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## Hydrogen peroxide effects on *Streptococcus thermophilus* CNRZ368 cell viability

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**Abstract** – *Streptococcus thermophilus* CNRZ368 is an anaerobic aerotolerant bacteria and its ability to survive under aerobic growth conditions raises the question of the existence of a putative defence system against oxidative stress. Thus, survival of CNRZ368 in the presence of increasing concentrations of hydrogen peroxide was studied. Moreover, the influence of the physiologic state of the cells, as well as that of a preexposition with sublethal doses of hydrogen peroxide, upon *S. thermophilus* CNRZ368 survival were determined. The results suggest that *S. thermophilus* displays a defence system against oxidative stress and that this system is inducible. © 2001 Éditions scientifiques et médicales Elsevier SAS

hydrogen peroxide / oxidative stress / *S. thermophilus* CNRZ368

### 1. Introduction

The oxidative stress response has been well characterized for some microorganisms and especially for the facultative aerobic Gram-negative bacteria *Escherichia coli* (for a review, [17]). In this organism, two distinct regulons, OxyR [4] and SoxR/S [9, 18], have been identified and are known to be induced by two distinct oxidative molecules, H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> respectively. The case of streptococci is less well documented since only the existence of some anti-oxidant enzymes have been reported [3, 8, 10, 13–15, 19]. Moreover, these bacteria lack catalase [16].

Our research focuses on the facultative anaerobic Gram-positive bacterium *Streptococcus thermophilus*, widely used in dairy product fermentation. Its ability to grow under aerobic conditions raises the question of the existence of a defence system against oxidative stress. Indeed, such a system might be necessary to rescue cells from damage caused by the inevitable reactive oxygen species produced under aerobic growth conditions.

The aim of our work was to test the effect of oxidative stress on *S. thermophilus* survival. For this purpose, H<sub>2</sub>O<sub>2</sub> was used as the oxidative agent:

hydrogen peroxide is notably known to react with reduced iron or copper ions to generate hydroxyl radicals (OH•) in the Fenton reaction [11, 12].

### 2. Materials and methods

#### 2.1. Strains and culture conditions

*S. thermophilus* CNRZ368 was purchased from the Centre National de la Recherche Zootechnique belonging to the INRA strains collection (France). D<sub>1</sub> was isolated in the progeny of strain CNRZ368 after serial subclonings. All experiments were performed on this clone. First, D<sub>1</sub> was grown overnight at 42°C in milk medium. Overnight culture was then diluted 100-fold in fresh TPPY [1] (0.7% tryptose, 0.7% proteose-peptone, 0.2% bacto yeast extract, 2% lactose) and grown at 42°C.

#### 2.2. Sensitivity to oxidizing agents

D<sub>1</sub> was grown overnight in milk medium, then inoculated into fresh TPPY medium. During exponential growth (OD<sub>600</sub> = 0.6), culture was exposed to oxidative stress by the addition of hydrogen peroxide (0.25 to 30 mM). Cells were left in contact with hydrogen peroxide for 15 min. Aliquots of the culture were diluted in TPPY, and appropriate dilutions plated on TPPY agar plates to determine cell viability.

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Plates were stocked in jars provided with the GENbox Anaer system (bioMérieux). Colonies were counted after 20-h incubation under anaerobic conditions. Percentage survival was determined by comparing viability with or without treatment. All experiments were performed at least in triplicate.

### 3. Results and discussion

#### 3.1. Effect of increasing concentrations of H<sub>2</sub>O<sub>2</sub> on survival

The ability of D<sub>1</sub> (derived from CNRZ368) to respond to 15-min exposure to a range of hydrogen peroxide concentrations (0.25–30 mM) was investigated and led to a depletion of cell viability (*figure 1A*). When D<sub>1</sub> was treated with low doses, the H<sub>2</sub>O<sub>2</sub> dose response curve was found to be biphasic: between 0 and 0.5 mM H<sub>2</sub>O<sub>2</sub> viability decreased, but then rose again above 0.5 to 1.0 mM (*figure 1B*). Further increases in the level of H<sub>2</sub>O<sub>2</sub> above 1 mM resulted in a further loss of viability. The kill curves seen in *figure 1* are very similar to those seen in *E. coli*, although *S. thermophilus* appears to be inherently more sensitive to H<sub>2</sub>O<sub>2</sub>.

Two hypotheses can be proposed to explain this biphasic shape: the curve may reflect two different

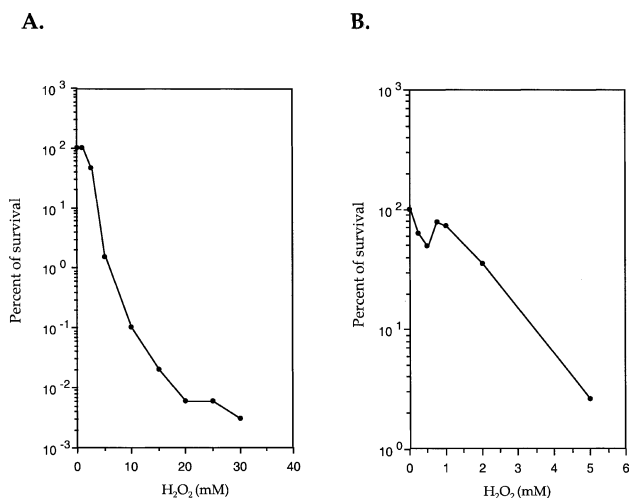
modes of killing by H<sub>2</sub>O<sub>2</sub>. Such an explanation has been proposed to account for two kinetically distinguishable modes of cell killing by H<sub>2</sub>O<sub>2</sub> in *E. coli*. In the first mode, cells are killed primarily by DNA damage caused by OH· radicals produced during the Fenton reaction. However, higher levels of H<sub>2</sub>O<sub>2</sub> are thought to scavenge OH·, thus suppressing mode one killing. As the concentration of H<sub>2</sub>O<sub>2</sub> rises still further, viability is decreased by a second mode of killing, although the cellular targets in this instance are not known [11, 12]. Alternatively, the curve shape could account for the induction of a protective response above a concentration of 0.5 mM H<sub>2</sub>O<sub>2</sub>. Above the concentration of 1 mM, viability again decreased, because of the saturation of the protective system.

#### 3.2. The influence of preexposure to H<sub>2</sub>O<sub>2</sub> on resistance to oxidative stress

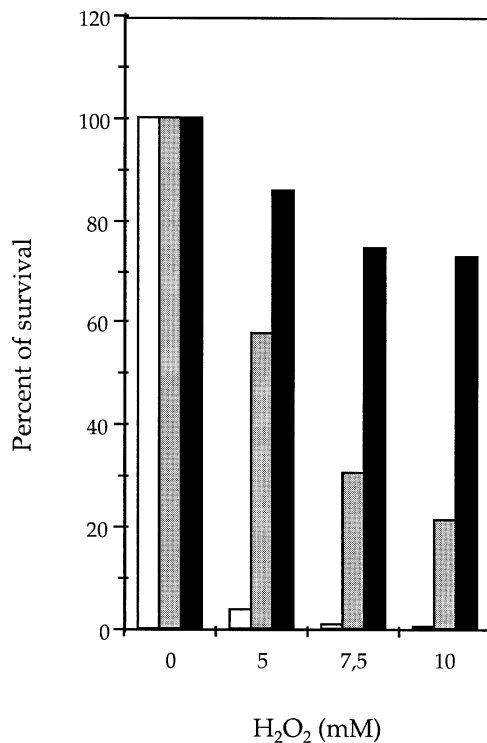
The ability of *E. coli* to resist stress has been shown to increase following prior exposure to low doses of the same stress [5]. Therefore, the viability of D<sub>1</sub> was tested with or without pretreatment with a sublethal level of H<sub>2</sub>O<sub>2</sub>. As shown in *figure 2*, a 0.25-mM pretreatment led to a 15-fold increase in survival when challenged with 5 mM H<sub>2</sub>O<sub>2</sub>, compared to strains that had not been pretreated. Moreover, when challenged with 10 mM H<sub>2</sub>O<sub>2</sub>, no more than 80% of the 0.25 mM H<sub>2</sub>O<sub>2</sub> pretreated population was killed, compared to 99% loss of viability for the non-pretreated control. A 1-mM H<sub>2</sub>O<sub>2</sub> preexposure led to even higher levels of survival to subsequent exposure: no more than 30% of the population was killed when subsequently challenged with 10 mM H<sub>2</sub>O<sub>2</sub>. Thus, D<sub>1</sub>, when exposed to sublethal levels of H<sub>2</sub>O<sub>2</sub>, was better able to cope with subsequent toxic doses. These results suggest that *S. thermophilus* cells respond to H<sub>2</sub>O<sub>2</sub>, probably by activating groups of genes encoding antioxidant and repair enzymes.

#### 3.3. The effect of growth phase on H<sub>2</sub>O<sub>2</sub> tolerance

In *E. coli* and *S. typhimurium*, stationary-phase cells are more resistant to environmental stress than exponentially growing cells [6]. To test whether this is the case for *S. thermophilus*, the effect of growth phase on H<sub>2</sub>O<sub>2</sub> tolerance was investigated. A culture of D<sub>1</sub> was split into five subcultures, and H<sub>2</sub>O<sub>2</sub> was added at four different time points during the ensuing growth period. One culture remained untreated

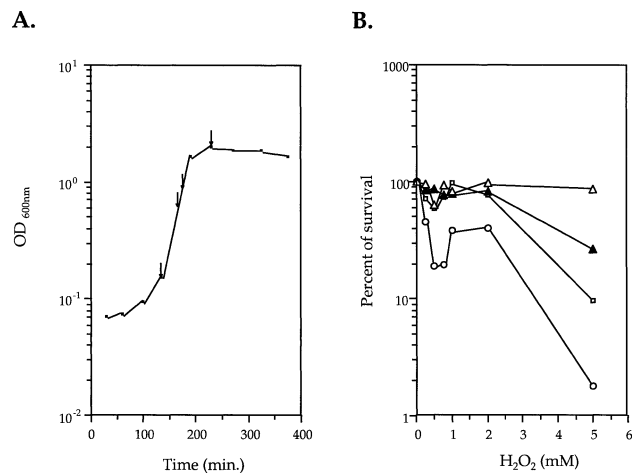


**Figure 1.** Sensitivity of D<sub>1</sub> isolate to killing by H<sub>2</sub>O<sub>2</sub>. Survival was determined by plating appropriate dilutions of samples after a 15-min exposure to H<sub>2</sub>O<sub>2</sub> of exponentially growing cells (OD<sub>600 nm</sub> = 0.6). Surviving fractions were calculated relative to colony counts without treatment. These experiments were performed in triplicate and the results of typical experiments are displayed in the figure. A. High concentrations of H<sub>2</sub>O<sub>2</sub>. B. Low concentrations of H<sub>2</sub>O<sub>2</sub>.



**Figure 2.** Effect of H<sub>2</sub>O<sub>2</sub> pretreatment on cells viability. Cells were preincubated for 15 min at 42°C in TPPY medium in the absence (□) or in the presence of 0.25 mM H<sub>2</sub>O<sub>2</sub> (▨) or 1 mM H<sub>2</sub>O<sub>2</sub> (■). Cells were then treated again with hydrogen peroxide at the indicated concentration and plated. Percentage of surviving cells is indicated on the graph.

and served to determine the growth rate, which proceeded in TPPY with a doubling time of 30 min<sup>-1</sup> (figure 3A). Figure 3B shows H<sub>2</sub>O<sub>2</sub> tolerance as a function of the growth phase. H<sub>2</sub>O<sub>2</sub> had little effect on the viability of stationary phase cells (OD<sub>600</sub> = 2.1), even up to 5 mM. In contrast, cells in early exponential phase (OD<sub>600</sub> = 0.15) were extremely sensitive to H<sub>2</sub>O<sub>2</sub>, and resistance increased throughout the growth period (OD<sub>600</sub> = 0.6; OD<sub>600</sub> = 0.85). Thus, nongrowing *S. thermophilus* cells appeared to be more tolerant to H<sub>2</sub>O<sub>2</sub> than their actively growing counterparts as found in *E. coli*. Moreover, since the resistance is settled down progressively through the growth curve, the oxidative stress response in *S. thermophilus* would have at least two modes of induction, one by H<sub>2</sub>O<sub>2</sub> and the other by starvation, as seen in *Bacillus subtilis* [7].



**Figure 3.** A. Growth curve of *S. thermophilus* D1 strain. B. Change in H<sub>2</sub>O<sub>2</sub> tolerance as a function of physiological state. Each point represents the average of a triplicate experiment. Cells were grown in TPPY medium. H<sub>2</sub>O<sub>2</sub> was added at different times during the ensuing growth period as a function of the OD<sub>600</sub>. Cells were treated at OD<sub>600nm</sub> = 0.15 (○), OD<sub>600nm</sub> = 0.60 (□), OD<sub>600nm</sub> = 0.85 (▲) and OD<sub>600nm</sub> = 2.10 (△).

#### 4. Conclusion

Our results suggest the existence of an inducible defence system against H<sub>2</sub>O<sub>2</sub> in *S. thermophilus* CNRZ368, possibly of the type OxyR-like regulon. Although the search for the structural homologue of OxyR (the regulator protein of the OxyR regulon) in *Streptococcus mutans* has failed [19], the hypothesis of a functional substitution for the OxyR protein can be drawn. Such functional substitution has already been identified in another Gram-positive bacteria as PerR in *Bacillus subtilis* [2].

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