The induction of oxidative enzymes in *Streptomyces coelicolor* upon hydrogen peroxide treatment

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Streptomyces coelicolor (Müller) became resistant to killing by hydrogen peroxide (H_2O_2) when pretreated with non-lethal concentrations of H_2O_2 . When rapidly growing cells were pretreated with $100\ \mu\text{M-H}_2\text{O}_2$, they became 7–10-fold more resistant to $20\ \text{mM-H}_2\text{O}_2$ than were naive cells. Activities of several oxidative defense enzymes were measured in cells treated with $100\ \mu\text{M-H}_2\text{O}_2$ in either exponential or stationary phase growth. The specific activity of catalase in crude extracts of cells pretreated in either phase increased about 40%. Peroxidase activity, in cell extracts and culture supernatants, respectively, of cells treated in the stationary growth phase increased two times and four times. Glucose-6-phosphate dehydrogenase increased by 60% at the exponential growth phase. Glutathione reductase increased 80% after treatment in the exponential phase and 4-fold in the stationary growth phase. However, superoxide dismutase activity decreased by 70%. Two mutants resistant to H_2O_2 were isolated after mutagenesis of spores with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. In addition to a dramatic increase in the survival rate in $20\ \text{mM-H}_2\text{O}_2$, both mutants exhibited increased activities of all the above enzymes except superoxide dismutase. The pleiotropic phenotype of the mutants suggests that there exists a global regulation of oxidative response in *S. coelicolor*.

Introduction

All aerobic organisms must cope with reactive oxygen species such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\text{HO}^\bullet$), produced as incomplete reduction products of molecular oxygen, and with singlet oxygen formed photochemically (Cadenas, 1989; Morgan *et al.*, 1986; Halliwell, 1990). Bacteria grown aerobically encounter these oxidants not only from inside the cell, but also from outside, as occurs, for example, when pathogenic bacteria are attacked by phagocytic cells (Hassett & Cohen, 1989). The reactive oxygen species can oxidize membrane fatty acids and proteins, and can damage DNA (Morgan *et al.*, 1986; Saran & Bors, 1990). The responses of bacterial cells to these oxidative stresses have been studied mostly in *Escherichia coli* and *Salmonella typhimurium* (Storz *et al.*, 1990; Farr & Kogoma, 1991).

Several oxidative defence proteins and their genes have been found in *E. coli* (Storz *et al.*, 1989, 1990; Farr

& Kogoma, 1991). These include defence (radical-scavenging) enzymes such as superoxide dismutase (SOD) (*sodA*, *sodB*), catalase (*katG*, *katE*), alkylhydroperoxide reductase (*ahpC*, *ahpF*) and glutathione reductase (*gorA*). Several DNA enzymes such as exonuclease III (*xthA*), endonuclease IV (*nfo*) and other endonucleases (*recB*, *recC*), as well as some metabolic enzymes such as NADH dehydrogenase (*ndh*) and glucose-6-phosphate dehydrogenase (*zwf*) are also involved in oxidative defence. These enzymes might repair DNA damage caused by H_2O_2 or $\text{HO}^\bullet$ radicals (Imlay *et al.*, 1988; Imlay & Linn, 1988), protect cells from membrane damage such as lipid peroxidation (Farr *et al.*, 1988), or lower the reducing equivalents to prevent generation of harmful $\text{HO}^\bullet$ radicals in the presence of metal and reducing equivalents (Imlay *et al.*, 1988). The evolution of such defence mechanisms in order to prevent or repair oxidative damage must have been critical to the survival of aerobic life forms (Halliwell & Gutteridge, 1989).

Induction of the defence proteins by treating cells with low doses of H_2O_2 or radical-producing oxidants provides the basis for adaptation as well as resistance to the oxidants. Several regulatory proteins mediate expression

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Abbreviation: SOD, superoxide dismutase.

of the genes for these proteins. The genes induced by superoxide-generating compounds such as paraquat, plumbagin or menadione include *sodA* and *zwf*, and are all controlled by the regulator SoxR (Tsaneva & Weiss, 1990). The genes induced by H_2O_2 include *katG*, *ahpC*, *ahpF* and *gorA*, and are all controlled by the positive regulator OxyR (Christman *et al.*, 1985, 1989; Morgan *et al.*, 1986). The so-called *soxR* and *oxyR* regulons seem to overlap other stress response regulons such as that for heat-shock, since some proteins are induced in common by different stresses (Greenberg & Dimple, 1989; VanBogelen *et al.*, 1987), and some cross-protection has been observed (Christman *et al.*, 1985; Jenkins *et al.*, 1988).

Compared with *E. coli* and *S. typhimurium*, little is known about these systems in *Bacillus subtilis*, and even less in other bacteria. In *B. subtilis*, several proteins are induced by H_2O_2 (Murphy *et al.*, 1987), and the sensitivity of *B. subtilis* to H_2O_2 is growth-cycle-dependent. Increased sensitivity in certain sporulation mutants has also been reported (Dowds *et al.*, 1987; Bol & Yasbin, 1990).

We have tried to elucidate the regulation of defences against oxidative stress in *Streptomyces coelicolor* (Müller). This paper presents a biochemical analysis of the adaptation to H_2O_2 in *S. coelicolor*, and some evidence for a global genetic system that controls the expression of many defence enzymes in this organism.

Methods

Media and culture conditions. Growth and maintenance of *S. coelicolor* (Müller) (ATCC 10147) were essentially as described by Hopwood *et al.* (1985). *S. coelicolor* was grown routinely in minimal or YEME media (Hopwood *et al.*, 1985) at 30 °C with shaking. The growth phase of cultures was determined by measuring growth as OD₆₄₀, dried mycelium weight or DNA content (Baserga, 1989).

For adaptation experiments in the early exponential growth phase, germinated spores were used to inoculate fresh minimal media to an OD₆₀₀ of 0.03–0.05. After cultures had been incubated with shaking for 3 h at 30 °C, H_2O_2 was added to a final concentration of 100 µM and the cultures were kept at 30 °C for 1 h. The cultures were then challenged with H_2O_2 at final concentrations up to 20 mM. Samples were withdrawn at 10 min intervals, and diluted immediately in minimal medium; aliquots were plated on nutrient agar and incubated for 2 d at 30 °C to count viable cells.

For adaptation experiments in the mid-exponential growth phase, spores were used to inoculate YEME media. Following growth for 36–40 h, mycelia were collected by centrifugation and resuspended in minimal medium. H_2O_2 was added to a final concentration of 100 µM and the cultures were incubated without shaking at 30 °C for 30 min. The cultures were then challenged with H_2O_2 as above. Protoplasts were obtained as described by Hopwood *et al.* (1985). Dilutions were made in P buffer (Hopwood *et al.*, 1985), and aliquots of diluted cells were plated on R2YE regeneration medium. They were incubated for 2 d at 30 °C to count viable and regenerated cells.

For enzyme assays, germinated spores were used to inoculate minimal medium and the cultures were grown for 40–48 h, when their OD₆₄₀ was 0.9–1.2 (exponential growth phase) or for 7–10 d (stationary growth phase). The production of both blue and red pigments was

regarded as an indication of entry into the stationary growth phase. The mycelium and DNA content of stationary phase cultures decreased by 20–60%.

Preparation of cell extracts. Mycelium was harvested by centrifugation at 3000 g for 10 min and was washed twice with 10 mM-potassium phosphate buffer (pH 7.0). The washed mycelium was suspended in 50 mM-potassium phosphate buffer (pH 7.0) containing 1 mM-phenyl-methylsulphonyl fluoride and was disrupted by abrasion with glass beads for 5 min in a mini-bead beater (Biospec) at the maximum power setting. The cell debris was pelleted at 12000 r.p.m. for 15 min in a microcentrifuge. Cells and extracts were maintained at 4 °C throughout the procedures described above.

Enzyme assays. Catalase activity in extracts was determined as described by Beers & Sizer (1952). The rate of disappearance of H_2O_2 was measured spectrophotometrically at 240 nm. The reaction mixture contained 12 µl 3% (v/v) H_2O_2 and an appropriate amount of enzyme in 50 mM-potassium phosphate buffer (pH 7.0) in a 1.0 ml total volume. The assay was done at 30 °C in quartz cuvettes of 1 cm path length. One unit of enzyme was defined as the activity that catalysed the degradation of 1 µmol H_2O_2 min⁻¹.

Peroxidase activity in cell extracts and culture supernatants was measured spectrophotometrically by monitoring the oxidation of *o*-dianisidine at 460 nm in the presence of H_2O_2 (Kapoor & Sreenivasan, 1988). The reaction mixture contained 10 µl 3 M-sodium acetate buffer (pH 4.8), 10 µl H_2O_2 and 10 µl 0.044% *o*-dianisidine, in a 1.0 ml total volume. One unit of enzyme was defined as the activity that increased the A_{460} by 0.01 min⁻¹.

The assay for superoxide dismutase (SOD) (Beauchamp & Fridovich, 1971) was based on the transfer of electrons by xanthine oxidase to nitroblue tetrazolium (NBT) via oxygen. This reduction of NBT is inhibited by SOD. The reaction mixture contained 0.1 mM-xanthine, 25 µM-NBT, 0.1 mM-EDTA and 0.01% (w/v) xanthine oxidase, in 50 mM-sodium carbonate buffer (pH 10.2). The initial rate of reduction of NBT in reaction mixtures with and without SOD was followed by measuring the A_{560} . The amount of SOD activity causing 50% inhibition was defined as one unit.

Glutathione reductase activity in crude extracts was measured by the modified procedure of Smith *et al.* (1988). This is based on the increase in A_{412} when 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) is reduced by reduced glutathione (GSH), produced by glutathione reductase. The reaction mixture contained 1 mM-EDTA, 0.3 mM-DTNB, 0.1 mM-NADPH and 1 mM-oxidized glutathione (GSSG), in 200 mM-potassium phosphate buffer (pH 7.5). A unit of activity was defined as the amount that catalysed the production of 1 µmol thiobis(2-nitrobenzoic acid) (TNB) min⁻¹.

Glucose-6-phosphate dehydrogenase was measured using protocols outlined in the *Worthington Manual* (Decker, 1977), as the rate of reduction of NADP monitored at 340 nm. The assay mixture contained 30 µl 32 mM-glucose 6-phosphate in 100 mM-Tris/HCl (pH 7.5), 10 µl 23 mM-NADP in 1% sodium bicarbonate, and enzyme solution in a 1.0 ml total reaction volume. One unit of enzyme was defined as the activity that catalysed the reduction of 1 µmol NADP min⁻¹.

Mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and screening for H_2O_2 -resistant mutants. Spores were treated with NTG (1 mg or 3 mg ml⁻¹) to a survival ratio of 0.1–10% and then plated on Bennet's medium (Jones, 1949), presoaked with 30 mM- H_2O_2 for 30 min. Spores of surviving colonies were collected to test resistance to H_2O_2 . About 10⁵ spores from each colony were spread in parallel rows on Bennet's medium. After 5 h, a strip of filter paper soaked with 3% (880 mM) H_2O_2 was placed at 90° across the centre of the inoculated streaks and incubation was continued for 2 d. Mutants that grew closer to the strip than the wild-type were collected. For disk tests, around 10⁶ spores from each mutant were plated on Bennet's medium; 5 h later 60 µl 30% H_2O_2 absorbed on a filter disk was placed at the centre of a

plate. Mutants that grew closer to the disc than the wild type were chosen.

Results and Discussion

Adaptation to H₂O₂ stress

We tested the sensitivity of *S. coelicolor* to H₂O₂ as well as its adaptation in early and mid-exponential growth phases. In the adaptation experiments, we pretreated cells with a non-lethal concentration (100 µM) of H₂O₂ for 1 h before exposure to a lethal concentration (20 mM). To obtain viable cell counts by plating, we grew cultures in minimal medium for 3 h after inoculation with germinated spores. The viability of cells in 20 mM-H₂O₂ was counted at 10 min intervals after plating. The results (Fig. 1) demonstrate that only 0.1% of the cells remained viable after 30 min in 20 mM-H₂O₂. However, when cells were pretreated with H₂O₂, survival was enhanced almost 10-fold.

The extent of adaptation was similar when cells were grown to mid-exponential phase and counted as regenerated protoplasts after H₂O₂ treatment. The results suggest that the pretreatment with 100 µM-H₂O₂ caused mycelial cells to adapt better to a higher dose of H₂O₂, showing a survival rate seven times higher than naive cells against 20 mM-H₂O₂ (Fig. 2). Since the colonies went through lysozyme treatment and regeneration in addition to H₂O₂ treatment, we cannot quantitatively estimate the H₂O₂ sensitivity or adaptation. However, the results point to qualitatively similar adaptations in early-exponential and mid-exponential growth phases.

The adaptation of H₂O₂ in *S. coelicolor* occurred under conditions slightly harsher than those previously described for *E. coli* (Dempsey *et al.*, 1983), *S. typhimurium* (Christman *et al.*, 1985) and *B. subtilis* (Dowds *et al.*, 1987). Pretreatment with 60 and 50 µM-H₂O₂ increased survival against 10 mM-H₂O₂ by approximately 50- and 100-fold, in *E. coli* and in *B. subtilis*, respectively.

Adaptation is accompanied by induction of several enzyme activities involved in defence against oxidative stress

The specific activities of several oxidoreductases and free-radical scavenging enzymes known to function in protection against oxidative stress were measured (Table 1). Cells in the exponential and stationary growth phases were treated with 100 µM-H₂O₂ for 30 min. The specific activity of catalase in crude extracts of treated cells increased about 40% above that in untreated cells at both exponential and stationary phases. This is a smaller response than the 4–5-fold induction of catalase activity in *E. coli* (Christman *et al.*, 1985) and *S. typhimurium* (Finn & Condon, 1975). The constitutive level of catalase

Fig. 1. Survival of *S. coelicolor* in early exponential growth phase following treatment with 20 mM-H₂O₂. Pregerminated spores were used to inoculate minimal medium, and were grown at 30 °C with shaking for 3 h. Some cells were pretreated with 100 µM-H₂O₂ for 1 h. Both pretreated (□) and naive (■) cells were then treated with 20 mM-H₂O₂. Samples taken at 10 min intervals were plated on nutrient agar. Each point represents the mean of three experiments with error bars showing standard deviations.

Fig. 2. Survival of *S. coelicolor* in mid-exponential growth phase following treatment with H₂O₂. Survival was measured by counting regenerated protoplasts (1) without any H₂O₂ treatment (regarded as 100%); (2) after treatment with 100 µM-H₂O₂ for 30 min; (3) after treatment with 20 mM-H₂O₂ for 30 min; or (4) after pretreatment with 100 µM-H₂O₂ for 30 min and then with 20 mM-H₂O₂ for another 30 min. Each result represents the mean of three experiments with error bars giving standard deviations.

activity in *S. coelicolor* was about 7-fold and 4-fold higher than that in *E. coli* and *B. subtilis*, respectively (data not shown).

Peroxidase activity was not induced in the exponential and stationary growth phases by treatment with 100 µM-H₂O₂ (Table 1). However, it increased 2-fold in extracts of treated cells from cultures more than 2 weeks old. Peroxidase activity in cell-free supernatants from cultures treated at this later stationary growth phase was 4-fold higher than in untreated cells. It is not known whether the activity in the culture supernatants originated from active secretion or from passive release due to cell damage.

Table 1. Changes in specific activity ($U\ mg^{-1}$) of defence enzymes following treatment with $100\ \mu M\ H_2O_2$

Exponential phase activities were measured in extracts from cells grown for 40–48 h in minimal medium until OD_{640} reached 0.9–1.2; stationary phase activities were measured from cells grown for 7–10 d in minimal medium until both red and blue pigments were produced. –, Untreated cells; +, cells treated with $100\ \mu M\ H_2O_2$ for 30 min. Values are means $\pm$ standard deviations from more than six experiments.

Enzyme	H_2O_2 treatment...	Exponential phase		Stationary phase	
		–	+	–	+
Catalase		634 $\pm$ 51	880 $\pm$ 114	690 $\pm$ 8	1003 $\pm$ 56
Peroxidase	Cellular	2.1 $\pm$ 0.3	2.0 $\pm$ 0.3 3.8 $\pm$ 0.2*	2.4 $\pm$ 0.2 7.7 $\pm$ 0.4*	2.9 $\pm$ 0.3
	Extracellular	ND	ND	7 $\pm$ 1*	26 $\pm$ 3*
Glucose-6-phosphate dehydrogenase		6.4 $\pm$ 3.0	10.5 $\pm$ 5.1	ND	ND
Glutathione reductase		508 $\pm$ 233	923 $\pm$ 226	200 $\pm$ 46	830 $\pm$ 128
Superoxide dismutase		28.5 $\pm$ 6.2	8.8 $\pm$ 1.6	NT	NT

NT, Not tested; ND, not detected.

* Activities from cells grown for 14–21 d in minimal medium.

Reduced glutathione is thought to play a role in preventing excessive damage from toxic oxygen species (Penninckx & Jaspers, 1982). Glutathione can be easily oxidized to the intermolecular disulphide, which subsequently becomes reduced by glutathione reductase (Meister & Anderson, 1983). The activity of glutathione reductase in cell extracts was induced almost 2-fold by H_2O_2 in the exponential growth phase and 4-fold in the stationary growth phase (Table 1). Since glutathione was not detected in *Streptomyces clavuligerus*, it is possible that the glutathione reductase activity that we measured in *S. coelicolor* is from another reductase that can use glutathione as a substrate. One plausible candidate is thioredoxin reductase (Y. Aharonowitz, Tel Aviv University, Israel, personal communication).

Reduced NADP, produced by glucose-6-phosphate dehydrogenase, can be used as reducing power during protection and repair against oxidative stress. The specific activity of glucose-6-phosphate dehydrogenase in the exponential growth phase was 60% higher in treated than in untreated cells (Table 1). Little activity was detected in extracts of stationary phase cells.

SOD activity in cell extracts decreased by 70% following treatment with $100\ \mu M\ H_2O_2$ in the exponential growth phase (Table 1). In contrast, SOD activity in *E. coli* (Gregory & Fridovich, 1973; Hassan & Fridovich, 1977) and *S. typhimurium* (Christman *et al.*, 1985) was induced following a shift from anaerobic to aerobic conditions or H_2O_2 stress. In *E. coli*, the activity of

Fig. 3. Survival rate of the wild-type and H_2O_2 -resistant mutants treated with $20\ mM\ H_2O_2$. Pregerminated spores from the wild-type (■) and from mutants N7 (□) and N24 (▲) were used to inoculate minimal media, and grown for 3 h. Cells were treated with $20\ mM\ H_2O_2$ and then analysed as described in the legend in Fig. 1. Each value is the mean from three experiments. The standard deviation is shown as a bar.

manganese SOD increased during oxidative stress, while that of iron SOD decreased (Hassan & Fridovich, 1977). Previous investigations showed that manganese SOD was induced by oxygen, redox-cycling compounds in aerobiosis, and iron chelators in anaerobiosis, suggesting that the regulation of SOD activity might be at the transcriptional and the post-transcriptional level (Touati, 1988). Since the superoxide radical is converted irreversibly to H_2O_2 *in vivo*, the failure of H_2O_2 to induce SOD activity in *S. coelicolor* is not surprising. The reduction in activity by H_2O_2 could have resulted from competition for metals among some H_2O_2 -induced oxidoreductases, which are metalloproteins.

The difference in the extent of induction depending on the growth phase is consistent with the growth-phase-dependent sensitivity to H_2O_2 observed in *B. subtilis* (Dowds *et al.*, 1987). This dependence suggests an interaction between the oxidation-inducible and the growth-phase-specific systems.

H₂O₂-resistant mutants overexpress several enzyme activities

As a preliminary to genetic investigation of the regulatory system for the response against oxidation in *S. coelicolor*, we isolated mutants resistant to up to $880\ mM\ H_2O_2$. The mutants arose during NTG mutagenesis at a frequency of about 1×10^{-5} per surviving cell. Two mutants showing the highest resistance by disc and strip tests were further characterized. They exhibited marked increases in survival rate against H_2O_2 (Fig. 3). Cross-protection against heat shock ($50\ ^\circ C$) was also greater in the two mutants compared with the wild-type (data not shown). The specific activity of oxidoreductases in the mutants were measured in the

Table 2. Comparison of specific activities (U mg⁻¹) of defence enzymes in the wild-type and in resistant mutants at different growth phasesGrowth phases were as in Table 1. Values are means $\pm$ standard deviations from more than three experiments.

Enzyme	Growth phase ...	Wild-type		N7		N24	
		Exponential	Stationary	Exponential	Stationary	Exponential	Stationary
Catalase		634 $\pm$ 51	690 $\pm$ 8	5339 $\pm$ 239	2596 $\pm$ 33	1718 $\pm$ 239	1390 $\pm$ 18
Peroxidase							
Cellular		2.1 $\pm$ 0.3	2.0 $\pm$ 0.3	2.3 $\pm$ 0.1	2.5 $\pm$ 0.1	2.1 $\pm$ 0.2	2.9 $\pm$ 0.1
			3.8 $\pm$ 0.2*		8.8 $\pm$ 0.2*		7.4 $\pm$ 0.3*
Extracellular		ND	6.8 $\pm$ 0.6*	ND	17.6 $\pm$ 1.0*	ND	25.1 $\pm$ 0.8*
Glucose-6-phosphate dehydrogenase		6.4 $\pm$ 3.0	NT	25.1 $\pm$ 0.9	NT	9.4 $\pm$ 0.5	NT
Glutathione reductase		508 $\pm$ 233	200 $\pm$ 46	993 $\pm$ 82	NT	947 $\pm$ 61	NT
Superoxide dismutase		28.5 $\pm$ 6.2	NT	21.6 $\pm$ 4.4	NT	24.0 $\pm$ 4.4	NT

NT, ND, *, as Table 1.

exponential and stationary growth phases without H₂O₂ treatment (Table 2). Both mutants contained higher levels than wild-type cells of all the enzymes tested except SOD.

Catalase activity in mutant N7 was 8-fold higher in the exponential growth phase and 4-fold higher in the stationary growth phase than in wild-type cells. In mutant N24, catalase activity was 3-fold higher in the exponential phase and 2-fold higher in the stationary phase. Intracellular peroxidase activity was about 2-fold higher in the later phase of stationary growth, whereas extracellular activity was 3–4-fold higher in the two mutants. Glutathione reductase activity was about 2-fold higher in the two mutants. Glucose-6-phosphate dehydrogenase activity was 4-fold higher in N7 and 50% higher in N24. In both mutants, the increase in catalase activity was much greater than that induced in wild-type cells by 100 μ M-H₂O₂. In the exponential growth phase, the level of catalase in mutants N7 and N24 was 6 times and 2 times greater than the H₂O₂-induced level in wild-type cells. SOD activity in the mutants was similar to that in naive wild-type cells. The extent of the increase in peroxidase and glutathione reductase activity in the mutants was similar to the extent of induction by H₂O₂ in wild-type cells. When the mutant cells were treated with 100 μ M-H₂O₂, the levels of all five enzymes did not change significantly (data not shown).

Considering the frequency with which these mutants arose, it is unlikely that separate mutations had occurred in the genes for each enzyme affected. The pleiotropic effect of the mutations is possibly due to a change in a regulatory gene controlling the response to H₂O₂ stress. It seems plausible to assume that there is global regulation of the oxidative response in *S. coelicolor*, as in *E. coli* and *S. typhimurium* (Christman *et al.*, 1985; Greenberg & Demple, 1989; Wu & Weiss, 1991). In *E.*

coli and *S. typhimurium*, the *oxyR* (Christman *et al.* 1989) and *soxR* (Tsaneva & Weiss, 1990) genes are known to be required for induction of a response regulon against H₂O₂ and superoxide, respectively. OxyR is a transcriptional activator for the genes *katG* (encoding catalase), *ahpCF* (encoding alkylhydroperoxide reductase) and *gorA* (encoding glutathione reductase), whereas SoxR is a global regulator governing *nfo* (encoding endonuclease IV), *zwf* (encoding glucose-6-phosphate dehydrogenase) and *sodA* (encoding manganese superoxide dismutase). However, since the enzymes induced in resistant mutants N7 and N24 encompass those from both OxyR and SoxR regulons in *E. coli*, we suppose that the make-up of the peroxide-inducible regulon in *S. coelicolor* is different from *E. coli*.

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