CHARACTERIZATION OF ANTIBIOTIC PRODUCTION BY *PSEUDOMONAS FLUORESCENS*

by

BRADLEY WAYNE GOODNER

Department of Biochemistry and Biophysics

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Dr. Jim Wild

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ABSTRACT

Characterization of Antibiotic Production

by *Pseudomonas fluorescens*

Bradley Wayne Goodner

Advisor: Dr. Jim Wild

The potential of using biological control agents to promote agricultural production is now being recognized by plant scientists. One agent, *Pseudomonas fluorescens* strain Pf-5, produces antibiotics which protect cotton seedlings from two particular seedling pathogens. In this project, the genes encoding for the enzymes which catalyze the production of one of these antibiotics, pyrrolnitrin, were possibly transferred to a new host. Modern recombinant DNA techniques were used to achieve this objective. A balance of positive and negative results led to the conclusion of possible success. Also, the survivability of this particular organism when introduced into a new environment was explored by way of a field study. The most exciting result from this experiment was that a plasmid introduced into *P. fluorescens* strain Pf-5 before soil inoculation was stably maintained in the cell population throughout the length

of the study period. Further experiments concerning the production of antibiotics by this organism are planned for the future.

The style and format of this thesis were the same as that used in the journal, Molecular and General Genetics.

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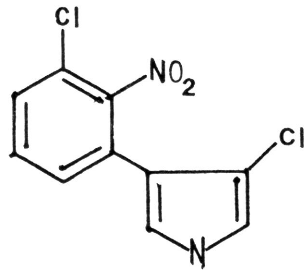
INTRODUCTION

Within the last few decades, plant scientists have become increasingly aware of the potential of using biological control to help combat plant pathogens. Biological control is defined to be the use of one organism to eradicate or decrease the spread of another organism which is a pathogen or pest. The spectrum of agents include bacteria, fungi, viruses, and insects. By incorporating biological control into current pest/pathogen management programs, agricultural production can be promoted and the dependence on synthetic chemical pesticides can be decresed to a more economical and safe level (Papavizas, 1981).

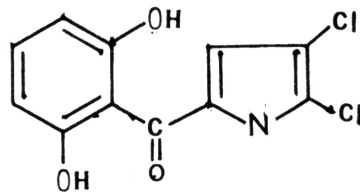
The soil inhabiting members of the genus *Pseudomonas* have been extensively studied for their biological control activity. Contributions to that activity include nutritional and environmental versatility, antibiotic synthesis, and production of fluorescent pigments which act as siderophores (Leisinger and Margraff, 1979; Kloepper et. al., 1980; Misaghi et. al., 1982; Schroth and Hancock, 1982). The first contribution allows the bacterium to aggressively inhabit many types of rhizospheres, while the last two decrease any competition from

other microbes for occupying a certain niche. As a result, those organisms are being considered for use in a wide range of agronomic situations. One in particular, Pseudomonas fluorescens strain Pf-5, was linked to cotton seedling protection by Howell (1979). In inhabits the root zone of cotton plants and produces antibiotics which suppress the growth of two important cotton seedling pathogens, Rhizoctonia solani and Pythium ultimum (Figure 1). One of the antibiotics, pyrrolnitrin, is synthesized and kept within the cells until lysis, yet upon release is inhibitory toward R. solani (Howell and Stipanovic, 1979). The other antibiotic, pyoluteorin, is released into the environment upon production and is effective towards P. ultimum (Howell and Stipanovic, 1980).

This particular organism is indicative of biological control agents in general, in that very little is known about the synthesis of these antibiotics or the regulation of that synthesis. These areas must be explored if scientists wish to understand, improve, and possibly manipulate biological control activities. One method of exploration is to use modern recombinant DNA techniques to isolate from the host those genes important for biological control activity and to transfer



pyrrolnitrin



pyoluteorin

Figure 1.

those genes to another organism where they can be more easily studied. This was the basic thrust of the research reported on here.

The objectives of this project were: 1) to identify the location of the genes needed for antibiotic production in *P. fluorescens* strain Pf-5, whether chromosomal or plasmid-borne, 2) to isolate these genes and clone them into an appropriate vector, and 3) to possibly transfer the genes needed for production of either or both antibiotics from *P. fluorescens* strain Pf-5 to some other microorganism. A review of the literature pointed to pyrrolnitrin as the antibiotic to concentrate on. First of all, pyrrolnitrin is synthesized under a variety of conditions while pyoluteorin production requires very special conditions (Bencini et. al., 1983). Second, pyrrolnitrin does not have the antibacterial activity that pyoluteorin does (Ohmori et. al., 1978). The fourth and final objective involved the actual use of *P. fluorescens* strain Pf-5 in a field situation. Many questions concerning the survivability of an introduced bacterial population were expected to be answered by this phase of the research.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* strains RRI (Bolivar et. al. 1977) and MM294 (Backman et. al., 1976) were provided by K. F. Foltermann. MM294 harbors the plasmid pBR322 (Bolivar et. al. 1977). *P. fluorescens* strain Pf-5 (Howell and Stipanovic, 1979) was provided by C. R. Howell.

Media. *E. coli* and *P. fluorescens* strains were maintained on TYE; 10 g tryptone (Difco Bacto-), 5 g yeast extract, 5 g NaCl, 15 g agar (Difco), and 1000 ml distillled deionized water. Selective isolation of *P. fluorescens* strain Pf-5 from cotton roots during the field study involved King's B medium (King et. al., 1954). Transformation of foreign DNA into recipient cells took place in L-broth; 10 g tryptone (Dico Bacto-), 5 g yeast extract, 10 g NaCl, and 1000 ml distilled deionized water. Pyrrolnitrin production tests were carried out on medium 523 (Kado and Heskett, 1970). Dilutions were made with a minimal salts solution; 0.1 M Tris pH 7.8, 0.142 g Na_2HPO_4, 0.05 g Na_2SO_4, 2.0 g KCl, 0.025 M $MgCl_2$, and 0.2% NH_4Cl in 1000 ml total volume.

Media was supplemented with antibiotics when required. All antibiotics were purchased from Sigma Chemical Company, St. Louis, Missouri.

Enzymes. The restriction endonucleases Bam Hl, Pstl, and Sall, were purchased from Bethesda Research Labs (Rockville, Maryland) and used according to the supplier's recommendations.

T4 DNA ligase was purchased from Bethesda Research Labs and used according to the supplier's recommendations.

Plasmid Isolation. Various procedures (Bidwell et. al, 1981; Holmes and Quigley, 1981; Kado and Liu, 1981; Machida et. al, 1982) were used to isolate plasmids from various bacterial strains.

Large-Scale Isolation of Chromosomal and Plasmid Vector DNA. Total chromosomal DNA was prepared from *P. fluorescens* strain PF-5 by a lysis-phenol extraction-dialysis method (W. D. Roof, personal communication).

The plasmid pBR322 was isolated from *E. coli* strain MM294 by first preparing a cleared lysate (Katz

et. al., 1973) and then subjecting this lysate to CsCl gradient ultra-centrifugation (Maniatis et. al., 1982).

Construction of Recombinant Plasmids. Aliquots of Pf-5 Chromosomal DNA were digested by BamHl, Pstl, and Sall, separately. The same three digestions were carried out on pBR322 aliquots. Digestions were confirmed by electrophoresis of small samples on horizontal submersible agarose gels (0.7%) prepared in Tris-borate buffer, pH 8.3 (0.089 M Tris base, 0.089 M boric acid, and 2.5 mM Na_2EDTA). After electrophoresis the gels were stained with ethidium bromide (0.5 mg/ml) and the DNA fragments visualized in UV light (302 nm).

After digestion with the same restriction endonuclease, the chromosomal fragments were ligated into the plasmid vector using T4 DNA ligase. The ratio of chromosomal DNA to plasmid vector DNA during ligation was approximately 7 to 1.

Transformation and Selection of Clones. Cultures of _E. coli_ strain RR1 were made compentent by $CaCl_2$ treatment (Morrison et. al., 1979). 100 µl of these cells were then mixed with 10 µl of a recombinant plasmid solution in a glass tube, shocked at 45°C for 3

minutes, added to 1 ml of L-broth, and aerated at 37°C for 1 hour. The cell suspension was then aliquoted out onto TYE plates containing antibiotics selective for those cells which took up a plasmid. Colonies that appeared after overnight incubation were replicated on TYE plates containing antibiotics selective against those cells which took up a plasmid with no fragment of Pf-5 chromosome inserted.

Visualization of Recombinant Plasmids from Colonies Showing Correct Antibiotic Response. A rapid disruption method (Maniatis et. al., 1982) was used on single colonies to obtain enough plasmid DNA to load on a single lane of an agarose gel. Electrophoresis and ethidium bromide staining were carried out as described earlier.

Pyrrolnitrin Production Test. Each *E. coli* strain RR1 clone carrying a recombinant plasmid was spot inoculated on medium 523 plates and allowed to grow for 5 days at 37°C. Each plate was then inoculated with a mycelial plug of *Rhizoctonia solani*, obtained from R. W. Jones. After 3 days incubation at room temperature, the plates were examined for fungal growth inhibition.

Preparation of Extracts and Characterization by Gas Chromatography. Those *E. coli* strain RR1 clones which gave positive inhibition results were grown up in 5 ml of TYE overnight. The cells were pelleted by centrifugation and resuspended in 2 ml of minimal salts solution. For each culture, 10 plates of medium 523 were inoculated by spreading 0.1 ml aliquots of the resuspended cells on each plate. Positive (*P. fluorescens* strain Pf-5) and negative (*E. coli* strain RR1/no recombinant plasmid) controls were also treated in the same manner. After 10 days incubation at 37°C, the culture plates were cut into approximately 1 cm^2 squares and extracted with 200 ml of 80% acetone for each set of 10 plates. Analysis of the acetone extracts was done by gas chromatography (methyl silicone column; argon-nitrogen carrier gas; electron capture detector).

Field Study of Survivability of *P. fluorescens* Strain Pf-5 when used as a Seed Treatment. 200 ml of King's B medium was inoculated with *P. fluorescens* strain Pf-5 and aerated for 36 hours. The cells were pelleted by centrifugation and resuspended in 14 ml of sterile water. Cotton seed (20 g) were allowed to imbibe this solution for 2 hours and then were air-dried

for 4 hours. A dilution series plating on King's B medium was carried out to determine the concentration of cells at each step.

The treated cotton seeds, along with 6.38 g of untreated seed, were planted June 4, 1982, in a research field located on Agronomy Road in College Station, Texas. The planting was done in 4 rows of 64 feet each, using a mechanical planter. The depth of planting was approximately 3 inches deep and the distance between seeds was 10 inches. The seeds were allowed to germinate and the cotton plants matured according to normal non-irrigated cotton farming practices.

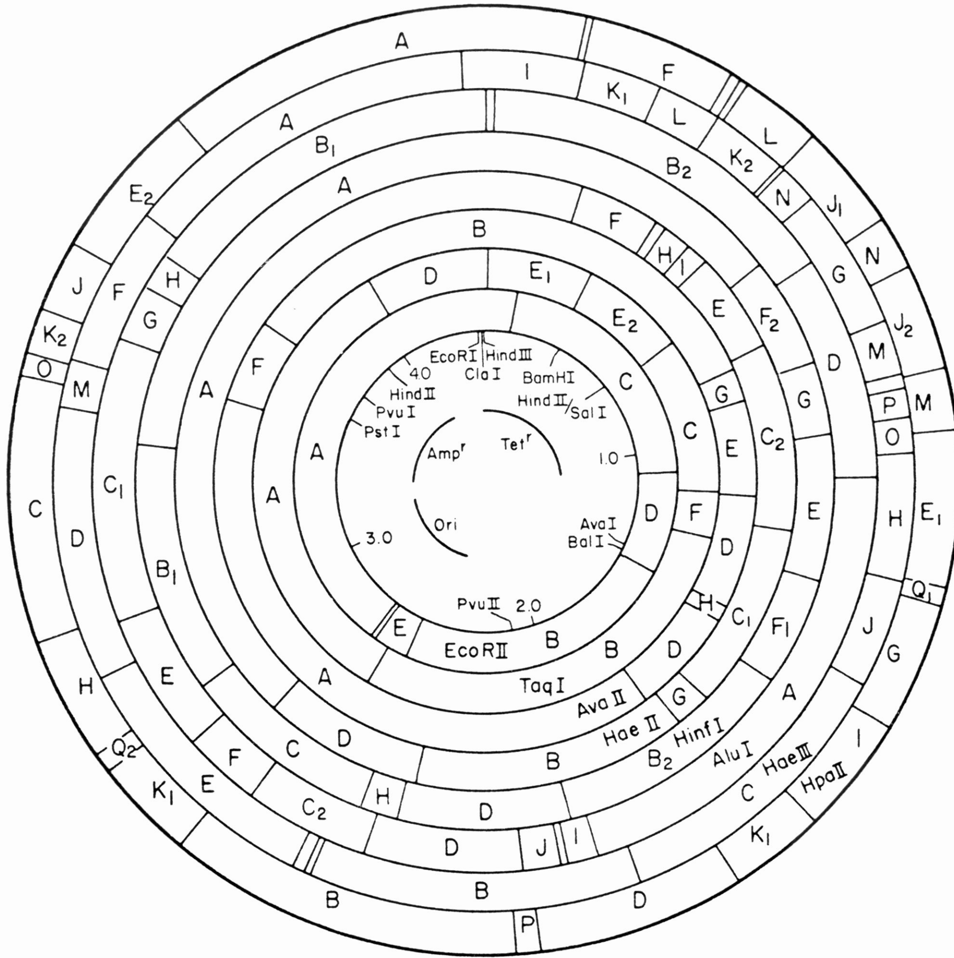
At regular intervals, treated and untreated cotton plants were collected. The roots were clipped and vortexed in minimal salts solution. A dilution series plating on King's B medium was carried out to enumerate the *P. fluorescens* strain Pf-5 present. Resistance to ampicillin, chloramphenicol, tetracycline, and kanamycin were used to distinguish the Pf-5 strain from other *P. fluorescens* strains. The tetracycline and kanamycin resistance markers had been transferred to Pf-5 by Bencini (personal communication) using conjugation of the plasmid RP4.

RESULTS

Preparation of DNA Fragment Library from *P. fluorescens* Strain Pf-5

Using both general plasmid isolation techniques and methods designed especially for large size plasmids, no plasmids were found in Pf-5. Bencini also failed in isolating any of these extrachromosomal elements (unpublished data). Yet the isolation of Pf-5 chromosomal DNA was a success with the final concentration as measured by absorbance at 260 nm being 300 mg/ml. The plasmid vector pBR322 was isolated from two different cultures of *E. coli* strain MM294. One culture had been "amplified" with chloramphenicol when the culture had reached 200 Kletts, while the other was allowed to reach stationary phase. The final concentration of plasmid DNA was 70 mg/ml in each case. Both covalently closed circular and open circular forms of the plasmid were observed after electrophoresis.

Complete digestions of Pf-5 chromosomal DNA using BamHl, Pstl, and Sal were performed. pBR322 ws also digested with these restriction endonucleases. Each one cleaves pBR322 at just one site (Figure 2). After liga-



pBR322

Figure 2.

tion of the Pf-5 fragments with their corresponding linearized vector and transformation of the ligation products into E. coli strain RR1, selection for those cells which took up recombinant plasmids was carried out. For the Sal1 and BamH1 trials, ampicillin resistance and tetracycline sensitivity was selected for. Tectracycline resistance and ampicillin sensitivity meant fragment insertion in the plasmid vector had occurred in the Pst1 trial. The frequency of fragment insertions was 35% for the Sal1 trial, 24% for BamH1, and 32% for Pst1. The total number of RR1 clones carrying recombinant plasmids isolated in the three trials was 78.

Identification and Characterization of E. coli Strain RR1 Clones Carrying Pyrrolnitrin Production Genes

Each E. coli strain RR1 carrying a recombinant plasmid was challenged with Rhizoctonia solani. RR1 with no recombinant plasmids, acting as a negative control, had no visible effect on the fungus. Two out of the seventy-eight clones, both from the Sal1 trial, showed signs of inhibitory activity and were designated 10-7 and 101-3. The inhibition seen for these two

clones was not as great as that seen for P. fluorescens strain Pf-5. Replcations of this test showed that the reproducibility of inhibition was questionable.

Using the rapid plasmid isolation procedure, a single plasmid was visualized for each of the two possible pyrrolnitrin producing RR1 clones. The size of the plasmids, extrapolated from a standard curve, were 9.53 kilbases for 10-7 and 8.24 kilobases for 10-13. Subtracting the size of the plasmid pBR322, 4.36 kilobases, gives Pf-5 fragment insert sizes of 5.17 and 3.88 kilobases for 10-7 and 10-13, respectively.

To confirm whether pyrrolnitrin production was actually occurring in the two RR1 clones, acetone extracts were made from 10 day-old plate cultures of the two clones. These extracts were subjected to gas chromatography analysis utilizing an electron capture detector. This took advantage of the presence of two chlorine atoms on the pyrrolnitrin molecule which are strong electron attractors. A small peak corresponding to the reference standard peak for pyrrolnitrin was seen in the extract of Pf-5, which acted as a positive control. No pyrrolnitrin peaks were seen for either of the two E. coli strain RR1 clones (Figure 3). In an effort to stimulate transcription of the Pf-5 fragments,

GC Data

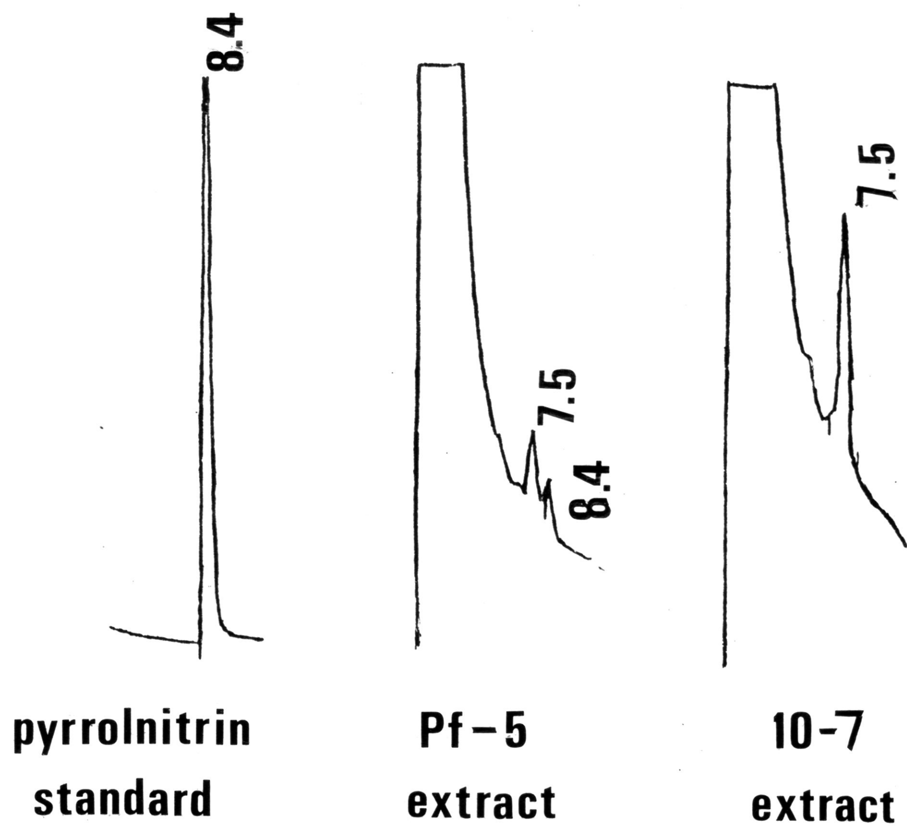


Figure 3.

the recombinant plasmids were cleaved with SalI and the Pf-5 fragments isolated. An attempt was then made to place these into another plasmid behind the lactose operon's promoter-operator region, but failed (unpublished data).

Use of P. fluorescens strain Pf-5 in a Field Situation

The concentration of Pf-5 cells in the imbibition solution was 4.06 X 10^9/ml. After two hours of imbibition, the average number of Pf-5 cells adhering to the cotton seeds was 2.66 X 10^7/seed, compared to the theoretical limit of 2.24 X 10^8/seed. After four hours of drying, the number was down to 1.4 X 10^7/seed. This was taken to be the inoculum concentration. The planting session went off without any problems. Thirty-three percent of the treated seeds germinated and produced seedlings, as opposed to 25% of the untreated seeds. The plants matured normally, despite the abnormally hot and dry conditions. The population of Pf-5 cells in the root zone of the cotton plants at various intervals after planting is shown in Table 1. As can be seen, the population started decreasing soon

Weeks after planting	# of cells/root system
1	11×10^3
2	5.1×10^3
4	4.5×10^3
6	3.1×10^3
9	no cells detected
12	0.2×10^3

Table 1.

after the initial planting time, reaching zero within 9-12 weeks. However, as the population was decreasing, it seemed to maintain the introduced plasmid. Finally, the dilution plates for the untreated plants contained, on the average, more contamination by soil fungi than did those dilution plates for the treated plants.

DISCUSSION

The failure to isolate any plasmids from P. fluorescens strain Pf-5 seems strange since most pseudomonads carry several. This could indicate a true absence of plasmids or that the plasmids present are so large that they were sheared during the procedures used (Currier and Nestor, 1976). Other possible explanations include nuclease degradation of the plasmid DNA (Williams et. al., 1980), and intermeshing of the plasmids amidst the bacterial chromosome (Kline et. al., 1975; Kline et. al., 1976).

One assumption formed the basis for the cloning experiments, that being that the genes encoding for all of the pyrrolnitrin production enzymes would be genetically linked. This idea was derived from the observation that, in many other bacteria, non-constitutive enzyme systems have their genes arranged in the form of an operon. A recent review (Halloway et. al., 197) reported very little evidence for this occurring on the chromosomes of Pseudomonas spp., but did point out that most of the large plasmids found in pseudomonads carry gene clusters encoding for degradative pathways. However, the positive cloning results obtained early in

this project seemed to suggest linkage of pyrrolnitrin production genes. Furthermore, the size of the fragments carried by the two positive-test E. coli strain RR1 clones corresponded to a linkage group of 4-5 genes if the size of the average bacterial gene is taken to be 1000 bases. This is in good agreement with the proposed pathway for pyrrolnitrin production from its precursor, tryptophan (Vournakis and Elander, 1983).

The negative results obtained with these two RR1 clones must also be considered. The poor reproducibility of R. solani inhibition and the absence of a pyrrolnitrin peak in the gas chromatography analysis lead one to believe that the positive results were artifactual or that the production of pyrrolnitrin in E. coli was very low due to weak expression of the cloned Pf-5 fragments. This latter explanation has precedence in other attempts to clone Pseudomonas genes into E. coli, especially genes for non-constitutive enzymes (Buckel and Zehelein, 1981; Schell, 1983; Jacoby et. al., 1978; Ribbons and Wigmore, 1978). In fact, it is easier to get expression of E. coli genes in Pseudomonas spp. than it is to do the opposite. This reflects the genetic versatility required for survival under wide ranging conditions by a soil bacterium as

opposed to the constant moderate environment inhabited by *E. coli* (Holloway et. al., 1979).

When all the results are taken into account, nothing definite can be said about the cloning experiment. In retrospect, the correct way to approach cloning of these genes would be to use a *Pseudomonas* host and a transformable vector which could replicate within that particular host. Vectors of this type are being constructed and soon will be widely available (Tait et. al., 1983). Plans have been made to repeat the cloning experiments in this new manner.

The field study provided many new insights. Foremost was the fact that a population of bacteria carrying an *in vitro* introduced plasmid could be put into a soil environment and still maintain the plasmid without selective pressure. This shows promise for future endeavors where agriculturally important recombinant plasmids will be considered for introduction into a soil bacterium. Second, the population data supports the idea that an unnaturally large inoculum population will decrease over time to its equilibrium position, which in this case was near zero. This apparent absence of indigenous soil fluorescent pseudomonads can be attributed to the extremely hot and dry conditions encoun-

tered during the growing season. Finally, the field results qualitatively confirm the biological activity of Pf-5, since the untreated plants' rhizospheres were heavily populated with soil fungi while most of the treated plants had no visible fungal inhabitants of their root zone as seen by the dilution series plates.

In conclusion, this type of research is needed if scientists are to fully understand the interactions between plants and their microbial neighbors. Direct application of this area of science can then greatly aid efforts to improve agricultural production in the years to come.

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