Quorum sensing mechanisms mediated by farnesol in *Ophiostoma piceae*: its effect on the secretion of sterol esterase

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Running title: Quorum sensing in *Ophiostoma piceae*
Abstract

*Ophiostoma piceae* CECT 20416 is a dimorphic wood-staining fungus able to produce an extracellular sterol-esterase/lipase (OPE) with great biotechnological interest. In this work we have studied the morphological change from yeast to hyphae of this fungus, associated to the cell-density related mechanism known as quorum sensing (QS), and how this affects the secretion of OPE.

The data presented here confirm that the molecule E,E-farnesol accumulates as the cell number is growing within the population. The exogenous addition of this molecule or spent medium to the cultures, increased 2.5 times the extracellular activity of OPE. This fact was not related to an increase in microbial biomass or in the expression of the gene coding for OPE, but to a marked morphological transition in the cultures. Moreover, the morphological transition also occurred when a high cell density was inoculated into the medium. The results suggest that E,E-farnesol regulates through QS mechanisms the morphological transition in the dimorphic fungus *O. piceae* and it is associated to a higher extracellular esterase activity. Furthermore, the identification and transcriptional analysis of genes *tup1* and *cyr1* involved in the response was carried out.

This is the first report on an enhanced production of a sterol-esterase/lipase with biotechnological interest by means of QS mechanisms. These results can be useful to increase the production of secreted enzymes of other dimorphic fungi with biotechnological interest.

**Keywords:** Sterol esterase, lipase, farnesol, quorum sensing, *Ophiostoma piceae*
1. Introduction

Triacylglycerol esterases, also known as lipases, have acylglycerols as their natural substrates, while sterol esterases hydrolyze fatty acid esters of sterols. In addition, both kinds of enzymes are able to carry out synthesis reactions in the presence of organic solvents (4, 21). They are widespread in nature, but those from microorganisms, especially fungi, have gained special interest due to their broad substrate specificity and their potential application for biotechnological applications (21).

*Ophiostoma piceae* is a wood-staining fungus, causal agent of pine and spruce discoloration. Although not being a pathogenic species, which associates with non aggressive bark beetles, the infection results in substantial economic losses in the forestry industry (24). Its ability to produce a morphological transition from yeast to hyphal forms, depending on fungal culture conditions, probably contributes to its adaptation to different environmental conditions (24). It has been recently reported the effect of inoculum size on culture morphology of fungi from the genera *Ophiostoma*, suggesting that the yeast- or hypha-like growth could be associated to quorum sensing (QS) activity (8).

The fungus *O. piceae* CECT 20416 produces an extracellular sterol esterase (OPE) with activity on triglycerides, esters of *p*-nitrophenol and cholesterol (11). This is the only esterase secreted by this strain, and represents a major protein in the enzyme crude obtained using a basal medium supplemented with olive oil. The enzyme is a glycosylated protein, with 8% N-linked carbohydrate and a molecular mass around 56.5 kDa (6, 11). Some applications have already been reported for this enzyme, either in hydrolysis reactions for pitch biocontrol in paper pulp manufacturing (11), as well as in the synthesis of sterol esters used as nutraceuticals (4).

QS processes allow the communication of individual cells within a microbial population and contributes in coordinating the behavior and physiology of the community.
These mechanisms are mediated by small diffusible molecules that accumulate as the cell number is growing within the population. When the QS molecule reaches a threshold concentration, microbes sense the extracellular signal, and the genes controlled by these mechanisms regulate their expression (7, 17). This phenomenon was first studied in bacteria (5, 13) and much later reported in fungi, particularly in the pathogenic yeast Candida albicans (19, 26), where the sesquiterpene alcohol farnesol (1-hydroxy-3,7,11-trimethyl-2,6,10-dodecatriene), was described as the first QS molecule in eukaryotic organisms (19). However, over the years, other molecules such as aromatic alcohols, tyrosol, dodecanol, γ-butyrolactone or γ-heptalactone, have been identified as mediators of QS processes in these organisms (1, 2, 14, 15, 18, 28, 33).

In C. albicans farnesol prevents the differentiation from yeast to hyphal growth, crucial to its pathogenicity (10, 26). These morphological changes are regulated at different levels, connecting diverse signal transduction pathways dependent on external stimuli. The morphological transition can be regulated through the conserved Ras and the adenylate cyclase (Cyr1) pathways (3). Recently, it has been shown that the protein kinase A (PKA) pathway can be also involved (25). In particular, farnesol inhibits the transcription of cyr1, provoking a decrease in the levels of intracellular cAMP (15), and also modulates the transcription levels of tup1, a major repressor of the morphological transition (22).

QS mechanisms have been described also in filamentous fungi from the genera Aspergillus (30, 33) and Penicillium (14, 27).

In this work we demonstrate the existence of QS mechanisms mediated by the signal molecule farnesol in the dimorphic fungus O. piceae, and how these mechanisms influence the secretion of a sterol esterase (OPE), an interesting biocatalyst for different biotechnological applications. We also analyzed the accumulation of the QS molecule during
fungal growth and its role on the yeast to mycelium differentiation. Finally, we studied the
signal transduction components that mediate the response.

2. Materials and Methods

Fungal strain and culture conditions

*O. piceae* (CECT 20416) was grown at 28 °C and 180 rpm in a medium containing: 10
g/L glucose, 2 g/L ammonium tartrate, 1 g/L KH₂PO₄, 1 g/L yeast extract, 0.5 g/L MgSO₄ •
7H₂O and 0.5 g/L KCl, and 1 mL mineral solution [100 mg/L B₄O₇Na₂ • 10H₂O, 70 mg/L
ZnSO₄ • 7H₂O, 50 mg/L FeSO₄ • 7H₂O, 10 mg/L CuSO₄ • 5H₂O, 10 mg/L MnSO₄ • 4H₂O, 10
mg/L (NH₄)₆Mo₇O₂₄ • 4H₂O], supplemented with peptone (0.5% (w/v)) and highly refined
olive oil (0.5% (w/v)) (Sigma, O1514) (11). Five-days-old cultures, inoculated from 2% malt
extract–glucose–agar, were used to inoculate 250 mL Erlenmeyer flasks with 50 mL of
culture medium (2.5% v/v inoculum), corresponding to a final cell density of 7×10⁵ cells/mL.

After 48 h incubation, different treatments were established using a battery of putative QS
signal molecules: Farnesol (Mixture of isomers, Sigma F203), E,E-farnesol (pure isomer,
Sigma 277541), γ-butyrolactone, N-(3-oxodecanoyl)-L-homoserine lactone, tyrosol, and
dodecanol were added to the cultures at a final concentration of 1 mM. E,E-farnesol was
assayed at two different final concentrations (100 µM and 1 mM) to corroborate the results
with a concentration 10 times below the solubility limit of farnesol in water, and to avoid any
possible toxic effect. Another treatment was established adding extracted medium (spent
medium obtained as described below) from 50 mL of cultures supernatants. A group without
these compounds was inoculated in the same conditions (2.5% v/v inoculum) as controls.

Samples were taken periodically to 240 h from three replicate flasks and experiments were
repeated twice. The mycelium was separated from the culture liquids by centrifugation at
8,000 g and 4 °C during 10 min.
In addition, the effect of using two different inoculum concentrations (1% and 4% v/v) was also analyzed. Cultures were inoculated from five-days-old pre-cultures were the proportion of yeast cells was very high and farnesol was not detectable.

**Growth evaluation**

Microbial biomass was calculated as dry weight (CDW) from 10 mL of culture. Culture morphology was determined at every sampling point on fresh samples from the culture, using a ZEISS Axioskop 2 microscope (Carl Zeiss Gmbh) with the 40X objective. When maximum differences in morphology were noticed, three fields of each replicate of each culture were inspected, and differentiated cells were quantified and normalized to 100% (yeast/filamentous cells). Cells with buds were counted as yeasts and spores and cells forming germ tubes were classified as hyphae, as previously reported (20).

**Analytical assays**

Generic esterase and lipase activities were assayed spectrophotometrically by $p$-nitrophenol ($\varepsilon_{410}=15,200 \text{ M}^{-1} \text{ cm}^{-1}$) release from $p$-nitrophenyl butyrate ($p$NPB) in 25 mM Tris–HCl buffer pH 7.2 (11). One unit of activity was defined as the amount of enzyme hydrolyzing 1 µmol of substrate per minute under the above conditions. Protein concentration was determined by the method of Bradford (Bio-Rad protein assay) using serum albumin as standard.

**Farnesol quantification**

Culture supernatants (10 mL) were extracted with 10 mL $n$-hexane–ethanol (90:10, v/v), dried and resuspended in acetonitrile for injection (29, 31). Reverse-phase high-performance liquid chromatography was carried out in a HPLC (Thermo Finnigan Surveyor
PDA Plus detector, Thermo Fisher) using a C18 column RP18 Aquapore (7 µm, 150 x 3.2 mm) (Life Technologies). A linear gradient of acetonitrile-water (0% to 100% over 20 min) was used as the mobile phase. Detection was done with an associated mass spectrometer (Thermoscientific LXQ, Thermo Fisher) selecting a characteristic ion of m/z 205 and daughter ions of m/z 121 and m/z 109. Standards concentrations of E,E-farnesol ranged from 1 µM to 250 µM. Extraction and quantification of farnesol was carried out from sterile media without inoculum as a negative control.

Supernatants (50 mL) from cultures with a high density inoculum (4% v/v) were extracted, dried, and resuspended in methanol at the second day of incubation, when the farnesol production was the highest. These extracts were used as inductor (spent medium) in fresh cultures.

Identification and quantification of the relative expression of QS-related genes

Homologous genes to those regulated by QS mechanisms in C. albicans, coding for the tup1 transcriptional repressor and the adenylate cyclase cyr1 (15, 22), were searched in the publicly available genome of O. piceae (16) and Ophiostoma ulmi (23). Once identified the genes with highest homology, a BLAST search against NCBI database was carried out for both genes. The sequences from the best hits (corresponding to phylogenetically related fungal species) were aligned, and primers for PCR amplification were designed in conserved regions: tup1 Fw 5’-GAGAGTTGTTTGTGTGCAC-3’ and tup1 Rv 5’-GACAGAGTTTGTGTCCCTGC-3’; cyr1 Fw 5’-GGACTAACCTGTGGGAGACATAC-3’ and cyr1 Rv 5’-ATTGGGCCCCTGGTAGTCC-3’.

The two sets of designed primers were used for amplification of O. piceae CECT 20416 genomic DNA and cDNA. Genomic DNA was extracted from 1 mL culture pellets, previously disrupted with liquid nitrogen, using DNeasy Plant Mini Kit (Qiagen) according to
manufacturer's instructions. RNA was extracted using the ‘RNeasy Plant Mini Kit’ (Qiagen) including the DNAse treatment specified by the manufacturer. Reverse transcription of RNA was done using the ‘GeneAmp RNA PCR Reagent Kit’ (Applied Biosystems).

The PCR amplifications were performed using a Mastercycler Pro S (Eppendorf) in a final volume of 50 µL with: 1X PCR Taq buffer, 2.5 U of Taq polymerase (Invitrogen), 15 mM MgCl₂, 0.25 mM of each primer, and 1mM of dNTPs (New England Biolabs). 100 ng of genomic DNA or cDNA were used as templates. Cycling parameters were 94 °C for 3 min followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1.5 min and a final extension of 10 min at 72 °C. PCR products were run in a 1% agarose gel and subsequently purified and inserted into pGEM-T easy cloning vector (Promega). After transformation of the recombinant vectors into the Escherichia coli DH5α strain, 3 clones containing the inserted fragments were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems).

To perform real time quantitative PCR (qPCR) primers were designed on the basis of the sequences of cyr1, tup1, the sterol esterase (ope) (gene Bank accession number AY899847) and the 18S rRNA gene from O. piceae (accession number KF531618), using the Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/). The primer sequences obtained were: ope Fw: 5' - CAGCTCTATCGCACTGTCGT -3' and ope Rv: 5' - TCCTCCTGGTCACCGATGAT -3'; cyr1 Fw: 5' - ACGACACCGAGTTGAGCATC -3' and cyr1 Rv: 5' - GAAGGTGACGCTTTCATGGT -3'; tup1 Fw: 5' - GGATATTGAGAACGGCCAGA -3' and tup1 Rv: 5' - AGGTAACCGTGCTGGATGTC -3'; 18S Fw: 5' - CGGAACGCAAGTTCTCTCTC -3' and 18S Rv: 5' - CCTACCTGATCCGAGGTCAA -3'.

qPCRs were performed using cDNA from three independent replicates of O. piceae control cultures or cultures induced with farnesol, inoculated with a medium cell density
(2.5%), at two sampling times (192 h and 240 h). The PCR cycling conditions were as follows: an initial step at 95 °C for 5 min and 45 cycles at 95 °C for 30 s and 55 °C for 30 s, followed by a denaturation step to check the absence of unspecific products or primer dimmers. SYBR green PCR master mix (Biorad) was used as the reaction mixture (10 µL), with the addition of 2.5 µL of sterile Milli-Q water, 1.25 µL of each primer 1 mM, and 5 µL of template cDNA, in a final volume of 20 µL. In all experiments, appropriate RT (-) and controls containing no template were done to detect any possible contamination. Each sample was amplified twice in every experiment. The PCR efficiencies for all the primer sets were calculated by performing a 10-fold serial dilution of positive control template to generate a standard curve, and by plotting the CT (Cycle Threshold) as a function of log10 of template. 18S rRNA gene was used as endogenous control gene to normalize the results. Quantification is relative to the control gene by subtracting the CT of the control gene from the CT of the gene of interest. CT values were transformed to log2 to generate the relative expression levels.

**Phylogenetic study**

Genomic DNA was extracted as described above, and the *O. piceae* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, were amplified from genomic DNA using universal primers (32) and sequenced as described below (sequence deposited at GeneBank under accession number KF531618). Sequences from *Ophiostoma* related species were retrieved from the databases and an unrooted phylogenetic tree was built using MUSCLE for alignment and the Maximum-Likelihood method (Fig. S1).
Results and Discussion

The ascomycete *O. piceae* causes important losses in the wood sector (24), but also secretes a sterol-esterase/lipase with high biotechnological interest (4, 12). In this work, the effect of several putative QS molecules on fungal growth and morphology, extracellular protein production and particularly, sterol esterase secretion, has been studied.

Growth evaluation and analytical assays

The evolution of fungal growth in the liquid culture, expressed as mg/mL of dry weight, showed that the stationary phase was reached between 48 and 72 h (Fig. 1). The maximum growth yield in the cultures occurred at 144 h (9 mg/mL). No significant differences were observed between the control and the cultures supplemented with the different signal molecules, indicating that the cell integrity it is not affected by the molecules added.

Figure 1: Fungal growth expressed as dry weight (black line corresponds to control cultures and the grey line to the farnesol supplemented cultures). The arrow indicates the moment of the addition of farnesol, final concentration 1mM.
The sterol esterase activity increased rapidly at the end of stationary phase, when glucose was depleted (11). Maximum activity was reached around 200 h of culture, in the presence or absence of signal molecules. The esterase activity was much higher in cultures supplemented with farnesol (~2.5 times more) (Fig. 2), as well as the extracellular protein concentration (Fig. 3). The increase in esterase activity and protein secretion was correlated with the morphological changes detected in the fungal cultures. These results were similar when the molecule added to the media was E,E-farnesol at 100 µM or 1 mM concentrations, and with the addition of an equivalent volume of “spent medium”. However, the other signal molecules tested, described as QS molecules in other microorganisms, γ-butyrolactone (28, 30), N-(3-oxodecanoyl)-l-homoserine lactone (5), tyrosol (2) and dodecanol (15), presented no effect on the parameters measured in O. piceae cultures, respect the control (data not shown). The different molecules were added to the cultures 48 h after inoculation in order to avoid growth inhibition, as described by other authors (14).

Figure 2: Time course of sterol esterase activity in O. piceae cultures by using p-nitrophenyl butyrate (pNPB) as substrate (black line corresponds to control and grey line to the farnesol supplemented cultures). The arrow indicates the moment of the addition of farnesol, final concentration 1mM.
Figure 3: Measurement of total secreted protein levels through growth of *O. piceae* cultures (black line corresponds to control and grey line to the farnesol supplemented cultures).

Microscopic analysis of the samples revealed a morphological change from yeast to hyphae along the time in the farnesol-induced cultures and with the addition of the “spent medium”. The maximum differences in morphology were observed around 120 h post-inoculation: the control cultures presented 86.3±3.6% of yeast cells, while in the case of 1 mM farnesol treatments presented much more hyphae, and only 30.1±10.5% of yeast cells (Fig. 4). In the case of 100 µM farnesol treatment the proportion of yeast cells was 42±5.2%, and in the treatment with the “spent medium” there were a 47±12.7% of yeast cells. In the case of the cultures inoculated with different inoculum concentrations (1% and 4% v/v), without exogenous signal molecules added, the morphological analysis at 120 h post-inoculation showed clear differences among them. When 1% inoculum was used, a large proportion of yeast cells (94±4.6%) were found, while the cultures with high inoculum density (4%) presented much more hyphae and only 58±10.7% yeast cells. Moreover, the cultures with a high inoculum density presented also higher esterase activity, 1.7-fold at 240 h post-inoculation, when no differences in dry weight were found.

The effect of farnesol or the “spent medium” was independent on the fungal biomass (Fig. 1). In this sense, it should be emphasized that OPE is the only esterase and the most abundant protein in *O. piceae* supernatants (11). The increase of secreted proteins seems to be
correlated with the morphological changes detected in the cultures supplemented with farnesol or the cultures with high inoculum density. OPE specific activity (esterase activity / protein mg) increases in the farnesol treated cultures along time, being eight times higher at the 240h (88 mU/mg) than at the 120h (10 mU/mg). Control cultures only increase 2.5 times from the 120h of culture (21 mU/mg) to the 240h (56 mU/mg). This may be due to a selective increased secretion of OPE coupled to the morphological transition along time.

Figure 4: Morphological changes of fungal cultures at 120 h after inoculation, in the absence (a) or presence of exogenous farnesol (b). The table at the bottom of the figure (c) indicates the percentage of hyphae or cell yeasts in the cultures also at 120 h after inoculation.
**Farnesol production**

No detectable amount of farnesol was found in control medium without fungal growth. E,E-farnesol is the unique isomer described with biological activity in fungi (19, 26), and was the only isomer identified in the *O. piceae* cultures. This was also the main isomer detected in the farnesol mixture of isomers (Sigma F203) added to cultures as QS molecule. The amount of farnesol found at the second day of growth in the cultures with high inoculum density (4%) was around 22 µM, while in the cultures with low inoculum density (1%) was not detectable. Figure 5 shows the accumulation of E,E-farnesol detected in the *O. piceae* cultures with 2.5% inoculum density measured at different times. The maximum farnesol concentration was reached between 24 and 48 h of incubation (~7 µM) and it decreased drastically during the stationary phase, suggesting a turnover of the molecule in the cultures. The scarce presence of the mycelial form of the fungi in the cultures with 1% and 2.5% inoculum could be explained by the low concentration of farnesol accumulated. This concentration may not be enough to produce the morphological differentiation; while in the higher density culture (4%) the farnesol concentration could lead to the morphological differentiation and to an increased extracellular esterase activity. The supernatants from the higher density cultures were the starting point for the production of the “spent medium”, its farnesol content could explain the biological activity when added to fresh cultures.
Figure 5: a) E,E-farnesol detected in control cultures at the different sampling times, b) Molecular structure of E,E-farnesol, c) HPLC-MS/MS chromatograms of the farnesol extracted from control culture at 48 h. The retention time corresponded with the E,E-farnesol standard and the farnesol mix of isomers (Sigma Aldrich, F203) where the E,E-farnesol was majority. Quantification was carried out with the daughter ions m/z 121 and 149 from the characteristic farnesol ion m/z 205.

The biological effect on the fungal morphogenesis mediated by QS mechanisms has also been described in other dimorphic fungi, such as C. albicans (19), O. ulmi (8), Cryptococcus neoformans (1) and Saccharomyces cerevisiae (18). Interestingly, other authors did not found any effect of farnesol in O. ulmi (8, 9, 20). In this sense, the O. piceae strain studied in this work is saprophyte and affiliates phylogenetically with the non-pathogenic species of the Ophiostoma clade, such as O. quercus, far from the pathogens O. ulmi and O. novo-ulmi (Fig S1). This fact may indicate a differential QS regulation mediated by farnesol in different clades and in close relatives that do not share the same habitat.
Figure S1. Un-rooted phylogenetic tree from the 18S ribosomal RNA gene partial sequence, the internal transcribed spacer 1, the 5.8S ribosomal RNA gene, the internal transcribed spacer 2, and the 28S ribosomal RNA gene partial sequence, of *O. piceae* CECT 20416 and related species.

**Identification and quantification of the relative expression of QS-related genes**

Orthologues to *tup1* and *cyr1* genes of *C. albicans* were identified “in silico” in the genomes of *O. piceae* (16) and *O. ulmi* (23) as close relatives of our strain, probably with a common ancestor (Fig. S1). These sequences presented an identity of 44% for *tup1*, and 66% for *cyr1* at the nucleotide level, and of 56% for *tup1* and 71% for *cyr1* at the amino acid level. After designing primers, homologues in *O. piceae* CECT 20416 were amplified by PCR and partially sequenced from cDNA and genomic DNA. The 820 bp sequence from *tup1* and the 363 bp sequence from *cyr1* lacked introns. The absence of introns has also been reported in the gene of the *O. piceae* sterol esterase (12). The sequences obtained had a nucleotide
identity of 76% for *tup1* and 87% for *cyr1* with the genes from the Canadian strain of *O. piceae* (16). The identity at the amino acid level rose to 98% and 97%, respectively.

Using the sequences of the QS-related genes *tup1* (accession number KF531616) and *cyr1* (accession number KF531617), the 18S ribosomal RNA partial sequence from *O. piceae* (accession number KF531618), and the sequence of the sterol esterase from *O. piceae* (*ope*), RT-qPCR analyses were carried out. Two different sampling points were analyzed, 192 h and 240 h, when induction of esterase activity and protein production in the farnesol supplemented cultures was more pronounced. The transcriptional levels of *cyr1* in the presence and absence of farnesol did not change significantly in our experiments (Fig. 6), in agreement with Hall et al. (15) that did not detected changes in gene expression, but in the activity levels of Cyr1p. However, the gene *tup1* showed a relative expression of 0.62 times the control at 192 h that recovered to 0.96 times the control at 240 h. These genes have been shown to be up- and down- regulated respectively, in the presence of farnesol in *C. albicans* (15, 22). These results may suggest a moderate repression of *tup1* that could be in concordance with the opposite effect of farnesol in *O. piceae*, where stimulates hyphae formation, and in *C. albicans*, where represses hyphae formation (22). Hence it would be interesting to know in future studies if the mechanisms behind this control are conserved.

![Figure 6: Relative expression of the genes identified in *O. piceae*, in the presence and absence of farnesol. RT-qPCR was carried out at 192 h (black bars) and 240 h (grey bars).](image-url)
The absence of differences in the gene expression of *ope* (Fig. 6), is in agreement with data from other *O. piceae* strain growing in a basal medium with olive oil. In this case it was not possible to detect an increase in the lipase gene transcription at the time sampled, although lipase activity was higher than in the culture without olive oil (16). Thus, the higher protein levels and lipase/esterase activity secreted in the cultures supplemented with farnesol in this study could be explained by the higher proportion of mycelia and active hyphal tips, where the secretion mainly occurs. Other authors reported similar results in the fungus *Penicillium decumbens*, where farnesol increased the hyphal size and, as a consequence, the secretion of hydrolytic enzymes by this fungus was higher (14).

**Conclusions**

In this work, we demonstrate the implication of QS mechanisms in the morphological transition from yeast to hyphae in the saprophytic fungus *O. piceae*. The results obtained suggest that E,E-farnesol is the molecule involved in the QS signaling, and its transduction could be mediated by the transcriptional repressor *tup1*. To the best of our knowledge, this is the first report on an enhanced production of a sterol-esterase/lipase with biotechnological interest mediated by QS mechanisms. These results can be useful to increase the production of secreted enzymes of other dimorphic fungi with biotechnological interest.

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