***Meloidogyne* Virulence Locus Molecular Marker for Characterization of Selected *Mi*-Virulent Populations of *Meloidogyne* spp. Is Correlated with Several Genera of Betaproteobacteria**

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Accepted for publication 8 October 2010.

ABSTRACT

Cortada, L., Sakai, H., Verdejo-Lucas, S., and Mizukubo, T. 2011. *Meloidogyne* virulence locus molecular marker for characterization of selected *Mi*-virulent populations of *Meloidogyne* spp. is correlated with several genera of betaproteobacteria. *Phytopathology* 101:410-415.

Resistance to root-knot nematodes in tomato is conferred by the *Mi* resistance gene to the three most important species of *Meloidogyne*: *M. arenaria*, *M. incognita*, and *M. javanica*. Nevertheless, the *Mi* gene is unable to inhibit the reproduction of selected and naturally *Mi*-virulent populations of root-knot nematodes. As pathogenicity assays are time consuming, molecular markers were developed for the easy identification of *Mi*-virulent populations of *Meloidogyne*. The sequence characterized

amplified region-*Meloidogyne* virulence locus (MVC) molecular marker is reported to differentiate *Mi*-avirulent and naturally *Mi*-virulent from selected *Mi*-virulent populations. This marker was used to compare acquired virulence in populations of *M. javanica* from Spain. The original populations used to develop the MVC marker were included as control for reference. Results showed that this marker did not amplify genomic DNA extracted from single juveniles or females of any of the populations tested either from Spain or Japan. In silico analyses performed with the recently published complete genome of *M. incognita*, indicated that the MVC marker is not correlated to a MVC or to any eukaryotic organism but to several betaproteobacteria genus from the family *Comamonadaceae*.

Resistance to *Meloidogyne arenaria*, *M. incognita*, and *M. javanica* in tomato is conferred by a single dominant gene named *Mi* (30). In nematology, virulence is defined as the ability of a nematode to infect and reproduce on a resistant plant, evolving into a fertile female (22). Populations able to overcome the resistance provided by the *Mi* gene are classified as naturally or selected *Mi*-virulent. The later appear after repeated exposure to the *Mi* gene (5,9,14,28,31), whereas naturally *Mi*-virulent populations reproduce significantly on resistant tomato cultivars despite never having been exposed to the *Mi* gene (15,19,20,33). Independent of their origin, *Mi*-virulent populations negate the use of resistance as a management option for root-knot nematodes.

The gene-for-gene model postulates that for every avirulence gene (*Avr*) present in the pathogen, there is a resistant gene (*R*) in the host that prevents infection. So far, *Avr* genes from nematodes have not been cloned (32) although there is genetic evidence that avirulence in certain species of plant-parasitic nematodes is transmitted to the progeny as a single dominant trait (13). This *Avr-R* gene model has been proved for *Globodera rostochiensis* and the *R* gene *H1* (2), for *M. hapla* and the bean cultivar NemaSnap (8), and more recently for the *Gp-RBP-1* protein from *G. pallida* and the *Gpa2* gene (23). Evidence to confirm this model in the root-knot nematode-resistant plant pathosystem are scarce (4), because the strictly parthenogenetic mode of reproduction of *M. arenaria*, *M. incognita*, and *M. javanica* does not permit the studying of the segregation of the *Mi*-virulent alleles (*avr*) in the progeny of these species. In 2001, Xu et al. (33) developed the

sequence characterized amplified region-polymerase chain reaction (SCAR-PCR) *Meloidogyne* virulence correlated (MVC) molecular marker to differentiate selected *Mi*-virulent populations of *Meloidogyne* from naturally *Mi*-virulent and *Mi*-avirulent populations; and it was suggested that selected *Mi*-virulent nematode isolates originated by similar mutational events and that they diverged genetically from those naturally *Mi*-virulent which have never been exposed to the selection pressure of the *Mi* gene.

The recent publication of the complete genome of *M. incognita* and *M. hapla* (4) offered the opportunity to correlate the sequences of the MVC locus with the genome of these species. In silico analyses performed with the original MVC-allelic sequences (GenBank accession nos. AB047761.1 to AB047767.1) did not reveal a correlation between these sequences and the genome of any nematode species including that of *Caenorhabditis elegans* or any eukaryotic organism. However, a close homology (*E* values = 0; maximum identities = 71 to 88%) was found with the genome of seven genera of betaproteobacteria belonging to the family *Comamonadaceae*: *Acidovorax* spp. (accession nos. CP001392.1 and CP000512.1), *Delftia* spp. (accession no. CP000884.1), *Polaromonas* spp. (accession no. CP000316.1), *Rhodopherax* spp. (accession no. CP000267.1), *Variovorax* spp. (accession no. CP001635.1), and *Verminephrobacter* spp. (accession no. CP000542.1). The objective of the present study was to demonstrate that the MVC molecular marker is correlated to the genome of a prokaryotic organism and not to a root-knot nematode virulence locus.

MATERIALS AND METHODS

Molecular analyses were performed with seven populations of the genus *Meloidogyne* from different geographic origins: (i) two Japanese laboratory-selected *Mi*-virulent populations, *M. javanica* (MJON-VI) and *M. incognita* (MIYN-VI) (33); and (ii) five

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doi:10.1094/PHYTO-04-10-0123

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populations from north-eastern Spain that differed in their ability to reproduce on *Mi*-resistant tomatoes: three *M. javanica* field-selected *Mi*-virulent populations (PG-76-P3, Brigeor-P3, and Monika-P3), one *Mi*-avirulent (Durinta-P3) (28), and one naturally *Mi*-virulent population (MJ-27) (19). Characteristics of the populations are described in Table 1. All the *Mi*-virulent populations from Japan have been maintained on resistant tomato plants (cv. Momotaro) since they were first identified as *Mi*-virulent (T. Narabu, *personal communication*). In the case of the Spanish populations, they have been maintained on resistant tomato cv. Monika and bioassays indicated that the ability of these populations to reproduce on *Mi*-resistant plants has remained stable along this period (29).

Water samples. Water samples were obtained from a potted resistant tomato plant (cv. Momotaro) infected by the MIYN-VI population. The infected plant was watered with distilled sterilized water and the draining water (150 ml approximately) was collected on a beaker and stored at 4°C, to restrict the growth of microorganisms. Three types of water treatments were investigated (Fig. 1A): (i) the drained water directly collected from the pot, which was called “nonfiltered water” (NF); (ii) the drained water filtered through a 5 µm pore-sieve of nylon to retain nematode second-stage juveniles (J2) and eggs (from now on referred as 5F sample), and (iii) an aliquot of the 5F sample filtered again through a 0.2 µm cellulose acetate filter (AstroPore Disk Capsule CALC. 20, 2,5 CMD; Fuji Film, Kanagawa, Japan)

TABLE 1. Characteristics of the populations of *Meloidogyne* spp. used for the molecular analyses

Species	Population	Origin	<i>Mi</i> -virulence ^a	Host	Reference
<i>M. javanica</i>	MJ-27	Barcelona, Spain	VI-natural	<i>Mi</i> -tomato cultivar	(19)
	PG-76-P3	Barcelona, Spain	VI-selected*	<i>Mi</i> -tomato rootstock	(28)
	Brigeor-P3	Barcelona, Spain	VI-selected*	<i>Mi</i> -tomato rootstock	(28)
	Monika-P3	Barcelona, Spain	VI-selected*	<i>Mi</i> -tomato cultivar	(28)
	MJON-VI	Okinawa, Japan	VI-selected**	<i>Mi</i> -tomato cultivar	(33)
	Durinta-P3	Barcelona, Spain	AV	<i>Mi</i> -tomato cultivar	(28)
<i>M. incognita</i>	MIYN-VI	Yamanashi, Japan	VI-selected*	<i>Mi</i> -tomato cultivar	(33)

^a VI (*Mi*-virulent) and AV (*Mi*-avirulent); * indicates *Mi*-virulent field selected populations and ** indicates *Mi*-virulent laboratory selected population.

Fig. 1. Types of samples used for DNA extraction. **A**, Water drained directly from a potted resistant tomato plant infected by the selected *Meloidogyne incognita* population (MIYN-VI). Water treatments: NF, draining water directly collected from the pot; 5F, originated from the NF water sample filtered through a 5 µm pore-sieve of nylon to retain second-stage juveniles (J2) and eggs; 0.2F, obtained by the filtration of an aliquot of the 5F water through a 0.2 µm cellulose acetate filter to retain microorganism(s). **B**, Egg masses, females, and J2 samples from the *M. javanica* (MJON-VI) and the MIYN-VI selected *Mi*-virulent populations.

that was identified as 0.2F sample; the last filtration step was performed in order to retain any possible microorganism(s) present in the sample (i.e., bacteria or fungal spores). For DNA extraction, one milliliter of the NF, 5F, and 0.2F water samples were pipetted separately into 1.5-ml Eppendorf tubes and spun at 13,000 rpm for 20 min at 4°C. Three samples of each were analyzed. Supernatant was removed and 4 µl of 0.1% SDS lysis buffer (24) was added to the tubes; samples were incubated at 50°C in a thermal cycler for 2 h followed by one cycle at 95°C for 10 min and 196 µl of sterilized water was added and vortexed (24).

Egg mass samples. Eight egg masses (EMs) of the MJON-VI population were used for molecular analyses. All the EMs were placed individually into screw-cap vials filled with 2 ml of sterile distilled water, and were left at room temperature to allow hatching of the J2 from the eggs (Fig. 1B). Vials were then vigorously shaken and J2s were left to settle at the bottom of the vials for 3 h. For every sample, 1 ml from each supernatant and pelleted sediment phase (Fig. 1B) was pipetted separately into 1.5-ml Eppendorf tubes, spun, and processed for DNA extraction as described for water samples.

Juvenile and female samples. DNA extraction was performed for 20 single J2 of the MJON-VI population collected from vials containing a single EM each (Fig. 1B). Juveniles were individually hand-picked with the aid of a sterilized needle and placed on a 5-µl drop of sterilized Milli-Q water on a glass slide where J2 were crushed with a sterilized filter paper chip (1 mm × 1 mm). Molecular analyses were additionally performed over 10 females of the MJON-VI population, three females of the MIYN-VI population, and 40 from each of the five nematode populations from Spain. For this purpose, females were dissected from infected roots of resistant tomato plants (cv. Momotaro and cv. Monika for Japanese and Spanish populations, respectively) with forceps under a stereo microscope. All females were stored at -80°C until DNA was extracted; as for J2, females were also squashed prior to DNA extraction. Paper chips with either the crushed J2 or the females were introduced individually into a 0.5-ml Eppendorf tube; DNA extraction and incubation were as indicated previously. DNA extraction from the females from the Spanish populations was performed according to Cenis (7).

PCR analyses. PCR conditions were those described by Xu et al. (33) at a final volume of 40 µl, including 20 µl of DNA template (0.001% SDS final concentration), 10× PCR buffer, and 0.5 units of *Ex Taq* DNA polymerase (Takara Biomedicals, Otsu, Japan); amplification was held for 40 cycles. Molecular analyses included the co-dominant marker MVC-F3/R2 that amplifies a fragment of ≈873 bp in all types of populations (*Mi*-avirulent, selected *Mi*-virulent and naturally *Mi*-virulent) and the dominant marker MVC-VF/R1 that exclusively amplifies a ≈1,032-bp band in the genome of the selected *Mi*-virulent populations (33). The

MVC-F3/R2 amplified products were digested overnight with the *NdeI* restriction enzyme (Takara Biomedicals) following the manufacturer's protocol. Nematode DNA extraction was tested by PCR amplification of the ITS region (10). For all reactions, 5 µl of the PCR products was resolved on 1.5% agarose gels in 0.5× TBE buffer. Electrophoresis was at 90V for 60 min. All PCR reactions were repeated twice.

DNA sequencing. PCR products obtained with the MVC-VF/R1 marker were cut from the gel and purified with a MinElute Gel Extraction Kit (Qiagen). DNA labeling was performed using BigDye Terminator version 1.1 (Applied Biosystems) followed by purification with Dye Ex 2.0 Spin Kit (Qiagen). Sequencing was performed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). DNA sequences were manually corrected using Bioedit version 7.0.9 program. The resulting sequences were then compared with the sequences available from public databases through a nucleotide BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

RESULTS

Amplification with the MVC molecular markers in the absence of nematodes. The expected DNA fragments were consistently amplified with the co-dominant MVC-F3/R2 and the dominant MVC-VF/R1 markers in all the NF and 5F water samples analyzed but not in the 0.2F samples: that is, in samples without nematodes (Fig. 2A and B) but presumably containing microorganisms that were retained on the 0.2 µm cellulose filter. DNA amplification for both markers also occurred in the supernatant phase obtained from the aqueous suspensions of individual EMs (Fig. 3A, lanes 1 to 8; Fig. 3B, lanes 1, 2, 4, and 5). Samples from the sedimented pellet phase that contained J2 and the remains of the gelatinous matrix (GM) from the EMs (Fig. 3) showed similar results.

The MVC-VF/R1 PCR-products obtained from the supernatant phase of the EM₄-water suspension (MVC/S-EM₄) (Fig. 3B, lane 2) were sequenced. A BLAST search on the public databases revealed that the 1,032-bp amplified fragment presented a high homology to the seven original MVC-allelic sequences (GenBank accession no. AB047761 to AB047767), but also to a ribosome small subunit-dependent GTPase A (*RsgA*) and a pterin-4- α carbinolamine dehydratase (PCD) from the betaproteobacteria species *Verminephrobacter eiseniae* EF01-2 (score = 951, *E* value = 0), *Acidovorax avenae* subsp. *citrulli* AAC00-1 (score = 771, *E* value = 0) and *Variovorax paradoxus* S110 (score = 574, *E* value = 3e-160); to a Ste24 endopeptidase (Ste24) and a transcriptional coactivator/pterin dehydratase from the species *Acidovorax ebreus* TPSY (score = 767, *E* value = 0) and *Delftia acidovorans* SPH-1 (score = 618, *E* value = 3e-173); and to a ribosome associated GTPase EngC and a PCD from the species *Rhodoferrax ferrireducens* T118 (score = 479, *E* value = 2e-131) and the genus *Polaromonas* sp. JS666 (score = 446; *E* value = 1e-121). In addition, the MVC/S-EM₄ sequence revealed a mutation in the target site of the restriction enzyme *NdeI* which could explain why no restriction fragments were obtained after digestion of the co-dominant PCR products from single J2 of the selected *Mi*-virulent population MJON-VI.

Absence of amplification of the MVC molecular markers in DNA from J2 and females. The co-dominant MVC-F3/R2 marker did not amplify any DNA fragment in any of the J2 individually analyzed from the MJON-VI population, or in a pool of DNA composed of DNA extractions from 10 single J2 from two independent EMs (10 J2/EM) of the same population, or in the two adult fertile females of the MIYN-VI population (Fig. 4). Similarly, the co-dominant MVC-F3/R2 marker did not amplify any DNA fragment when using the three field-selected *Mi*-virulent populations PG-76-P3, Brigeor-P3, and Monika-P3, the naturally *Mi*-virulent population (MJ-27), and the *Mi*-avirulent population Durinta-P3 as DNA template (Fig. 5A and B). For all

Fig. 2. *Meloidogyne* virulence locus (MVC) marker profile of draining-water samples collected from a potted plant infected by a laboratory selected *Mi*-virulent population of *Meloidogyne incognita* (MIYN-VI). **A**, Co-dominant molecular marker MVC-F3/R2. **B**, Dominant molecular marker MVC-VF/R1. Lanes: (1 to 3) 0.2F water, (4 to 6) 5F water, (7 to 9) NF water, (10) Supernatant of egg mass no. 3 (EM₃), and (11) Milli-Q sterilized water. Lane M indicates 100-bp DNA ladder.

nematode samples, amplification of the ITS region was successfully performed in order to check DNA quality (data not shown).

DISCUSSION

Recent advances in the sequencing of the complete genome of *M. incognita* and *M. hapla* and of several betaproteobacteria species from the family *Comamonaceae*, together with the experimental evidence presented in this paper indicates that the MVC locus is not related to a root-knot nematode *Avr* gene. The SCAR-MVC molecular marker repeatedly failed to amplify DNA from J2 and females from *Mi*-avirulent, laboratory and field selected *Mi*-virulent and naturally *Mi*-virulent populations of *M. incognita* and *M. javanica*, including two of the Japanese populations originally used to develop this marker (33). Conversely, the genomic DNA amplified by the primer pair MVC-VF/R1 was closer to a prokaryotic than to a eukaryotic genome. Amplification of DNA from a microorganism(s) and not from the nematode itself, would explain why the results reported by Xu et al. (33) have been considered as nonreproducible for non-Asiatic isolates of *Meloidogyne* spp. (3). In agreement with this hypothesis, the results presented in this paper could also explain why the MVC molecular markers only amplified DNA from minipreps of EMs, but not from single J2 in the original paper (33). Possibly, no amplification occurred in J2 as microorganism(s) adhered to the cuticle surface would have been washed away while hand picking them from the sterile water suspension; in a similar way, adult females might have been protected inside the root tissue from attachment of the microorganism(s). As Xu et al. (33) did not find any significant correlations at the nucleic acid level for the MVC-allelic sequences in the public databases available in 2001, they concluded that the MVC locus was intimately related to the acquired *Mi*-virulence in *Meloidogyne* spp. The recent sequencing of the genomes of *Acidovorax avenae* subsp. *citrulli* AAC00-1, *A. ebreus* TPSY, *Delftia acidovorans* SPH-1, *Polaromonas* sp. JS666, *Rhodoferrax ferrireducens* T118, *Variovorax paradoxus* S110, and *Verminephrobacter eiseniae* EF01-2 (DOE Joint Genome Institute at <http://www.jgi.doe.gov/genome-projects/pages/projects.jsf>; 6,25) allowed us to detect the homology between the MVC-amplified sequences and those from diverse proteobacterial enzymes.

The amplification of several genomic bands with the co-dominant molecular marker in the aqueous suspensions of the

EMs suggests that the microorganism(s) were highly polymorphic or that different species of microorganisms were amplified. Further studies will be necessary to determine the microbiologic origin of the genomic DNA amplified by the MVC molecular marker in the selected *Mi*-virulent populations from Japan. Regardless of the origin or the identity of the microorganism(s) detected, they must have been very abundant as DNA amplification occurred readily in all the aqueous suspensions samples from the EMs and the drained-water samples from the potted plant.

It is well known that bacteria play an important role in the protection of nematode eggs against microorganisms (18). Up to 70 bacterial isolates have been reported in association with the GM of the EMs of *M. hapla* from infected tomato roots, with *Acidovorax* being the second most abundant genus (17). Although in silico analyses established the highest similarity with *V. eiseniae* EFO1-2, an endosymbiont of the earthworm *Eisenia foetida* (21), it is unlikely that the MVC-amplified genomic DNA corresponded to an endosymbiont bacterium as no amplification occurred in individual J2. Homology was also found to the strains *A. avenae* subsp. *citrulli* AAC00-1, *A. ebreus* TYP5, and *D. acidovorans* SPH-1. These bacterial genera are frequently present in waste water treatment plants, aquatic environments, soil, sludge, or siderurgic-polluted environments (11), and some species can be found as a group of plant pathogens (16,21). The enzymes showing the highest homology with the sequence amplified from the aqueous suspension of an EM present a zinc-

Fig. 4. *Meloidogyne* virulence locus (MVC)-F3/R2 marker profile of the *Mi*-virulent laboratory selected population of *Meloidogyne javanica* (MJON-VI) and field selected population *M. incognita* (MIYN-VI) from Japan. Lanes: (1 to 10) individual juveniles (J2) from one egg mass (EM₁) of the MJON-VI population, (11) pool of DNA from 10 J2 of EM₁, (12) pool of DNA from 10 J2 of EM₂, (13 to 14) individual females of the MIYN-VI population, (15) Mili-Q sterilized water, and (16) supernatant of EM₃. Lane M indicates 100-bp DNA ladder.

Fig. 3. *Meloidogyne* virulence locus (MVC) marker profile of egg masses (EMs) of a laboratory selected *Mi*-virulent population of *Meloidogyne javanica* (MJON-VI). **A**, Co-dominant molecular marker MVC-F3/R2. **B**, Dominant molecular marker MVC-VF/R1. (S) Indicates samples from supernatant phase obtained from the aqueous suspension of an EM, and (P) samples obtained from the sedimented pellet containing J2 and remains of the EM. Lanes: (1 and 9) EM₃, (2 and 10) EM₄, (3 and 11) EM₅, (4 and 12) EM₆, (5 and 13) EM₇, (6 and 14) EM₈, (7 and 15) EM₉, (8 and 16) EM₁₀, and (17) Mili-Q sterilized water. Lane M indicates 100-bp DNA ladder.

binding motif and have been described in several bacteria and plants.

Genetic differences were found between the MVC/S-EM4 and the MJON-VI sequences. Despite the MVC/S-EM4 sequence having been obtained from a MJON-VI infected plant, this sequence was genetically closer to betaproteobacteria proteins than to the original MJON-VI sequence (accession no. AB047762). The fact that *NdeI* was able to digest the genomic sequences of the MJON-VI populations in Xu et al. (33) experiments but not in the ones described here, can be explained by a mutation in the target sequence of the restriction enzyme. The maintenance and replication of this population originating from a single EM for the last 10 years may have changed the bacterial community associated to the population MJON-VI or selected a mutated bacterial strain through several generations.

Therefore, the MVC molecular marker could not be used to characterize at the molecular level selected *Mi*-virulence in populations of *M. javanica* acquired after repeated exposure to the *Mi* gene in the field (28). Many efforts have been made during the past years to characterize *Mi*-virulent populations through molecular approaches (1,26). The protein *MAP-1*, the first candidate coding for a nematode *Avr* gene (27), was found in *Mi*-avirulent populations of *M. arenaria*, *M. incognita*, and *M. javanica* subjected to the *Mi*-selection pressure although not in *M. hapla* or *M. fallax*. Nevertheless, no functional evidence correlating the *Avr* candidate protein *MAP-1* to virulence or avirulence has been established, as this protein did not reveal genetic polymorphisms between two closely related *Mi*-virulent and *Mi*-avirulent isolates of *M. javanica* (12). Recently, the new candidate *Cg-1* gene has been related to avirulence in *M. javanica*, as *Cg-1* is required in the nematode for the expression of the *Mi*-mediated resistance response in the plant (13). The *Cg-1* marker has also been detected in the three most important species of *Meloidogyne* affected by the *Mi*-mediated resistance, suggesting that this gene was probably present in a common nematode ancestor (12,13). However, the *Cg-1* has been amplified from some *Mi*-virulent isolates of *M. javanica* and the copies of *Cg-1* in *M. incognita* are different in sequence from those in *M. javanica* (V. M. Williamson, personal communication), suggesting that *Cg-1* does not explain all virulence to *Mi*. Hence a universal molecular marker that can distinguish root-knot nematode strains that are *Mi*-virulent from those that are *Mi*-

avirulent has not been obtained yet. Under this perspective, virulence will still need to be determined by pathogenicity assays until new molecular markers are generated.

ACKNOWLEDGMENTS

L. Cortada acknowledges the Spanish Instituto Nacional de Investigaciones Agrarias (INIA) for support through a pre-doctoral grant. Thanks are given to I. Iwahori and T. Narabu for providing the nematode populations, P. Manzano, F. J. Sorribas, and C. Ornat for critical reading of the manuscript, and C. Lorente for suggestions.

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Fig. 5. *Meloidogyne* virulence locus (MVC) marker profile of the field selected *Mi*-virulent populations of *M. javanica* (PG-76-R3, Brigeor-P3, and Monika-P3), the naturally *Mi*-virulent *M. javanica* population (MJ-27), and the *Mi*-avirulent *M. javanica* (Durinta-P3), all from north-eastern Spain; and the laboratory selected *Mi*-virulent *M. javanica* population (MJON-VI) from Japan. A, Co-dominant molecular marker MVC-F3/R2. B, Dominant molecular marker MVC-VF/R1. Lanes: (1) PG-76-R3, (2) Brigeor-P3, (3) Monika-P3, (4) MJ-27, (5) Durinta-P3, (6) pellet of egg mass EM₄ (MJON-VI), and (7) Mili-Q sterilized water. Lane M indicates 100-bp DNA ladder.

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