Role of Calcium Carbonate in Protecting the Colony Forming Ability of
Bacillus simplex TWW-04 Exposed to Oxidative Stress

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Abstract: About 72% of cells in a population of Bacillus simplex TWW-04 in early exponential phase lost their colony forming ability after being exposed to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) for 90 min compared to 38% in another population exposed to H\textsubscript{2}O\textsubscript{2} and calcium carbonate (CaCO\textsubscript{3}). Interestingly, both H\textsubscript{2}O\textsubscript{2} and CaCO\textsubscript{3} added separately or combined had oxidative effect shown through induced protein carbonylation and induction of Catalase (Cat) and Glutathione peroxidase (GPX), while Peroxidase (Per) responded to the addition of H\textsubscript{2}O\textsubscript{2} only. The addition of CaCO\textsubscript{3} increased the protein of both whole cells and total proteins in the periplasmic fraction. It prevented lipid peroxidation, despite some alterations in cellular fatty acid composition. Scanning Electron Microscopy (SEM) showed no evident morphological alterations when CaCO\textsubscript{3} was added with or without H\textsubscript{2}O\textsubscript{2}. The counteracting effect of adding CaCO\textsubscript{3} to H\textsubscript{2}O\textsubscript{2} containing cultures and its effect on maintaining colony forming ability proved to be neither via enzyme induction nor chemical reaction but through membrane interaction. It was assumed that loss of colony forming ability is attributed to carbonylation of proteins essential for growth, but our evidence suggests that protein carbonylation is irrelevant and that the highly susceptible part responsible for cell colonization is lipoproteins. H\textsubscript{2}O\textsubscript{2} and CaCO\textsubscript{3} appear to act as both toxic and signaling compounds.

Key words: Colony forming ability • calcium carbonate • hydrogen peroxide • oxidative damage

INTRODUCTION

Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is a powerful oxidant which received wide attention as an antimicrobial agent in process streams [1], wastewater contaminant [2], by-product inside cellular compartments [3] and for having an effective role in treating organic contaminants in soil and groundwater [4]. Bacterial cells are capable of countering the oxidative effect of internal and external H\textsubscript{2}O\textsubscript{2} by releasing enzymes [5, 6]. Scavenging processes are more rapid than the flow of H\textsubscript{2}O\textsubscript{2} across membranes. E. coli was reported to reduce endogenous H\textsubscript{2}O\textsubscript{2} to submicromolar levels to enable the cells to continue growing in environments containing high concentrations of H\textsubscript{2}O\textsubscript{2} which exceeds what could be tolerated internally [3]. H\textsubscript{2}O\textsubscript{2} is believed to pass freely in and out of the cells carefully controlling transmembrane concentration gradients [7]. Recent studies point out that some membranes have poor permeability to H\textsubscript{2}O\textsubscript{2}, differences in permeability could be explained either by changes in membrane lipid compositions or by diffusion-facilitating channel proteins or a combination of both [8].

Calcium carbonate (CaCO\textsubscript{3}) is a reported inducer for fungal catalase and glucose oxidase [9, 10]. Though commonly used in fermentation media for producing these two enzymes, yet its role in fungal cultures is ubiquitous. CaCO\textsubscript{3} was reported to be neither responsible for maintaining pH stability nor for the need of calcium salts in the media, but was reported to be more related to a shift from glycolysis to the pentose phosphate pathway in fungi [11, 12].

In an attempt to increase catalase induction in the bacteria under study, CaCO\textsubscript{3} was added to bacterial cultures for more tolerance against H\textsubscript{2}O\textsubscript{2}, we noticed that the extent of reduction of colony forming units was not as drastic as in cultures containing H\textsubscript{2}O\textsubscript{2} alone. It was reported that some cultures lose their colony forming ability upon exposure to stress factors [13] and some regain their colony forming ability after the addition of a counteracting compound to the media [14]. The loss
of colony forming ability was attributed to enhanced
carbonylation of proteins essential for growth [15] and
postulated to be due to oxidative damaging mechanisms
which target membrane proteins [16].

The aim of this study was to test the effect of
simultaneous addition of CaCO₃ with H₂O₂ in the culture,
examining the colony forming ability and some parameters
related to oxidative damage in an attempt to understand
the implications of CaCO₃ interactions with Bacillus
simplex TWW-04 cells in the presence of high
concentrations of H₂O₂ and to highlight its role in
defense during oxidative stress.

MATERIALS AND METHODS

Bacterial cells and cultivation: Bacillus simplex TWW-04
was used to inoculate LB media, static cultures were
incubated at 30°C for 24 h. Two % of preculture inoculum
(vol/vol) was used to inoculate flasks containing 50 ml LB
media, 250 mM of H₂O₂ (30%) and different concentrations
of oven sterilized CaCO₃ (0.5, 1.5, 2.5, 3.5, 4.5 and 5.5) were
added after sterilization. Cultures were incubated for
90 min at 30°C and centrifuged rapidly to stop the action
of H₂O₂. The optimum CaCO₃ concentration obtained was
used in the following experiments. Cultures were divided
into 4 groups: control (A), +H₂O₂ (B), +CaCO₃ (C), +H₂O₂ and +CaCO₃ (D).

Colony Forming Ability (CFA): To measure the number of
colonies, 100 µl aliquots of 10⁻⁶ time diluted cultures were
spread onto LB-agar plates and incubated for 24 h at 30°C.

Scanning Electron Microscopy (SEM): Cultures C and D
were centrifuged at 3000 rpm×15 min to collect the cells.
Cells were resuspended in physiological saline. A small
drop of the suspension was placed on a carbon coated
copper grid, dried and was coated with gold. The samples
were observed using scanning electron microscope JOEL
JEM 100cx at magnification power of 5000X.

Degree of carbonylation: The introduction of carbonyl
groups into amino acid residues of proteins to determine
the extent of oxidative modification was performed
according to Levine et al. [17]. The degree of
 carbonylation was expressed as nmol of protein
carbonyls per milligram of protein.

Lipid peroxidation: Lipid peroxidation was estimated
as thiobarbaturic acid reactive substance (TBARS)
according to Yoshika et al. [18], test was performed for all
4 group cultures.

Fatty acid composition: Fatty acid methyl esters were
extracted with hexane. The relative percentages of the
fatty acids were determined from the peak areas of the
methyl esters using Fatty acid methyl esters were
extracted with hexane. The relative percentages of the
fatty acids were determined from the peak areas of the
methyl esters using hp 6890 gas chromatograph
instrument with FID detector, equipped with: innowas-
crosslinked polyethylene glycol column 30 m; i.d. 0.32
mm; 0.5µm film thickness. Oven temperature was
programmed at 150°C for 1 min, elevated to 235°C with a
rate of 17°C min⁻¹, then raised to 245°C with a rate of 1°C
min⁻¹ and held at 245°C for 5 min. The carrier gas was
Nitrogen (1.5 ml min⁻¹) Injection temperatures was
260°C. Total fatty acid and fatty acid composition
were performed for all 4 group cultures.

Preparation of periplasmic and cytosolic fractions and
assay of some of the oxidative enzymes: Cultures were
centrifuged at 4°C for 30 min at 10000 rpm, the pelleted
cells were washed and centrifuged in phosphate buffer pH 7 for 15 min at 39000 rpm in a cooling ultracentrifuge SORVALL ULTRA 80 at 4°C, this process was done twice to obtain periplasmic cell fractions.

Catalase (cat), peroxidase (per) in periplasmic fraction and Glutathione peroxidase (GPX) in cytosolic fraction were assayed.

Catalase was determined by monitoring the degradation of H₂O₂ in 1 min at 25°C using a Schimadzu UV 2100 spectrophotometer at 240 nm. The assay mixture contained enzyme extract and 1% H₂O₂ [19]. Peroxidase was assayed by monitoring the rate of formation of purpurogallin from pyrogallol in 20 seconds at 25°C spectrophotometrically at 420 nm [20]. GPX was assayed according to Paglia et al., [21]. Protein concentrations were determined by the method of Lowry [22] using bovine serum albumin as a standard. All samples were examined for their colony forming ability after incubation with hydrogen peroxide to ensure that the bacteria were viable at the chosen concentration and time of the experiments. All results are the mean value of replicates and tests were performed as separate experiments for all 4 group cultures.

RESULTS AND DISCUSSION

The loss of colony forming ability for bacterial cells has been reported for cultures treated with H₂O₂ [13]. The cell membrane, being the primary target for damage and sensing/signaling for damage control [23], is assumed to have a role in colony aggregation. Bacillus simplex TWW-04 has been the subject of a number of previous studies for its ability to tolerate extreme concentrations of H₂O₂ [16, 24]. It showed loss of colony forming ability after exposure to oxidative stress, its viability was detected by studying the membrane permeability via dielectric properties [25]. Periplasmic Cat was the major enzyme released to counteract the damaging effect of H₂O₂ [26]. A number of substances maybe used to induce catalase, the use of CaCO₃ was exploited in this study because it was reported for its contribution in simultaneous glucose oxidase and catalase induction in fungi [10], though not documented in bacteria. The addition of CaCO₃ in the cultures containing H₂O₂ caused a noticeable increase in Cat, total cell protein and colony forming ability, but this parallel increase dropped at concentrations above 3.5% (Fig. 1). The result obtained was expected for Cat induction and protein concentration as they were reported before [10], but it unexpectedly interfered with the colony forming ability. Upon the increase in CaCO₃ concentrations, cell toxicity might have occurred as Ca²⁺ is known to cause cell toxicity [8], this might explain the adverse effect represented by a sharp drop of all tested parameters upon using higher concentrations of CaCO₃ (4.5 and 5.5%).

Cat, Per and GPX levels for cultures B, C and D showed that Cat and GPX were induced by both H₂O₂ and CaCO₃ while the release of Per responded only to the
presence of H$_2$O$_2$ (Fig. 2). The colony forming ability of cultures B decreased by 72% compared to a decrease of only 37.7% in D. This result shows that the addition of CaCO$_3$ to cultures containing H$_2$O$_2$ increased the CFA by 14% (Fig. 2 and 3). Cell dry weight was increased evidently when CaCO$_3$ was added to Aspergillus niger [27] and this indicates a relation between microbial biomass and CaCO$_3$. The use of 3.5% of CaCO$_3$ was considered the optimum concentration needed to counteract the damage exerted by hydroxyl radicals and was used in the following experiments.

There are different views for explaining the action of CaCO$_3$ as a protective agent against oxidative stress: by interacting with hydrogen peroxide through a chemical reaction, thus giving a chance for the bacterial enzymatic systems to be fully expressed and counteract the damage, or through an increase in enzyme induction as in fungi or through physical blocking of membrane channels, which governs the entrance of H$_2$O$_2$ into bacterial cells.

The induction of Cat, Per and GPX were reported to differ in their time of expression, each according to their affinity for H$_2$O$_2$, Cat being the first to respond to high concentrations after which Per and GPX follow, calcium is known for its contribution as a central signaling compound in cellular homeostasis, same as H$_2$O$_2$ [8], therefore, its likely to act as a start signal for induction of oxidative stress enzymes.

In plants, intracellular H$_2$O$_2$ mediates the salt stress signal transduction pathway by activating the expression of GPX resulting in cross-resistance of several biotic and abiotic stresses [28] which is considered different from the signaling pathway induced by extracellular H$_2$O$_2$ [8]. The bacteria under study proved to be tolerant to high concentrations of salt which reached 5 M [16]. The expression of GPX in Bacillus simplex TW-04 could be through cross-resistance with salt, same as in plants and is only initiated by intracellular H$_2$O$_2$. Cat and Per, having high affinity to H$_2$O$_2$ would act on high concentrations of extracellular H$_2$O$_2$ leaving GPX to act on intracellular H$_2$O$_2$ because of its lower affinity.

Treatment with H$_2$O$_2$ either promotes or inhibits disulfide bonding of select proteins in a concentration-dependent manner. Many of the targeted proteins are involved in translation and energy production [29]. This clearly demonstrates that oxidation by Reactive Oxygen Species (ROS) is not simply destructive but rather a specific targeted modification [30]. Bienert et al. [8], was stated that protein structure and consequently protein function can be altered by intra-and intermolecular oxidation of two cysteine residues by the formation of disulfide linkages. While intramolecular disulfides lead to alterations of enzyme activity by conformational changes. Such conformational changes can expose or shape the reaction centers of enzymes, shape DNA binding motifs or uncover localization signals like in the case of transcription factors.

Biological membranes are rich in specific lipids and protein, known as lipid rafts, these rafts are highly dynamic [31]. The physical state of the membrane is the key to microbial tolerance through remodeling of membrane composition [23]. In biological systems, H$_2$O$_2$
reacts with various cellular targets thereby causing cell damage, or even cell death. But at low concentrations, certain cellular changes occur which potentially affect the proteins and lipids present in the cell membrane as the primary target. Protein carbonylation is considered an indication for oxidative modification [17] which is proposed to be the main cause for loss of colony forming ability under H$_2$O$_2$, stress [15].

Therefore, it was crucial to investigate the effect of H$_2$O$_2$ and CaCO$_3$ on proteins and lipids. The degree of protein carbonylation in cultures exposed to H$_2$O$_2$ and CaCO$_3$ separately were increased. The effect of adding H$_2$O$_2$ on the level of protein carbonylation is well established [17], while the effect of adding CaCO$_3$ is not well defined, but it could be explained by the release of the CO$_3^{2-}$ radical, which attacks the membrane protein. This is in agreement of Arai et al., [32] who suggest an interaction between bicarbonate and iron or heme derivatives leading to the generation of highly reactive oxygen species and/or generation of highly reactive carbonate radical anion, bicarbonate radical or peroxynitrite carbonate which facilitates low-density lipoproteins oxidation in blood vessels of humans. The Bacillus under study was reported to contain heme catalase [26], in analogy, the same correlation could be applied, the carbonate radical might be responsible for the increase in protein carbonylation in cultures containing CaCO$_3$ (an increase from 5.37 in control cultures to 11.08 µmol mg$^{-1}$ protein) which is close to the increase induced by the addition of H$_2$O$_2$ (12.55 µmol mg$^{-1}$ protein), a similar increase is exhibited in cultures containing both H$_2$O$_2$ and CaCO$_3$ (12.44 µmol mg$^{-1}$ protein).

The addition of CaCO$_3$ simultaneously with H$_2$O$_2$ prevented some of the oxidative damage of whole cell protein (increase from 20.6 to 25.3 mg ml$^{-1}$ in cultures exposed to H$_2$O$_2$ and both CaCO$_3$ and H$_2$O$_2$, respectively) and prevented extreme loss in colony forming ability (72% to 37.7%), neither via prevention of protein carbonylation, nor by increase in protein concentration (Table 1 and 2), but through prevention of lipid peroxidation which decreased from 16.2 to 12.38 nmol ml$^{-1}$ upon the addition of CaCO$_3$ (Fig. 4). The degree of fatty acid unsaturation is regarded as a universally conserved adaptation response in the majority of microbes in response to growth temperature. It is also reported a cellular fatty acid composition in cases of salt stress, acid and oxidative stress [33]. This is in agreement with Nishida et al. [15] who stated the need for polyunsaturated fatty acid (PUFAs), specifically eicosapentaenoic acid (EPA) in E. coli transformants which caused resistance against oxidative damage of H$_2$O$_2$ and prevented loss of colony forming ability via partial blocking of the plasma membrane by the EPA-containing phospholipids. Shigapova et al., [23] suggest rapid remodeling of membrane composition for short-term thermostolerance in E. coli, a hyperfluid state forming non-bilayer structures or particular lipid molecular species involved directly in lipid-protein interactions, these in return act as stimulus for activation of related genes. Pedersen et al. [34] indicated that calcium ions are strongly bound to the interfacial region inducing a “condensing effect” on the bilayer, this is reflected by an increase in acyl chain order parameter and hydrophobic thickness. Calcium ions are known to function as a second messenger in living cells, it has a pronounced effect on membrane structures and therefore, is responsible for implications during signal transduction events.

Changes in biophysical membrane properties leads to changes in length of unsaturated fatty acids, the amount of unsaturated versus saturated fatty acids, protein composition, degree of phosphorylation and glycosylation of lipids, all this leading to changes in membrane permeability [35]. When hydrogen peroxide was added to the media in high concentrations, Bacillus simplex TWW-04 cells were proved viable but not through classical colony forming techniques due to physical alterations in cell membrane after exposure to H$_2$O$_2$ [25], therefore it is postulated that the lipid bilayer in the cell membrane is involved in cell colonization.

In conclusion, there is proportional evidence linking loss of colony forming ability to the presence of some oxidants or variation in environmental conditions and other evidence attributing the regain of colony formation to the membrane fatty acids. The role of CaCO$_3$ in cultures containing H$_2$O$_2$ is divided into two parts: the carbonate radical interacting with heme catalase increasing the oxidation, while the Ca$^{2+}$ has both signaling and “condensing effect”, thus aiding in prevention of loss of colony forming ability, increasing cellular proteins and protecting cellular lipids. Low concentrations of CaCO$_3$ are favorable for oxidative enzyme induction, because of their action as hydroxyl scavenger, this also aids in saving bacterial cells, thus increasing colony formation and cell growth. At high concentrations, CaCO$_3$ causes cell toxicity. Still, the precise correlation between CaCO$_3$ and membrane lipids and its role in metabolic pathways in bacteria are in dire need for further elucidation.
REFERENCES