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# Protection against oxidative stress in *Escherichia coli* stationary phase by a phosphate concentration-dependent genes expression

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ABSTRACT

*Escherichia coli* gradually decline the capacity to resist oxidative stress during stationary phase. Besides the aerobic electron transport chain components are down-regulated in response to growth arrest. However, we have previously reported that *E. coli* cells grown in media containing at least 37 mM phosphate maintained *ndh* expression in stationary phase, having high viability and low NADH/$NAD^+$ ratio. Here we demonstrated that, in the former condition, other aerobic respiratory genes (*nuoAB*, *sdhC*, *cydA*, and *ubiC*) expression was maintained. In addition, reactive oxygen species production was minimal and consequently the levels of thiobarbituric acid-reactive substances and protein carbonylation were lower than the expected for stationary cells. Interestingly, defense genes (*katG* and *ahpC*) expression was also maintained during this phase. Our results indicate that cells grown in high phosphate media exhibit advantages to resist endogenous and exogenous oxidative stress in stationary phase.



In *Escherichia coli*, the three types of respiratory components are: (1) dehydrogenases, which carry out the oxidation of organic substrates and feed electrons into mobile quinone pool, (2) quinones, which deliver reducing equivalents to the terminal oxidases, and (3) oxidases, which reduce the terminal electron acceptors [1]. Bacteria alter the composition of the respiratory chain as part of its ability to adapt to different growth conditions. The amount of each component is strictly regulated to optimize the respiratory chain, and to maintain the redox balance, according to the available substrates and the physiological needs of the cell.

During stationary phase, *E. coli* cells down-regulate the expression of genes involved in aerobic electron transport to decrease oxidative stress [2], since aerobic respiration is a primary source of endogenous ROS. However, stationary cells are particularly vulnerable to oxidative damage given that strong injuries may be produced when they lack from energy and material to repair or replace the damaged molecules.

We have previously found that the respiratory NADH dehydrogenase-2 activity and the expression of *ndh* gene, encoding this enzyme, were unusually maintained in late stationary phase when phosphate concentration in the culture medium was >37 mM [3]. In the former conditions, we have also demonstrated that cells were more viable and had a lower NADH/$NAD^+$ ratio than cells grown in sufficient phosphate media (2 mM). In order to evaluate the effect of phosphate in stationary phase, several genes expression of aerobic metabolism, ROS production and oxidative damage to proteins and lipids were assayed in either high or sufficient phosphate media.

## Materials and methods

### *Bacterial strains, media, and growth conditions*

The *E. coli* strains are listed in Table 1. The fusion Φ(*ahpC-lacZ*) was transduced by a P1 *vir* lysate into MC4100. Bacteria were grown in Luria broth (LB, Sigma) or in MT minimal medium [12]. MT contains: 5.8 g (99 mM) NaCl, 3.7 g (50 mM) KCl, 0.15 g (1.02 mM) $CaCl_2{\cdot}2H_2O$, 1.1 g (21 mM) $NH_4Cl$, 0.142 g (1 mM) $Na_2SO_4$, 0.272 g (2 mM) $KH_2PO_4$, 12.1 g (100 mM) Tris (Tris [hydroxymethyl] aminomethane), 0.27 mg (1.7 μM) $FeCl_3$, and 0.2 g (0.8 mM) $MgSO_4{\cdot}7H_2O$, per L of distilled water. We have defined the MT medium supplemented with 40 mM phosphate buffer pH 7 as MT + P [3]. Phosphate buffer was prepared with sodium phosphate salts (Sigma). In all experiments the carbon source was 0.5% glycerol. For strains carrying the *ndh-lacZ* and *ahpC-lacZ* transcriptional fusions, the minimal media were supplemented with 0.1 mM tryptophane. When required, the following antibiotics were used: 40 μg $mL^{-1}$ ampicillin, 30 μg $mL^{-1}$ chloramphenicol and 50 μg $mL^{-1}$ kanamycin. Cells were grown aerobically at 37 °C with linear shaking. Growth was monitored by measuring $OD_{560nm}$.

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**Table 1**
Strains used in this work.

| Strains | Relevant genotype | Source or reference |
|---|---|---|
| MC4100 | *araD Δlac rpsL flbB deoC ptsF rbsR relA1* | [4] |
| LSB006 | MC4100 Φ(*ndh-lacZ*) | [3] |
| TSDH00 | MC4100 λ[Φ(*sdhC-lacZ*)] | [5] |
| IMW33 | MC4100 λ[Φ(*nuoAB-lacZ*)] | [6] |
| MC4100(λVLH114) | MC4100 λ[Φ(*cyoA-lacZ*)] | [7] |
| MC4100(λGC101) | MC4100 λ[Φ(*cydA-lacZ*)] | [7] |
| MC4100(λMO2) | MC4100 λ[Φ(*ubiC-lacZ*)] | [8] |
| GS022 | MC4100 λ[Φ(*katG-lacZ*)] | [9] |
| ECL1Φ(*ahpC-lacZ*) | ECL1 Φ(*ahpC-lacZ*) | [10] |
| LSB020 | MC4100 Φ(*ahpC-lacZ*) | This work |
| JRG3533 | MC4100 Φ(*sodA-lacZ*) | [11] |

*Membrane preparation*

Bacteria were harvested at different times of growth and membranes were prepared by sonication according to Rapisarda et al. [13].

*Enzymes activities*

β-Galactosidase activity was assayed by hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONGP)[1] and expressed in Miller Units (MU) [14]. The membrane NADH and succinate dehydrogenase activities, were monitored at 570 nm by the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as an artificial electron acceptor [15]. Reactions were performed at 37 °C with appropriate membrane suspensions (~20 μg protein $mL^{-1}$) in 50 mM phosphate buffer pH 7.5 containing 50 μg $mL^{-1}$ MTT, 3 mM KCN, and 0.1 mM NADH or 10 mM succinate as substrate, respectively. Catalase activity was measured by the decrease in the absorbance at 240 nm of hydrogen peroxide [16] in cell-free extracts prepared by sonication [13]. The initial rate of decomposition of 20 mM $H_2O_2$, at 25 °C was determined using a molar extinction coefficient $43.6 \times 10^{-3}$ $mM^{-1}$ $cm^{-1}$. Catalase specific activity was defined as μmol $H_2O_2$ decomposed $min^{-1}$ mg $protein^{-1}$.

*Measurement of intracellular oxidation level*

Aliquots of cells growing aerobically were washed and resuspended in 50 mM sodium phosphate buffer pH 7, then 10 μM 2′,7′-dichlorofluorescein diacetate ($H_2$DCFDA, the oxidant-sensitive probe dissolved in dimethyl sulfoxide) was added and incubated for 30 min [17]. After incubation, the cells were washed, resuspended and sonicated in the same buffer. Then the fluorescence intensity was measured using ISS-PCI spectrofluorometer (Champaign, IL, USA) (excitation, 490 nm; emission, 519 nm). The fluorescence intensity was normalized by protein concentration and the results were expressed as a percentage of values obtained in exponential phase cells for each medium.

*Measurement of thiobarbituric acid-reactive substances (TBARS)*

TBARS were determined in cell extracts as described by Rice-Evans et al. [18]. Briefly, 1 mL of cell suspension was precipitated with 1 mL of 20% TCA (w/v), centrifuged and the supernatant was mixed with 2 mL of a saturated solution containing thiobarbituric acid in 0.1 M HCl and 10 mM butylated hydroxytoluene. The sample was then heated for 60 min at 100 °C. A 1.5 mL aliquot was then removed, chilled and mixed with 1.5 mL of butanol. The organic fraction was removed and absorbance at 535 nm was measured. TBARS content (expressed in nmol mg $protein^{-1}$) was determined using a molar extinction coefficient 156 $mM^{-1}$ $cm^{-1}$ [18,19].

*Measurement of protein carbonyl content*

The content of carbonyl groups in proteins was evaluated with dinitrophenylhydrazine (DNPH) [20]. Cells extracts were treated with 10 mM DNPH prepared in 2 M HCl, the mixture was incubated for 1 h at room temperature with vortexing every 10–15 min. Proteins were precipitated by adding 20% trichloroacetic acid (TCA) and then sedimented by centrifugation. The pellet was washed at least three times with an ethanol/ethyl acetate mixture (1/1) to remove any unreacted DNPH and redissolved at 37 °C with 6 M guanidine hydrochloride in 5% (v/v) phosphoric acid and absorbance at 370 nm was measured. Carbonyl content was expressed in μmol mg $protein^{-1}$ using a molar extinction coefficient of 22 $mM^{-1}$ $cm^{-1}$ [19].

*Determination of cellular tolerance to $H_2O_2$*

Cells grown in MT or MT + P for 48 h were further challenged to different $H_2O_2$ concentrations for 1 h at 37 °C with shaking. During the treatment, the tolerance was evaluated by viability, counting the CFU on LB-agar plates after 24 h at 37°C. The percentage of survival was calculated as (CFU of hydrogen peroxide treated culture/CFU of untreated culture) × 100. Sensitivity to the treatment was also determined by spotting 1/10 serial dilutions of bacterial suspensions on LB-agar plates.

*Protein determination*

Protein concentration was determined by the method of Lowry et al. [21].

## Results

*Unusual respiratory genes expression in the stationary phase*

We have previously reported that *ndh* gene expression was maintained in the stationary phase when cells were grown in media containing >37 mM phosphate (e.g. MT + P medium) [3]. In order to study whether other respiratory chain genes were expressed in a phosphate-dependent way, some chromosomal transcriptional fusions were assayed. The *nuoAB* (coding NDH-1), *sdhC* (succinate dehydrogenase), *ubiC* (chorismate pyruvate-lyase) and *cydA* (cytochrome *bd*) genes had similar expression profiles to *ndh* during the growth (Fig. 1). In MT + P, a high expression was observed for at least 72 h of culture. In sufficient phosphate medium (MT, 2 mM), these genes expression became negligible at 48 h. In agreement with that, membranes NDH-2 and succinate dehydrogenase activities of MT + P cells were maintained for at least 72 h (Table 2). However, these activities in membranes of MT cells were negligible at 48 h. From the studied genes, only *cyoA* (cytochrome *bo*) was not expressed during stationary phase in MT + P (not shown).

*Low oxidative stress and damage in the stationary phase mediated by a critical phosphate concentration*

Stationary phase cells grown in MT + P present lower NADH/$NAD^+$ ratio (similar to exponential) than cells grown in MT [3]. In MT + P cells, the viability decreased only one order of magnitude at 96 h (from $10^9$ to $10^8$ CFU $mL^{-1}$) whereas in MT viability was reduced three orders of magnitude (from $10^9$ to $10^6$ CFU $mL^{-1}$). The

[1] *Abbreviations used:* ONGP, *o*-nitrophenyl-β-D-galactopyranoside; $H_2$DCFDA, 2-7-dichlorofluorescein diacetate; TBARS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid.

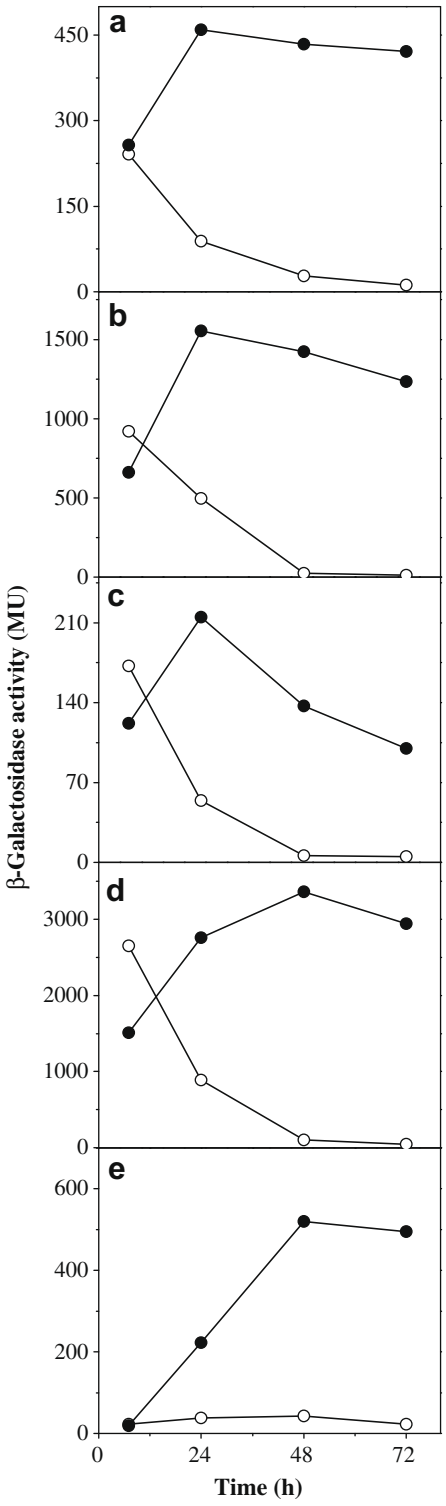


**Fig. 1.** Expression of respiratory chain genes. *ndh* (a), *sdhC* (b), *nuoAB* (c), *ubiC* (d), and *cydA* (e) genes expression was measured in strains with the corresponding chromosomal fusion as β-galactosidase activity at the indicated times of growth in MT (open symbols) or MT + P (closed symbols). Data are representative of at least five separate experiments performed in duplicate.

relationship between cell viability and the intracellular oxidation level (ROS) was studied. The ROS formation was determined using the fluorescent probe $H_2$DCFDA. In MT cells at 72 h, ROS increased 6-fold with respect to exponential phase whereas in MT + P cells this production was not observed (Fig. 2a). In concordance with ROS production, in MT cells TBARS level was around 5-fold higher than in MT + P in the stationary phase (Fig. 2b). It is important to mention that a marked increased in ROS and TBARS were observed as soon as 24 h in MT. The generation of carbonyl groups in proteins, a consequence of ROS and TBARS, was used as a marker to monitor protein oxidative damage. The protein carbonylation content was similar in both media up to 48 h, but a significant increase was only observed in MT cells at 72 h (Fig. 2c).

*Unusual defense genes expression in the stationary phase*

The low oxidative damage let us to think that the high phosphate concentration in culture media would induce the antioxidant defense genes expression against oxidative stress (generated mainly by respiratory chain). The expression of defense genes, *sodA* (manganese-containing superoxide dismutase), *katG* (HPI catalase) and *ahpC* (alkyl hydroperoxide reductase), were assayed in the different phosphate concentration media (Fig. 3). Expression of *katG* and *ahpC* genes in MT + P stationary cells was elevated and invariable for 72 h of culture. However, expression in MT decreased at 24 h and it became negligible at 48 h. In MT + P cells, catalase activity was constant through stationary phase whereas in MT cells it was decreasing during this phase (Table 2). On the contrary, the *sodA* expression diminished gradually in the two tested media (Fig. 3c).

*Protection against exogenous oxidative stress mediated by a critical phosphate concentration*

The ability to tolerate the exogenous oxidative stress generated by $H_2O_2$ was studied in both MT and MT + P cells grown for 48 h (Fig. 4). MT + P cells were highly tolerant to exogenous peroxide since the high viability was maintained in the presence of 10 mM $H_2O_2$ while MT cells were sensitive to this treatment. It is important to mention that in MT cells an elevated loss of viability was observed at 20 min of incubation with $H_2O_2$ (Fig. 4a). Serial dilutions of control and treated MT + P cultures showed the same growth in LB-agar plate whereas MT cultures treated with peroxide presented a reduced growth from the first dilution (Fig. 4b). It should be noted that at 48 h of growth, the $OD_{560nm}$ (2.5 ± 0.2) was similar for both cultures but the viability on plates was lower in MT (see above and control spots in Fig. 4b).

## Discussion

Here we proved that the presence of at least 37 mM phosphate in the culture media, maintains in late stationary phase *nuoAB*, *sdhC*, *ubiC*, and *cydA* expressions in agreement with the maintenance of *ndh* expression previously showed [3], whereas *cyoA* gene was not expressed under these conditions. As well, NDH-2 and succinate dehydrogenase activities were maintained under these conditions.

The respiratory chain activity is commonly associated to the generation of oxidant species thus these genes are down-regulated in response to growth arrest to reduce the ROS formation [2]. ROS include both, oxygen radicals (e.g. superoxide anion radical $O_2^{\cdot-}$ and hydroxyl radical OH$^{\cdot}$), and nonradicals compounds (e.g. $H_2O_2$) [22]. When biological membranes are exposed to hydrogen peroxide or to alkoxyl radicals trigger the fragmentation of lipids generating aldehydes (then, TBARS levels increase) and leading to the impairment of membrane function [23,19]. On the other hand, protein oxidation as carbonyls accumulation has been associated to aldehydes generated during lipid peroxidation [24]. Thus, carbonyls in proteins are relatively difficult to induce compared to other oxidative modifications [25]. In spite of the maintenance of

**Table 2**
Enzymatic activities.

| Time of culture (h) | NADH dehydrogenase-2 (nmol MTT $min^{-1}$ mg $protein^{-1}$) | | Succinate dehydrogenase (nmol MTT $min^{-1}$ mg $protein^{-1}$) | | Catalase (μmol $H_2O_2$ $min^{-1}$ mg $protein^{-1}$) | |
|---|---|---|---|---|---|---|
| | MT | MT + P | MT | MT + P | MT | MT + P |
| 7 | 1251 ± 42 | 1284 ± 65 | 654 ± 24 | 602 ± 22 | 15 ± 2 | 16 ± 3 |
| 24 | 257 ± 21 | 1241 ± 21 | 285 ± 19 | 652 ± 31 | 26 ± 4 | 27 ± 2 |
| 48 | 102 ± 14 | 1297 ± 54 | 71 ± 16 | 624 ± 28 | 17 ± 2 | 26 ± 1 |
| 72 | ND[a] | 1179 ± 39 | ND | 598 ± 19 | 15 ± 3 | 27 ± 4 |

*Note:* values are average ± SD.
[a] ND, not detectable.

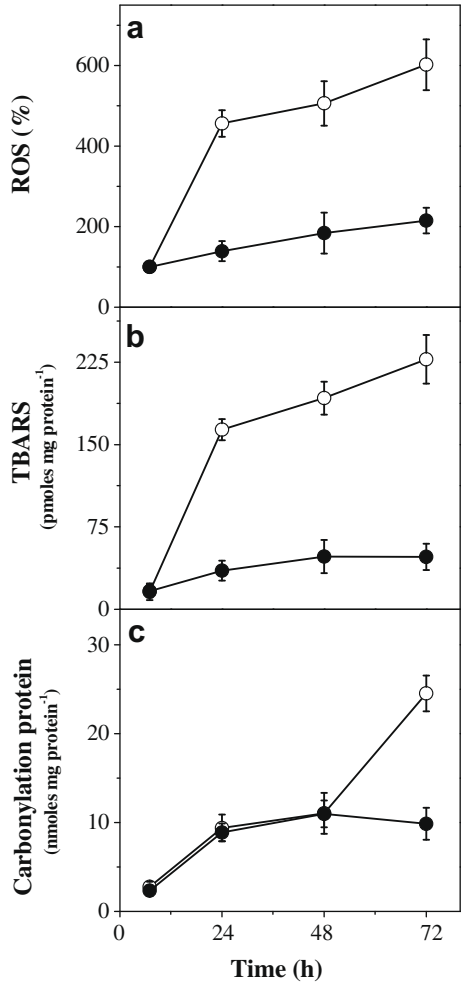


**Fig. 2.** Oxidative stress and damage in MC4100 cells. Intracellular ROS (a), TBARS (b) and protein carbonylation (c) were determined at the indicated times of growth in MT (open symbols) or MT + P (closed symbols). ROS level was expressed as percentages of the values at 7 h for each medium. Data are the average ± SD of four independent experiments.

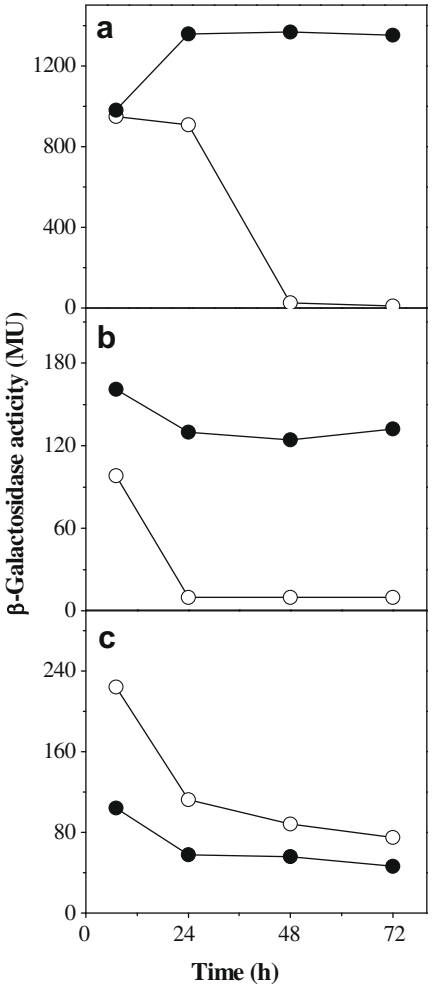


**Fig. 3.** Expression of oxidative stress defense genes. *ahpC* (a), *katG* (b), and *sodA* (c) genes expression was measured in strains with the corresponding chromosomal fusion as β-galactosidase activity at the indicated times of growth in MT (open symbols) or MT + P (closed symbols). Data are representative of at least three separate experiments performed in duplicate.

respiratory genes expression, our results showed that the growth in MT + P avoids the increase of intracellular oxidation level at least for 72 h. As was expected for stationary phase, the oxidative damage to lipids and proteins in MT cells was elevated. In these cells the delay observed between TBARS and proteins oxidation may be due to the necessity of a peroxidized lipids accumulation.

*Escherichia coli* has developed a defense system including superoxide dismutase, catalase and alkyl hydroperoxide reductase that act as primary scavengers of endogenous superoxide and peroxides [26,27,9]. We studied the presence of defense enzymes, to be able to understand the fitness state in which is the cells grown in MT + P. The *katG* and *ahpC* expression and catalase activity were maintained through the stationary phase. In contrast, *sodA* level in stationary phase was similar in the two phosphate concentrations media, maybe by a slow rate of superoxide generation which undergoes a spontaneous dismutation to peroxide.

The high viability and tolerance to $H_2O_2$ when cells were grown in MT + P could be explained by an improved resistance to oxida-

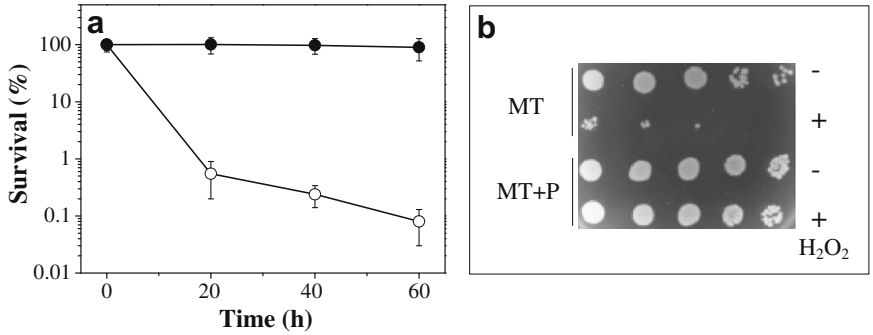


**Fig. 4.** Tolerance to exogenous $H_2O_2$. MC4100 cells grown for 48 h in MT or MT + P were challenged with 10 mM $H_2O_2$. (a) Aliquots were taken each 20 min for 1 h to measure viability. Relative survival was calculated as percentage of parallel controls of each culture without $H_2O_2$. Data from MT (open symbols) or MT + P (closed symbols) are expressed as average ± SD of four independent experiments. (b) After 1 h of assay, serial dilutions of control or treated cultures were spotted. Experiments were repeated four times with similar results.

tive stress. One reason could be that the maintained respiratory chain activity, especially NDH-2 activity [3], triggers the formation of ROS and consequently induces antioxidant defenses. Such mild stress may promote a better adaptation to ROS, explaining the decrease in oxidative damage. In addition, other respiratory components such as ubiquinone and cytochrome *bd* were proposed as antioxidants [28,29].

Taking together, the critical phosphate concentration in the culture media maintains in stationary phase a cellular fitness similar to late exponential phase, showing an improved protection against endogenous and exogenous oxidative stress.

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