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Oxidative damage induced by H₂O₂ reveals SOS adaptive transcriptional response of *Dietzia cinnamea* strain P4

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Abstract

The oxidative stress response of the highly resistant actinomycete *Dietzia cinnamea* P4 after treatment with hydrogen peroxide (H₂O₂) was assessed in order to depict the possible mechanisms underlying its intrinsic high resistance to DNA damaging agents. We used transcriptional profiling to monitor the magnitude and kinetics of changes in the mRNA levels after exposure to different concentrations of H₂O₂ at 10 min and 1 h following the addition of the stressor. Catalase and superoxide dismutase genes were induced in different ways, according to the condition applied. Moreover, alkyl hydroperoxide reductase *ahpCF*, thiol peroxidase, thioredoxin and glutathione genes were upregulated in the presence of H₂O₂. Expression of peroxidase genes was not detected during the experiment. Overall results point to an actinomycete strain endowed with a set of enzymatic defenses against oxidative stress and with the main genes belonging to a functional SOS system (*lexA*, *recA*, *uvrD*), including suppression of *lexA* repressor, concomitantly to *recA* and *uvrD* gene upregulation upon H₂O₂ challenge.

Keywords *Dietzia cinnamea* · Hydrogen peroxide · Catalase gene · Superoxide dismutase gene · SOS system

Introduction

Reactive oxygen species (ROS) derived from the reduction of oxygen to superoxide (O⁻²), hydrogen peroxide (H₂O₂) or the hydroxyl radical (·OH) are highly reactive and able to efficiently damage nucleic acids, membrane lipids and proteins (Farr and Kogoma 1991; Imlay 2002). Among these compounds, H₂O₂ is continuously formed in the aerobically growing cell by the autoxidation of redox enzymes. It can also enter from the environment, where it can be generated both by chemical processes and by the deliberate action of

competing organisms. Because H₂O₂ is acutely toxic, bacteria elaborate scavenging enzymes to keep the intracellular concentrations at sub lethal levels (Mishra and Imlay 2012).

In bacteria, oxidative stress levels are sensed by specific transcriptional regulators that are able to activate a set of enzymes involved in defense mechanisms when the ROS concentration exceeds these critical levels (Zheng and Storz 2000; Pomposiello and Demple 2002). The cell defenses involve the detoxification of the harmful agents by catalase and superoxide enzymes, protection of macromolecules, e.g. DNA by DNA-binding proteins, and repair and removal of damaged molecules by alkyl hydroperoxide reductase and thiol peroxidase enzymes. The latter are controlled by specific transcriptional regulators, such as LexA/RecA, which are member of the SOS system, OxyR, Spx, OhrR and PerR (Farr and Kogoma 1991; Zuber 2009).

The actinomycete *Dietzia cinnamea* P4 is a Gram-positive bacterium having a genome with high G + C content. The species is widely distributed in nature, as it has been isolated from soil, deep-sea sediment, soda lakes, reed rhizomes, skin and the intestinal tracts of marine fish (Yassin et al. 2006). *D. cinnamea* strain P4 was isolated in a study on the diversity of hydrocarbon degraders in a tropical rainforest soil in Brazil, with no history of oil contamination (Evans et al. 2004). Information from the *D. cinnamea* P4 genome

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sequences, recently published, suggests that the protection mechanisms against oxidative stresses are in a complex system encompassing diverse enzymes. In addition, results from previous study suggested that *D. cinnamea* P4 is quite resistant to stressors such as H_2O_2 (Procópio et al. 2012). The identification of well-known enzymes involved in the defense against reactive oxygen species (ROS) and hydrogen peroxide sensor and response regulator proteins indicates the presence of a coordinated protection mechanism in *D. cinnamea* P4.

In this study, we performed a comprehensive transcriptional analysis of selected presumed protective genes in response to high level of H_2O_2 , in particular genes of the SOS system. The results represent a first step towards a better understanding of the function of the defense system adopted by *D. cinnamea* P4 to oxidative stress conditions.

Materials and methods

Growth, stress conditions and survival experiments

A culture (pre-inoculum) of 10 ml was prepared from an isolated colony of *D. cinnamea* P4, previously growth in Luria–Bertani agar (LB agar: tryptone 1%, NaCl 0.5%, yeast extract 0.5%, agar 1.5%, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) or in LB broth at 28 °C under agitation. After the culture reached an OD₆₆₀ of 1.0 (begin of exponential phase), it was centrifuged at 8.000×g and the pellet resuspended in 10 ml of phosphate buffered saline (PBS: NaCl 0.08%, KCl 0.02%, Na_2HPO_4 0.14%, KH_2PO_4 0.024%, pH 7.4). The culture was split into two aliquots, of which one was left untreated and the other was treated with hydrogen peroxide to a final concentration of 100 mM during 10 min and 1 h, both under agitation. For survival tests, H_2O_2 was used at two different final concentrations: 50 mM and 100 mM. Control cells were processed as treated cells in terms of cell survival and gene expression measurements. Gene expression was calculated considering the ratio of treated and untreated cells at 0 time or at the corresponding time, always considering the highest constitutive control value. Following the treatments, samples from bacterial suspensions were diluted and 0.1 ml aliquots plated onto LB agar plates. After overnight incubation at 28 °C for 5 days, the colony-forming units were counted and data were processed to provide arithmetic means of replicate systems. Surviving fractions in the treated populations were determined by using the ratio between the number of viable cells before and after each treatment.

Quantitative real-time PCR

In order to analyze the mRNA transcriptional levels under hydrogen peroxide stress, we isolated the total RNA from the *D. cinnamea* P4 cultures, either untreated or treated with 100 mM H_2O_2 treatment, as described above. The RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol, with the addition of one initial step of liquid nitrogen freezing for cellular lysis. Then, the total RNAs were treated with DNase I (Promega, SP, Brazil). The integrity of the RNAs was checked by agarose gel electrophoresis and the yield was estimated using a Nanodrop UV spectrometer (Thermo Scientific, Wilmington, DE, USA). About 100 ng of RNA from each sample were used for cDNA synthesis using random hexamers by the ImProm-I Reverse Transcription System (Promega) according to the manufacturer's protocol. The yield of cDNAs was estimated by Nanodrop UV spectrometer and their concentrations adjusted to about 1 ng. For real-time quantitative RT-PCR, 5 µl of cDNA was mixed with LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche, IN, USA) mix, 0.5 µg of forward and reverse primers of each gene to be analyzed in a final volume of 20 µl in three replicates. In addition, no-template controls, in three replicates for each gene to be analyzed, were included. Expression of the 16S rRNA gene was used as the reference gene in order to normalize the expression of the tested genes. Then, these were correlated to the amounts of corresponding transcripts in the non-stressed grown cells. The real-time quantitative RT-PCR reaction was carried out with the LightCycler 1.5 (Roche), following the protocol: one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 10 s, followed by 60 °C for 10 s, 72 °C for 25 s. The specificity of the amplification was verified at the end of the PCR run through uses of the LightCycler 4.1 software, and the normalized relative fold change in mRNA levels were calculated for the gene of interest in each sample using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Sequence analysis and phylogenetic studies

Alignments of the deduced amino acid sequences of the selected putative proteins of strain P4 with those of related organisms were performed by Clustal-W (Thompson et al. 2002). Phylogenetic analyses of the deduced amino acid sequences were inferred using the maximum-parsimony method and MEGA5 software (Tamura et al. 2011). Bootstrap analysis was used to evaluate the tree topology by 1000 re-samplings.

Results

Survival of *D. cinnamea* P4 challenged with H₂O₂

To investigate the survival upon oxidative stress, the strain P4 was treated with 50 mM and 100 mM H₂O₂ during 30 min, 1 and 2 h. Analysis of the colony-forming unit

Fig. 1 Effect of different concentrations of H₂O₂ on the survival of *Dietzia cinnamea* P4 strain: control (untreated, black square); gray square (50 mM) and light gray (100 mM)

numbers showed P4 to be quite resistant to the stressor. The different H₂O₂ concentrations had little effect on the P4 CFU numbers after 30 min or 1 h of exposure, while lethal dose 10% (LD₁₀) was only attained after at least 2 h exposure with 50 mM H₂O₂ or more (100 mM) (Fig. 1).

Genes involved in the response to oxidative stress

The in silico analysis of the *D. cinnamea* P4 genome revealed the presence of three putative catalase genes (Table 1), three of these were identified as *katE* (ZP_08022106, ZP_08022939, ZP_08025226), designed in this study as *katE1*, *katE2* and *katE3*, and one possible *katMn* (ZP_08022130). These genes are localized in different positions on the chromosome and do not seem to share genetic links. Phylogenetic analysis of the putative P4 catalases and catalases of other related organisms showed that the four putative sequences are distinct from each other (Fig. 2). In order to estimate the involvement of each catalase in *D. cinnamea* P4 oxidative stress response, we evaluated the effect of the sublethal H₂O₂ concentration (100 mM) defined previously on the expression of *katE1*, *katE2*, *katE3* and *katMn* by real time PCR (See Material and methods). After 10 min, *katE1* was overexpressed 1.91-fold, whereas

Table 1 Levels of gene expression related to response to exposure to H₂O₂ and the SOS system of strain *D. cinnamea* P4 after 10 min and 1 h

Access number	Gene	Description	100 mM H ₂ O ₂ (increase)		100 mM H ₂ O ₂ (repression)	
			10 min	1 h	10 min	1 h
ZP_08023119	<i>ahpF</i> ^a	Alkyl hydroperoxide reductase	ND	3.65 (0.58)	ND	ND
ZP_08023120	<i>ahpC</i>	Alkyl hydroperoxide reductase	ND	207.94 (0.06)	ND	ND
ZP_08024224	<i>tpx</i>	Thiol peroxidase	ND	3.71 (0.77)	ND	ND
ZP_08024195	–	Glutathione S-transferase	ND	15.63 (1.21)	ND	ND
ZP_08023504	<i>trxB</i>	Thioredoxin reductase	ND	8.98 (3.06)	ND	ND
ZP_08024053	<i>trxA</i>	Thioredoxin	ND	33.51 (0.96)	256.0 (0.96)	ND
ZP_08024106	<i>gpx1</i>	Glutathione peroxidase	ND	2.52 (1.31)	ND	ND
ZP_08023200	<i>gpx2</i>	Glutathione peroxidase	ND	119.53 (1.49)	ND	ND
ZP_08021853	<i>sodC</i>	Cu/Zn superoxide dismutase	ND	5.79 (0.85)	ND	ND
ZP_08023526	<i>sodA</i>	Superoxide dismutase	14.25 (0.55)	8.77 (1.04)	ND	ND
ZP_08022106	<i>katE1</i>	Catalase	1.91 (1.00)	13.93 (0.67)	ND	ND
ZP_08022939	<i>katE2</i>	Catalase	36.76 (0.06)	9.40 (1.64)	ND	ND
ZP_08025226	<i>katE3</i>	Catalase	ND	ND	ND	ND
ZP_08022130	<i>KatMn</i>	Mn catalase	32.00 (0.36)	8.19 (0.26)	ND	ND
ZP_08024268	<i>alkD</i>	DNA alkylation repair enzyme	8.00 (0.55)	8.98 (4.04)	ND	ND
ZP_08023084	–	Peroxidase	ND	ND	ND	ND
ZP_08022314	<i>lexA</i>	LexA repressor	ND	ND	58.3 (2.31)	128.0 (0.72)
ZP_08022556	<i>recA</i>	Recombinase A	2.9 (0.60)	ND	ND	ND
ZP_08025300	<i>uvrA</i>	Excision repair protein	ND	ND	ND	ND
ZP_08022138	<i>uvrD</i>	Excision repair protein	2.7 (0.99)	3.03 (0.55)	ND	ND

Standard errors of mean are indicated in parentheses

ND no detectable signal

^a*ahpF* gene showed repression of 1.2-fold after 1 h of exposition, compared to time zero

Fig. 2 Phylogenetic tree of *Dietzia cinnamea* strain P4 catalase with those from different organisms. The tree was constructed based on the neighbor-joining method. Bootstrap analyses were performed with 1000 repetitions. The scale bar represents the estimated sequence divergence

katE2 exhibited an increase of 36.76-fold and *katMn* 32.0-fold. After 1 h, *katE1* showed an increase to 13.93, while *katE2* and *KatMn* showed 9.40- and 8.19-fold, respectively. The expression of *katE3* transcript was not detected at any time during both treatments with H_2O_2 , and we could not detect any expression signal of the putative peroxidase of P4 in both H_2O_2 treatments (Table 1).

Two putative alkyl hydroperoxide reductase genes, *ahpC* (ZP_08023120) and *ahpF* (ZP_08023119) are present in the P4 genome in the same chromosome localization, in tandem. The analysis of transcriptional levels of P4' *ahpCF* showed that both putative genes were not expressed when strain P4 was treated with H_2O_2 (100 mM) during the first 10 min. However, after 1 h of exposure to these conditions, the *ahpC* transcript was markedly increased by 207.94-fold, while the transcriptional increase of the *ahpF* gene was less pronounced, i.e. 3.65-fold (Table 1). Two putative thioredoxins, *trxA* (ZP_08024053) and thioredoxin reductase, *trxB* (ZP_08023504), also were differentially expressed in the presence of 100 mM H_2O_2 . Although no signal has been detected for both genes after 10 min of treatment with H_2O_2 , after 1 h the results show an increase in transcriptional levels of *trxA* by 33.51-fold and of *trxB* by 8.98-fold. In addition, the putative thiol peroxidase *tpx* (ZP_08024224) showed enhanced expression only after 1 h of treatment, at about 3.7-fold (Table 1). Thioredoxins and thiol peroxidase,

together with NADH-dependent butanol dehydrogenase, belong to the putative Spx-regulon, which has antioxidant functions (Zeller et al. 2006). The TrxA and TrxB proteins have a function in the defense against oxidative stress by directly detoxifying H_2O_2 (Spector et al. 1988), by acting as hydrogen donors for peroxidases (Chae and Rhee 1994) or by reactivating oxidatively damaged proteins, while Tpx enzymes probably act in peroxide detoxification (Mostertz et al. 2004).

The *D. cinnamea* strain P4 has two different presumed superoxide dismutase genes in the genome, one Mn superoxide dismutase, *sodA* (ZP_08023526) and one Cu/Zn superoxide dismutase, *sodC* (ZP_08021853). The transcriptional profile of putative *sodA* gene in 100 mM H_2O_2 showed an increase in mRNA levels (14.25-fold) after 10 min, but exhibited a decrease to 8.77-fold at 1 h, when compared with the untreated. On the other hand, the expression analysis of *sodC* did not yield any signal after 10 min of treatment and showed an increase to 5.79-fold after 1 h (Table 1). The induction of expression of superoxide dismutase enzymes in bacteria under conditions of oxidative stress has been well described in the scientific literature. SodAB enzymes are currently described as being upregulated in the presence of hydrogen peroxide (Grifantini et al. 2011; Schroeter et al. 2011). The results were not surprising, as these enzymes may contribute to peroxide resistance in streptococci, as

The annotation of the strain P4 genome also revealed the presence of two putative glutathione peroxidase genes

(ZP_08024106 and ZP_08023200), identified here as *gpx1* and *gpx2*, respectively, next to a single glutathione S-transferase (ZP_08024195). Although the results position both genes in distinct groups (Fig. 3a), they showed extensive similarity. Importantly, the catalytic triad, Cys₄₁, Gln₇₁ and Trp₁₃₁, was present in their sequences (Fig. 3b) (Maiorino et al. 1995). The expression analysis did not exhibit any signal of expression during the first 10 min of H₂O₂ treatment. However, after 1 h in the presence of H₂O₂ (100 mM), the *gpx1* transcript showed a moderate expression of 2.52-fold. The expression levels of the *gpx2* gene showed a significant increase to 119.53-fold, while the putative glutathione S-transferase exhibited an elevated expression of 15.63-fold

Fig. 3 Sequence analysis of glutathione peroxidases of *Dietzia cinnamea* strain P4 with those from different organisms. **a** The tree was constructed based on the neighbor-joining method. Bootstrap analyses were performed with 1000 repetitions. The scale bar represents the estimated sequence divergence. The label “bullet” indicates P4’ORF. **b** Alignments between deduced amino acid residues of P4’glutathione peroxidase and related organisms. The asterisk indicates the conserved catalytic triad Cys₄₁, Gln₇₁ and Trp₁₃₁. Sequences are labeled using the NCBI code. The different colors represent the classes of the amino acids based on the characteristic of the side chain

(Table 1). Similar to the SodC protein, the glutathione peroxidase is described as an agent of defense against deleterious effects of various oxidants inside host cells (Wu et al. 2005; Arenas et al. 2010), which is in agreement with our results of differential expression under conditions of oxidative stress.

SOS regulon

Analysis of the P4 genome further confirmed the presence of putative genes encoding enzymes of the SOS system, consisting of the putative LexA repressor, *lexA* (ZP_08022314), recombinase A, *recA* (ZP_08022556) and the excision repair proteins, *uvrAD* (ZP_08025300, ZP_08022138). However, no putative sequences were found for the error-prone polymerases PolIV(*dinB*) and Pol IV (*umuD'C*), which are also controlled by the SOS response in *Escherichia coli* (Fuchs et al. 2004). During the treatment of strain P4 with H₂O₂ (100 mM), the *lexA* gene was repressed by 58.3-fold at 10 min and 128.0-fold after 1 h. The *recA* expression was detected only after 10 min of exposure to H₂O₂, by 2.9-fold. Concerning the excision repair proteins, *uvrA* and *uvrD*, only the *uvrD* gene showed increased expression, 2.7- and 3.03-fold at 10 min and 1 h, respectively. The regulatory LexA and RecA proteins have control over genes belonging to the SOS regulon (Miller et al. 1996). This system controls the cellular response to DNA damage through genes that are transcriptionally induced by such damage (Love et al. 1985). Surprisingly, the *lexA* gene of strain P4 was not induced under the conditions of oxidative stress, but strongly suppressed at both times (Table 1), while the *recA* gene was shown to be induced only after the first 10 min. This may indicate that, in our stressing conditions, low amounts of activated RecA protein is sufficient to inactivate just few LexA proteins repressing genes, such as *uvrD*, but are not sufficient enough to trigger a full SOS response. This justifies the lack of LexA and UvrA overexpression, even in the presence of 100 mM H₂O₂. This partial induction of the SOS response may be also explained by the concomitant overexpression of other enzymatic defenses such as alkyl hydroperoxide reductase and catalases.

Discussion

In a previously study Procópio et al. (2012) demonstrated that strain P4 to be more resistant than the *E. coli* K12A15 type strains to chemical agent tested and also extremely refractory to induced mutagenesis ($p < 0.05$), indicating that it is endowed with efficient DNA repair or tolerance mechanisms, particularly when considering its resistance to H₂O₂ and UV radiation.

Present results point to selected catalase and peroxidase enzymes as the most obvious candidates for detoxifying H₂O₂ and UV radiation effects. *In silico* results are similar to those presented by Bidaud et al. (2012), who also reported the presence of four catalase genes, three heme catalase genes and a single manganese catalase gene in the *Rhodococcus equi* genome. The four catalases analyzed in this study showed different behavior under the conditions described. Only *katE1* showed a time-dependent response, with an increase in expression levels over time, while *katE2* and *katMn* had higher transcriptional levels during the first 10 min and a decrease after 1 h. Finally, *katE3* and peroxidase genes did not show any differential gene expression. Differential expression of multiple catalases under different stress conditions was also described in *Aspergillus nidulans* (Kawasaki and Aguirre 2001). In addition, the transcriptional response of *Burkholderia cenocepacia* strain J2315 also showed differential expression levels and unexpected results among the catalases genes, with the *katB* and *katA* genes exhibiting no significant expression in their levels (Peeters et al. 2010). The *katE1*, *katE2* and *katE3* sequences share conserved catalytic residues and metal ligand amino acids, which are present in monofunctional heme catalases (Loewen et al. 2000). The *katMn* gene product showed the conserved catalytic residues and metal ligand amino acids of members of the nonheme catalases, which are characterized by the presence of manganese ions, instead of ferric heme in their active site (Amo et al. 2002). A single peroxidase gene was detected in the strain P4 genome (ZP_08023084), which showed similarity with the peroxidase of *Rhodococcus opacus* B4 strain (data not shown). In addition, together with catalases and the peroxidase enzymes, the products of the alkyl hydroperoxide reductase genes are part of a coordinated regulation of genes associated with protection against oxidative stress called the PerR regulon (Grifantini et al. 2011). The results show that the strong induction of the *ahpCF* genes of strain P4, concurrently with catalase genes in hydrogen peroxide stress conditions, is consistent with findings in other scientific papers, which suggest a role of *ahpCF* with catalase and hydroperoxidase in protective and detoxification functions (Helmann et al. 2003; Mostertz et al. 2004; Peeters et al. 2010; Zhou et al. 2010; Schroeter et al. 2011).

These unexpected gene expressions of the SOS regulon of *D. cinnamomea* may denote a distinct evolved strategy to counteract oxidative stress in this gram-positive strain. In P4 case, under our stress conditions, a full SOS was not observed as sufficient expression of catalase and peroxidase enzymes may actually be sufficient to counteract most of oxidatively induced damage. *E. coli* presents bimodal pattern of killing upon H₂O₂ challenge, mode-one at low H₂O₂ doses, in which lesion recovery relies on DNA repair, and mode-two, which occurs at higher H₂O₂ doses, and seems

to independent of DNA repair. In this case, P4 presents its resistance to ROS as high responsive expression of detoxifying enzymes. Thus multiple other cell targets are implied in cell death, but DNA damage would not be crucial (Imlay and Linn 1986). The P4 strain is not at all sensitive to low H_2O_2 concentrations, neither in terms of survival, nor in terms of induced mutagenesis (Procópio et al. 2012 and this work). This suggests that at low H_2O_2 doses DNA damage is minor or even not being produced, probably by the already high constitutive levels of scavenging enzymes involved in H_2O_2 detoxification. These are further induced at high H_2O_2 concentrations, which may account to the high cell survival level, even upon 100 mM H_2O_2 for 2 h treatment. Interestingly, if DNA damage happens to occur, it seems to be undertaken by RecA and UvrD (minorly), as a backup for the ongoing arsenal of H_2O_2 detoxifying enzymes. In fact, classical UvrABC complex does not seem to be induced at these conditions, but rather a recombinational-based tolerance strategy depending on RecA and UvrD gene products. Lestini and Michel (2007) reported that UvrD has multiple functions at inactivated DNA replication forks, and that UvrD is able to counteract deleterious action of RecA protein. This evidence may support the notion that recombinational tolerance, rather DNA repair itself, may undertake eventual DNA damage in an error-free manner. A series of other evidences corroborate this notion (i), the absence putative DNA sequences for error-prone polymerases in *D. cinnamomea* genome; (ii) even *E. coli* displays significant H_2O_2 -induced mutagenesis independently from umuDC (Imlay and Linn 1987), while P4 stain remains refractory to mutagenesis (Procópio et al. 2012) and (iii) LexA is repressed denoting that if a classical SOS system can be induced in P4 stain, it is not fully induced in conditions, therefore, DNA repair is not being highly demanded, however a small but significant amount of double strand breaks generation cannot be discarded, which could be undertaken by RecA and UvrD. Low H_2O_2 concentrations can generate DNA clustered lesions which can ultimately lead to these doubled strand breaks (Sharma et al., 2016 esta referência está no outro arquivo), consistent with CG-rich genomes.

Conclusion

In conclusion, the *D. cinnamomea* P4, which is well adapted to tropical soil, may encounter a wide range of environmental stressors in its natural setting. Strain P4 showed an elaborate molecular mechanism which is probably employed to defend against the stress exerted by H_2O_2 . The transcriptional profiles revealed differential expression of diverse classes of enzymes putatively involved in the stress response, e.g. catalases, superoxide peroxidases, thiol and glutathione oxidases, and enzymes of the SOS system. Our data contribute

to a better understanding of the molecular mechanisms involved in the physiological responses to oxidizing agents present in the ecological niche of a tropical environment. The identification of the set of enzymes employed by strain P4 to make the transition from successful acclimation/resistance to an induction of oxidative stress tolerance will help to shed light on the cumulative effect of environmental stress conditions.

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