ISOLATION OF *Streptomyces* STRAIN INHIBITORS OF TOXIGENIC FUNGI. PARTIAL CHARACTERIZATION OF THEIR CHITINOLYTIC SYSTEM

(Caracterización parcial del sistema quitinolítico de cepas de Streptomyces inhibidoras de hongos toxicogénicos)

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SUMMARY

*In the search of potential biocontrollers for toxigenic fungi we isolated 27 soilborne Streptomyces spp. and performed confrontation tests against toxigenic strains of **Aspergillus parasiticus** (aflatoxins producer), **Fusarium sporotrichioides** (T-2 and HT-2 toxin producer) and **Fusarium graminearum** (deoxynivalenol and nivalenol producer).*

The growth of at least one of the toxigenic fungi was inhibited by 63 % of the Streptomyces isolates, 41 % of the Streptomyces strains inhibited the growth of at least two tested fungi, and 36 % of the Streptomyces were effective against all three fungi. Moreoever, the ability of isolates to degrade colloidal chitin was analysed and the chitinolytic complex of two of the Streptomyces strains were further characterized. Sixty six % of the Streptomyces spp. degraded colloidal chitin in plate assays. Chitinase secretion of two isolates was assayed by colorimetric methods and by activity gels, and the degradation products from different substrates were analysed by thin layer chromatography.

Results suggested that one isolate (Streptomyces sp. C112) has endochitinase activity but not N-acetylglucosaminidase activities, and the complex of chitinolytic enzymes of the other strain (Streptomyces sp. C103) include one N-acetylglucosaminidase activity.

RESUMEN

*En la búsqueda de biocontroladores potenciales de hongos toxicogénicos, se aislaron desde el suelo 27 cepas de Streptomyces spp. y se desarrollaron pruebas de confrontación contra cepas toxicogénicas de: **Aspergillus parasiticus** (productor de aflatoxinas), **Fusarium sporotrichioides** (productor de toxinas T-2 y HT-2) y **Fusarium graminearum** (productor de deoxinivalenol y nivalenol). El desarrollo de al menos uno de los hongos toxicogénicos fue inhibido por el 63 % de los Streptomyces aislados, 41 % de las cepas de Streptomyces inhibieron el crecimiento de al menos dos de los hongos probados, y el 36 % de los Streptomyces fue efectivo contra los tres hongos. Además, fue analizada la capacidad de los aislamientos de degradar quitina coloidal y posteriormente fueron caracterizados los complejos quitinolíticos de dos de las cepas de Streptomyces. El 66 % de los Streptomyces spp. degradaron quitina coloidal en las pruebas en placa. La secreción de quitinasas de dos aislamientos fue ensayada empleando métodos colorimétricos y geles de actividad, y los productos de degradación a partir de diferentes sustratos fueron analizados por cromatografía en capa delgada.*

Los resultados indicaron que uno de los Streptomyces (C112), posee actividad endoquitinasa pero no N-acetilglucosaminidasa y que el complejo de enzimas quitinolíticas de la cepa (C103) incluye una actividad de N-acetil-glucosaminidasa.

INTRODUCTION

Mycotoxins, toxic secondary metabolites, are a major cause of contamination in cereal grains and animal feed (Pittet, 1998). Their global occurrence is considered to be a major risk factor affecting human and animal health as it is estimated that 25 % of the world's crop production is contaminated to some extent with mycotoxins (Fink-Gremmels, 1999). Unacceptably high and potentially harmful levels of several mycotoxins such as deoxynivalenol (DON), aflatoxins, T-2 toxin, fumonisins and zeralenone were detected in cereals and related products in Argentina in recent years (Dalcero *et al.,* 1997; Dalcero *et al.,* 1998).

Both health risks and reduced profitability of contaminated crops create a need to prevent the formation of mycotoxins (Desjardins & Hohn, 1997; Bock & Cotty, 1999). Therefore, in past years, there has been an increasing interest for new methods of biological control where antagonistic organisms or their antimicrobial metabolites may act as natural controllers of toxigenic fungi (Desjardins & Hohn, 1997).

The genus *Streptomyces* comprises potential candidate species to control fungal contamination, since they are abundant in soil where they compete with filamentous fungi for food and space. Some of these species are included among the main producers of bioactive metabolites and extracellular enzymes whose effectiveness against bacteria, fungi, some protozoa and nematoda has been observed (Trejo-Estrada *et al.,* 1998; Hiraga *et al.,* 1999). *Streptomyces* can readily utilise chitin as sole carbon source and are amongst the major chitinase producers in soil (Miyashita *et al.,* 1997). As fungal cell walls are the main sources of chitin in soil, it is assumed that the chitinolytic activity of *Streptomyces,* among other producers, play an important role in the decomposition of chitin in soil as well as the control of fungal population in this system (Vionis *et al.,* 1996). Therefore, the knowledge of the mechanisms employed by the chitinolytic systems is very important.

Aiming at the isolation of potential biocontrollers for toxigenic fungi we have undertaken the isolation of *Streptomyces* strains from soil. We evaluated the capacity of such isolates to inhibit the growth of three species of toxigenic fungi. We further analysed their chitinase production and we partially characterized the chitinolytic system of two selected isolates.

MATERIALS AND METHODS

(1) Organism and growth conditions

Streptomyces spp. were isolated from soil samples in the southern region of Santa Fe State (Argentina) on glycerol-asparagine-salt-agar medium (Panthier *et al.,* 1979) and were grown on Potato Dextrose Agar (PDA) for 5 days at 28°C (Fulgueira *et al.,* 1996). The morphological and physiological characteristics of the isolated strains were analyzed by Bergey's Manual of Systematic Bacteriology (Kreig & Holt, 1989).

The toxigenic fungi used were ***Aspergillus parasiticus*** NRRL 2999 (aflatoxin producer), ***Fusarium sporotrichioides*** (ex - *F.tricinctum*) NRRL 3299 (T-2 and HT-2 toxins producer), obtained from the Northern Regional Research Laboratory (USA) and ***Fusarium graminearum*** C136-92 (deoxynivalenol and nivalenol producer) isolated from field contaminated wheat grains. *F. sporotrichioides* and *F. graminearum* were inoculated on V-8 juice agar and incubated for ten days under fluorescent light to stimulate production of conidia (Fulgueira *et al.,* 1996). For *A. parasiticus* PDA was used. Conidial suspensions were prepared in sterile distilled water (10^7 conidia/ml^{-1}).

(2) Plate confrontation assays

Each *Streptomyces* isolate was streaked in a straight line on the surface of PDA plates. After 48 hours incubation, 200µl of previously prepared fungal conidia suspensions were spread on the surface of the plate. After 7 day-incubation at 28°C the diameter of the inhibition halos was recorded.

(3) Analytical determinations of chitinolytic activity

Plate assays for determination of chitinolytic activity were performed on Czapek Dox medium without sucrose supplemented with 0.2% of colloidal chitin. After inoculation of the *Streptomyces* isolates, incubation proceeded at 28°C up to twenty days until chitin degradation zones could be observed around the colonies. The clearing zones diameters were measured.

To induce chitinase production in solution, the isolates were inoculated in Czapek Dox liquid medium containing 0.2% colloidal chitin as sole carbon source (Chernin *et al.,* 1995). The flasks were incubated at 28°C for 3, 5, 7, 9 and 11 days. After incubation the cultures were filtered into Millipore filters (0.22µm) and dialysed with a MWCO 6-8000 D membrane for 48 hours against distilled water. Sodium azide was added to a final 0,02% concentration.

Chitinase activity of the *Streptomyces* grown in Czapek Dox liquid medium containing 0.2% colloidal chitin, was assayed by the following colorimetric methods: (1) the reaction mixture contained 0.5 ml of swollen chitin (5 mg/ml in 50 mM acetate buffer pH 5.5), 250µl enzyme sample and 0.5 ml of 50 mM acetate buffer pH 5.5 (de Siqueira Pinto *et al.,* 1997). After incubation at 37°C for 4 hours, it was centrifuged at 2000 g for 5 min, and the
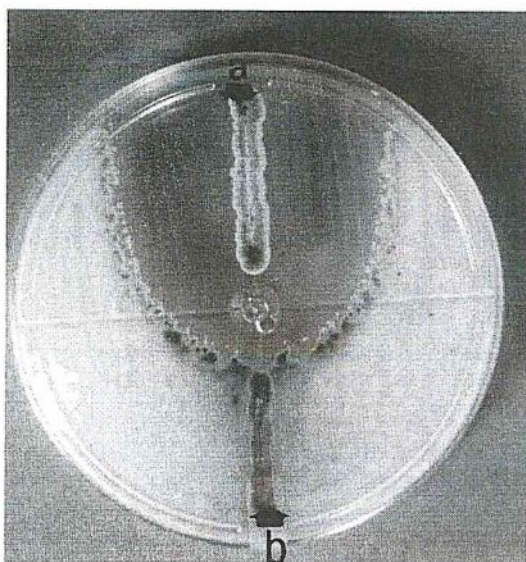
Figure 1.- Confrontation plate assays. **A**. Inhibition zone produced by a *Streptomyces* isolate against *F. graminearum* grown for 7 days on PDA plate. **B**. Same as in A, using a non antagonistic *Streptomyces* isolate.

Figure 2.-: Plate assays for chitinolytic activity. **A**. Clearing halos formed by action of *Streptomyces* chitino-lytic isolate on Czapek Dox medium supplemented with 0.2% (W/V) colloidal chitin, after 20 days incubation. **B** and **C**, same as in A, with isolates of *Streptomyces* with no chitinolytic activity.

amount of N-acetylglucosamine (GlcNAc) released in the supernatant was determined (Reissig *et al.*, 1955) using GlcNAc as a standard. One unit (U) was defined as the amount of enzyme that catalyses the release of 1 µMol of GlcNAc / h / ml at 37°C; **(2)** the reaction mixture contained 30 µl of 4 mM *p*-nitrophenyl β-D-N-acetylglucosamine (suspended in 10 mM sodium phosphate buffer pH 6.0 containing 10% dimethylformamide), 120 µl of 10 mM sodium phosphate buffer pH 6.0 and 50 µl of dialysed culture filtrate. The mixture was incubated at 37°C for 30-min (Irhuma *et al.*, 1991). One unit (U) was defined as the amount of enzyme that catalyses the release of 1 µMol of *p*-nitrophenol / h / ml at 37°C; **(3)** the following substrates were used: N,N'-diacetylchitobiose, N,N',N''-triacetylchitotriose and N,N',N'',N'''-tetraacetylchito-tetraose. The reaction mixture contained 10 µl of 4 mM of each substrate, 40 µl of 50 mM sodium acetate buffer (pH 5.4) and 20 µl of enzyme sample (de Siqueira Pinto *et al.*, 1997). After incubation at 37°C for 2 hours. the amount of GlcNAc produced was determined as described for assay 1. One unit (U) was defined as the amount of enzyme that catalyses the release of 1 µMol of GlcNAc / h / ml at 37°C.

(4) Analysis of hydrolysis products

The reaction mixtures for chitinase activity were performed as described above, using colloidal chitin and N,N'-diacetylchitobiose, N,N',N''-triacetylchitotriose and N,N',N'',N'''-tetraacetylchitotetraose as substrates. After the incubation samples were concentrated by speed
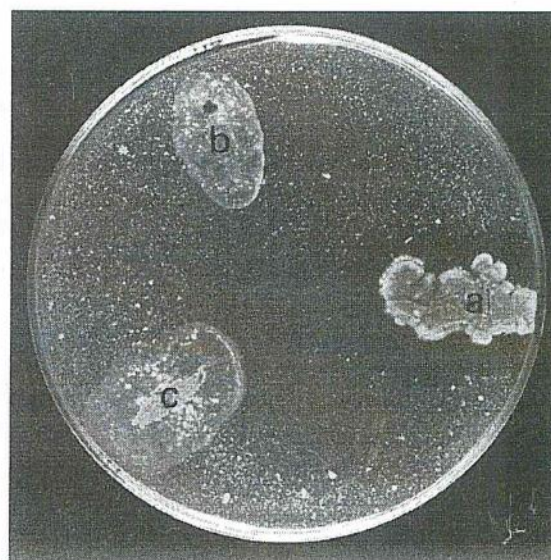
vacuum and spotted onto thin layer chromatography plates (TLC). The chromatogram was developed in a solution of n-butanol / acetic acid / ether / water (9:6:3:1) and sugars were visualised by spraying a solution consisting of 2% aniline, 2% diphenylalanine and 15% phosphoric acid dissolved in acetone, followed by incubation for 4 hours at 60°C (Lato *et al.*, 1969).

(5) Electrophoresis procedures

To detect chitinase activity, SDS-PAGE was performed in a 12 % (W/V) polyacrilamide gel containing 0.01% (W/V) glycol chitin and 0.1% SDS, prepared according to Molano *et al.* (1977). Gel loading buffer was as described by Laemmli (1970), but β-mercaptoethanol was omitted and samples were boiled before running. After electrophoresis, gels were incubated for 2 h at 37°C by shaking, in 50 mM sodium acetate buffer pH 5.4 containing 1% Triton X-100. Chitinase lytic zones were revealed by incubation of the gel in a freshly prepared 0.01% calcofluor white solution (Fluorescent Brightener 28, SIGMA) in 500 mM Tris-HCl pH 8.9. The calcofluor white solution was removed, the gels were incubated for about 1 h at room temperature in distilled water (Trudel & Asselin, 1989) and visualised under UV light.

RESULTS AND DISCUSSION

We isolated twenty-seven strains of *Streptomyces*

Table 1.- Comparative table of the growth inhibition produced by *Streptomyces* spp. strains against toxigenic fungi spread on PDA plates (incubation: 7 d at 28°C) and their chitinolytic activity on Czapec Dox plates supplemented with 0,2 /W/V) colloidal chitin (20 d. incubation)

Streptomyces STRAINS	INHIBITION ZONES DIAMETERS (mm) *			CHITINOLYTIC HALOS (mm) *
	F. sporotrichioidesm	F. graminearum	A. parasiticus	
C 101	15.8 ± 0.2	20.0 ± 0.1	0	25.0 ± 0.1
C 102	0	0	0	21.0 ± 0.1
C 103	42.0 ± 0.2	40.0 ± 0.1	31.5 ± 0.1	28.0 ± 0.0
C 104	0	22.3 ± 0.2	0	0
C 105	17.5 ± 0.1	21 ± 0.1	14.5 ± 0.0	0
C 107	0	0	14.3 ± 0.1	22.1 ± 0.1
C 110	10.5 ± 0.1	9.8 ± 0.1	15.5 ± 0.0	0
C 111	0	0	0	0
C 112	0	13.7 ± 0.1	0	27.5 ± 0.1
C 113	7.3 ± 0.1	0	0	0
C 114	14.3 ± 0.1	22.2 ± 0.1	0	0
C 201	0	0	0	16.0 ± 0.1
C 202	44.0 ± 0.1	38.1 ± 0.1	28.0 ± 0.0	16.5 ± 0.1
C 203	0	0	0	21.5 ± 0.1
C 205	0	0	0	0
C 206	0	0	20.5 ± 0.1	15.5 ± 0.2
C 207	0	0	14.2 ± 0.1	20.0 ± 0.0
C 208	20.3 ± 0.1	24.5 ± 0.2	19.5 ± 0.1	12.5 ± 0.2
C 209	0	0	0	0
C 210	10.1 ± 0.1	0	0	13.5 ± 0.2
C 212	0	0	0	14.0 ± 0.0
C 301	28.5 ± 0.1	32.3 ± 0.2	19.0 ± 0.1	11.0 ± 0.2
C 302	13.3 ± 0.1	0	15.5 ± 0.1	15.0 ± 0.2
C 303	0	0	0	16.5 ± 0.1
C 304	0	0	0	19.5 ± 0.1
C 305	0	12.0 ± 0.1	20.3 ± 0.1	0
C 306	0	0	0	22.0 ± 0.0

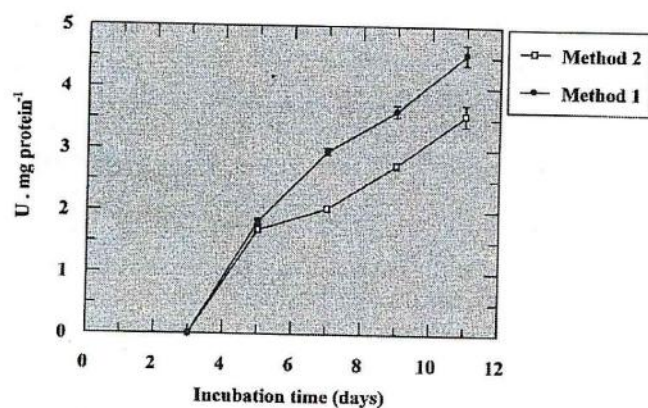* Values shown are the mean of three replicates ± SE

Figure 3.- Secretion of chitinases by *Streptomyces* isolate C103 on Czapek Dox broth supplemented with 0.2% (W/V) colloidal chitin assayed by colorimetric methods. One unit (U) was defined as the amount of enzyme that catalyses the release of: 1 µMol of GlcNAc /h /ml at 37°C (Method 1 -●-) or 1 µMol of *p*-nitrophenol / h / ml at 37°C (Method 2 -□-). The values shown are the mean of three replicates. Standard deviations are represented.

Figure 4.- Degradation products of colloidal chitin and oligosaccharides after incubation with culture supernatant of *Streptomyces* isolate C112. **Lane 1**, standard mixture of mono, di-, tri- and tetra- oligosaccharides. **Lanes: 2**, reaction mixtures with colloidal chitin; **3**, colloidal chitin, standard; **4**, reaction mixtures with tetramer (chitotetraose); **5**, tetramer standard; **6**, reaction mixtures with trimer (chitotriose); **7**, trimer standard; **8**, reaction mixtures with dimer (chitobiose); **9**, dimer standard.

from soil samples. In confrontation tests (Figure 1 shows an example), 63% of the isolates exhibited antagonistic effects against at least one of the toxicogenic fungi tested, *A. parasiticus*, *F. sporotrichioides* or *F. graminearum* (Table 1). Forty one percent of the *Streptomyces* isolates inhibited the growth of at least two tested fungi, and 36 % of the *Streptomyces* were effective against all three toxigenic fungi. Although the sizes of growth inhibition zones displayed by different *Streptomyces* isolates were variable, in general, they behaved as moderate antagonists according to Reddi's criterion (Reddi & Rao, 1971). However, 37.8% of the *Streptomyces* isolates revealed strong antagonism according to the same criterion.

To know if the antagonistic *Streptomyces* strains could produce chitinolytic enzymes, we analyzed the chitin degradation capacity of the isolates. On plate assays (Figure 2), two thirds of the strains were able to produce degradation halos after incubation up to twenty days. Table 1 shows the degradation halo diameters which were obtained in this assay. For several of the isolates the degradation halos were very large suggesting high activity and secretion of chitinases.

The chitinolytic enzymes were divided into three principal types (Sahai & Manocha, 1993; Chernin *et al.*, 1995). Cleavage by endochitinases occurs randomly at internal points over the entire length of the chitin microfibril, releasing mainly the dimer diacetylchitobiose. Exochitinases or chitobiosidases catalyse the progressive release of diacetylchitobiose units in a stepwise fashion while a third enzyme, β-1,4-*N*-acetylglucosaminidase, is responsible for the production of GlcNAc using dimers,
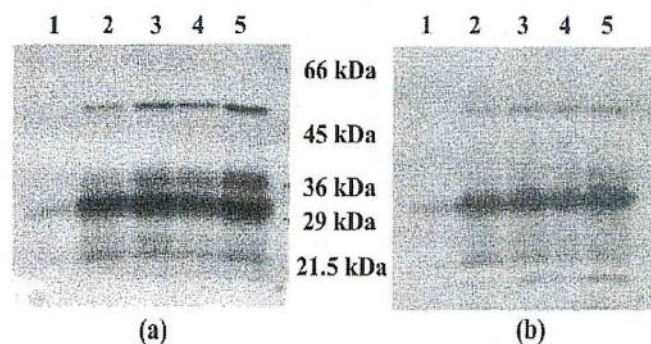
trimers, or tetramers as substrate.

To further analyze the *Streptomyces* chitin degradation capacity, two of the isolates (C103 and C112) that produced the most prominent halos in plate assays, were grown on liquid medium with chitin to assess secreted chitinase activity against different substrates. Assays 1 and 2 were utilised for two selected *Streptomyces* strains, assay 3 was performed to characterize the chitinolytic system of one of the strains selected before.

In the course of 11 days of cultivation, *Streptomyces* isolate C103 secreted chitinase active against colloidal chitin and also hydrolysed the chromogenic substrate *p*-nitrophenyl β-D-*N*-acetylglucosamine. Figure 3 shows the chitinase specific activity towards both substrates. This activity was detected after three days in several oligomers of GlcNAc and was performed by thin layer chromatography (Figure 4). Hydrolysis of colloidal chitin and chitotetraose produced mainly one spot corresponding to the dimer (chitobiose). Hydrolysis of chitotriose released chitobiose and GlcNAc, and the chitinolytic complex analyzed was not capable of degrading chitobiose to produce GlcNAc. Therefore, the presence of a glucosaminidase is considered unlikely in the chitinolytic system secreted by *Streptomyces* C 112. According to the culture supernatants of both strains, C103 and C112, were determined after their separation by SDS-PAGE. The activity of the renatured chitinases was detected by the degradation of glycol chitin incorporated into gels (Figure 5). Multiple bands corresponding to the chitinolytic complex could be observed. Some authors have reported the multiple nature of microbial chitinases and it seems to be a characteristic
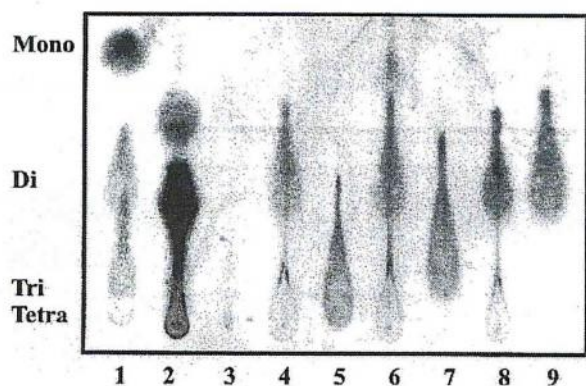
Figure 5.- Extracellular proteins of two *Streptomyces* isolates, C112 and C103, analyzed in chitinase activity gels. **Lanes 1 to 5** correspond to 3, 5, 7, 9 and 11 days of growth, respectively. Equal volume samples were used. **A.** *Streptomyces* isolate C112. **B.** *Streptomyces* isolate C103

fof the genus *Streptomyces* (Miyashita *et al.*, 1997; Vionis *et al.*, 1996). It was suggested that this complex mixture of chitinases may be necessary for maximum activity acting synergistically in the degradation of native chitin (Okazaki *et al.*, 1995; Romaguera *et al.*, 1992). The most important chitinase activities presented an apparent molecular mass between 29 and 36 kDa. Activities of similar molecular mass produced by *Strepto-myces* strains have also been reported (Romaguera *et al.*, 1992; Okazaki *et al.*, 1995). Despite the very similar profiles of chitinolytic enzymes shown by isolates C103 and C112, several differences were detected

in degradation bands in both isolates. Five distinct enzymes with apparent masses of 50, 36, 30, 24 and 22 kDa were detected in the culture medium of strain C112. In strain C103 degradation bands of 50, 30 and 22 kDa were also found, whereas the 36 kDa bands were only slightly visible at 11 days of incubation. Moreoever, an additional chitinolytic activity with a molecular mass lower than 21.5 kDa was observed. Taking into account the different behaviour of two *Streptomyces* isolates, we could attribute endochitinase activity to the 36 kDa enzyme produced by strain C112, and *N*-acetyl-β-1,4-glucosaminidase activity to the protein with a molecular mass lower than 21.5 kDa.

This work allowed to isolate *Streptomyces* spp. strains with the ability to suppress the growth of toxigenic fungi in vitro. These isolates might be useful in biological control to inhibit toxigenic fungi growth as a preharvest agent to prevent disease during plant development and/or as a postharvest agent during seed storage to suppress mycotoxin accumulation when kernels are dried inadequately. Among the *Streptomyces* isolates, two chitinolytic strains that showed different mechanisms to degrade chitin were selected. Since their complex of chitinolytic enzymes has not yet been purified, it is difficult to consider other characteristics of the individual enzymes. The complexity of the chitinolytic enzyme system, might contribute significantly to the antagonistic activity of the strains described. Moreoever, although lysis of fungal cell walls of toxigenic fungi by *Streptomyces* is a possible mechanism of suppression, other studies also indicate that other metabolites can also produced (Fulgueira, 1998). The mechanisms of suppression are now under investigation.

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