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Phenotypic Fingerprints of *Ralstonia solanacearum* Biovar 3 Strains from Tobacco and Tomato in China Assessed by Phenotype MicroArray Analysis

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ABSTRACT
Ralstonia solanacearum that causing devastating disease in Solanaceae crops, is a notorious pathogen worldwide. A collection of the pathogen originating from tobacco and tomato in China was analyzed by using BIOLOG Phenotype MicroArray (PM) and assigned to biovar. Phenotypic fingerprints of two different original strains were also compared to explore their phenotypic diversities. All tested isolates of *Ralstonia solanacearum* oxidized both disaccharides and sugar alcohols and assigned to biovar 3. Using PM plates 1 to 8, 758 different assays were tested, including 190 different carbon substrates, 95 nitrogen substrates, 59 phosphorus substrates, 35 sulfur substrates, 94 biosynthetic pathways and 285 nitrogen pathways. Phenotypic fingerprints of the pathogen from tobacco and tomato were nearly the same. *Ralstonia solanacearum* was able to utilize 19% of tested carbon substrates, 43% of nitrogen substrates, 100% of sulfur substrates and 95% of phosphorus substrates. Most informative utilization ways for carbon substrates were organic acids and carbohydrates and for nitrogen were various amino acids. Those findings showed useful information for biology and physiology study of *R. solanacearum*.

Key words: Biolog phenotype microArray, metabolic fingerprint, bacterial wilt

INTRODUCTION

Ralstonia solanacearum (Smith) is a notorious soil-borne bacterial pathogen with a large host range of more than 200 species in 50 families and a worldwide distribution (Hayward, 1995). It is a major threaten in the production of several important economy crops, particularly for the Solanaceae crops of tobacco, tomato, potato and pepper in the world (French and Sequeira, 1970). In recent five years, *R. solanacearum* is one of the wide spread and most important bacterial phytopathogens in China, mainly in flue-cured tobacco and tomato growing seasons (Chen *et al*., 2013; Peng *et al*., 2007). Symptoms of bacterial wilt include leaf wilting, yellowing and necrosis (Swanson *et al*., 2005). Typically, diseased stem and tuber sections exude whitish bacterial exudates (Genin and Boucher, 2002). The bacterium survives in infested soil, infected plants, susceptible weed hosts and volunteer crops and spreads through contaminated irrigation water and infected planting material (Hayward, 1991).

Ralstonia solanacearum exhibits great diversities in host range, geographical distribution, pathogenicity and physiological properties (Horita and Tsuchiya, 2001; Poussier *et al*., 1999). The species of the pathogen was subdivided into five races based on its host range (Buddenhagen *et al*., 1962; He *et al*., 1983), into six biovars according to the utilization of disaccharides and hexose alcohols (Hayward, 1964; Hayward *et al*., 1990; He *et al*., 1983) and into four phylotypes divisions indicating the phylogenetic relationships (Fegan and Prior, 2005; Prior and Fegan, 2005). Most bacterial wilt in host of tobacco and tomato caused by *R. solanacearum* in China belongs to Race 1 biovar 3 (R1Bv3) (Deng *et al*., 2004; Xue *et al*., 2011; Zou and Xiao, 2008). The R1Bv3 strains in tobacco and

tomato has also been reported in several other countries in Asia, such as Japan, Kero and India (Chandrashekara *et al*., 2012; Jeong *et al*., 2007; Liu *et al*., 2012; Tsuchiya and Horita, 1998). Recently, technique such as phenotypic analysis was used to investigate the phenotypic diversity of various microorganisms which could also help scientists to know the phenotype diversities between isolates of *R. solanacearum* from hosts of tobacco and that of tomato.

Recently, a high throughput Phenotypic MicroArray (PM)/Omnilog system, was developed by the company Biolog (Hayward, CA) to assay nearly 1000 metabolic phenotypes (Bochner *et al*., 2001). In the system, microorganism cells in test medium including a tetrazolium dye are inoculated into 96-well plates, where the plates include various substances to analyze for the use of carbon, nitrogen, sulfur, phosphorus substrates and biosynthetic pathways. Cell respiration and growth result in the reduction of the tetrazolium dye and lead to blue color. The intensity of the color is recorded and analyzed by Omnilog software which yield a quantitative analysis of all data. The software sketches kinetic data of color formation against time for each well and ascribes artificial color to each plot.

In this study, *R. solanacearum* strains originating from host of tobacco and tomato in China was assigned to biovar and characterized by phenotypic analysis. Phenotypic fingerprints were also compared between different strains to explore their phenotypic diversities. Although few management methods are available to control bacterial wilt in tobacco or tomato, a detailed understanding of the phenotypes of *R. solanacearum* is valuable to develop management practices to decrease the impacts of the disease.

MATERIALS AND METHODS

Origin and collection of *Ralstonia solanacearum* isolates: All isolates of *R. solanacearum* used in this study were collected from infected tobacco stem with typical bacterial wilt symptoms. The infected stems were sampled in 2011 during the disease epidemic season from tobacco fields in Guizhou Province of China (Table 1). Single-stem isolates were purified from 9 different commercial fields. To isolate *R. solanacearum* from tobacco stems, infected tobacco plants were washed with distilled water five times and dried under laboratory conditions. Pieces (8×8 mm) of tissue were cut from margins of the lesions on stems. Tissues pieces were then surface disinfected in a 0.05% NaClO (w/v) solution for 2 min, rinsed in distilled water for three times and placed on a semi-selective medium (SMSA) (Buddenhagen and Kelman, 1964). Plates were incubated at 30°C in the dark for 2-4 days. Colonies with morphology similar to *R. solanacearum* were purified. Additional three strains from host of tomato were donated by Professor Wang and Professor Wei from Nanjing Agricultural University, Nanjing, China (Table 1). All strains were stored as suspensions in sterile water at 25°C for long-term storage and retrieved at 28°C using the YPGA medium (peptone, 5 g L^{-1}; yeast extract, 5 g L^{-1}; glucose, 10 g L^{-1} and agar, 16 g L^{-1}; pH 7.2).

Table 1: Strains of *Ralstonia solanacearum* used in this study

Strain	Host plant	Geographic origin	Biovar
R1	Tobacco	Guizhou	3
R2	Tobacco	Guizhou	3
R3	Tobacco	Guizhou	3
R4	Tobacco	Guizhou	3
R5	Tobacco	Guizhou	3
R6	Tobacco	Guizhou	3
R7	Tobacco	Guizhou	3
R8	Tobacco	Guizhou	3
R9	Tobacco	Guizhou	3
R10	Tobacco	Guizhou	3
R11	Tobacco	Guizhou	3
R12	Tobacco	Guizhou	3
X1	Tomato	Jiangsu	3
X2	Tomato	Jiangsu	3
X3	Tomato	Jiangsu	3

Molecular identification of *Ralstonia solanacearum*: A loopful of suspect colonies on SMSA plate was swabbed from the agar, re-suspended in 150 μL of distilled water, boiled for 12 min in an Eppendorf tube, cooled to room temperature on ice for 8 min, centrifuged at 9000 g for 2 min and the supernatant was utilized for PCR. The flagella C gene (*flic*) fragments (Schonfeld *et al*., 2003) were amplified using the *R. solanacearum*-specific primers as described by Schonfeld *et al*. (2003) which yielded a 400 bp product. PCR amplifications were conducted by thermocycler (Biorad MyCycler™; BioRad, CA, USA) in a 30 μL reaction system that contained 6 μL of boiled supernatant, 3 mM $MgCl_2$, 0.5 μM of each primer, 1 unit of *Taq* DNA polymerase (Takara, Dalian, China), 200 μM (each) deoxynucleoside triphosphate (dNTP) in 1×PCR buffer. PCR conditions were performed as follows: 95°C for 10 min, 35 cycles of 30 sec at 95°C, 120 sec at 63°C and 10 min at 72°C and a final extension at 72°C for 10 min. Products (4 μL) were loaded on 1.0% agarose (Biowest®, Spain) gels, electrophoresed at 100 V for 30 min and checked under UV transillumination (254 nm).

Biovar determination: Strains of suspect pathogen were analyzed into biovars based on their ability to utilize three disaccharides (lactose, maltose and cellobiose) and three hexose alcohols (mannitol, sorbitol and dulcitol). The characterization of the biovars was conducted according to the method as outlined in previous reports (Hayward, 1964; He *et al*., 1983).

Phenotypic characterization: One isolate of *R. solanacearum* from tobacco and another from tomato were chosen randomly from the strains isolated above for this study. All materials, reagents and media for the phenotypic study were bought from Biolog. Isolates were streaked on Biolog Universal Growth medium plus blood agar (BUG+B) plates and incubated at 30°C in darkness for 48 h. Cells were scraped from the surface of the plates and re-suspended in appropriate medium containing Dye Mix C; 120 μL of a 1:200 dilution of an 85% transmittance cells suspension were added to each well of the PM plates. Plates 1-8 which test for phenotypes of carbon,

nitrogen phosphorus, sulfur, as well as for biosynthetic pathways, were used in this study. IF-0 GN Base was prepared for PM plates 1 and 2. IF-0 GN Base plus 20 mM sodium succinate, pH 7.1 and 2 μM ferric citrate was utilized for plates 3-8. After inoculation under clean beneath, plates were incubated in the Omnilog incubator for 72 h. Data were collected every 15 min by the Biolog incubator and analyzed using Kinetic and Parametric software (Biolog). Phenotype diversities were performed based on the area difference under the kinetic curve of dye formation.

RESULTS

Isolation, identification of the pathogen and assignment to biovar: All suspect *R. solanacearum* strains were isolated from tobacco stems with symptoms of typical wilt. No more than two isolates per field site were conducted in our study. By incubation of the infected tissues nieces on selective media of SMSA, a total of 12 single-tissue isolates of *R. solanacearum* were isolated. All isolates exhibited the typical colony morphology on the test medium. Moreover, all strains presented fragments of the expected targeting *flic* product (400 bp size) (data not shown). These results confirmed the correct assignment of the isolates from diseased tobacco stems to *R. solanacearum*.

The color changed from blue to yellow, indicating the oxidization of sugars by the tested bacterial isolates. Result in biovar test presented that all *R. solanacearum* isolates metabolized disaccharides (lactose, maltose and cellobiose) and sugar alcohols (mannitol, sorbitol and dulcitol) within several days' incubation. Therefore, all isolates of *R. solanacearum* tested in this study belong to biovar 3 (Table 1).

Phenotypic characterization: Phenotypic characterization of *R. solanacearum* was conducted by using the PM plates 1-8. A total of 758 different growth conditions were analyzed, including 190 different carbon substrates, 95 nitrogen substrates, 59 phosphorus substrates, 35 sulfur substrates, 94 biosynthetic pathways and 285 nitrogen pathways. Isolate of *R. solanacearum* from tobacco had nearly the same metabolic fingerprints as that from tomato. Two isolates were able to utilize 19% of tested carbon substrates, 43% of nitrogen substrates, 100% of sulfur substrates and 95% of phosphorus substrates (Fig. 1). *R. solanacearum* is very efficient in metabolizing phosphorus compounds (56/59 tested, plate PM4, Wells A1-E12). The phosphorus substrates that were poorly metabolized by *R. solanacearum* were adenosine 3', 5'-cyclic monophosphate (plate PM4, Well A12), uridine 3', 5'-cyclic monophosphate (plate PM4, Well D12) and methylene diphosphonic acid (plate PM4, Well E8). Meanwhile, nearly all S-containing compounds (35/35 tested, plate PM4, Wells F1-H12) tested could be assimilated by the pathogen. The two isolates of *R. solanacearum* presented 94 different biosynthetic pathways (94/94 tested, plate PM5, Wells A3-H12).

Using data from PM1 and PM2 (carbon substrates), isolate of *R. solanacearum* X1 from tomato was compared with isolate R1 from tobacco. They presented the ability to use 49 and 36 different carbon substrates, respectively. Thirty-six compounds supported the growth of the two pathogens; these were D-galactose, D-saccharic acid, L-aspartic acid, succinic acid, D-trehalose, dulcitol, D-sorbitol, glycerol, D-glucuronic acid, D-mannitol, D, L-malic acid, L-glutamic acid, tween 20, acetic acid, D-fructose, a-D-glucose, L-asparagine, tween 40, sucrose, L-glutamine, tween 80, fumaric acid, bromosuccinic acid, mucic acid, L-alanine, methylpyruvate, L-malic acid, pyruvic acid, D-galacturonic acid, pectin, g-amino-N-butyric acid, b-hydroxybutyric acid, quinic acid, L-tartaric acid, L-histidine and L-pyroglutamic acid. In particular, X1 showed metabolic activity on several other carbon compounds (L-proline, D-gluconic acid, D, L-lactic acid, D-aspartic acid, a-ketoglutaric acid, citric acid, m-inositol, propionic acid, L-serine, L-threonine, sodium butyrate, D-tartaric acid, N-acetyl-L-glutamic acid), whereas, R1 was unable to utilize these chemicals.

Data from PM3 plate presented the ability of the two strains of *R. solanacearum* in 95 different nitrogen substrates metabolize (Fig. 1). One striking result was that the apparent growth of *R. solanacearum* in the negative control without any nitrogen substrate (PM3, Well A1). Forty-one compounds supported the growth of the pathogen; these were ammonia, nitrite, nitrate, urea, L-arginine, L-glutamic acid, L-asparagine, L-aspartic acid, L-alanine, L-glutamine, glycine, L-leucine, L-histidine, L-isoleucine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine, D-alanine, D-aspartic acid, D-glutamic acid, L-citrulline, L-ornithine, L-pyroglutamic acid, glucuronamide, xanthine, allantoin, g-amino-N-butyric acid and some combination of some amino acids. In comparison, fifty-four out of 95 nitrogen substrates supported the growth of *R. solanacearum* at the level of the negative control, indicating that *R. solanacearum* cannot utilize these substrates; these were biuret, L-cysteine, L-lysine, L-methionine, L-tryptophan, D-asparagine, D-lysine, D-serine, D-valine, L-homoserine, N-phthaloyl-L-glutamic acid, N-acetyl-L-glutamic acid, N-amylamine, N-butylamine, hydroxylamine, ethylamine, methylamine, ethanolamine, ethylenediamine, agmatine, putrescine, histamine, b-phenylethylamine, tyramine, formamide, acetamide, D, L-lactamide, D-glucosamine, D-galactosamine, D-mannosamine, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, adenine, adenosine, cytidine, cytosine, guanine, guanosine, thymine, thymidine, uracil, uridine, inosine, xanthosine, uric acid, alloxan, parabanic acid, D, L-a-amino-N-butyric acid, e-amino-N-caproic acid, D, L-a-amino-caprylic acid, d-amino-N-valeric acid, a-amino-N-valeric acid and Gly-Met.

Using data from PM6 to PM8 (nitrogen pathways), isolate of *R. solanacearum* X1 from tomato was compared with isolate R1 from tobacco. They presented 127 and 120 different nitrogen pathways, respectively. One hundred and twenty

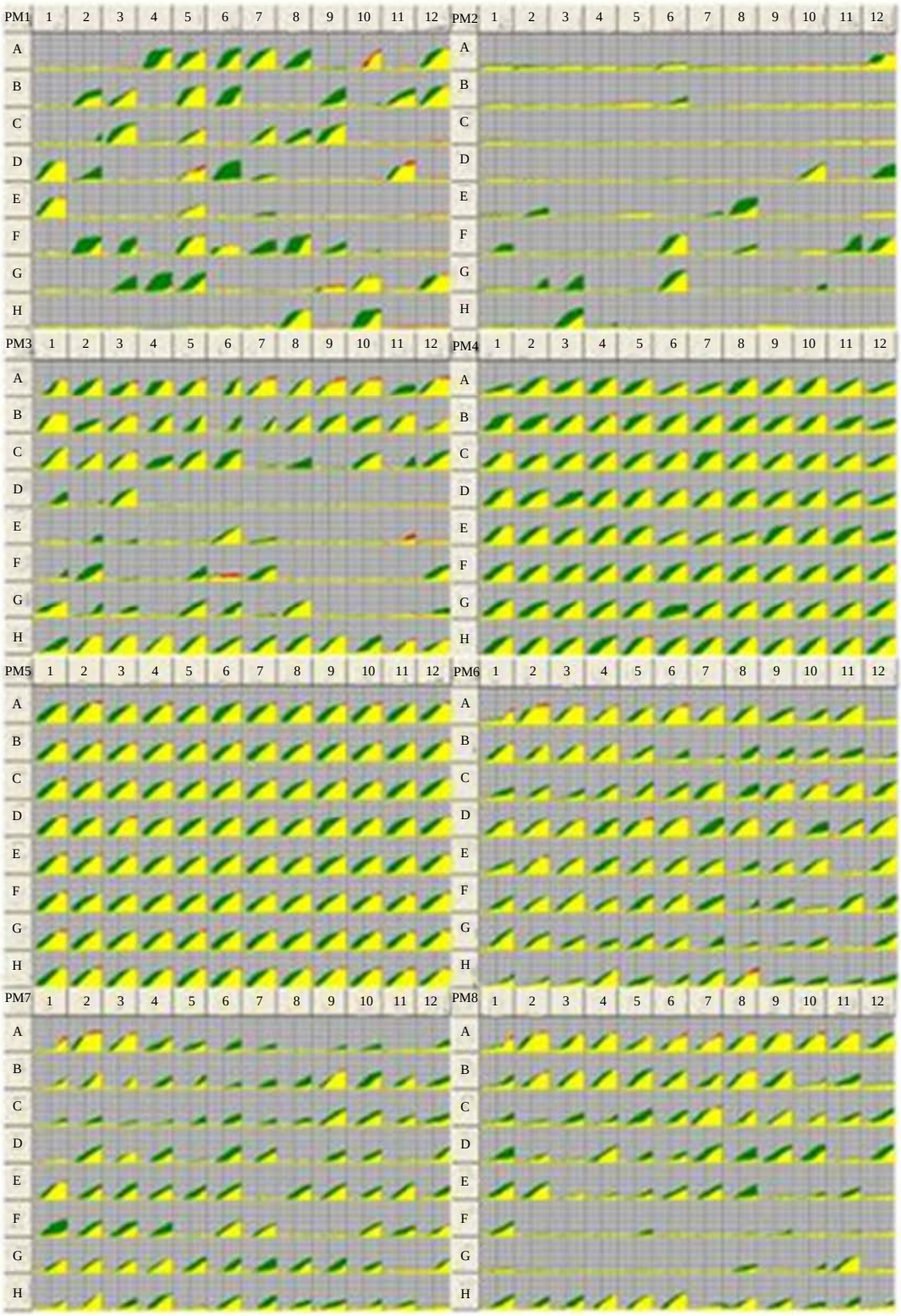

Fig. 1: Data for biolog phenotype microArray PM 1-8 plates comparing one isolate of *R. solanacearum* from tomato and another one from tobacco. (Increased utilization of the isolate of *R. solanacearum* from tomato compared to the other from tobacco was indicated by green areas in the growth curve for each substrate, reduced utilization was noted by red areas)

kinds of combination of different amino acids supported the growth of the two pathogens (Fig. 1). In particular, X1 showed metabolic activity on several other combinations (Glu-Ser, Thr-Glu, Thr-Met, Tyr-Glu, Pro-Glu, Ser-Asp and Val-Glu), whereas, R1did not have those nitrogen pathways.

DISCUSSION

Despite many molecular biology and genetic studies have been conducted to the sequenced pathogen *R. solanacearum*, phenotypic characterization in bacterial populations is still poorly understood. Direct high-throughput testing of phenotypes using the Phenotype MicroArray (PM) system (Biolog) (Bochner *et al*., 2001) has provided a much easy way for biology, genomic and population studies of microorganisms (Guard-Bouldin *et al*., 2007; Mols *et al*., 2007; Viti *et al*., 2007). Here, metabolic abilities of the pathogen from hosts of tobacco and tomato were systematically analyzed and compared using PMs (Biolog). Significant metabolic diversity between the two strains was found.

In comparing *R. solanacearum* data from our study with data for other bacterial species (*Pseudomonas aeruginosa* and *Ralstonia pickettii*) reported on Biolog website, it appears that *R. solanacearum* presents higher carbon substrates use ability, especially for chemicals tested on PM1, such as amino acids. Additionally, most of the nitrogen, sulfur and phosphorus substrates were also utilized by the pathogen. These data indicate a general view of the lifestyle of the pathogen *R. solanacearum* which is a notorious bacterium that has a fair ability to colonize both plants and soils (Wei *et al*., 2011). The phenotype characters of a wide range of nutrient substrates found in this study has great adaptive value. In between, the informative plates were PM1/PM2 (carbon substrates) and PM3 (nitrogen substrates). Additionally, important utilization patterns for carbon substrates were organic acids and glucoside while for nitrogen were various amino acids. These substrates are commonly tested in many plant exudates (Fan *et al*., 2001; Lung *et al*., 2008). It appears to play an important role to support the infection of *R. solanacearum* nearing roots of solanaceous plants and the survival of the pathogen in soil in various conditions. Additionally, phenotypic characters for utilization of those substrates could also have positive value in plant-microbe interaction study.

Since the PM technique analyzes strains for high-throughput characters including different environmental stressors, it becomes possible to determine whether it has a chance bacterial wilt management in agriculture. Enhancing the amount of carbon and nitrogen sources that could not be metabolized by *R. solanacearum*, or decreasing the amount of these sources that could be utilized by the pathogen in the field may reduce the damage caused by bacterial wilt. These imagines could be proved in the next study in the near future. In conclusion, phenomics study of *R. solanacearum* in our study increases our understanding of the pathogen.

Bacterial wilt caused by *R. solanacearum* is a great threat to many important crops in China, especially for tobacco and tomato (Lu, 1998; Xu *et al*., 2009). Huge economic losses happened each year for tobacco and tomato throughout the country (Lu, 1998; Li *et al*., 2004; Xu *et al*., 2009). Isolates of *R. solanacearum* have been assigned to biovar 2, biovar 3, biovar 4, biovar 5 and biovar 6 in China (Xue *et al*., 2011), with host plants of peanut, tobacco, potato, casuarina, eggplant, ginger, mulberry, ramie, pepper, sesame, night shade, sweet potato and tomato. While for host of tobacco and tomato, just biovar 3 and biovar 4 of the isolates were detected in China (Liu *et al*., 2008; Xue *et al*., 2011). In our study, only biovar 3 isolates were found from tobacco in Guizhou province and from tomato in Jiangsu province. This finding is in accordance with the results detected by other scientists mentioned above. Biovar 3 of *R. solanacearum* should be a major pathogen for bacterial wilt in tobacco and tomato in China. Due to high prevalence and varies of the pathogen, many strategies for bacterial wilt management showed very limited success. Phenotypic diversities of *R. solanacearum* might be one of the reasons for the difficulty. Additionally, the relationship between biovars and pathogenicity of *R. solanacearum* is still unclear, further analysis using more isolates from different biovars is needed.

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