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Potential biological control Mechanisms of *Bacillus paralicheniformis* TRQ65 against phytopathogenic fungi

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ABSTRACT

Background/Objective. *Bacillus paralicheniformis* TRQ65 was isolated from wheat (*Triticum turgidum* subsp. *durum*) rhizosphere in commercial fields in the Yaqui Valley, Mexico. This strain was one of the most abundant bacteria in the rhizosphere. The objective of this study is to explore the potential biological control action mechanisms of *Bacillus paralicheniformis* TRQ65 against phytopathogenic fungi of agricultural importance, through genome sequencing and mining.

Materials and methods. The biocontrol activity of this strain was quantified through *in vitro* dual assays evaluating inhibition zones against 11 agronomically important fungi. A whole-genome analysis was conducted as genomic mining to evaluate its potential for biological control.

Results. Strain TRQ65 showed biocontrol activity against 45% of the studied fungi, where the highest inhibition was against *Botrytis cinerea*, 43.8% ± 9% on day 5. Based on genome sequencing and mining (antiSMASH), this bioactivity could be associated with the biosynthesis of lichenysin, bacillibactin, and/or fengycin.

Conclusion. This research provides the first insight into the potential biological control activity of strain TRQ65. Further studies need to be carried out to validate *Bacillus paralicheniformis* TRQ65 as an active ingredient in sustainable bacterial inoculants for eco-friendly agriculture.

Keywords: Biological Control Agents, Agriculture, *Bacillus*, Fengycins, Lichenysins, Bacillibactin.

INTRODUCTION

Sustainable agricultural practices are crucial since the growing population is estimated to reach ~ 10 billion people by 2050 (FAO, 2018). The conventional methods for food production have highly impacted agricultural land availability, agronomical soil degradation, the loss of agrochemical efficiency, and increased incidence of plant diseases (Lowry *et al.*, 2019; Pellegrini and Fernández, 2018). Plant diseases generate yield losses of 15-25% but in some cases, up to 85% of crop yield have also been reported (Gupta *et al.*, 2018; Mehta 2014). The most important phytopathogenic organisms agronomically are usually fungi, representing approximately 70% of plant diseases (FAO, 2016). Thus, among the most important phytopathogenic fungi due to their incidence, severity, and economic losses in subtropical and tropical regions, especially in Mexico, are *Rhizoctonia*, *Botryosphaeria*, *Bipolaris*, and *Fusarium* (Rodríguez-Guadarrama *et al.*, 2018; Villa-Rodriguez *et al.*, 2021; Mondragón-Flores *et al.*, 2021).

Plant disease management worldwide in traditional agriculture is mainly focused on the intensification of the applied pesticides. Where, the use of pesticides worldwide has increased by almost 100% from 1992 to 2016 (FAO, 2018), while in Mexico the increase has been 76% in the same period (Roser *et al.*, 2019). However, only about 0.1% reaches the agricultural crop (Gill *et al.*, 2014); thus, the remaining pesticide is lost, resulting in negative impacts including environmental degradation, destruction of non-target organisms, the contamination of soils and aquifers with recalcitrant residues, soil degradation, and salinization (Tudi *et al.*, 2021). The loss of biodiversity and chemical and microbial degradation in soil result in an increase in sensitivity and/or tolerance to potential external effects generating serious economic and environmental impacts (Chappell *et al.*, 2019; Gálvez-Gamboa *et al.*, 2018). Therefore, sustainable yield-focused strategies must be developed under an integral ecosystem approach to ensure the efficiency, quality, and yield of crops (Dietemann *et al.*, 2024).

In this sense, biopesticides have been a topic of interest worldwide (Thakur *et al.*, 2024). In Mexico, 6.2% of commercial pesticides used are of biological origin (biological control agents), among which the most frequently used biological control agents are *Bacillus thuringiensis*, *Phaeoacremonium nidulans*, and *Bauveria bassiana* (Pérez-Olvera *et al.*, 2011). The genus *Bacillus* occupies 85% of the commercially available bacterial biopesticides (Villarreal-Delgado *et al.*, 2017). Biological control agents are beneficial organisms that help reduce the

harmful effects of plant pathogens. They achieve this through antagonistic actions using various mechanisms; such as mycoparasitism, production of lytic enzymes, induction of systemic response of the plant, and/or production of δ -endotoxins, siderophores, and lipopeptides among others (Valenzuela Ruiz *et al.*, 2020).

In recent decades, the introduction of genomic, phylogenomic, and bioinformatics tools has contributed significantly to a better understanding of the interactions between microorganisms and plants (Sharma *et al.*, 2020). The study of microbial species at the genetic level has not only allowed the identification of mechanisms and genes involved in root colonization, the adaptation of the host, and the improvement of plant growth (Sheppard *et al.*, 2018; Youseif, 2018) but also, it has allowed the realization of very important advances in the characterization and taxonomic affiliation of taxa associated with beneficial effects in crops (Jagadeesan *et al.*, 2019). Thus, the objective of this study is to explore the potential biological control action mechanisms of *Bacillus paralicheniformis* TRQ65 against phytopathogenic fungi of agricultural importance, through genome sequencing and mining.

MATERIALS AND METHODS

Microbial strains and culture conditions

Bacillus paralicheniformis TRQ65 was isolated from the wheat (*Triticum turgidum* subsp. *durum*) rhizosphere in commercial fields of the Yaqui Valley, Mexico (27.3692°, 110.3886°). The strain is stored in the Colección de Microorganismos Edáficos y Endófitos Nativos (COLMENA) in Mexico (www.itson.edu.mx/COLMENA) (de los Santos Villalobos *et al.*, 2021). To prepare bacterial cultures, 1×10^4 colony-forming units (CFU) of TRQ65 were inoculated into 10 mL of Nutrient Broth (NB). After 24 hours of incubation at 28 °C and 180 rpm, the suspension was centrifuged at 4000 rpm for 5 minutes. The bacterial pellet was then re-suspended in sterile distilled water (autoclaved at 121 °C and 15 psi for 15 minutes) and adjusted to a concentration of 1×10^8 CFU/mL (Villa-Rodriguez *et al.*, 2021). The antagonistic activity of TRQ65 was tested against 11 phytopathogenic fungi: *Fusarium brachygibbosum*, *Botryosphaeria rhodina*, *Penicillium expansum*, *Penicillium crustosum*, *Geotrichum candidum*, and *Cladosporium* sp. (provided by the Michoacan University of San Nicolás de Hidalgo); *Botrytis cinerea*, *Mucor rouxii*, and *Rhizoctonia solani* (provided by the Ensenada Center for Scientific Research and Higher Education); and *Neocosmospora solani* and *Bipolaris sorokiniana* (from COLMENA). The fungi were grown on Potato Dextrose Agar (PDA) and incubated in light for 120 hours at 28°C. Spores were collected using

distilled water with 0.01% Tween 20 and adjusted to a concentration of 1×10^8 spores/mL (Villa-Rodriguez *et al.*, 2019).

***In vitro* dual confrontation assay of *Bacillus paralicheniformis* TRQ65 against phytopathogenic fungi**

A qualitative dual assay was carried out on Petri dishes containing Potato Dextrose Agar (PDA), where strain TRQ65 was tested against the fungal strains listed earlier. Each fungal strain was inoculated with 1×10^8 spores at the center of the Petri dish, while 1×10^8 CFU of TRQ65 was placed at equidistant points, 2 cm away from the fungal inoculum (de los Santos Villalobos *et al.*, 2012). The inhibition zones formed around the fungal pathogens were measured quantitatively over 7 days, with three replicates for each condition. Statistical analysis was performed using Statgraphics Plus version 5.1 software. Data were analyzed using one-way ANOVA and Tukey's Range Test (HSD) ($p \geq 0.05$), and the results are presented as the mean of the replicates or independent experiments.

Bacterial genome sequencing and mining

Genomic DNA was isolated from a fresh culture of strain TRQ65, which was grown in Nutrient Broth (NB) at 28°C with shaking at 180 rpm for 24 hours, reaching a concentration of 1×10^8 CFU/mL, as described by Valenzuela-Aragon *et al.* (2019). The extracted DNA was then sequenced using the Illumina MiSeq platform (2 x 300 bp) at a 30x coverage depth, and the Next Generation Sequencing library preparation was carried out by using the TruSeq DNA Nano Kit for Illumina® Platforms, following the manufacturer's specified protocols (LANGEBIO-CINVESTAV unidad Irapuato). The quality of the sequencing reads was evaluated using FastQC version 0.11.5 (Andrews, 2010). To remove adapter sequences and low-quality bases, Trimmomatic version 0.32 (Bolger *et al.*, 2014) was applied. For de novo assembly, SPAdes version 3.10.1 (Bankevich *et al.*, 2012) was used with the "--careful" option to minimize errors. The assembled contigs were arranged using Mauve Contig Mover version 2.4.0 (Darling *et al.*, 2004). To detect plasmids within the genome, PLACNETw (<https://castillo.dicom.unican.es/upload/>) (Vielva *et al.*, 2017) was utilized. Genome annotation was performed using the RAST server version 2.0 (<http://rast.nmpdr.org>) (Aziz *et al.*, 2008; Overbeek *et al.*, 2013), employing the RASTtk pipeline with default settings. Contigs were scaffolded using MeDuSa, a multi-draft-based scaffolder (Bosi *et al.*, 2015). Lastly, antiSMASH version 5.0 (<https://antismash.secondarymetabolites.org>) (Blin *et al.*, 2019) was used to identify potential biosynthetic gene clusters related to biocontrol activity in the genome of the studied bacterial strains.

RESULTS AND DISCUSSION

In vitro dual assay of *Bacillus paralicheniformis* TRQ65 against phytopathogenic fungi

Strain TRQ65 showed biocontrol activity against 45% of the studied phytopathogenic fungi (*Fusarium brachygibbosum*, *Neocosmospora solani*, *Botryosphaeria rhodina*, *Botrytis cinerea*, and *Bipolaris sorokiniana*) (Table 1). *Bacillus paralicheniformis* TRQ65 inhibited the mycelial growth of *Botrytis cinerea* in 43.8% ± 9%. This biocontrol activity started on day 4 and decreased until day 7. Furthermore, Świątczak *et al.* (2023), reported *Bacillus paralicheniformis* as a biological control agent against *Botrytis cinerea* in canola plants with 13% on inhibition. Similarly, Asif *et al.* (2023), evidenced biological control of *B. paralicheniformis* against *Elsinoe* sp., *Phytophthora capsica*, *Botrytis cinerea*, *Rhizoctonia solani*, *Coniella* sp., *Thielaviopsis* sp., and *Phytophthora sojae* attributing its phytopathogen inhibition mainly to fengycin and bacillibactin production.

Table 1. Inhibition zone (percentage) of *Bacillus paralicheniformis* TRQ65 against agriculturally important fungal plant pathogens.

Phytopathogen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
<i>Fusarium brachygibbosum</i>	ND	ND	ND	13% ± 0%	16% ± 1%	ND	ND
<i>Neocosmospora solani</i>	22% ± 3%	17% ± 1%	19% ± 5%	7.6% ± 3%	ND	ND	ND
<i>Botryosphaeria rhodina</i>	ND	ND	7% ± 1%	ND	ND	ND	ND
<i>Botrytis cinerea</i>	ND	ND	ND	ND	43.8% ± 9%	34% ± 5%	31% ± 5%
<i>Bipolaris sorikionania</i>	ND	ND	ND	14% ± 3%	11% ± 2%	8% ± 0%	ND

Tukey HSD test ($n=3, p \geq 0.05$). The variations correspond to the standard deviation. ND = Not detected.

On the other hand, the biocontrol of *B. paralicheniformis* TRQ65 against *Fusarium brachygibbosum*, *Neocosmospora solani*, *Botryosphaeria rhodina*, and *Bipolaris sorokiniana* showed variable results, where this activity started being quantifiable on day four, one, three, and four, respectively, and disappear on day

five, four, three, and six, respectively (Table 1). In this sense, De la Lastra *et al.* (2021), reported *B. paralicheniformis* ability to inhibit the fungal radial growth of the pathogenic *Fusarium* isolates, reaching from 73 to 84% inhibition. This biocontrol activity may be associated with diffusible metabolites biosynthesized by strain TRQ65 (Kulimushi *et al.*, 2017; Cawoy *et al.*, 2015), where its inhibition success is highly dependent on the interacting fungal species (Andrić *et al.*, 2020; Beauvais and Latgé 2018). However, research has shown that phytopathogens exhibit remarkable adaptability to antimicrobial metabolites produced by biological control agents, despite initial growth inhibition (Bonaterra *et al.*, 2022). Initially, these phytopathogens may be susceptible to antibiosis or competitive exclusion by biocontrol agents (Khan *et al.*, 2018), then they exert sophisticated mechanisms to counteract the initial inhibitory effects of biological control agents, including the synthesis of degradative enzymes capable of catabolizing or modifying inhibitory compounds, the upregulation of efflux pump systems to extrude toxic metabolites, and alterations in target site morphology or biochemistry to reduce susceptibility (Köhl *et al.*, 2019). For instance, some *Fusarium* species have demonstrated the ability to produce detoxifying enzymes that neutralize antifungal metabolites produced by biological control agents (Costa Almeida *et al.*, 2022). Thus, the efficacy of biological control can be compromised over time due to various factors, including environmental fluctuations, depletion of inhibitory compounds, complex microbial interactions within the rhizosphere, and the development of resistance by phytopathogens (Lal *et al.*, 2020). The dynamic nature of these interactions emphasizes the importance of unraveling the molecular mechanisms behind pathogen adaptation to explain the observed loss of efficacy (Barber *et al.*, 2024).

Bacterial genome analysis

During the last decades, biological control bacteria from the genus *Bacillus* with protective activities toward economically important plant pathogens have been highlighted (Finkel *et al.*, 2017; Fira *et al.*, 2018; Köhl *et al.*, 2019). Thus, several studies have been carried out to explore their potential action modes. In this sense, the genome of *B. paralicheniformis* TRQ65 was sequenced and mined to identify promising biosynthetic gene clusters associated with its biocontrol activity.

After sequencing, the genome of *B. paralicheniformis* TRQ65 showed 5,079,308 total reads [2×300 base pairs (bp)]. In the quality analysis and adapter remotion, only 8.42% was dropped. After *de novo* assembly, a draft genome of strain TRQ65 consisted of 4,475,481 bp; 45.5% G+C content; 676,421 bp N50; 3 L50; and 32 contigs (>200 bp). The ordered and scaffolding generated 25 scaffolds. In addition, no plasmids were observed, to our understanding, the presence of plasmids has not been reported for this species (Vielva *et al.*, 2017).

The RAST server’s genome annotation of *B. paralicheniformis* TRQ65 showed 91 RNAs, and 4809 predicted coding DNA sequences (CDS) distributed into 340 subsystems. The most abundant subsystem was amino acids and derivatives (341 CDS), followed by carbohydrates (338 CDS), protein metabolism (198 CDS), cofactors, vitamins, prosthetic groups, and pigments (147 genes), nucleosides and nucleotides (101), and dormancy and sporulation (96) (Figure 1). Furthermore, relating to biocontrol secondary metabolism (9 CDS) were identified including the production of auxin biosynthesis (4 CDS). A previous study carried out by Rojas Padilla *et al.* (2020), signaled that strain TRQ65 can produce indole acetic acid by 39.29 ± 0.30 ppm, and solubilize phosphate by 1.37 ± 0.05 %. In addition, iron acquisition and metabolism (44 CDS), including the production of siderophores (33 CDS) as bacillibactin, was detected in the genome of *B. paralicheniformis* TRQ65.

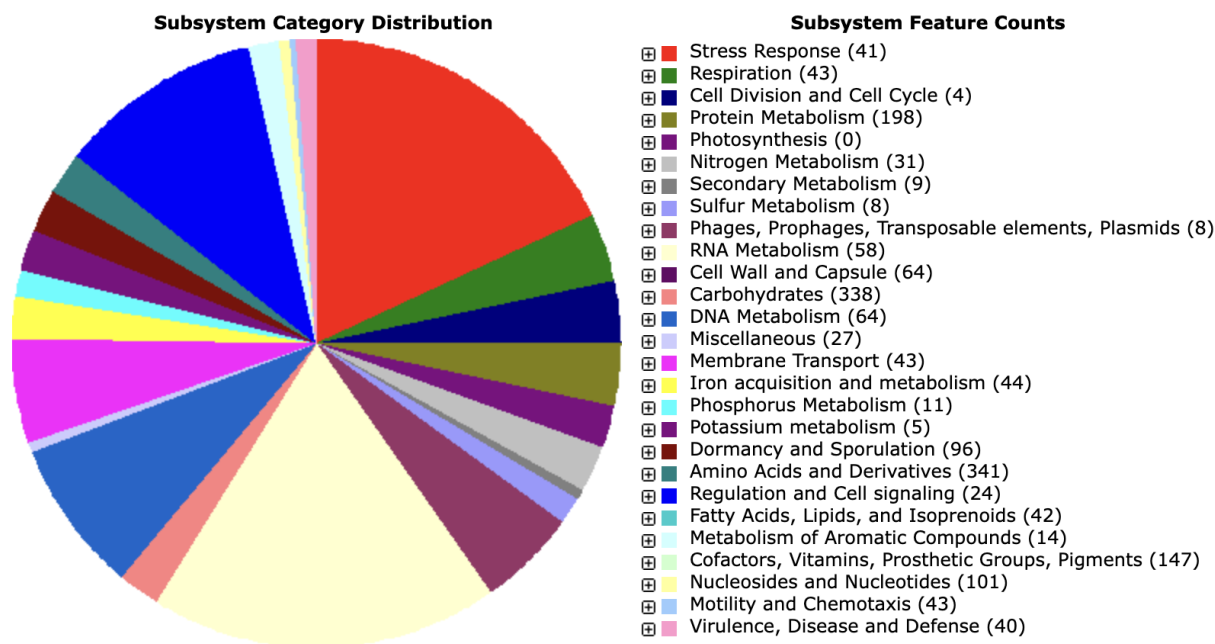


Figure 1. Subsystem category distribution of coding DNA sequences (CDS) in the *B. paralicheniformis* TRQ65 genome, following the RAST pipeline.

Fira *et al.* (2018) state that bacteria belonging to the genus *Bacillus* are well-known as producers of a wide array of antagonistic compounds of different structures, among the most important are non-ribosomally synthesized peptides and lipopeptides. To explore potential biosynthetic gene clusters associated with biocontrol activity, the genome of *B. paralicheniformis* TRQ65 was mined by antiSMASH (<https://antismash.secondarymetabolites.org>) (Blin *et al.*, 2019). In the

genome of strain TRQ65, gene clusters associated with biocontrol were detected: i) lichenysin (100% identity), an anionic lipopeptide biosurfactant with cytotoxic, antimicrobial, and hemolytic activities (Coronel *et al.*, 2017); ii) bacitracin (88% identity), cyclic lipopeptide antibiotic that is active against Gram-positive and some Gram-negative bacteria by inhibiting bacterial cell-wall biosynthesis (Liu *et al.*, 2018); iii) fengycin (93% identity), a lipopeptide with fungi-toxic activity against filamentous fungi, which interact with lipid layers and alter the structure of the cell membrane and permeability (Li *et al.*, 2019); iv) the siderophore bacillibactin (100% identity) a cyclic compound that contains catechol groups, which have a high affinity for iron (Fe^{3+}), in which it makes a non-suitable environment for phytopathogen colonization as it competes for iron (Félix-Pablos *et al.*, 2022); and v) amyloliquocidin (93% identity) an antimicrobial peptide against Gram-positive bacteria. (Gerst *et al.*, 2022). Based on the action modes of the biosynthetic gene clusters mentioned before, only lichenysin, fengycin and bacillibactin (Figure 2) have shown antifungal activities. Thus, we inferred that these three compounds could be involved in the biocontrol of the studied phytopathogenic fungi, these molecules are now being strongly studied by our team.

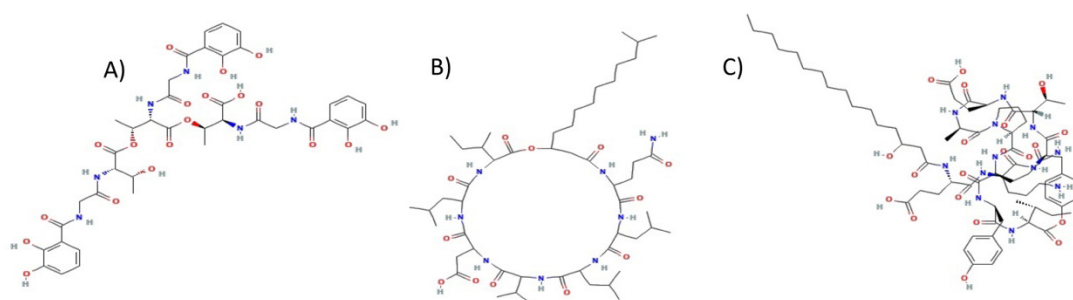


Figure 2. A) Bacillibactin structure (National Center for Biotechnology Information, 2024). Bacillibactin is built around a trilactone core formed by the cyclization of three molecules of 2,3-dihydroxybenzoic acid (DHB) linked to a central scaffold of threonine residues. The trilactone ring is formed through ester bonds between the hydroxyl groups of the threonine and the carboxyl groups of DHB. Each DHB moiety in bacillibactin contains catechol (2,3-dihydroxybenzoate) functional groups. These catechol groups are responsible for the high-affinity binding of Fe^{3+} . B) Lichenysin structure (National Center for Biotechnology Information, 2024). Lichenysin contains a cyclic peptide ring consisting of 13 amino acid residues. Attached to the cyclic peptide core is a lipid tail, typically a β -hydroxy fatty acid chain. The peptide chain forms a cyclic structure through an amide bond between the carboxyl group of one amino acid and the amine group of another, creating a stable ring structure. C) Fengycin structure (National Center for Biotechnology Information, 2024). Fengycin consists of a decapeptide forming a cyclic structure through a lactone linkage. Attached to the peptide ring is a β -hydroxy fatty acid, which can vary in length, usually between 14 and 18 carbon atoms. The exact sequence of amino acids can vary slightly depending on the specific isoform of fengycin.

Furthermore, bacillibactin is a high-affinity siderophore with a trilactone core composed of 2,3-dihydroxybenzoic acid (DHB) units linked to threonine residues, where catechol groups chelate Fe³⁺ ions with high specificity, forming stable complexes that support essential metabolic processes under iron-limited conditions (Khan *et al.*, 2016; Dimopoulou *et al.*, 2021; Timofeeva *et al.*, 2022). Lichenysin, an amphiphilic lipopeptide surfactant with a cyclic peptide ring and hydrophobic fatty acid chain, exhibits stability under extreme conditions and has demonstrated inhibition of fungal pathogens such as *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Neocosmospora keratoplastica* (Gudiña and Teixeira, 2022; Yeak *et al.*, 2022; Olmedo *et al.*, 2022; Armenova *et al.*, 2024). Fengycins, particularly fengycin A and B, are lipodecapeptides with antifungal activity against filamentous fungi, disrupting fungal cell membranes, perturbing mycelial growth, and inducing systemic resistance in plants. Studies have demonstrated their effectiveness against *Botrytis cinerea*, *Pythium ultimum*, and *Monilinia fructicola*, showcasing their ability to suppress spore germination and cause fungal cytoplasm leakage (Vanittanakom *et al.*, 1986; Koumoutsi *et al.*, 2004; Deleu *et al.*, 2005; Ongena *et al.*, 2005; Liu *et al.*, 2011; Geissler *et al.*, 2019; Andrić *et al.*, 2020). This study provides the first insight into the potential biological control mechanism used by *Bacillus paralicheniformis* TRQ65 as a biological control agent; however, this study's results indicate moderate biological control against the studied fungi. Thus, further research is necessary to explore the biological control ability, identified through genomic mining to complement the confrontation analysis results of low inhibition.

CONCLUSIONS

Bacillus paralicheniformis TRQ65 is a biological control agent that inhibits the mycelial growth of *Fusarium brachygibbosum*, *Neocosmospora solani*, *Botryosphaeria rhodina*, and *Bipolaris sorokiniana*. Based on genome mining, this bioactivity could be related to the biosynthesis of lichenysin, fengycin, and/or bacillibactin. Thus, further studies are currently being conducted by our research team to improve the ability of *B. paralicheniformis* TRQ65 and validate its use as an active ingredient in bacterial inoculants to migrate toward sustainable agriculture.

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