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CutC is induced late during copper exposure and can modify intracellular copper content in *Enterococcus faecalis*

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ABSTRACT

Copper is a micronutrient that is required for proper metabolic functioning of most prokaryotic and eukaryotic organisms. To sustain an adequate supply of copper, a cell requires molecular mechanisms that control the metal content to avoid copper toxicity. This toxicity comes primarily from the reactivity of copper, which can lead to the generation of free radicals. In bacteria, two independent systems are responsible for maintaining the balance of copper within the cells (Cop and Cut family proteins). Previous studies describe CutC as a member of the Cut family that is probably involved in copper homeostasis. However, the role of CutC in copper homeostasis is still unclear. In this work, a homolog of CutC was studied in *Enterococcus faecalis*, a bacterial model for copper homeostasis. The molecular 3D model of efCutC shows the presence of triose phosphate isomerase (TIM) barrel motifs, previously described in CutC crystals from other organisms, which illustrates the conservation of amino acids with the potential ability to coordinate copper. Through quantitative real-time PCR (qPCR), it was demonstrated that *efcutC* expression is induced late by copper stimulus, Interestingly this transcriptional response directly correlates with a significant increase in the intracellular copper concentration when the protein is absent in the bacteria, suggesting its participation in mechanisms related to efflux of the metal. Our results describe efCutC as a protein able to respond transcriptionally to copper and to participate in the control of copper homeostasis in *E. faecalis*. This bacterium is the first reported organism containing a *cop* operon and an active member of the Cut protein family.

1. Introduction

Copper is a trace element required by prokaryotic and eukaryotic organisms [1]. In nature, copper can be found in an oxidized (Cu^{+2}) or reduced (Cu^{+1}) state. Thus, it can act as an electron donor or acceptor in the reduction or oxidation of different substrates. Within biological systems, copper is used as a cofactor in metallo-enzymes involved in cellular respiration processes, iron transport and protection from free radicals [2,3]. Such properties make this transition element essential to carry out a series of biochemical processes in a normal fashion. On the other hand, excess copper can induce the generation of free radicals through the Haber–Weiss and Fenton reactions [4], which are capable of damaging cell membranes, proteins, and DNA [5]. This duality of copper's behavior explains the need for specific regulatory systems that maintain a cellular homeostasis of the metal. Uptake, internal traffic, storage and efflux are the four main steps required to handle this metal in the cell's environment [6]. In bacteria, one of the most studied systems that control copper content is codified by the *cop* operon [7]. This operon codes for proteins able to control intracellular copper traffic, mediate the uptake and efflux of the metal, and regulate the operon's own expression.

A second group of proteins that also has a crucial role in maintaining copper levels within the cell is the *cut* family [8]. One of the most studied members of this family is the CutC protein. Although its role in copper homeostasis is not well understood, there is increasing evidence suggesting that this gene might be differentially expressed in response to copper, as it could be directly or indirectly involved in protection from excess amounts of this metal. Transcriptional analysis in the plant pathogen *Xylella fastidiosa* shows an induction of *cutC* under increasing copper concentrations [9]. The opposite behavior has been observed in the nematode *Caenorhabditis elegans*, where the gene is repressed in response

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to elevated copper concentrations [10]. The crystal structure of human CutC assumes a typical TIM barrel fold present in cytoplasmic proteins (previously described in *Shigella flexneri*) [11,12], with two conserved cysteine residues for potential participation in copper coordination. All these antecedents suggest that CutC probably participates in regulation of copper homeostasis; however, it is still unclear how the capacity of *cutC* to respond to copper levels may affect the intracellular concentration of the metal.

In this context, using *Enterococcus faecalis* as a model for copper homeostasis, the goal of this work is to show a temporal correlation between the expression of *cutC* and the effect of its absence on the metal content over time, thus strengthening the link between the behavior of the *cutc* gene and copper homeostasis in this bacterium.

2. Materials and methods

2.1. Bioinformatics analysis

The efCutC molecular 3D model was obtained by automatic sequence comparison from the human CutC crystallographic information (PDB id. 3IWP) using the SWISS-MODEL program. It was displayed with VMD v1.8.6 software [13,14]. Homologous CutC sequences from *Lactobacillale* order organisms were obtained from the National Center for Biotechnology Information (NCBI) website by BlastP using the efCutC sequence as a template (EF2667) [15]. Global protein alignments were performed using Clustal W (BioEdit software) [16]. A secondary structure prediction of efCutC was generated using the PROTEUS website [17].

2.2. Strains, growth conditions and copper/iron/hydrogen peroxide treatments

E. faecalis strains were grown on N medium (Peptone 1%, yeast extract 0.5%, Na_2HPO_4 1% and glucose 1%) [18]. For all the treatments, the strains were precultured overnight in N medium broth at 37 °C. The next day, the cells were diluted in two parallel cultures (control and treatment) to a final $OD600_{nm}$ of 0.05 and then grown at 37 °C and 160 rpm. The treatment culture was supplemented with a nonlethal concentration of 0.5 mM of $CuSO_4$ [18], 2.4 μM of H_2O_2 or 0.5 mM of $FeCl_3$:NTA (1:1). After 15, 45, 90 and 135 min of incubation with $CuSO_4$, 45 min with H_2O_2 and 6 h with $FeCl_3$:NTA, 6 mL of each culture were collected by centrifugation and washed with phosphate buffered saline (PBS: 0.15 M NaCl, 50 mM glycine (pH 3.5) and 1.0 mM EDTA) at a pH of 6.8. Finally, the washed cells were resuspended in 1 mL of PBS. Aliquots were taken for RNA extraction and copper content quantification.

2.3. RNA extraction, cDNA synthesis and quantitative real-time PCR (qPCR)

The total RNA from the control and the treatment aliquots was extracted using a Qiagen RNeasy mini kit (Qiagen). The residual DNA was removed with RQ1 RNase-free DNase (Promega), according to the manufacturer's recommendations [18]. The RNA integrity was assessed by gel electrophoresis. For cDNA synthesis, 2 μg of total RNA were reverse-transcribed using Moloney Murine Leukemia Virus Reverse (Promega, USA) with random primers (Invitrogen). All of the PCR primers used were designed with Primer3 plus software [19], employing the *E. faecalis* V583 genome sequence [20] as a template (Table 1). The qPCR reactions and the data analysis were performed as described previously [21], using the *gdh* gene (EF1004) for normalization [18,22]. In all of the cases, qPCR reactions were carried out in triplicate using two independent RNA samples. The results were expressed as the fold change between copper-treated and control cultures. The statistical analyses were assessed by the REST 2008 algorithm [23].

Table 1
List of primers used in qPCR experiments.

Gene	Id[a]	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
cutC	EF2667	CCCCAAGCCATTCAAAAAG	CGCCAGCGTATGCTAAGAC
copY	EF0297	TGAAACGGTGGCAGATGAA	CATTGGCATTGACCAGGAA
copA	EF0298	TCCAGTGGCTATGGGTGTG	GTAAGGCGTTTAGTAAGACAG
copZ	EF0299	TTGATGGGATGAAATGTGATG	GCCAAGTGAACCGCAACA
sod	EF0463	GTCACGCAAACCATACATTC	CACAACTAACCAAGCCCAAC
cat	EF1597	CGCCCATTTTTTTCATTCGTG	GACGGAACGACAACGGAAT
trx	EF1405	GCAAGCACCCATCTTAGAAC	CTTTTGTATGAACACCGACTG
gdh	EF1004	GCGGCTATTATGACCACAG	TCTTCAGAATAAAGGCGGA

[a] Id code for *E. faecalis* V583 genome.

2.4. Mutant cutC generation

Mutant *cutC* strains of *E. faecalis* OG1RF were constructed using the pTEX4577 vector system, as previously described [24]. Briefly, a fragment of 400 bps corresponding to a central portion of the gene was amplified by PCR. The amplicon was first cloned in pGEM-T Easy (Promega) and then assembled in pTEX4577. The resulting construct was transferred to *E. faecalis* OG1RF by electroporation, and the transformants were selected in N medium agar plates supplemented with 2 mg/mL of Kanamycin. The selected mutants were verified by PCR and Southern Blot. Finally, different restriction patterns between wild-type and mutant *cutC* were confirmed by a pulsed-field gel electrophoresis.

2.5. Quantification of copper

After the copper treatments, an intracellular copper quantification for each aliquot was performed by atomic absorption spectrometry (AAS), as described previously [18]. The results were expressed as copper atoms normalized per μg of protein and represent the average value of three measurements for two independent biological replicates. The statistical analysis was done by an ANOVA test ($p < 0.05$).

3. Results and discussion

The proteins involved in copper homeostasis are structurally and functionally conserved from eukaryotic to prokaryotic organisms. Such is the case for copper efflux ATPases, chaperons, and proteins related to the control of oxidative stress generated by an excess of the metal [6]. In this context, our first approach was to identify whether CutC exhibits the structural characteristics described in other organisms for this metalloprotein. Previous bioinformatics searches indicated the presence of a putative CutC protein in *E. faecalis* (efCutC) [25]. It has been reported that homologues of this protein exhibit the classical folding of the TIM barrel family of proteins, highly conserved among prokaryotes and eukaryotes [10,11]. The 3D projection generated in this work was consistent with data previously reported in other species; eight beta sheets form a central pore surrounded by seven alpha-helices, which is typical in several cytoplasmic proteins (Fig. 1A). In particular, the residues Cys6, Glu25, Cys27, Met57, His123 and Glu204 stand out because they are arranged in a form similar to the copper coordination motif described in the human crystal CutC protein [10,11]. In addition to this information, it has been documented that TIM family proteins are able to coordinate divalent metals, as is the case for phosphotriesterase (linked to the detoxification of organophosphates) and aminoacylase (linked to amino acid synthesis), which use zinc as a cofactor and can even be inhibited by copper and cadmium [26,27].

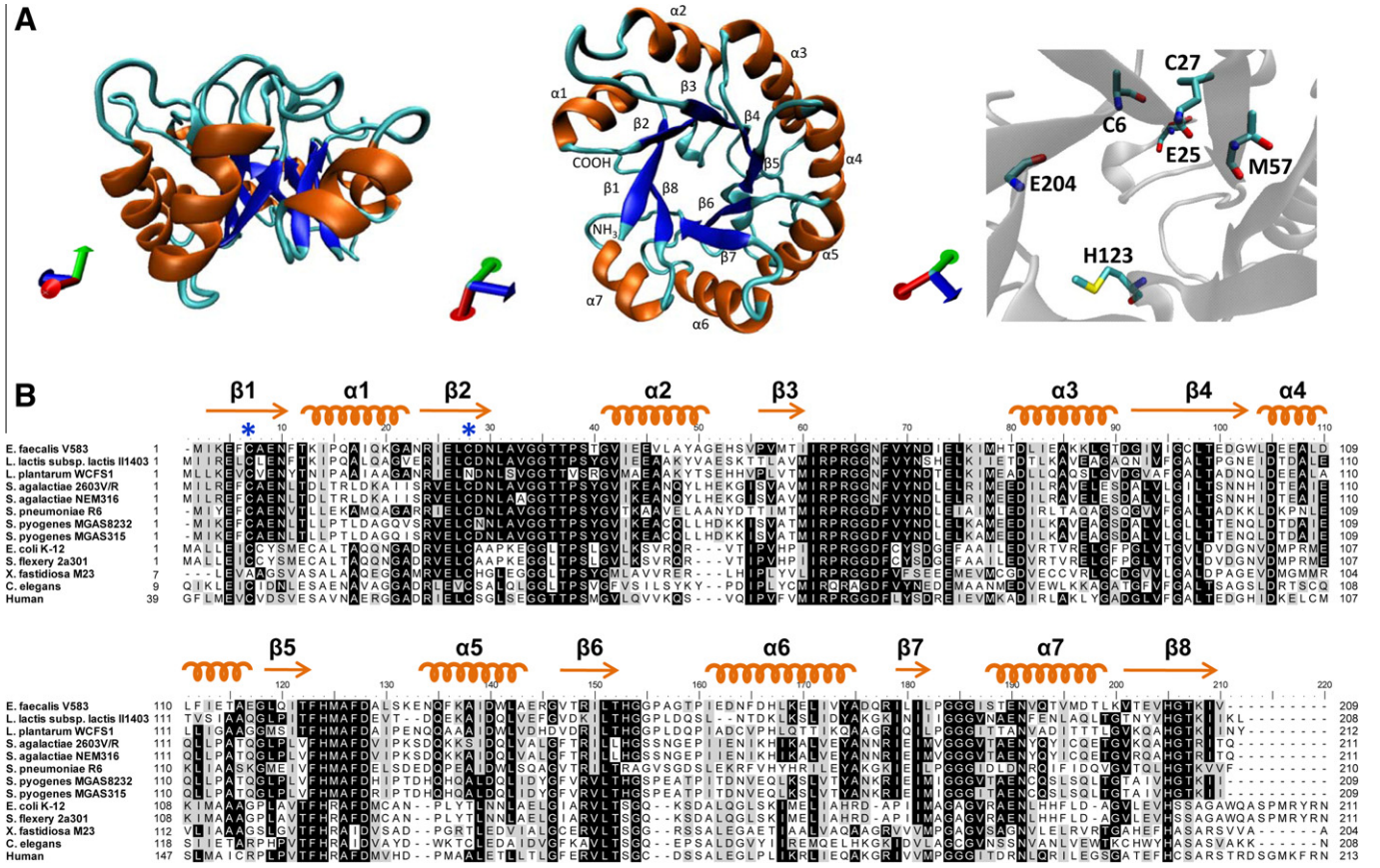

Fig. 1. Bioinformatic characterization of efCutC. (A) 3D molecular model of efCutC. Orange and blue identify alpha helix and beta sheet structures, respectively. The stereo view represents the putative copper binding motif. (B) Global alignment of CutC proteins, *Lactobacillale* order species: *E. faecalis* V583, *L. lactis subsp. lactis* Il1403, L. plantarum WCFS1, *S. agalactiae* 2603 V/R, *S. agalactiae* NEM316, *S. pneumoniae* R6, *S. pyogenes* MGAS315 and *S. pyogenes* MGAS8232. Similar and identical residues are marked in grey and black, respectively. Asterisks indicate cysteine residues potentially involved in metal coordination.

Global alignment shows that the homologs of efCutC found in species of the *Lactobacillale* order retained a high degree of conservation of their primary structures (identity values > 45%). The elevated identity of the copper binding motif (Fig. 1B) suggests the existence of a common structure for the coordination of the metal. A similar configuration has been previously described in the copper binding motifs C-x(2)-C-x(21)-C and C-x(18)-C present in *Saccharomyces cerevisiae* metallothioneins [28]. Furthermore, the genomic organization of *efcutC* as a monocistronic operon is conserved within the *Lactobacillale* order (data not shown) as is also the case for the *cop* genes, which are highly conserved in their genome in different species of the *Lactobacillale* order [25].

Several studies indicate that genes encoding components involved in copper homeostasis or that are related to a secondary response to the generation of oxidative stress alter their expression levels in response to metal exposure [29–31]. qPCR experiments show that a prolonged exposure (135 min) to a nonlethal concentration of copper generated a significant increase, nearly threefold, of the *efcutC* transcript levels (Fig. 2A). This result is consistent with observations reported for cells of the plant pathogen *X. fastidiosa* that were exposed for 24 h to 3, 4, 5, 6 and 7 mM $CuSO_4$ [9]. The abundance of all *E. faecalis* transcripts for *cop* genes was augmented approximately 30-fold after 15 min of copper exposure and remained around the same levels during the 135 min of treatment (Fig. 2B). It has been reported that the transcription factor CopY recognizes the motif (TACA-x(2)-TGTA), a palindromic sequence in the promoter region of *cop* genes that is highly conserved in species of the *Lactobacillale* order [25]. This binding site is absent in the promoter of *efcutC* (Supplementary S1). This information, in addition to the differences in expression of the *cop* genes and *efcutC* suggests the presence of independent regulatory transcriptional mechanisms for these genes that are activated by copper exposure.

Our data allow us to propose that *E. faecalis* produces an early transcriptional response (*cop* genes) followed by a second late response (*efcutC*) when confronted with an increase in the availability of extracellular copper. The first, early response is probably linked directly to management of the copper inside the cell, and the second one might be associated with the consequences of increasing intracellular metal levels, such as changes in the redox status of the cell. To verify the possible effects of oxidative stress generation during copper exposure, three general transcription markers for oxidative stress were quantified: thioredoxin (*trx*), superoxide dismutase (*sod*) and catalase (*cat*) (Fig. 2C) [32]. The results show that after 90 min of copper exposure, the transcript levels for the *trx* gene were augmented approximately 2-fold. Thioredoxins, given their capacity to reduce thiol groups, are generally linked to a wide response against different reactive oxygen species (ROS) generated by the presence of several metals, including copper [32]. On the other hand, several studies in bacteria have reported an overexpression of *cat* when cells are exposed to hydrogen peroxide [33,34]. In the same way, *Helicobacter pylori* cultures treated with iron have shown an induction of *sod* [35], and this response is directly related with the presence of superoxide generated by the metal exposure [32]. In this line of evidence, our qPCR experiments showed that the exposition of *E. faecalis* to nonlethal concentrations of hydrogen peroxide and iron increased the transcript levels of *cat* and *sod*, respectively, in absence of changes

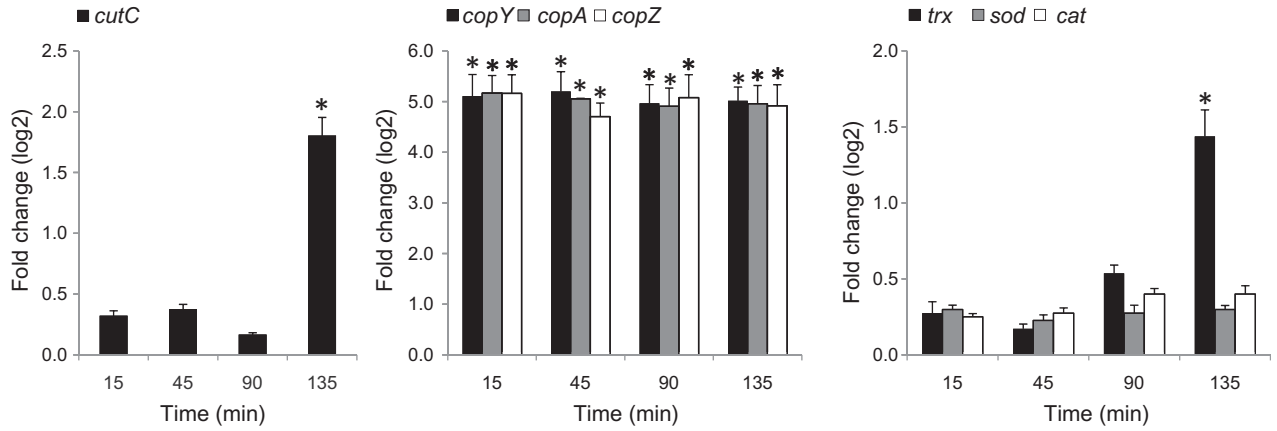

Fig. 2. Changes in the relative abundance of *efcutC* and *cop* genes and ROS markers (*sod*, *cat*, *trx*) induced by copper exposure. The values were determined by qPCR and expressed as the fold change between the copper treatment and the control culture for each time. All transcript levels were normalized by EF1004 quantification. Asterisks represent significant differences between the conditions (REST2008).

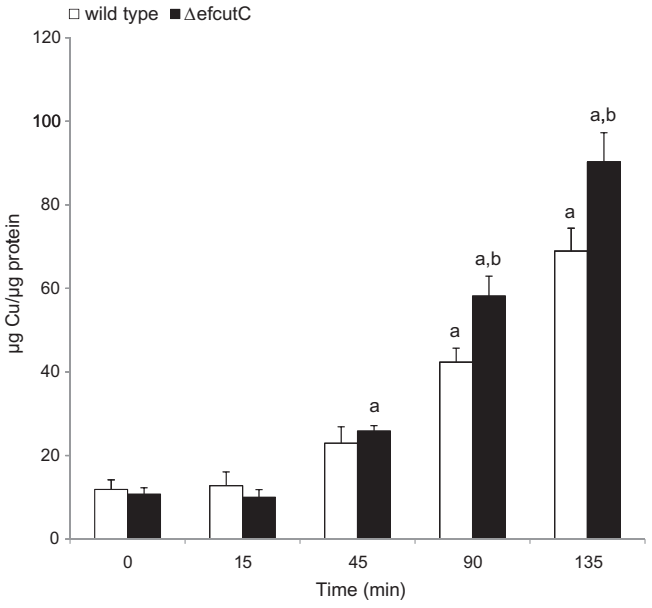

Fig. 3. Intracellular copper content as a function of time for wild-type and efCutC mutant strains exposed to copper. All values were determinate by AAS and are expressed as the direct copper content normalized by the total protein quantification. a and b indicate statistically significant differences (ANOVA test, $p < 0.05$), a: between the control (time 0) and the time of copper exposure for the same strain, b: between both strains at the same time of treatment.

in the expression of *efcutC* (Supplementary Table 1). These results indicate that the effects of prolonged copper exposure could activate the expression of *efcutC*. This activation was not observed when *E. faecalis* was exposed to other oxidative stress inductors, suggesting that the induction of *efcutC* is linked specifically to the stimulus of copper. This particular behavior has been previously described for several genes involved in copper homeostasis [36]. Several hypotheses have been proposed to explain copper induced cellular toxicity. Most often, the basis for these theories is the propensity of copper ions to participate in the generation of ROS [37,38]. Our results suggest that the transcriptional changes of *efcutC* and *trx* are induced by copper, sharing the same direction and time occurrence. In general, the induction of *trx* is an indicator of the activation of a wide array of mechanisms that respond to different types of damage generated by ROS [39]. In addition, the presence of thioredoxins is essential in the biogenesis of iron-sulfur clusters [40], and recent experiments describe that copper could directly damage iron-sulfur clusters in *Escherichia coli* [41]. In this context, further investigations are necessary to identify the specific mechanisms through which copper might induce the expression of *efcutC*.

The absence of different components involved in copper trafficking has been proven to influence copper homeostasis, substantially affecting the cellular copper content [6,18]. To understand the effects of efCutC in the regulation of copper homeostasis and the possible relationship of copper homeostasis with efCutC expression changes, we compared the intracellular copper content between *E. faecalis* wild-type and efCutC mutant strains (Fig. 3). The absence of efCutC generates a statistically significant increase (approximately 1.3 times) in copper content after 90 and 135 min of exposure to the metal in the efCutC mutant. A similar phenotype is observed in *E. faecalis* when the copper efflux P-type ATPase protein (CopA) is absent, in which the intracellular metal content increases nearly 1.6 times after 2 h of exposure to 0.5 mM $CuSO_4$, which is directly related to a decrease in cell viability [18]. The increment in intracellular copper levels as a consequence of the absence of efCutC suggests that the protein could be involved in mechanisms of homeostasis regulation related to metal efflux. The viability of the efCutC mutant exposed to copper is not altered (Supplementary S2), and a possible cause for this might be the active presence of CopA or other mechanisms of resistance to high copper levels. These results suggest that under copper exposure, the presence or absence of certain elements (such as CopA or CutC) can cause differences in the accumulation of the metal inside the cell, establishing different thresholds of copper tolerance in *E. faecalis* that depend on the activity of these proteins.

4. Conclusion

The elevated intracellular copper levels that result from a prolonged exposure to the metal induces the expression of *efcutC*. When efCutC is not present, the increase in transcript abundance is temporally correlated with an increase of the intracellular copper concentration, suggesting that efCutC may participate directly or indirectly in copper efflux as a chaperone or as a copper-user protein involved, for instance, in metabolic processes capable of generating sufficient energy (ATP) required by the efflux ATPases systems (CopA), suggesting an indirect participation in copper homeostasis. Finally, along with the characterization of CutC as a component involved in copper homeostasis, our results position *E. faecalis* as the first bacteria with an active member of the Cut protein family and a Cop operon able to respond to copper and modify the cellular metal content, making this bacteria an

interesting model to study the complementation of the homeostatic mechanisms controlled by these proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.109.

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