.5 M NaCl were challenged with 50 µM H_2O_2, and at the indicated time intervals, aliquots of cells (0.5 ml) were labeled with 10 µCi/ml of ^{35}S-methionine (1083 Ci/mmol, 40.07 TBq/mmol, NEN) as previously described [4, 11]. Whole cell extracts were analyzed by one-dimensional slab electrophoresis, blotted and autoradiographed [10]. An equal number of counts (50,000 cpm) from each of the radiolabeled cell extracts was loaded to the gel. Sodium dodecyl sulfate-polyacrylamide gels (15%) were used (SDS-PAGE). For the determination of catalase activity, cells were grown to midlog phase in minimal medium and lysed by sonication. Catalase activity was localized on 7.5% native polyacrylamide gels [2]. In these experiments equal amounts of protein (100 µg) were loaded onto the gel. Protein concentration was estimated using the Biorad protein dye concentrate according to the manufacturer's protocol.

Results

D. halophila cells grown in medium containing 1 M or 2.5 M NaCl were treated with various concentrations of H_2O_2 for 45 min, and appropriate dilutions were plated. The results (fig. 1) demonstrated that the overall resistance to the oxidative stress caused by H_2O_2 was much higher when cells were grown in 1 M NaCl than in 2.5 M. These differences could be explained by the different concentration of catalase in cells grown with different NaCl

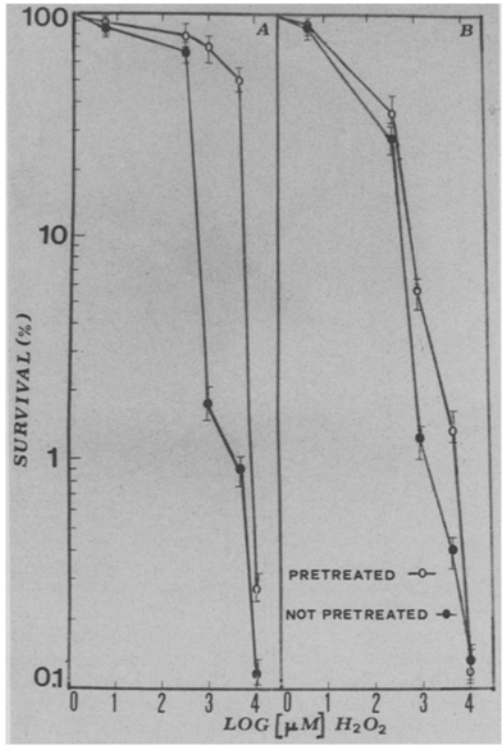

Figure 1. Survival of *D. halophila* after H_2O_2 treatment. Cells growing in minimal medium containing 1 M (*A*) or 2.5 M (*B*) NaCl were challenged directly with increasing concentrations of H_2O_2 for 45 min or after prior treatment with 50 µM H_2O_2 for 45 min. Each point represents the mean of three separate experiments. Error bars are means ± SD.

concentrations in the medium, which would effectively reduce the dose of H_2O_2 to which the cells were exposed. To test this possibility, cell extracts were assayed for catalase activity. No differences in the enzyme levels were observed, indicating that the resistance is not mediated by catalase (data not shown). Among the H_2O_2 concentrations examined, 50 µM was found to be the highest which caused no significant inhibition of the growth of *D. halophila*, irrespectively of the NaCl concentration of the medium. Interestingly, pretreatment of bacteria with 50 µM H_2O_2, following exposure to lethal doses of H_2O_2, resulted in a higher survival rate, particularly in cells grown in 1 M NaCl as compared to those in 2.5 M (fig. 1). The levels of catalase activity were found to be unaltered in the pretreated cells, indicating that resistance may be conferred by other inducible proteins.

In order to investigate whether the protective concentration of H_2O_2 induces the synthesis of any H_2O_2-inducible proteins (hips), as well as to discern whether growth in different NaCl concentrations may influence the synthesis of these hips, cells growing in 1 M or 2.5 M NaCl were challenged with 50 µM H_2O_2. At the indicated time intervals, cells were labeled with ^{35}S-methionine and whole cell extracts were analyzed by SDS-PAGE and autoradiographed. A time-course of H_2O_2-induced alterations of protein synthesis patterns in *D. halophila* grown in 1 M or 2.5 M NaCl is shown in figure 2. Visual

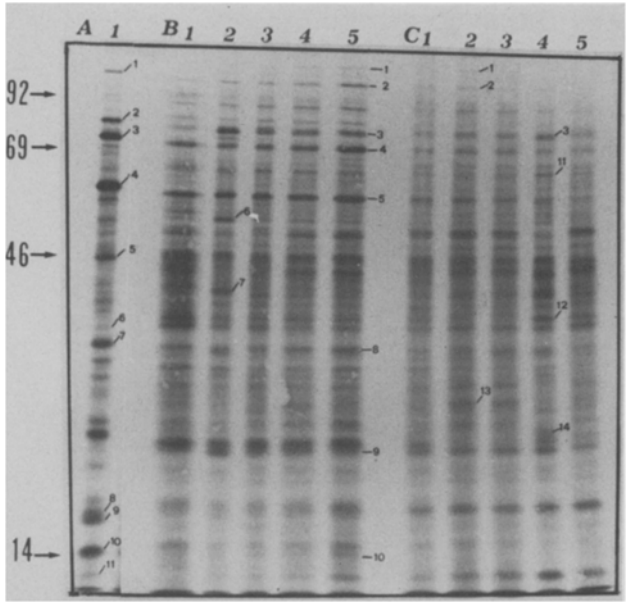

Figure 2. Proteins induced in *D. halophila* cells following H_2O_2 treatment. Cells growing in minimal medium containing 1 M (panel B) or 2.5 M (panel C) NaCl were labeled with ^{35}S-methionine, before (lanes 1) or 5 min (lanes 2), 15 min (lanes 3), 30 min (lanes 4) and 60 min (lanes 5) after treatment with 50 μM H_2O_2. Each lane received 50,000 cpm. The positions of hips are indicated by numbered bars (panel B and C). The ^{35}S-methionine labeled proteins synthesized by *D. halophila* (lane 1) following a 15 min heat shock treatment at 47 °C are shown in panel A. The positions of hsps in *D. halophila* are indicated by the numbered bars (panel A). Molecular weights (kDa) of standard proteins are indicated on the left.

examination of the autoradiographs revealed that cells grown in 1 M NaCl induced a number (about 10) of hips (denoted by the numbered bars). Most of these proteins (hips 1,2,4,5,8,9 and 10) achieved a maximum level of induction 60 min after the H_2O_2 treatment (fig. 2, panel B). Three other induced polypeptides (hips 3,6 and 7) reached their maximum level 5 min after H_2O_2 treatment, and their synthesis was then gradually reduced (fig. 2 panel B). The levels of induction of the different hips appear to differ appreciably; some hips were only slightly induced (1,4,5 and 8) while other hips (2,3,6,7,9 and 10) were significantly induced (fig. 2, panel B).

When cells grown in 2.5 M NaCl were challenged with 50 μM H_2O_2 the induction kinetics and the extent of induction, and also the number of hips, were quite different. Visual examination of the autoradiographs (fig. 2, panel C) indicated that at different time-points following exposure to H_2O_2 about seven polypeptides (denoted by the numbered bars) were transiently induced. Three proteins (hips 1,2 and 3), with apparent molecular masses identical with those of the respective proteins seen in 1 M NaCl (fig. 2, panel B), were slightly induced, and then gradually their levels were reduced to levels similar to those in the controls. Interestingly, the hips 4,5,6,7,8,9 and 10 (fig. 2, panel B) are induced exclusively in cells grown in 1 M NaCl, whereas hsps 11,12,13 and 14 (fig. 2, panel C) are visible only in cells grown in 2.5 M NaCl.

It has been reported that in enteric bacteria there is an overlap among H_2O_2-inducible proteins (hips) and hsps[2,13,17]. Therefore it was of interest to determine whether this may happen in the moderately halophilic bacteria as well. The data shown in figure 2 (panel A, lane 1) clearly show that five hsps (numbers 2,3,4,7 and 10) have the same apparent molecular masses as the hips 1,3,4,8 and 10 (fig. 2, panel B).

Discussion

In the present report, we examined the response of *D. halophila* cells to oxidative stress caused by H_2O_2. In general, it was observed that *D. halophila* displays the characteristic features also found in enteric bacteria[2,3,17]. Exposure of the above bacteria to non-lethal doses of H_2O_2 induced the synthesis of about 30 proteins, while our data showed the induction of about fourteen proteins, a number which is close to that observed in *B. subtilis*[14]. These differences are likely to reflect the greater resolution of the two-dimensional gel electrophoresis rather than a significant difference in the number of stimulated gene products.

Our data demonstrated that the death rate of *D. halophila* following lethal doses of H_2O_2 is influenced by the NaCl concentration of the growth medium (fig. 1). It has been shown that oxidative stresses can damage lipids and proteins, and can also disrupt membrane-mediated transport processes[1,5,8]. Thus, these variations could be attributed to changes in the outer membrane of the bacteria which somehow mitigate the toxic effect. Indeed, it has been reported that fatty acid, phospholipid and protein composition in moderately halophilic bacteria is altered during growth in different NaCl concentrations[12,16].

Our data suggest that during the adaptive response to non-lethal doses of H_2O_2 the cells growing in 1 M NaCl responded faster and produced larger quantities of hips for a longer period of time compared to cells growing in 2.5 M NaCl. Since pretreatment with 50 μM H_2O_2 enhanced the proportion of cells resistant to lethal doses of H_2O_2, particularly in cells grown in 1 M NaCl, it could be argued that the sustained synthesis of some of the hips may be involved in alleviating the toxic effects of H_2O_2. The data shown in figure 2 indicates that the overlap among H_2O_2 and heat shock-induced proteins in *D. halophila* is extensive. However, each stress induces a distinct group of proteins along with some proteins that appear to be common. Collectively taken, these data suggest that this overlap does not result from common intracellular signals, but rather that the genes coding for the proteins that can be induced by the two different stresses contain the cis-acting regulatory sequences required for these forms of regulation. The means by which this is achieved is not known yet. However, recent studies suggest that induced changes in DNA supercoiling may provide a simple means of achieving such a regulatory overlap[15].

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Lithium and phorbol ester modify the activating capacity of ascidian spermatozoa

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Abstract. In this paper we have shown, using the whole-cell voltage clamp technique, that two parameters of the fertilization current in ascidian eggs may be modified by exposing spermatozoa to lithium or to phorbol ester. When spermatozoa were pre-treated in 250 mM lithium sea water for up to 30 min there was a significant increase in the mean initial slope of the fertilization current, from 116 ± 90 to 169 ± 84 pA/s ($p < 0.05$). The peak current increased from 1371 ± 1079 to 1719 ± 1052 pA ($p > 0.05$). Pre-treatment in 200–600 nM phorbol 12-myristate 13-acetate also increased the activating capacity of ascidian sperm, as monitored by a significant increase in the initial slope current in control eggs; however, there was no increase in peak current. Furthermore, we have shown, using NH_4Cl, that an increase in intracellular pH alone is insufficient to change the activating capacity of spermatozoa. This is the first report showing that the kinetics of an egg activation event depend upon the physiological status of the spermatozoon.
Key words. Fertilization channels; ascidian eggs, sperm-borne factors.

Although progress has been made in elucidating the role of second messengers in the activation of eggs, particularly those of Ca, pH and inositol tri-phosphate ($InsP_3$) little is known about the primary trigger signal from the spermatozoon (see Dale[1], for general references). There are two current hypotheses. One school suggests that the interaction between an externally located receptor in the egg plasma membrane with a complementary molecule on the spermatozoon is the primary trigger, and that this signal is transduced to the egg cytoplasm by G-proteins[2]. The second hypothesis is that the spermatozoon contains a soluble activating factor that is released into the egg cytoplasm following gamete fusion[3–8].

In *Ciona intestinalis* eggs, as in other eggs[9,10], the first indication of fertilization is a current that is inward at a holding voltage of − 80 mV. Single channel recordings have shown that the activation of large conductance, non-specific ion channels generate this current[11]. Although in ascidians the channel precursors giving rise to this current are homogeneously distributed over the egg surface, it appears that the spermatozoon only activates a limited number of channels close to the site of fusion[12]. Micro-injection of extracts of spermatozoa, $InsP_3$ or Ca^{2+} into both regulative and mosaic eggs induces several activation events[3–8,14]. Furthermore, $InsP_3$ increases in the spermatozoon during the acrosome reaction[15], a prerequisite for gamete fusion, and there is evidence that sea urchin spermatozoa contain enough $InsP_3$ to activate eggs[5]. In this study we have exposed ascidian spermatozoa to Li^+ and phorbol ester, agents known to alter the phosphoinositide cycle[16], in an attempt to modify their activating capacity. Since it has been shown in the sea urchin that the magnitude of the conductance change at activation is sperm-dependent[13], we have used the fertilization current in ascidian eggs to monitor sperm activating capacity.

Materials and methods

Gametes of the ascidian *Ciona intestinalis* in natural sea water at 22 °C were used. The chorion was removed manually using steel needles and recordings were carried out on glass slides. Micropipettes of 1–2 µm diameter, used to whole cell clamp the eggs (130 µm diameter), were filled with an intracellular-like medium (200 mM