



Irreversible thelytokous reproduction in *Muscidifurax uniraptor*

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Abstract

Vertically transmitted bacteria of the genus *Wolbachia* are obligatory endosymbionts known to cause thelytokous (asexual) reproduction in many species of parasitic Hymenoptera. In these species production of males can be induced, but attempts to establish sexual lines have failed in all but one genus. We have found three reproductive barriers between antibiotic-induced males and conspecific females of *Muscidifurax uniraptor* Kogan and Legner (Hymenoptera: Pteromalidae): males do not produce mature sperm, females are reluctant to mate, and a major muscle is absent from the spermatheca. These findings suggest that *Wolbachia*-induced thelytokous reproduction in *M. uniraptor* is irreversible, and are consistent with the idea that since sexual reproduction has ceased, selection on sexual traits has been removed leading to the disappearance or reduction in these traits. Because under these circumstances asexual reproduction is irreversible, the host has become totally dependent on the symbiont for reproduction.

Introduction

Agents such as cytoplasmic microorganisms, that are typically inherited through the maternal lineage, encounter biological disaster when they find themselves passed to a male zygote: they then leave no descendants beyond the lifetime of their male host. Hence, a cytoplasmic gene which can induce its female host to parthenogenetically produce exclusively female progeny can produce a clonal asexual population of hosts, all of which are capable of vertically transmitting the cytoplasmic gene to the next generation (Hamilton, 1967; Cosmides & Tooby, 1981).

Wolbachia is an intracellular bacterium belonging to the alpha sub-division of the purple bacteria (O'Neill et al., 1992). This symbiont is known to affect sex ratio and cause reproductive disorders in many insects and other arthropods. The bacteria are located mainly in the reproductive tissues of their hosts and are transferred from the female to her offspring through the egg cytoplasm. *Wolbachia* have been found in

more than 16% of all the insects surveyed (For review, see Stouthamer et al., 1999). Four major phenomena are known to be associated with the presence of *Wolbachia*: (1) Cytoplasmic incompatibility, which occurs between infected and uninfected strains, or between strains harboring different infection types, and result in reduction of uninfected offspring (Wade & Stevens, 1985; Hoffman et al., 1986; O'Neill & Karr, 1990; Breeuwer & Werren, 1993). (2) Feminization, the diversion of genetic males into phenotypic females, has been described in several Isopod species (Rigaud et al., 1991; Juchault et al., 1994). (3) Male-Killing, early death of male hosts during embryogenesis (Hurst et al., 1999). (4) Parthenogenesis, thelytokous reproduction in which females produce female offspring without fertilization by males, has been found in species of parasitic Hymenoptera (see Stouthamer, 1997, for review). The evolutionary advantage of each of these phenomena is obviously acceleration of transmission. By causing thelytokous parthenogene-

sis, *Wolbachia* ensures 100% vertical transmission to its host's offspring.

When *Wolbachia* induce thelytokous reproduction, the production of males can be induced by antibiotic treatments. However, *Trichogramma* is the only reported genus in which a sexual line could be established from an originally asexual one (Stouthamer et al., 1990). We have attempted to establish a sexual line of *Muscidifurax uniraptor* (Kogan and Legner (Hymenoptera: Pteromalidae), a pupal parasitoid of synanthropic filth-breeding Diptera. This species is an important natural enemy of the housefly (*Musca domestica* L.) and the stable fly (*Stomoxys calcitrans* (L.)). *Muscidifurax uniraptor* is a parthenogenetic species in which *Wolbachia* endosymbionts have been found to be the cause for thelytokous reproduction (Stouthamer et al., 1993). Repeated failures to establish a sexual line prompted this study in which we tested whether sexual reproduction was still possible in this species. Two closely related sexual species, *M. raptor* Girault and Sanders, and *M. raptorellus* Kogan and Legner were used as a control in the study. We tested the sexual functioning of antibiotic-induced males and conspecific females at three levels: (1) sperm production and viability; (2) sperm transfer; and (3) sperm storage.

Materials and methods

Insect cultures. Both the asexual species *M. uniraptor* and the sexual species *Muscidifurax raptor* were obtained from J. H. Werren, Rochester NY in 1997. *Muscidifurax uniraptor* is known only from Puerto Rico (Kogan & Legner, 1970) and was originally collected there (Legner, 1985). *Muscidifurax raptor* was used for the comparison of reproductive tract. The sexual species *M. raptorellus* was obtained from C. Geden, Gainesville FL. This species was used in the copulation experiment, because it is considered to be the most closely related species to *M. uniraptor* as determined by their identical mitochondrial DNA genes (Taylor et al., 1997). All *Muscidifurax* species were reared in the laboratory on *Musca domestica* pupae in a rearing chamber under standard conditions of 25 ± 2 °C, L16:D8, and 50% r.h.

Antibiotic treatments. *Muscidifurax uniraptor* females (0–24 h old) were starved overnight and then were allowed to feed on 100 mg ml^{-1} rifampicin (Sigma) in honey, as described by Zchori-Fein et al.

(1995). Following 24 h feeding, the wasps were supplied with hosts for oviposition. After three weeks, emerged F1 males were collected for the experiments.

Sperm production and viability. Sperm production was tested by dissecting the reproductive tracts of ten males from each species on a microscope slide in PBST (50 mM Na_2HPO_4 ; 50 mM NaCl; 5 mM KCl; 2 mM CaCl_2 , pH 7.8, with 0.01% Triton X-100). The slides were viewed under a light microscope. Sperm viability was tested using the LIVE/DEAD sperm viability kit (Molecular Probes) as followed: Stock dye solution of $5 \mu\text{l}$ 1:500 CYBR 14 in DMSO, $5 \mu\text{l}$ 1:10 propidium iodide in tris-diluents buffer (NaCl (1.1%), glucose (0.1%), L-arginine-HCL (0.01%), and L-lysine (0.01%) dissolved in 0.05 M tris (hydroxy methyl)-amino-methane buffer at pH 8.7 [Moritz, 1984]), and $90 \mu\text{l}$ tris-diluents buffer, was added to the dissected males on a microscope slide, testes were torn to allow solution penetration. After 2 min incubation at room temperature, the preparation was covered with a cover slip and viewed under a fluorescence microscope, where live and dead sperm appear green and red, respectively.

Copulation experiments. One virgin female and one virgin male (0–2 days old) were placed together in a small glass tube, and the pairs were observed for 4 h under a dissecting microscope with no illumination. To test whether the symbionts render the females less receptive, 0–2 days old virgin *M. uniraptor* females were fed on honey mixed with 100 mg ml^{-1} rifampicin and food coloring. After 48 h these females were tested as described above. All experiments were repeated with ten pairs.

Spermatheca ultrastructure. Spermathecae were dissected and fixed on a microscope slide as described for male reproductive tracts. A drop of 1:100 FITC labeled phalloidin (Sigma), a specific actin fluorescence dye, in PBST was applied for 1 h, the preparation was washed, mounted, and viewed under a fluorescence microscope.

Results

Sperm production and viability. Mature sperm production was found in the seminal vesicles of *M. raptor*, but could not be detected in *M. uniraptor* seminal vesicles (Figure 1). Sperm viability was studied us-

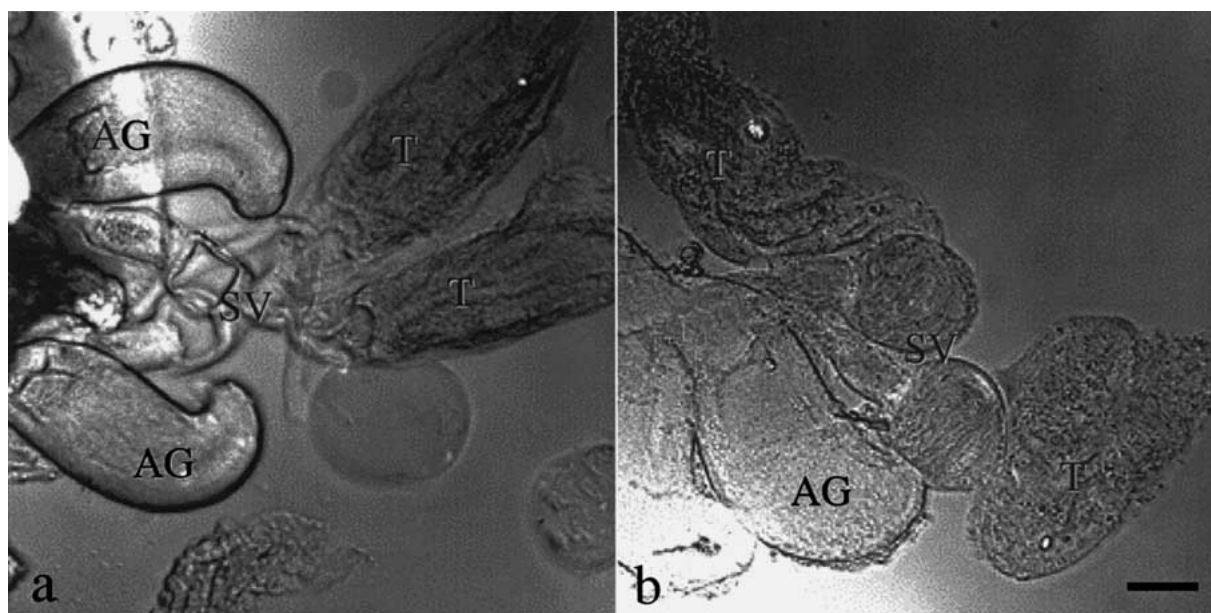


Figure 1. Male reproductive tracts of *M. uniraptor* and *M. raptor*. (a) *Muscidifurax uniraptor* male gonads with empty seminal vesicles. (b) *Muscidifurax raptor* male gonads with seminal vesicles filled with mature sperm. AG = accessory gland; SV = seminal vesicle; T = testes. Bar length = 25 μ .

ing a combination of the fluorescent dyes CYBR 14 (green) and propidium iodide (red), which differentially stain live and dead sperm, respectively. Under the fluorescent filter, live sperm were found in various developmental stages in the testes of both species, as well as in the seminal vesicles of *M. raptor*, but could not be detected in *M. uniraptor* seminal vesicles (data not shown).

Copulation experiments. When a female and a male of *M. raptorellus* were placed together in a vial, the male approached the female and mounted her. He then palpated her antennae with his own; if she was receptive she flattened her antennae (down), lowered her valvilli and allowed the male to copulate with her. This usually took less than 2 min after the confinement, after which the females were not receptive again during the 4 h observation. Van den Assem & Povel (1973) also found that *Muscidifurax* females copulated only once within 15 min of pair confinement. All courtship attempts between conspecific *M. raptorellus* sexes ended up in mating (Table 1). In contrast, in the presence of males of both species, *M. uniraptor* females showed no signs of receptivity, and were never observed to mate, whether the bacteria were eliminated or not (Table 1). This non-receptive behavior did not prevent the males from mounting the females,

tapping their antennae and making numerous mating attempts during 4 h of observation. Forty-eight hours after observation, the females were dissected in a drop of water and their spermathecae were removed. Viable sperm were found in all spermathecae dissected from *M. raptorellus* females mated to conspecific males. Sperm were not observed in spermathecae in the other crosses presented in Table 1.

The same trend was observed when *M. uniraptor* females were confined with *M. raptor* males where 45% of the males made mating attempts, but all females were non-receptive, non of the crosses ended up with mating, and no sperm was ever found in the spermathecae dissected (data not shown).

Spermatheca ultrastructure. The above results indicate that no sperm reach the spermatheca of *M. uniraptor* females. Spermatheca function in the Chalcidoidea depends on a longitudinal muscle that runs along the spermathecal duct. This muscle plays an important role both in drawing sperm into the spermathecal reservoir after copulation, and in straightening, and thereby unblocking the duct so that sperm could be released to fertilize the eggs (King, 1962; Wilkes, 1965). The spermathecae were studied using a fluorescent phalloidin dye specific for F-actin filaments. The longitudinal muscles surrounding the primary duct of

Table 1. Number of paired *Muscidifurax* wasps exhibiting sexual behavior in different crosses

Crosses (females × males)	Number of pairs (out of 10)		
	Courting	Mating attempts	Mating
<i>M. raptorellus</i> × <i>M. raptorellus</i>	10	10	10
<i>M. raptorellus</i> × <i>M. uniraptor</i>	9	9	8
<i>M. uniraptor</i> × <i>M. raptorellus</i>	7	6	0
<i>M. uniraptor</i> × <i>M. uniraptor</i>	10	3	0
<i>M. uniraptor</i> (AT) × <i>M. raptorellus</i>	4	2	0
<i>M. raptorellus</i> (AT) × <i>M. uniraptor</i>	10	10	10
<i>M. uniraptor</i> (AT) × <i>M. uniraptor</i>	10	3	0
<i>M. raptorellus</i> (AT) × <i>M. raptorellus</i>	10	10	10

AT – antibiotic treated.

the spermatheca were visible in *M. raptor*, but not in *M. uniraptor* (Figures 2b, c; 2e, f).

Discussion

In recent years a wide-array of maternally transmitted elements that alter their hosts' reproduction to their own advantage has been described (Werren et al., 1988; Werren & O'Neill, 1997). The maternally inherited bacteria *Wolbachia* are a striking example of selfish cytoplasmic elements, and as such, they represent an excellent model to test possible conflicts of interests between nuclear and cytoplasmic genes. The results of our study, combined with those of others (Table 2), indicate how *Wolbachia*-induced thelytoky promotes irreversible thelytoky and prevent the nuclear-cytoplasm genomes conflict. In a landmark study, Stouthamer et al. (1990) treated four species of *Trichogramma* with antibiotics, and induced the production of males. They then established isofemale lines by allowing mating between the artificially-induced males and antibiotic-treated females, and followed the mode of reproduction of some of these lines for up to 23 generations. Using this method they were able to permanently restore the mode of reproduction from thelytoky to arrhenotoky. The second parasitic wasp in which the sexual functioning of antibiotic-induced males was extensively studied was *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae). In this asexual species, antibiotic-induced males produce sperm and sometimes mate with conspecific females, but insemination does not occur (Zchori-Fein et al., 1992). In another aphelinid, *Aphytis lingnanensis* Compere antibiotic-induced males of the asexual line were found to carry sperm, and observation of

the spermathecae showed that successful insemination occurred. However, mated daughters of antibiotic-treated females failed to produce female offspring (Zchori-Fein et al., 1995). The question of whether the females fail to use that sperm (pre-zygotic barrier), or whether a post-zygotic barrier is involved, remains unanswered. It is important to note that when the induced males of *A. lingnanensis* asexual line mated with females of the sexual line, no sperm were found in the dissected spermathecae (Argov et al., 1995). In contrast, antibiotic-induced males of the asexual line of *Apoanagyrus diversicornis* (Howard) (Hymenoptera: Encyrtidae) mated readily with females from the sexual line, which consequently produced fertile female offspring, but asexual line females were reluctant to mate with males of either line (Pijls et al., 1996). Thus, the sexual functioning of both antibiotic-induced males and conspecific females is characterized by phenomena ranging from production of undeveloped sperm, to the successful production of daughters in crosses between males from asexual lines and females from closely related sexual lines.

In *Wolbachia* infected *Trichogramma* spp., sperm can successfully fertilize an egg (Stouthamer & Kazmer, 1994). However, all other *Wolbachia* infected species that have been studied, show different levels of sexual functionality disorder (Table 2), suggesting differences in time since infection. For example, recent infection can be suggested in *Aphytis lingnanensis* where induced males of an asexual line appear to function normally, but asexual females display greatly reduced sperm storage efficiency (Zchori-Fein et al., 1995). Ancient infection can be suggested in *Encarsia formosa*, where induced males have no mature sperm (Hunter, 1999), and females are not receptive (Zchori-

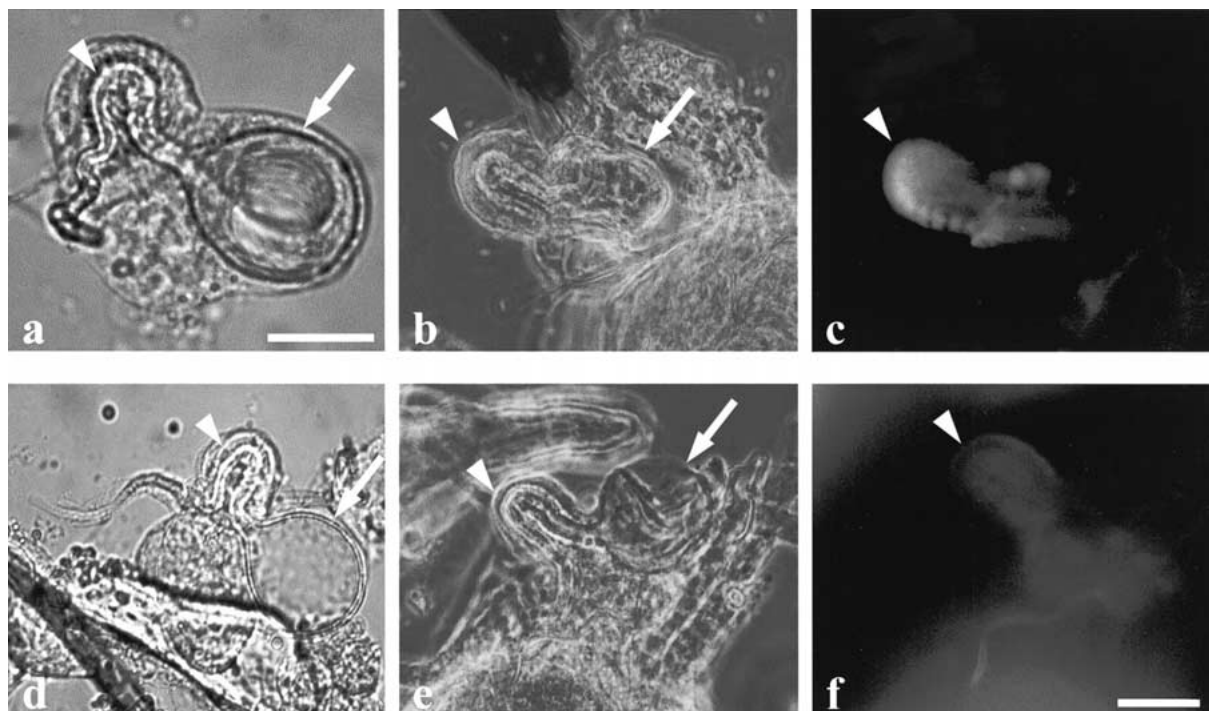


Figure 2. The spermathecae of mated *M. uniraptor* and *M. raptor*. (a) *Muscidifurax raptor* spermatheca under light phase microscope. The spermatheca reservoir is filled with sperm; (b and c) *Muscidifurax raptor* spermatheca with F-actin specific fluorescent phalloidin dye. (b) under light phase microscope, (c) under fluorescence filter. Note the longitudinal muscle around the primary duct. (d) *M. uniraptor* spermatheca under light phase microscope. The spermatheca reservoir is clear and empty; (e and f) *M. uniraptor* spermatheca with F-actin specific fluorescent phalloidin dye. (e) under light phase microscope, (f) under fluorescence filter. No muscles appear around the primary duct. Arrow- Spermatheca reservoir, Arrow head-spermathecal duct. Bar length = 50 μ .

Table 2. Sexual function of various parasitic wasps in which thelytoky is induced by *Wolbachia*

	Mating behaviour	Sperm production	Sperm storage	Fertilization	Males sire offspring with closely related lines
<i>Trichogramma</i> spp. (Stouthamer, 1990)	Males court Females receptive	yes	yes	yes	yes
<i>Aphytis lingnanensis</i> (Zchori-Fein et al., 1995)	Males court Females receptive	yes	yes (54%)	no	no
<i>Apoanagyrus diversicornis</i> (Pijls et al., 1996)	Males court Females non-receptive	yes	no	no	yes
<i>Telenomus nawai</i> (Ashmead) (Arakaki et al., 2000)	Males court Females receptive	yes	?	no	yes
<i>Muscidifurax uniraptor</i> (This study)	Male court Females non-receptive	no	no	no	no
<i>Encarsia formosa</i> (Zchori-Fein et al., 1992; Hunter, 1999)	Males court Females ?	no	no	no	?
<i>Aphytis diaspidis</i> Howard (Y. Gottlieb, unpubl.)	Males court Females receptive	?	?	no	?
<i>Diplolepis rosae</i> (Stille & Dävring, 1980)	Males rarely court Females ?	?	?	no	?

? incomplete data.

Fein et al., 1992). Comparing polymorphism data, which are currently available only for *Trichogramma* spp. (Stouthamer & Kazmar, 1994) would be one way to test these predictions.

Here we found that at least three obstacles exist to establish a sexual line of *M. uniraptor*, which may indicate ancient infection: (1) Males do not produce mature sperm. The failure of sperm produced in testes to reach the seminal vesicles may be due to a block between the testes and the seminal vesicles, a sperm motility problem or any number of other physical or physiological defects. (2) Females are not sexually receptive, and (3) an absence of a major muscle in *M. uniraptor* spermathecae suggests that this organ is vestigial, and has lost the ability to convey and store sperm efficiently. Each of these factors is, in itself, sufficient to form an irreversible reproductive barrier between the sexes.

Muller (1949) predicted that a random mutation will tend to destroy the genetic basis of a character from which selection has been removed. It is therefore expected that once selection favoring a trait or suit of traits has been relaxed, the probability of losing these traits through mutation is greatly increased. Supporting this prediction, Yokoyama & Yokoyama (1990) found that there is a high rate of nonsynonymous substitution in blind cave fish visual pigment gene, and Hoch & Howarth (1989) showed intermediate stages between surface and cave morphologies in *Solonamia* (Homoptera: Fulgoroidea) species. Genes responsible for characters involved in sexual reproduction are expected to accumulate mutations that make them non-functional in the absence of selection for maintenance of function. However, in the presence of *Wolbachia*, asexual reproduction is believed to be initially facultative. With the relaxation of selection favoring sexual traits, eventually asexual reproduction becomes obligatory. The loss of sexual traits observed in *M. uniraptor* is consistent with these theoretical predictions. Furthermore, *Wolbachia* was shown to be the cause of relaxed selection in host sexual behaviour and developmental requirements of *Wolbachia* infected autoparasitoids (Hunter, 1999). The mechanism involved in diploidy restoration in *Wolbachia* induced thelytokous species is gamete duplication (*Diplolepis rosae* (L.), Stille & Dävring, 1980; *Trichogramma* spp., Stouthamer & Kazmer, 1994; *M. uniraptor*, Y. Gottlieb, unpubl.), which results in complete homozygosity at all loci. Such a mechanism allows for the immediate expression of recessive mutations, and

hence potentially accelerates the loss of sexual traits and the ability to reproduce sexually.

Although our results show that elimination of the bacteria does not reinstate spermathecal function nor does it change the receptivity of females, the possibility that the reproductive barriers are somehow induced by the actual presence of *Wolbachia* in the developing embryo cannot be ruled out. Because we were unable to establish a sexual line of *M. uniraptor*, we could not directly test whether non-infected females have normal sexual behavior and functioning spermathecae. However, evidence exists to suggest that this is not the case. Firstly, reproductive barriers are not the same in different *Wolbachia*-induced thelytokous Hymenoptera species (Table 2), and it appears unlikely that *Wolbachia* induce different reproductive defects in each of its hosts. Secondly, in *Trichogramma*, *Wolbachia*-induced asexual reproduction is reversible (Stouthamer et al., 1990).

Vertically transmitted symbionts were recently described as 'reproductive parasites' which manipulate the reproduction of hosts in ways that enhance transmission of the microorganism, even at the expense of the host's reproductive success (Werren & O'Neill, 1997). Under such a scenario, the host is expected to develop defense mechanisms against its parasite. In the *Wolbachia*-*M. uniraptor* system, we expect a causally linked series of evolutionary significant consequences: the introduction of thelytoky by gamete duplication fosters the rapid accumulation of mutations in genes that are not under selection. Since in thelytoky the selection on genes functioning exclusively in sexual reproduction is relaxed, and mutations influencing sex-related traits are no longer deleterious, reproductive barriers frequently accumulate. Once the host becomes *dependent* on the symbiont for reproduction, the conflict between cytoplasmic and nuclear genes no longer exists, and the two genomes are expected to 'cooperate' for their mutual benefit.

Mutualistic endosymbiosis such as intracellular bacteria in sap-sucking insects or nitrogen fixing bacteria in plants, was described as 'slavery', where the host captures bacteria for its own benefit (Douglas & Smith, 1989). We are suggesting here that in thelytoky-inducing symbionts, the host is being captured by the bacteria for its own transmission benefit, leaving no other way of reproduction for its host.

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