The response of *Escherichia coli* K12 upon exposure to hypochlorous acid and hydrogen peroxide

B Pietersen[1#], VS Brözel[2*] and TE Cloete[1]
[1] *Environmental Biotechnology Laboratory, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, South Africa*
[2] *Department of Microbiology, University of the Western Cape, P/Bag X17, Bellville 7535, South Africa*

Abstract

The aim of the work reported here was to investigate the growth-inhibitory activity of HOCl and H_2O_2 toward *Escherichia coli* K12 during both logarithmic and stationary phases of the growth cycle, as well as the response of *E. coli* K12 to these oxidants. Stationary phase cultures were exposed to sub-inhibitory oxidising stress, and the minimum inhibitory concentrations (MIC) were determined during the ensuing 24 h. The effect of oxidant on logarithmically growing cultures was also determined. Stationary phase cultures of *E. coli* K12 responded to H_2O_2 stress, both the MIC and survival following exposure to high concentrations increasing following exposure to stress. By contrast stationary phase cells did not become more tolerant of high concentrations of HOCl following HOCl stress. Logarithmically growing *E. coli* K12 did not display increased tolerance to either inhibitory or lethal concentrations of H_2O_2 or HOCl following the relevant oxidising stress.

Introduction

Hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) are oxidising bactericides used in various applications to prevent, control or decrease bacterial activity. HOCl was first employed as a wound disinfectant by Hueter in 1831, as hand disinfectant by Semmelweis in 1847, and its bactericidal activity was confirmed by Koch in 1881 (Wallhäußer, 1988). HOCl is used widely as an antimicrobial agent for the control of microbial activity in recreational and industrial water systems, for sanitary applications and for surface disinfection. H_2O_2 is used amongst others in industrial water systems to control biofouling, in swimming pools and for the sanitation of surfaces and pipelines in food and other industries (Baldry and Fraser, 1988; Characklis, 1990; Cloete et al., 1992).

Although much work on the mechanism of HOCl action in eukaryotic cells has been done, its mechanism of antibacterial action is not yet clear. HOCl is generated in white blood cells as part of the mechanism of pathogen control (Schraufstätter et al., 1990). HOCl does not enter freely into eukaryotic cells but attacks surface and plasma-membrane proteins, impairing transport of solutes and the salt balance (Schraufstätter et al., 1990). It oxidises thiol groups and inhibits plasma membrane ATPases. It appears to impair protein synthesis in cells at low concentrations for ca. 2 h following exposure, thereby affecting replication of DNA and cell division (McKenna and Davies, 1988; Schraufstätter et al., 1990). It does not, however, cause any damage to eukaryotic genomic material. The stability and antimicrobial activity of HOCl is dependent on pH (Wallhäußer, 1988). It dissociates at pH greater than 7, and the undissociated moiety is the antibacterial agent (Hoffman et al., 1981). Above pH 7.5 it therefore loses its antibacterial activity. The antibacterial activity of chlorine dioxide and of chlorine gas in aqueous environments is also via HOCl because both react with water to form HOCl (Wallhäußer, 1988).

H_2O_2 is omnipresent in aerobic niches as it is formed, along with superoxide, as a by-product during aerobic metabolism (Fridovich, 1978). H_2O_2 reacts with a wide array of biological macromolecules such as DNA, proteins and membrane lipids (Tao et al., 1989). For example, H_2O_2 penetrates cells, causing site-directed damage due to metal-dependant ·OH formation (Schraufstätter et al., 1990; Storz et al., 1990). It causes DNA strand breaks and hydroxylation of bases in intact DNA, resulting in termination of replication (Schraustätter et al., 1990). In eukaryotes H_2O_2 also inhibits mitochondrial ADP-phosphorylation (Schraustätter et al., 1990). Bacteria respond to a wide range of environmental stresses including cold, heat, osmotic pressure, UV radiation and oxidising stress. Stress responses generally lead to tolerance of cells to further exposure to otherwise lethal levels of the same stress (Völker et al., 1992; Watson, 1990). A variety of bacteria, including *Escherichia coli* (Storz et al., 1990) and *Bacillus subtilis* (Hartford and Dowds, 1992) respond to oxidising stress by producing oxidant-degrading enzymes as well as DNA-repair enzymes (Ahern, 1993; Storz et al., 1990).

The aim of the work reported here was to investigate the growth-inhibitory activity of HOCl and H_2O_2 toward *E. coli* K12 during both logarithmic and stationary phases, as well as the response of *E. coli* to these oxidants.

Materials and methods

Cultures and media used

E. coli K12 was obtained from Prof. WOK Grabow, Dept. of Medical Virology, University of Pretoria, and was maintained on R2A agar slants (Reasoner and Geldreich, 1985) containing 1% glycerol, and subcultured monthly. R2A medium was made up as follows (per litre): 0.5 g peptone (Biolab); 0.5 g yeast extract (Biolab); 0.5 g Casamino acids (Difco); 0.5 g glucose (BDH); 0.5 g starch (BDH); 0.3 g Na pyruvate (Merck); 0.3 g K_2HPO_4 (Merck); and 0.05 g $MgSO_4$ (Saarchem). For solid R2A medium, 15 $g \cdot \ell^{-1}$ agar (Biolab, bacteriological grade) was added. H_2O_2 (8.8 $M \cdot \ell^{-1}$) was from Saarchem. HOCl was prepared fresh as an aqueous solution by dissolving $Ca(OCl)_2$ (Olin) in autoclaved deionised water.

* To whom all correspondence should be addressed.
☎ (021) 959-2976; Fax: (021) 959-2266; E-mail: volker@mbiol.uwc.ac.za
Present address: Institute for Pathology, University of Pretoria, PO Box 2034, Pretoria 0002, South Africa.
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Determination of the minimum inhibitory concentration

E. coli K12 was cultured in 100 mℓ R2A broth in 250 mℓ Erlenmeyer flasks with orbital shaking at 100 r·min⁻¹ for 24 h at 30°C. The MICs of the two bactericides were determined as described previously (Brözel et al., 1993). Briefly, 10 µℓ volumes of standardised suspensions of washed cells were inoculated into duplicate tubes of half-strength tryptic soy broth (Biolab) containing various concentrations (e.g. 100 to 200µM·ℓ⁻¹ in increments of 10 µM·ℓ⁻¹) of freshly added bactericide. Tubes were incubated at 30°C for 24 h. The lowest concentration of bactericide-inhibiting growth was taken to be the MIC.

Reaction during 24 h following exposure to sub-inhibitory oxidising stress

E. coli K12 was cultured for 24 h in R2A broth (omitting Na pyruvate in the case of H_2O_2treatment) at 28°C by shaking at 120 r·min⁻¹. Twenty-four hour old cultures were exposed to sub-inhibitory oxidising stress by adding H_2O_2 or HOCl to one fourth of the MIC (220 µM·ℓ⁻¹ H_2O_2 or 210µM·ℓ⁻¹ HOCl). The MICs were determined at 5, 10, 20, 30, 60 min, and 2, 3, 4, 6, 8, 14 and 24 h after exposure to stress. The culturable count was determined at the same times as the MICs by plating serial dilutions onto triplicate plates of R2A agar. Plates were incubated at 37°C for 72 h.

Effect of oxidising stress on survival following oxidant treatment

Twenty-four hour old cultures of *E. coli* K12 (cultured in R2A broth for 24 h at 28°C with shaking at 120 r·min⁻¹) were exposed to 880 mM·ℓ⁻¹ and 1 760 mM·ℓ⁻¹ of H_2O_2 and to 840 mM·ℓ⁻¹ and 1 680 mM·ℓ⁻¹ of HOCl for 60 min. The numbers of surviving cells (culturable count) were then determined by plating serial dilutions onto triplicate plates of R2A agar. Plates were incubated at 37°C for 72 h. The effect of pre-exposure to oxidant was determined by treating cultures with 88 µM·ℓ⁻¹ H_2O_2, or with 84 µM·ℓ⁻¹ HOCl for 60 min. These cultures were then exposed to 880µM·ℓ⁻¹ and 1 760 µM·ℓ⁻¹ of H_2O_2 and to 840 µM·ℓ⁻¹ and 1 680 µM·ℓ⁻¹ of HOCl for 60 min and the numbers of surviving cells determined as described above. The percentage surviving cells was calculated by dividing the number of surviving cells by the initial number of cells and multiplying by 100.

Determination of the effect of oxidant on the growth rate

The growth rate of *E. coli* in R2A broth at 28°C while shaking at 120 r·min⁻¹ was determined by following the absorbance at 550 nm (A_{550}). Broths were inoculated with 1 ml of a culture in the late log phase and the A_{550} was determined at 15 min intervals. To determine the effect of H_2O_2 on the growth rate, various early-logarithmic phase cultures were exposed to a series of final concentrations of H_2O_2 of 0, 220, 440, 880, 1320 and 1760 µM·ℓ⁻¹. The extent of the bacteriostatic effect of H_2O_2 was determined by neutralisation of H_2O_2 at various times after addition by the addition of bovine catalase (Sigma) at a final concentration of 200 U·mℓ⁻¹. To determine whether *E. coli* K12 exhibited an oxidising stress response during logarithmic growth phase, logarithmically growing cultures were exposed to 88 µM·ℓ⁻¹ H_2O_2 10 min prior to inoculation into fresh R2A broth. Cultures were then challenged with 0 and 880 µM·ℓ⁻¹ H_2O_2, and the A_{550} was determined at 15 min intervals.

To determine the effect of HOCl on the growth rate, various early-logarithmic phase cultures were exposed to a series of final concentrations of HOCl of 0, 140, 280, 420, 560 and 840 µM·ℓ⁻¹. The extent of the bacteriostatic effect of HOCl was determined by neutralisation of HOCl at various times after addition by the addition of $Na_2S_2O_3$ (0.01 % (m/v) final concentration) (LeChevalier et al., 1988). To determine whether *E. coli* exhibited an oxidising stress response during logarithmic growth phase, logarithmically growing cultures were exposed to 84 µM·ℓ⁻¹ HOCl 5 min prior to inoculation into fresh R2A broth. Cultures were then challenged with 0 and 420µM·ℓ⁻¹ HOCl, and the A_{550} was determined at 15 min intervals.

Determination of cellular catalase following exposure to H_2O_2

The levels of cellular catalase following exposure to oxidising stress were determined as follows. Logarithmically growing cultures were exposed to 140 µM·ℓ⁻¹ H_2O_2 and 1 mℓ samples were taken at 10, 20, 30, 60 and 90 min following exposure to stress. Cells were harvested by centrifugation at 10 000 x g, washed in 0.5 M phosphate buffer (PB) (pH 7.0), and resuspended in 500 µℓ of PB. Cells were lysed in suspension by ultrasonication for 4 x 15 s. The total protein concentration was determined by the method of Lowry et al. (1951). Catalase was quantified by monitoring the rate of disappearance of H_2O_2 spectrophotometrically at 240 nm, according to the method of Beer and Sizer (1952). Cell lysates were supplemented with 1 500µℓPB, and 1 000µℓH_2O_2 (8.8 mM·ℓ⁻¹) was added at t_0. One unit of catalase decomposed 1 µM·min⁻¹ of H_2O_2 per mg of protein at pH 7.0 (Beer and Sizer, 1952).

Results

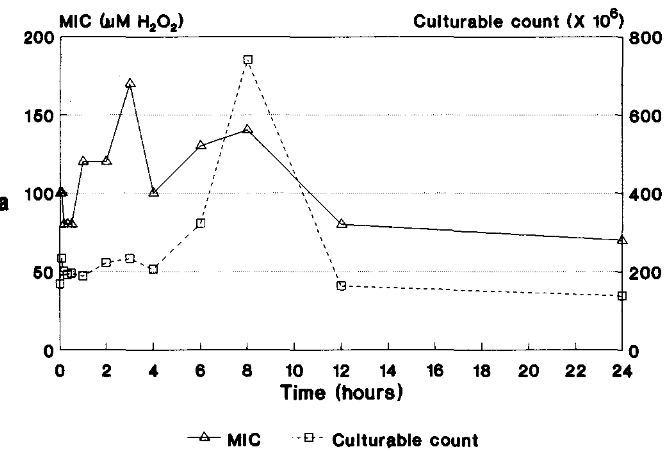
Reaction during 24 h following exposure to sub-inhibitory oxidising stress

E. coli K12 demonstrated stress response to H_2O_2 (Fig. 1a), as reported in the literature (Storz et al., 1990). The increased level of protection of stationary phase cells against H_2O_2 persisted for 45 and 480 min after exposure to stress. This increased level of protection was lost after 10 h, cells becoming more susceptible to H_2O_2 than they were before exposure (Fig. 1a). There was no decrease in the culturable count following exposure to sub-inhibitory stress. Therefore the sub-inhibitory concentration applied did not cause any detectable death of cells.

E. coli K12 in stationary phase showed an increased degree of protection towards HOCl within 120 min following exposure to HOCl stress (Fig. 1b). Cells were, however, sensitised to HOCl between 30 and 60 min following exposure to stress. The sub-inhibitory concentration of HOCl applied did not cause any detectable cell-death as reflected by the plate count. The increase in the culturable count 6 h after exposure to stress was due either to growth or to breaking up of cell aggregates.

Effect of oxidising stress on survival of oxidant treatment

Stationary phase *E. coli* K12 did respond to H_2O_2 stress by exhibiting increased resistance to lethal concentrations of H_2O_2 60 min following stress (Fig. 2). Pre-stressed cultures grew in the presence of 0.88 mM H_2O_2 whereas 40% of the non-stressed



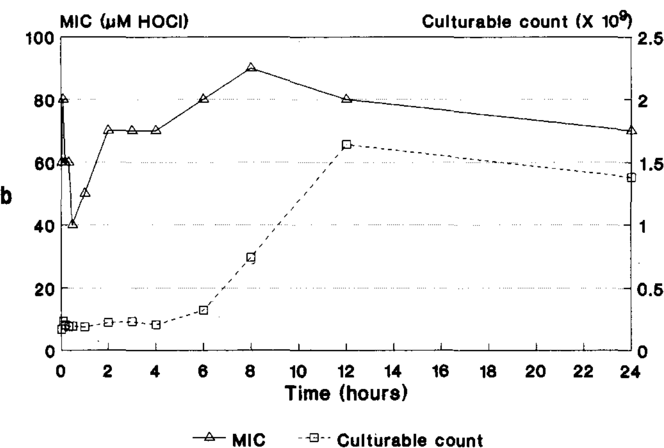


Figure 1

Minimum inhibitory concentrations (MIC)(µM) as well as culturable counts of Escherichia coli *K12 cultures during 24 h following exposure to a sub-inhibitory concentration of H_2O_2 (a) or HOCl (b)*

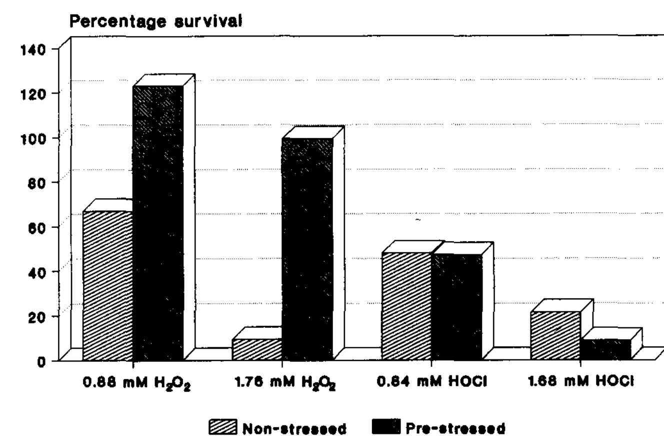


Figure 2

Survival of stressed and unstressed cultures of Escherichia coli *K12 following exposure to 0,88 and 1.76 mM H_2O_2, and to 0.84 and 1.68 mM HOCl for 60 min*

culture lost its culturability. The response of *E. coli* K12 following exposure to HOCl stress did, however, not lead to increased protection to lethal concentrations of HOCl (Fig. 2). *E. coli* K12 in stationary phase was more susceptible to high concentrations of HOCl following exposure to HOCl stress than before.

Determination of the effect of oxidant on the growth rate

Logarithmically growing cultures exposed to H_2O_2 reverted to a lag phase, returning to logarithmic growth a while later (Fig. 3a). The duration of the lag phase was proportional to the concentration of H_2O_2 added. Where H_2O_2 was inactivated at certain times following addition, logarithmic growth resumed ca. 20 min following inactivation (Fig. 3b). Where H_2O_2 was inactivated 10 min following its addition, the time to recommencement of logarithmic phase was 30 min, for the 30 min inactivation delay it was 50 min, and for the 60 min inactivation delay it was 80 min. This indicated a fixed period for repair of damage to the cells, irrespective of the initial concentration of H_2O_2. Pre-stressed cultures displayed the same lag period following secondary exposure to 0.88 mM·ℓ^{-1} H_2O_2 as did unstressed cultures, indicating a lack of additional protective mechanisms in cells stressed while in logarithmic growth phase (Fig. 3c).

Logarithmically growing cultures exposed to HOCl also reverted to a lag phase, returning to logarithmic growth, albeit with a much longer lag phase than in the case of H_2O_2 (Fig. 4a). The duration of the lag phase was again proportional to the concentration of HOCl added. Where HOCl was inactivated at certain times following its addition, cultures reverted to logarithmic growth only 4 hours following inactivation (Fig. 4b). Prestressed cultures exhibited a similar lag period following re-exposure to HOCl but showed a lower growth rate in early log phase, than did unstressed cultures (Fig. 4c). This indicated that the cells were not induced to produce any additional protective mechanisms, but rather that they were sensitised by the initial exposure to HOCl.

Cellular catalase following exposure to H_2O_2

The level of catalase increased from 1.4 to 2.1 units per mg protein within 10 min following exposure to H_2O_2, and remained between 2.1 and 2.4 units per mg protein during the 90 min assay period (Fig. 5). The mean level of cellular catalase was 1.4 units per mg *E. coli* K12 protein, much lower than in *Pseudomonas aeruginosa* (15 units per mg protein) (B Pietersen and VS Brözel, unpublished results).

Discussion

H_2O_2 was demonstrated to be primarily bacteriostatic, high concentrations being required to bring about cell death. This bacteriostatic activity was dependant on the presence of the oxidant since the cells resumed growth 20 min following depletion of H_2O_2. The mechanism of repair of H_2O_2-induced damage was found to be active even following exposure to high concentrations. The stress response to H_2O_2 of the *E. coli* K12 strain used was not found to be as marked as reported in the literature (Storz et al., 1990). The increased level of protection of stressed stationary phase cells was not sustained for very long following exposure to subsequent stress, and they appeared more susceptible to H_2O_2 after depletion of the stress response than they were before exposure. This indicated that the stress response was energy intensive, rendering cells more susceptible in the long term. Whereas

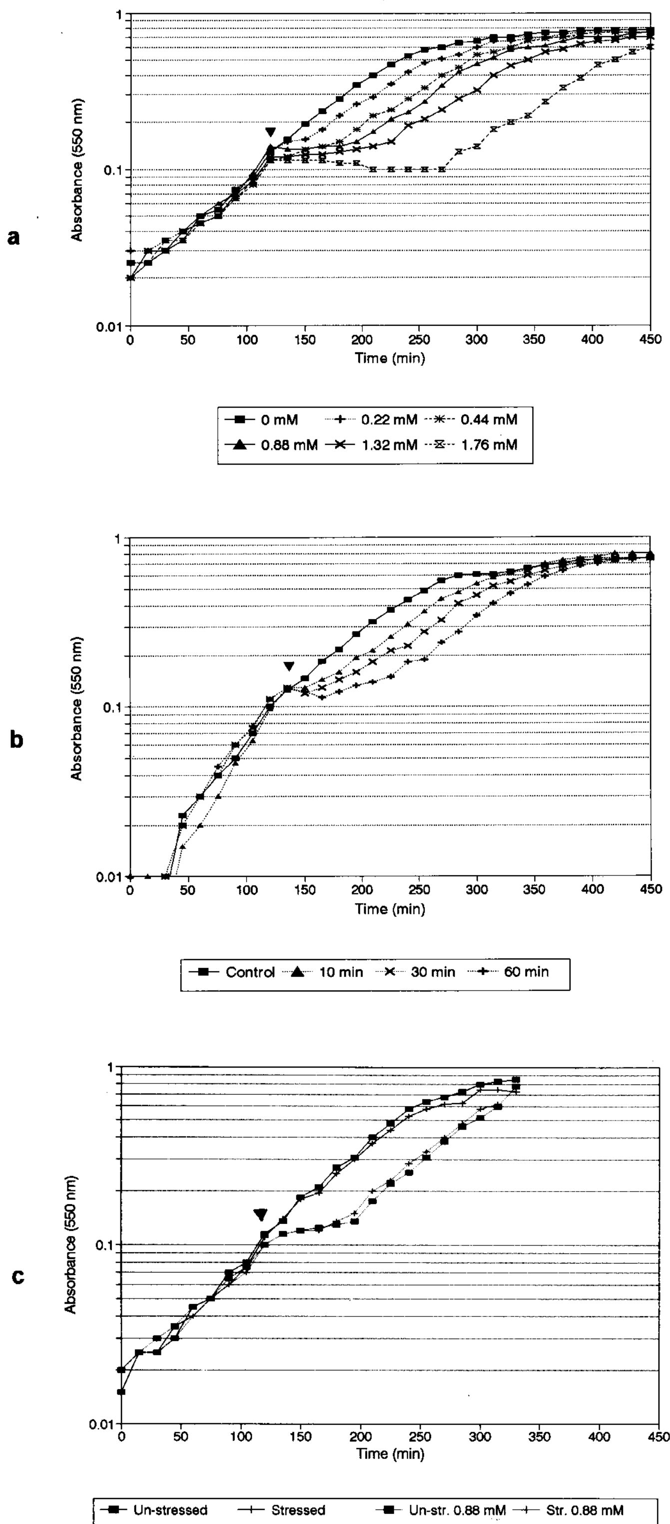


Figure 3

Response of Escherichia coli *K12 following exposure to various concentrations of H_2O_2. The growth rates (a), recovery of growth rate following inactivation of 0.88 mM H_2O_2 by addition of catalase (200 $U{\cdot}m\ell^{-1}$ final concentration) at 10, 30 and 60 min following addition of H_2O_2 (b), and growth rates of stressed as well as unstressed cultures (c) were determined by measuring absorbance at 550 nm. The times of addition of H_2O_2 are indicated by the arrows*

cultures in stationary phase did exhibit increased tolerance to H_2O_2 following stress, logarithmically growing cultures did not exhibit increased levels of tolerance to H_2O_2 despite the concurrent induction of increased catalase levels. This indicated that catalase is not the sole protective factor. Volkert et al. (1994) have shown that an *E. coli* mutant, equally sensitive to H_2O_2 in logarithmic phase as the parent strain, was more resistant when entering the stationary phase. They attributed this to loss of a stationary phase H_2O_2-sensitising mechanism.

It is possible that certain of the repair enzymes are only expressed in older or non-dividing cells, so that logarithmically growing cultures will contain only few cells with operative repair mechanisms.

The 20 min lag following degradation of H_2O_2 supported the notion that additional protective factors play a role in the H_2O_2 stress response, and further that these are only inducible once cell division has stopped, or alternatively thus require some time for induction. This may be because the natural colonic habitat of *E. coli* is anaerobic (Storz et al., 1990), and only cells exposed to air, i.e. following faecal shedding, must survive the damaging effects of H_2O_2 formed in the presence of oxygen. Once a microcolony has formed in an aerobic environment, however, cells can afford protection to each other from H_2O_2, so that the high level of protection required by single cells is no longer necessary. This would explain why the stress response declined 10 h after exposure to stress.

Hypochlorous acid also proved to be primarily bacteriostatic and only bacteriocidal at high concentrations. Its bacteriostatic activity was, however, found to be longer-lived than that of H_2O_2, cells only resuming growth 4 h following depletion of HOCl. This corresponds with chemical data as HOCl has a significantly higher oxidising potential than H_2O_2 (McKenna and Davies, 1988). This indicated either that HOCl inflicted different cellular damage than did H_2O_2, or that HOCl did not induce production of repair enzymes, if any, as rapidly as did H_2O_2. The stress response of stationary phase *E. coli* K12 to HOCl has not been reported previously. Its induction was slower than in the case of H_2O_2, so that at least some of the regulatory genes involved must be different. HOCl has a different mode of antibacterial action than has H_2O_2 (Schraufstätter et al., 1990), so that it is unlikely that the protective mechanism to HOCl overlaps much with that induced by the H_2O_2 stress response. Macrophages produce HOCl as part of their antimicrobial activity (Schraufstätter et al., 1990) so that it is conceivable that *E. coli*, being part of the mammalian flora, has evolved a protective mechanism to this oxidant.

Both H_2O_2 and HOCl were found to be primarily bacteriostatic towards *E. coli* K12. Both oxidants elicited a stress response in stationary phase cells, cultures being more resistant following initial exposure to low concentrations of the relevant oxidant. However, no stress response could be demonstrated in logarithmically growing cells, neither to H_2O_2 nor to HOCl.

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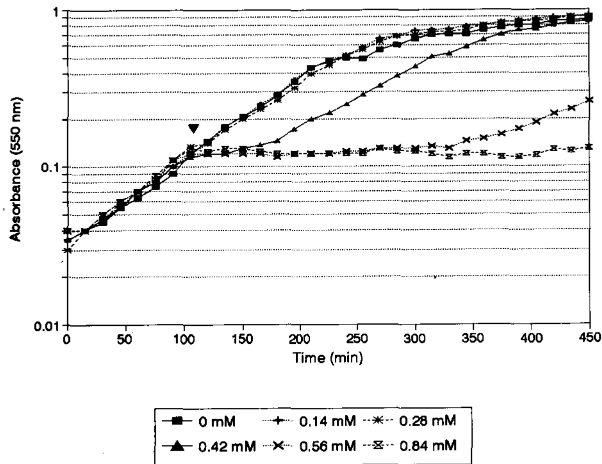

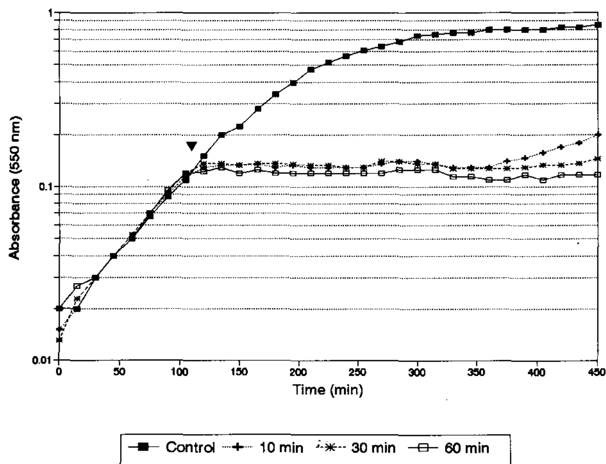

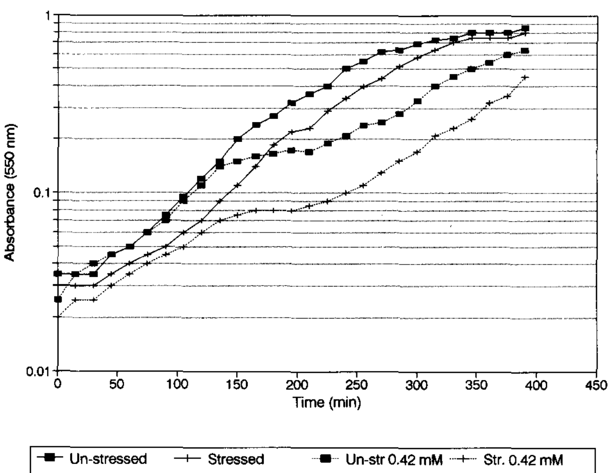

Figure 4

Response of Escherichia coli *K12 following exposure to various concentrations of HOCl. The growth rates (a), recovery of growth rates following inactivation of 0.84 mM HOCl by addition of $Na_2S_2O_3$ (0.01 % (m/v) final concentration) at various times (b), and growth rates of stressed as well as unstressed cultures (c) were determined by measuring absorbance at 550 nm. The times of addition of HOCl are indicated by the arrows*

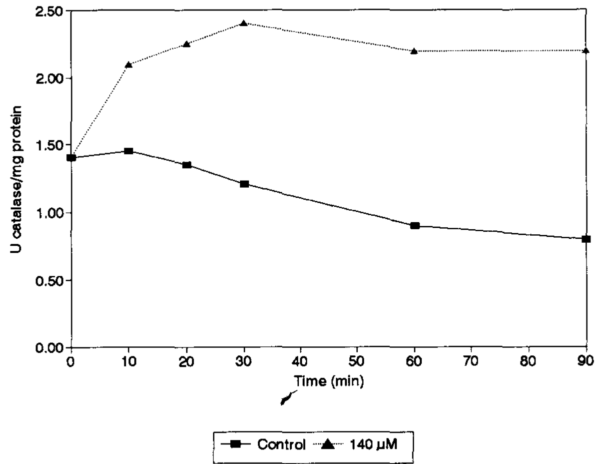

Figure 5

Effect of H_2O_2 stress on cellular catalase levels in Escherichia coli *K12. Logarithmically growing cultures were exposed to 140 $\mu M \cdot \ell^{-1}$ H_2O_2. Cell extracts were prepared at various times following addition of H_2O_2, and catalase was assayed as described in* ***Material and Methods***

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