


## Minireview

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### Summary

**Coping with oxidative stress originating from oxidizing compounds or reactive oxygen species (ROS), associated with the exposure to agents that cause environmental stresses, is one of the prerequisites for an aerobic lifestyle of *Bacillus* spp. such as *B. subtilis*, *B. cereus* and *B. anthracis*. This minireview highlights novel insights in the primary oxidative stress response caused by oxidizing compounds including hydrogen peroxide and the secondary oxidative stress responses apparent upon exposure to a range of agents and conditions leading to environmental stresses such as antibiotics, heat and acid. Insights in the pathways and damaging radicals involved have been compiled based among others on transcriptome studies, network analyses and fluorescence techniques for detection of ROS at single cell level. Exploitation of the current knowledge for the control of spoilage and pathogenic bacteria is discussed.**

### Introduction

The genus *Bacillus*, belonging to the phylum *Firmicutes*, comprises a diverse group of Gram-positive and Gram-variable bacteria. *Bacillus* spp. are rod-shaped aerobic or facultative anaerobic organisms that can form endospores. Vegetative cells and spores of *Bacillus* spp. can be isolated from a wide range of environments (Claus and Berkeley, 1986). The most well-known and best-studied members of the *Bacillus* genus are *Bacillus subtilis*, *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis*. *Bacillus subtilis* is widely used as model

organism for Gram-positive bacteria and besides being a major food spoilage organism, it is used in food fermentations (Murooka and Yamshita, 2008) and as a probiotic (Sorokulova *et al.*, 2008). *Bacillus cereus* is a notorious food-borne pathogen that causes two distinct types of diseases: emesis and diarrhoea (Stenfors Arnesen *et al.*, 2008). *Bacillus thuringiensis* is widely applied as biopesticide and it is also known to cause various human diseases (Ghelardi *et al.*, 2007). *Bacillus anthracis* is well known, because it causes the mammalian and human disease anthrax that can be present in three clinical forms: cutaneous, pulmonary and gastrointestinal (Kolsto *et al.*, 2009).

*Bacillus* spp. may encounter oxidative agents and conditions in a range of settings. To prevent spoilage and (re)contamination of food by *Bacillus* spp., food-processing equipment is regularly cleaned and disinfected with oxidative compounds such as hydrogen peroxide and sodium hypochlorite (Block, 2000). Furthermore, *B. anthracis* encounters oxidizing compounds, including superoxide (O<sub>2</sub><sup>-</sup>), hydrogen peroxide and nitric oxide (NO), upon germination and growth inside macrophages (MacMicking *et al.*, 1997; Fang, 2004; Shatalin *et al.*, 2008). *Bacillus* spp. that encounter oxidizing conditions react by inducing an oxidative stress response, including upregulation of catalases and thioredoxins. The response of *Bacillus* spp. to oxidative agents such as hydrogen peroxide, hypochlorite, paraquat, diamide and peracetic acid is addressed in this minireview as the primary oxidative stress response. In addition, it has been noted that exposure to heat, acid and high salt concentrations, which are also widely used to prevent food spoilage and contamination by bacteria including *Bacillus* spp., also cause an oxidative stress response. This so-called secondary oxidative stress response is activated when bacterial cells are exposed to harsh, unfavourable conditions while the cells are still actively respiring. Recent studies showed the impact of secondary oxidative stresses and that it was suggested to act as a general mechanism in cellular death when bacteria are exposed to toxic agents and conditions such as bactericidal antibiotics, heat and acid (Kohanski *et al.*, 2007; Mols *et al.*, 2009; 2010).

The aim of this minireview is to provide an overview in the responses of *Bacillus* spp. to primary and secondary

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oxidative stress and to give insight in the differences between these primary and secondary oxidative stress responses observed in recent studies. Furthermore, the importance and implications for the secondary oxidative stress responses in the general stress response and mechanisms of cellular death are discussed.

### Primary oxidative stress responses

The response of *Bacillus* spp. upon exposure to chemicals that are oxidizing and used or studied because of their oxidizing chemical properties is briefly discussed below. For a more detailed overview in the different oxidative stimulons we recommend recent reviews by Zuber (2009), Duarte and Latour (2010), Faulkner and Helmann (2011) and Antelmann and Helmann (2011).

#### *Response of Bacillus to hydrogen peroxide*

Hydrogen peroxide is often used as a model chemical for experiments designed to study oxidative stresses. It is inevitably formed as a by-product of oxidative phosphorylation and other reactions. The antimicrobial properties of hydrogen peroxide provide a first line of defence against invading microbes along wound sites in plants and in mammal macrophages. Furthermore, it is used in households and industries as bleaching agent and antibacterial disinfectant.

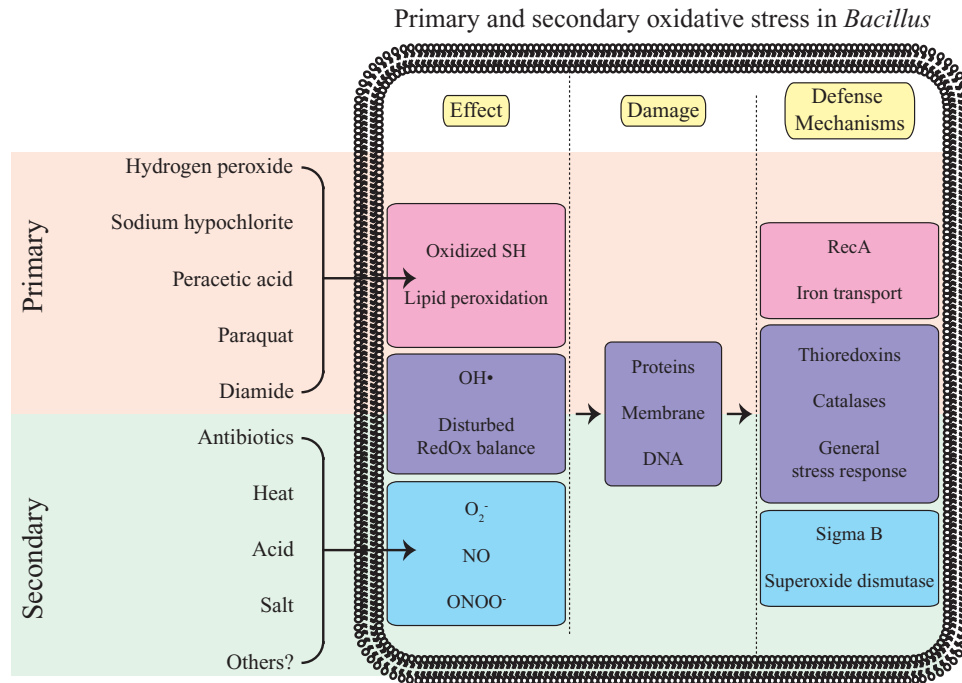
Upon exposure of *B. cereus* to mild and lethal hydrogen peroxide concentrations the expression of numerous genes was affected (Ceragioli *et al.*, 2010), including genes involved in the common response to general stresses. For example, genes involved in protein protection, refolding and turnover including *groES*, *dnaK* and *clp* proteases were upregulated. The exposure to hydrogen peroxide leads to the induction of oxidative stress-associated genes and mechanisms. Genes encoding catalases, thioredoxin reductases and peroxidases were induced to remove hydrogen peroxide from the cells or extracellular environment. The involvement of iron and manganese in the response of *B. cereus* to hydrogen peroxide was indicated by the induction of *perR* and iron and manganese uptake systems. Imbalance in iron homeostasis has also been reported for *B. subtilis* (Zuber, 2009) and manganese may provide protection against hydrogen peroxide (Inaoka *et al.*, 1999). The SOS response, which is activated when DNA is damaged, and other DNA repair and protection mechanisms were induced upon exposure to hydrogen peroxide. *Bacillus subtilis* showed similar responses as *B. cereus* when exposed to hydrogen peroxide, including the induction of the PerR and OhrR regulons (Helmann *et al.*, 2003). On the other hand, the  $\sigma^B$  regulon of *B. subtilis* was induced

upon peroxide stress while in *B. cereus* this was not the case (Ceragioli *et al.*, 2010).

The putative functions of the induced genes suggest that exposure to hydrogen peroxide leads to protein and DNA damage. The reaction of hydrogen peroxide with thiol and methionine residues of proteins, concomitantly leading to damage, has been verified by measuring oxidation of sulfhydryl groups (Ceragioli *et al.*, 2010). Additionally, hydrogen peroxide may cause damage to proteins harbouring iron–sulfur clusters leading to elevated levels of free iron that can bind adventitiously to proteins. Free iron also initiates the generation of highly reactive hydroxyl radicals (OH $\cdot$ ) in the Fenton reaction. Consequently, OH $\cdot$  oxidizes biomolecules including lipids, DNA and proteins (Imlay, 2008; Zuber, 2009). Damage to DNA was experimentally verified by establishing mutation rates in hydrogen peroxide treated and untreated *B. cereus* cells, showing that exposed cells demonstrated higher mutation rates (Ceragioli *et al.*, 2010). In contrast, no indications were found for lipid peroxidation and staining with propidium iodide (a fluorescent probe that enters cells through compromised membranes) did not show damage to the cell membranes in the conditions tested. Even more unexpected was the lack of free radicals upon exposure to primary oxidative stress, while these are observed after exposure to heat and acid stress. Primary oxidative stress may lead to OH $\cdot$  formation; however, the threshold needed for detection may not be reached in these experiments as OH $\cdot$  formation may be low from autolysis of hydrogen peroxide (Mohan *et al.*, 2009; Ikai *et al.*, 2010). Furthermore, differences in (threshold) levels of free radicals resulting from primary and secondary oxidative responses may originate from the localized formation and inflicted damage of for example OH $\cdot$  (Imlay, 2003; Mohan *et al.*, 2009).

#### *Response to other oxidative agents*

Besides hydrogen peroxide other chemicals, such as peracetic acid and sodium hypochlorite, are also used for their oxidative properties to clean and disinfect food-contacting surfaces. Exposure of *B. cereus* to peracetic acid showed similar responses as observed for hydrogen peroxide (Ceragioli *et al.*, 2010). The nature of peracetic acid, i.e. being a mixture of hydrogen peroxide and acetic acid, is perhaps the reason for the large overlap between the responses to hydrogen peroxide and peracetic acid exposures. Sodium hypochlorite treatments led to the induction of genes involved in the metabolism of sulfur-containing amino acids, i.e. cysteine and methionine, in *B. cereus* (Ceragioli *et al.*, 2010). Correspondingly, elevated levels of sulfhydryl-group oxidation were found after exposure to sodium hypochlorite.



**Fig. 1.** Schematic representation of the response of *Bacillus* spp. to primary and secondary oxidative stress. Effects and defence mechanisms specific for primary oxidative stress are indicated in pink boxes. The secondary oxidative stress-related effects and defence mechanisms are shown in blue boxes. Overlapping effects, the possible inflicted damage and defence mechanisms are depicted in purple boxes.

Exposure of *B. anthracis* to the O<sub>2</sub><sup>-</sup>-generating compound paraquat leads to the induction of siderophore production and iron transport-associated genes (Passalacqua *et al.*, 2007). Paraquat resistance of *B. subtilis*, on the other hand, is dependant on  $\sigma^M$  (Cao *et al.*, 2005), which is normally associated with cell envelope stress (Eiamphungporn and Helmann, 2008). Another well-known oxidative compound is diamide. Diamide is known to cause thiol oxidation and disulfide stress in *B. subtilis*. Exposure to diamide leads to activation of the PerR regulon, including the activation of vegetative catalase and alkylhydroperoxide reductase (Leichert *et al.*, 2003). Furthermore, the induction of thioredoxin and thioredoxin reductase genes *trxA* and *trxB*, which are regulated by the disulfide stress regulator Spx (Nakano *et al.*, 2003), was noted. To prevent irreversible thiolation *B. subtilis* protects its proteins by S-cysteinylation (Hochgrafe *et al.*, 2007; Pöther *et al.*, 2009). Thiol-reactive electrophiles are generated from the oxidation of amino acids and other biomolecules (Marnett *et al.*, 2003). When *B. subtilis* is exposed to exogenously added electrophiles, such as formaldehyde and methylglyoxal, the displayed response resembles the response to diamide exposure (Liebeke *et al.*, 2008; Nguyen *et al.*, 2009). Spx and PerR regulons are stimulated upon formaldehyde exposure and the induction of the LexA regulon indicates DNA damage. Furthermore, common mechanisms are induced to resist diamide and electrophile-associated thiol stress

(Antelmann *et al.*, 2008), possibly including the recently discovered low-molecular-weight thiol bacillithiol (Gaballa *et al.*, 2010).

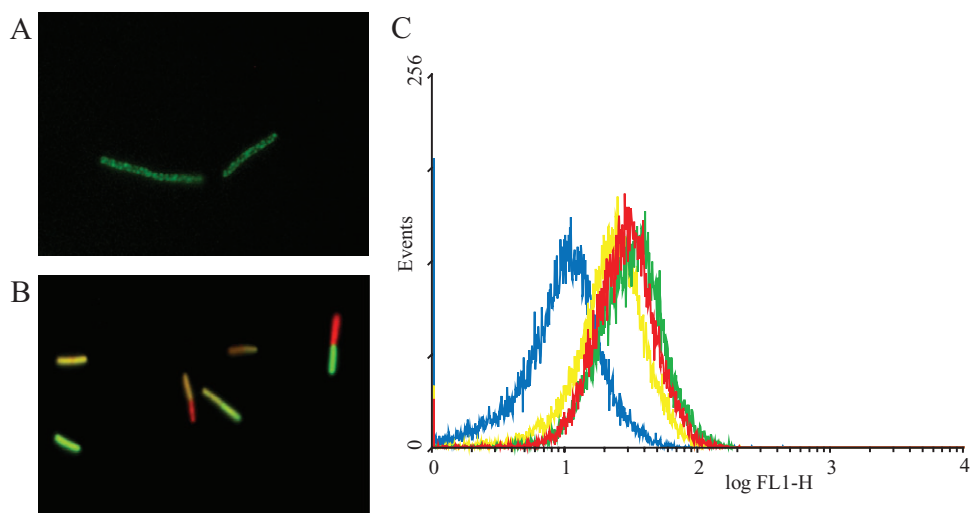
In general, the exposure of *Bacillus* spp. to oxidative agents, such as hydrogen peroxide, paraquat and sodium hypochlorite, leads to oxidized sulfhydryl groups, lipid peroxidation, the generation of reactive oxygen species (ROS) and a disturbed NAD<sup>+</sup>/NADH balance (Fig. 1). Subsequently, the cell responds to the damage to DNA, membranes and proteins by inducing thioredoxins, catalases, general stress responses and the SOS response.

### Secondary oxidative stress responses

The response of *Bacillus* spp. upon exposure to conditions, including low-pH, high-temperature and other stressful conditions, that lead to secondary oxidative stress is discussed in the following section.

#### *Response of Bacillus to acid stress-associated secondary oxidative stress*

Recently, we have reported that upon exposure to low-pH environments *B. cereus* displays an oxidative stress response (Mols *et al.*, 2010). The induction of genes generally associated with oxidative stress, including superoxide dismutase, catalase and thioredoxins, was noted when *B. cereus* cells were exposed to growth inhibiting,



**Fig. 2.** Fluorescence microscopy images and flow cytometry histograms of stressed *Bacillus* cells. *Bacillus subtilis* cells were exposed to 54°C and stained with HPF. The green fluorescence indicates the formation of hydroxyl and/or peroxynitrite (A). *Bacillus cereus* cells were exposed to pH 4.5 and stained with cFDA and MitoSOX. The green fluorescence indicates esterase activity (often used as indicator for cells that are alive) and the red fluorescence shows the formation of superoxide. The orange or yellow cells indicate intermediate responses with superoxide formation and cFDA-derived fluorescence (B). *Bacillus cereus* cells exposed to 50°C for 0 (blue), 10 (green), 20 (yellow) and 30 (red) min, stained with HPF and analysed with a flow cytometer (C). The shift in fluorescence to the right indicates a higher fluorescent signal corresponding to the formation of hydroxyl and/or peroxynitrite.

bacteriostatic, or bactericidal levels of acidity. Upon exposure to lethal pHs two strains independently showed the formation of excess radicals. Using the fluorescent probes HPF, which reacts with  $\text{OH}\cdot$  and peroxynitrite ( $\text{ONOO}^-$ ), and MitoSOX that reacts with  $\text{O}_2^-$ , we could link the formation of highly reactive oxygen spp. to the physiological outcome of the stress exposure, i.e. upon exposure to lethal acid stress  $\text{OH}\cdot$  and/or  $\text{ONOO}^-$  were formed (Fig. 2). Like *B. cereus*, *B. subtilis* also shows an oxidative stress response upon exposure to mildly acidic conditions (Wilks *et al.*, 2009). Genes generally related to oxidative stress, such as *katA*, encoding a catalase, and *namA*, coding for an antioxidant enzyme, were upregulated upon a mild acid shock.

Conditions with a low pH may cause acid-specific damage to cells, including the protonation of biomolecules such as proteins and DNA. Bacteria generally respond to this damage by inducing intracellular pH homeostasis mechanisms and general stress responses, including protein chaperones and DNA repair (Cotter and Hill, 2003). However, numerous genes that are induced by *Bacillus* spp. upon exposure to acid conditions signify damage related to secondary oxidative stress. Most apparent is the switch in electron transport chain components and the conceivably disturbed  $\text{NAD}^+/\text{NADH}$  balance. Although cells were grown and exposed to acid in aerobic conditions, the changes in metabolism towards anaerobic pathways were apparent (van der Voort and Abee, 2009). Whether the exposure to low pH leads to this switch or that the change in metabolism is caused by

the secondary oxidative stress remains to be elucidated. Furthermore, more data are needed to establish an explanation for how exposure to low pH gives rise to an oxidative stress through electron chain malfunctioning. Such dysfunction may originate from the hyperactivation of the electron transfer chain by induction of the TCA cycle and the mistranslation of proteins as proposed for the origin of bactericidal antibiotic-induced radical-mediated cell death (Kohanski *et al.*, 2009).

#### *Secondary oxidative stress upon exposure to heat and other toxic agents and conditions*

The response of *Bacillus* spp. to heat and other stresses have been studied for years and a secondary oxidative response has been noted previously (Hecker and Volker, 2001; Hecker *et al.*, 2007). As well as upon exposure to acid stress, when exposed to a heat shock *B. cereus* displays a secondary oxidative response. It is known that a mild heat shock, besides the upregulation of the general stress response including  $\sigma^B$ , proteases and chaperones, induces oxidative stress-related genes, such as catalase and thioredoxin (van Schaik *et al.*, 2007). Also at single cell level a secondary oxidative stress was apparent in heat-stressed *B. cereus* cells (Fig. 2C). A burst of  $\text{OH}\cdot$  and/or  $\text{ONOO}^-$  was shown in aerobically grown cells that were exposed to lethal heat and acid stress (Mols *et al.*, 2009). Additionally,  $\text{O}_2^-$  was also shown to be formed upon exposure to bactericidal heat stress (A. Subires, M. Mols and T. Abee, unpubl. results). Similar results were found for *B.*



*subtilis*: the induction of genes generally involved in oxidative stress (Helmann *et al.*, 2001) and the importance of oxidative stress protection in the response to other stresses has been recognized (Hecker and Volker, 2001). The formation of ROS, including OH· and O<sub>2</sub><sup>-</sup>, upon the exposure to heat stress of *B. subtilis* has been experimentally verified (Fig. 2A). Both acid and heat stress lead to the formation of radicals and the induction of a secondary oxidative stress response. Exposure to high concentrations of salt is known to induce catalase genes and catalase activity in *B. cereus* (den Besten *et al.*, 2009). However, the formation of OH· and/or ONOO<sup>-</sup> was limited upon exposure to high salt concentrations (Mols *et al.*, 2009).

Bactericidal antibiotics are known to cause the formation of OH· radicals in *Escherichia coli* and *Staphylococcus aureus* (Kohanski *et al.*, 2007). In *B. subtilis*, a burst of free radicals was also initiated upon exposure to bactericidal antibiotics such as kanamycin (our unpublished results). Additionally, the exposure to rifampicin, a bactericidal antibiotic known to inhibit transcription, leads to increased amounts of catalases, Dps and MrgA, which are known to bind to DNA to prevent damage caused by oxidative stress (Bandow *et al.*, 2002). The peptidoglycan synthesis inhibitors enduracidin and bacitracin induced the expression of numerous Spx-regulated genes, indicating that thiol-oxidative stress may be induced by these bactericidal antibiotics (Rukmana *et al.*, 2009).

*Bacillus* spp. exposed to a range of toxic agents and conditions induce secondary oxidative stress. When lethal stresses are applied the formation of ROS is apparent, suggesting that these ROS may be part of a common mechanism of cellular death in respiring *Bacillus* species.

#### Putative origin of secondary oxidative stress

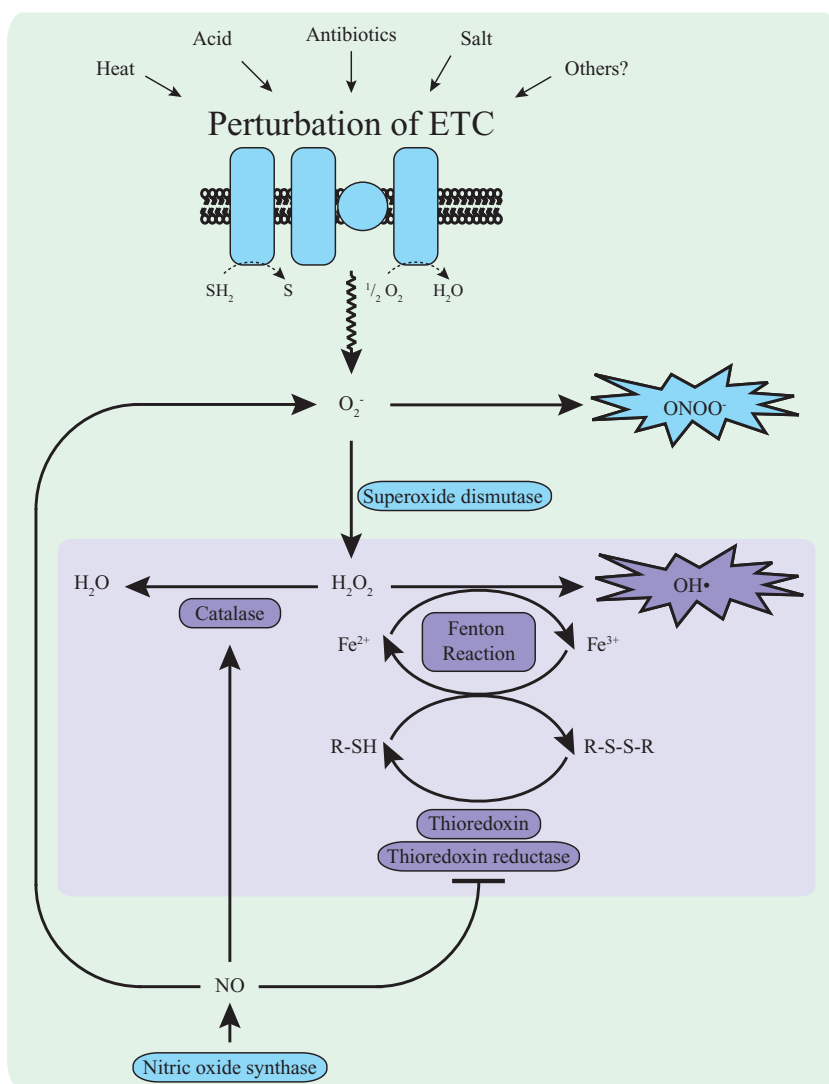
In general, when actively respiring *Bacillus* cells are exposed to conditions that abruptly affect the electron transport chain an oxidative stress response can be observed. This oxidative stress encompasses the formation of ROS, including superoxide (O<sub>2</sub><sup>-</sup>), hydroxyl (OH·) and peroxynitrite (ONOO<sup>-</sup>) (Fig. 3). Transcriptome analyses indicated that environmental stresses indeed affect the electron transfer chain (Mols *et al.*, 2010). Most likely the electron transfer chain is hampered to such extent that the premature leakage of electrons to oxygen, forming O<sub>2</sub><sup>-</sup>, cannot be prevented (Imlay, 2003). To a certain threshold *Bacillus* spp. can cope with O<sub>2</sub><sup>-</sup> by converting it to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) using superoxide dismutases and subsequently to water using catalases (Imlay, 2008). When cells cannot dissipate O<sub>2</sub><sup>-</sup>, it may damage proteins containing iron-sulfur clusters elevating free iron levels in the cell (Kohanski *et al.*, 2007). Free iron can react with hydrogen peroxide, forming the highly toxic OH· radicals in the

Fenton reaction (Imlay *et al.*, 1988). Furthermore, O<sub>2</sub><sup>-</sup> can rapidly react with NO to form another highly toxic oxidative compound: ONOO<sup>-</sup> (Beckman and Koppenol, 1996).

Nitric oxide is formed by a reaction catalysed by nitric oxide synthase (bNOS). Indirect indications for the formation of NO upon low pH exposure can be inferred from the upregulation of NO dioxygenase and NO-dependant regulator *dnrN* in acid-stressed *B. cereus*. The induction of bNOS activity, which is possibly regulated at protein level (Shatalin *et al.*, 2008), may initially have a positive effect on surviving oxidative stress. bNOS-derived NO may inhibit thiol reduction leading to the inhibition of the OH·-forming Fenton reaction (Shatalin *et al.*, 2008; Sudhamsu and Crane, 2009). Other positive effects of NO to counteract oxidative damage are the induction of catalase activity (Shatalin *et al.*, 2008) and the inhibition of the aerobic electron transfer chain (Husain *et al.*, 2008). However, the presence of NO has drawbacks. It facilitates the formation of ONOO<sup>-</sup>, which may have a damaging effect that could lead to cell death. Although some of the key players in this mechanism of cellular death are identified, the exact target of environmental stresses in the electron transfer chain that initiates the formation of O<sub>2</sub><sup>-</sup> remains to be elucidated. Kohanski and colleagues (2007) revealed that the formation of OH· radicals upon exposure to bactericidal antibiotics plays a crucial role in the inactivation of bacteria. They therefore postulated that the formation of OH· was a common mechanism of cellular death. In our studies we showed that indeed OH· and/or ONOO<sup>-</sup> are formed upon exposure of *B. cereus* to stresses, including heat and acid (Mols *et al.*, 2009; 2010). However, the crucial role as a mechanism of cellular death could not be established for OH· radicals. The importance of the formation of ROS can be inferred from the increased resistance to acid and heat stress of anaerobically grown and exposed cells compared with actively respiring cells (Mols *et al.*, 2009). However, the fact that the bacterial cells are still killed without the presence of oxygen, which is required to generate ROS, proves that the formation of OH· and/or ONOO<sup>-</sup> is not the only mechanism of cellular death.

#### Concluding remarks

The oxidative response shown by *Bacillus* spp. upon exposure to stress conditions resembles the response shown when the cells are exposed to oxidative compounds (Fig. 1). Several genes are commonly induced when *Bacillus* cells are exposed to oxidative stress or environmental stresses such as heat and acid. Catalase and thioredoxin encoding genes and genes involved in the general stress response, including Clp proteases and chaperones, are commonly upregulated by oxidative stress and other stresses. The induction of these genes



**Fig. 3.** Schematic representation of the putative origin of ROS upon exposure of *Bacillus* spp. to environmental stresses. Environmental stresses, including heat, acid, antibiotics and high salt concentrations, can cause perturbation of the electron transfer chain resulting in the formation of superoxide ( $O_2^-$ ).  $O_2^-$  can be converted into hydrogen peroxide ( $H_2O_2$ ) subsequently leading to a response similar to the response to primary oxidative stress (shown in purple box). The role of nitric oxide (NO) in the formation of the highly reactive hydroxyl radicals ( $OH\cdot$ ) and peroxynitrite ( $ONOO^-$ ) should be emphasized. NO inhibits the formation of reduced thiols ( $R-SH$ ) that recycle  $Fe_3^+$  to  $Fe_2^+$  and, thus, inhibits the  $OH\cdot$ -forming Fenton reaction. NO also activates catalase that breaks down  $H_2O_2$  to oxygen and water ( $H_2O$ ). A drawback to these protective mechanisms of NO is that NO itself may react with  $O_2^-$  to form highly reactive  $ONOO^-$ .

indicates common malfunctions and damage to biomolecules such as proteins, lipids and nucleic acids. This damage may be caused by similar perturbations caused by primary oxidative stress and other stresses, such as the formation of  $OH\cdot$  radicals and the disturbance of the  $NAD^+/NADH$  balance inside the cells. There are, however, differences notable between the responses to primary and secondary oxidative stresses, e.g. the formation of excess  $OH\cdot$  and/or  $ONOO^-$  inside the cells. On the other hand, there are chemicals, such as electrophilic quinones, that next to a primary oxidative nature can induce a secondary oxidative stress response by thiol depletion (Liebeke *et al.*, 2008). Therefore, a clear cut between primary and secondary oxidative stress responses cannot be made in all cases.

In conclusion, there are two distinct types of oxidative stress responses in *Bacillus* spp.: a primary oxidative stress response when cells are exposed to oxidative com-

pounds and a secondary oxidative stress response when cells are exposed to environmental stresses. Although both types share several aspects, they differ in origin, type of ROS formed and induced protection mechanisms. The generation of a secondary oxidative stress has been stated as a common mechanism of cellular death in actively respiring bacteria. However, several aspects are still unknown and insight in the exact reactions causing the perturbation of the electron transport chain and the resultant generation of ROS is needed. Insights in these mechanisms and key factors involved in the generation of ROS under different stress conditions could be exploited to identify novel targets for control of microbial growth.

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