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Antifungal activity of *Stenotrophomonas maltophilia* in *Stomoxys calcitrans* larvae

Atividade antifúngica de *Stenotrophomonas maltophilia* em larvas de *Stomoxys calcitrans*

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Abstract

The microbiota present in *Stomoxys calcitrans* larvae may assist their survival in contaminated environments through production of inhibitory substances. Bacteriological identification methods, the polymerase chain reaction (PCR) and scanning electron microscopy (SEM) were used to detect a bacterium naturally present in mucus and macerated *S. calcitrans* larvae. The antifungal activity was determined based on the results from disk diffusion tests on an artificial solid medium. The bacterium was identified as *Stenotrophomonas maltophilia* and presented antifungal activity against *Beauveria bassiana sensu lato* isolates CG 138, CG 228 and ESALQ 986. This result suggests that the larval microbiota is a factor that can compromise the use of *B. bassiana s.l.* fungus for biological control of *S. calcitrans* larvae.

Keywords: *Stenotrophomonas maltophilia*, *Stomoxys calcitrans*, antifungal activity.

Resumo

A microbiota presente em larvas de *Stomoxys calcitrans* pode auxiliar na sua sobrevivência em ambientes contaminados, devido à produção de substâncias inibidoras. Métodos bacteriológicos de identificação, reação em cadeia da polimerase (PCR) e microscopia eletrônica de varredura (MEV) foram utilizados para detectar uma bactéria naturalmente presente no muco e macerado de larvas de *S. calcitrans*. A atividade antifúngica foi baseada nos resultados obtidos no teste de difusão em meio sólido artificial. A bactéria foi identificada como *Stenotrophomonas maltophilia* e apresentou atividade antifúngica contra os isolados CG 138, CG 228 e ESALQ 986 de *Beauveria bassiana sensu lato*. Estes resultados sugerem que a microbiota larval é um fator que pode comprometer o uso de *B. bassiana s.l.* no controle biológico de larvas de *S. calcitrans*.

Palavras-chave: *Stenotrophomonas maltophilia*, *Stomoxys calcitrans,* atividade antifúngica.

Introduction

Stomoxys calcitrans (Linnaeus, 1758) (Diptera: Muscidae), known as the stable fly, parasitizes different animal species for its blood meal. This parasitism supports transmission of pathogenic microorganisms that cause losses in livestock. The fly's immature stages can be found in decomposing plant material (cane sugar, coffee straw or grass), in association with urine, feces and uneaten animal feed (BITTENCOURT; CASTRO, 2004; BITTENCOURT, 2012). Accumulation of sugarcane by-products contributes towards stable fly outbreaks and consequently towards population imbalance (LEITE et al., 2013). Oda and Arantes (2010) conducted a study with the aim of identifying the causes of high *S. calcitrans* infestation in the municipality of Planalto, SP, Brazil. They observed that larvae were found in the cane fields with the vinasse, and that inadequate vinasse management in the sugar cane processing plant and poor management of organic matter by farmers favored expansion of the *S. calcitrans* population.

In an ecological context, microorganisms should be considered to be important components of insect biology (DILLON; DILLON, 2004). According to Romero et al. (2006), *S. calcitrans* larvae are dependent on the presence of natural symbiotic microbiota. Lysyk et al. (1999) evaluated *S. calcitrans* larval survival in rearing

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medium supplemented with pure or mixed bacteria cultures. They reported that addition of *Acinetobacter* sp., *Empedobacter breve*, *Flavobacterium odoratum* or *Escherichia coli* favored larval development. In another study, microbiota with pathogenic potential for humans and animals and bacteria used for biological control of arthropods were isolated from adult flies; 33 bacterial species from the families Bacillaceae, Enterobacteriaceae and Micrococcaceae were identified (CASTRO et al., 2008).

The bacterium *Stenotrophomonas maltophilia*, formerly classified as *Pseudomonas* sp. and *Xanthomonas* sp. (PALLERONI; BRADBURY, 1993; OLIVEIRA-GARCIA et al., 2002), can be found in a variety of geographical regions, environments and decomposing organic materials (rivers, wells, reservoir lakes, soil, raw milk, frozen fish, eggs, animal carcasses, hospital equipment and environment, semen, frozen bovine embryos, human anatomical sites and urine) (KONEMMAN et al., 2001; ALMEIDA et al., 2005). It has also been reported in goats and crocodiles (HARRIS; ROGERS, 2001) and in fish like *Seriola quinqueradiata* (FURUSHITA et al., 2003) and *Ictalunes punctatus* (DU et al., 2011). Depending on the strain isolated, it may have pathogenic activity against both humans and animals (CHANSANG et al., 2010).

Beauveria bassiana sensu lato (*s.l.*) (Balsamo) Vuillemin (REHNER; BUCKLEY, 2005; MEYLING et al., 2012) is an entomopathogenic anamorphic fungus found in soil samples and in a variety of insects (MEYLING; EILENBERG, 2007). Among 171 biopesticides surveyed by Faria and Wraight (2007), *B. bassiana s.l.* represented 33.9% of the fungi most commonly used as the basis for mycoinsecticides and mycoacaricides.

This study aimed to identify and report for the first time the *S. maltophilia* bacteria present in mucus and macerate of *S. calcitrans* larvae, using bacteriological methods, the polymerase chain reaction (PCR) technique and scanning electron microscopy (SEM), and to evaluate the bacterial antifungal activity by means of the disk diffusion test.

Material and Methods

Stomoxys calcitrans colony

Adult flies were maintained in plastic cages supplied with citrated bovine blood (0.38%). Immature stages were maintained using an autoclaved rearing medium composed of sugarcane pulp (66 g), wheat flour (25 g), sodium bicarbonate (1 g), ground meat (8 g) and distilled water (127 ml) (CHRISTMAS, 1970; MORAES, 2007).

Mucus and macerate of Stomoxys calcitrans larvae

Groups of nine 9-day-old larvae were used for collecting mucus and obtaining larval macerate. The larvae were washed in sterile distilled water and placed in sterile cryogenic tubes (1.2 ml) for mucus production (1 hour). After removing the larvae, 900 µl of cold sterile phosphate buffer solution (PBS) was added (0.1 M; NaCl 1.5 M; pH 7.4). Larval macerate was obtained by placing larvae in sterile microcentrifuge tubes (1.5 ml) with 900 µl of buffer solution (PBS) followed by maceration using a cone stick and storage in sterile cryogenic tubes (1.2 ml). The entire procedure was performed under a laminar flow hood. The samples were manipulated on ice and stored in an ultrafreezer (–80 °C).

Bacterial identification

The bacterial isolate from the mucus and macerate of *S. calcitrans* larvae was transferred to Petri dishes (9 cm) containing brain and heart infused agar (BHI) (52.0 g/l, MERCK®) and was incubated at 27 °C for 48 hours. After bacterial growth, the plates were kept under refrigeration (4 °C) (KONEMMAN et al., 2001).

Macroscopic characteristics (colony aspects) and microscopic characteristics (optical microcopy after Gram staining) were evaluated and the catalase test (KONEMMAN et al., 2001) and molecular biology techniques were applied, in order to identify the bacterium species.

Fragments of the 16S rRNA gene (~1500 bp) were amplified from genomic DNA. The purified PCR products were sequenced directly using the sequencing primers 27f (AGA GTT TGA TCC TGG CTC AG) (FURUSHITA et al., 2003); 108r (CAG ATT CCC ACG CGT TAC GC) and 420r (TTA CAA CCC TAA GGC CTT C) (LEYS et al., 2004); Amp2 (AAG GAG GTG ATC CAR CCG CA) (WANG et al., 1996); 16S1203f (GAG GTG GGG ATG ACG TCA AGT CCT C); and 16S1110r (TGC GCT CGT TGC GGG ACT TAA CC) (SOARES-RAMOS et al., 2003).

The 16S rRNA gene sequence was automatically aligned using ClustalW, and then corrected manually. The sequences were subjected to BLAST analysis (ALTSCHUL et al., 1997) with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov), and the closest relatives were included in the phylogenetic analysis. Phylogenetic analysis of 16S rRNA was performed using the neighbor-joining method and Kimura two-parameter (K2P) with the MEGA5 software package (TAMURA et al., 2011). A total of 1000 bootstrap replicates were performed.

Scanning electron microscopy

Groups of six 9-day-old *S. calcitrans* larvae were washed in sterile distilled water, fixed in super skipper solution [kerosene (17 pt), glacial acetic acid (11 pt), 95% ethanol (50 pt), isobutyl alcohol (17 pt) and dioxane (5 pt)] for 30 seconds, washed through immersion in Carl's solution [95% ethanol (28 pt), 45% formaldehyde (11 pt), glacial acetic acid (4 pt) and H_20 (57 pt)] and maintained in this same solution for 24 hours (GRODOWITZ et al., 1982). The larvae were dehydrated in a standard ethanol series (15 minutes in each of 30, 50, 70, 80, 85, 90 and 95% and three periods of 100%), dried in a CO_2 critical-point drier and coated with gold particles (10 milliampere/90 seconds) for production of electron micrographs in a JEOL JSM-JEM 1011 scanning electron microscope, with the aim of viewing the bacterial structures (GRODOWITZ et al., 1982; OLIVEIRA-GARCIA et al., 2002; DEDAVID et al., 2007).

Beauveria bassiana sensu lato

Beauveria bassiana s.l. CG 138, CG 228 and ESALQ 986 isolates were cultured in potato dextrose agar (PDA; 39 g/l; DIFCO®) and 1% yeast extract (YE; 1%; MERC®) in the dark at 25 °C and 70-80% relative humidity (RH) for 15 days (FERNANDES et al., 2009). For the isolate CG 138, the host was: (Coleoptera: Curculionidae); origin: Recife, Pernambuco, Brazil; latitude: 08°05'S; longitude: 34°55'W; year: 1989. For the isolate CG 228, the host was: (Coleoptera: Chrysomelidae); origin: Manaus, Amazonas, Brazil; latitude 03°07'S; longitude: 60°10'W; year: 1991. For the isolate ESALQ 986, the host was: (Acari: Ixodidae); origin: Piracicaba, São Paulo, Brazil; latitude: 22°43'S; longitude: 47°38'W; year: 1990 (FERNANDES et al., 2009).

To prepare the fungal suspensions, the isolates were removed from the Petri dishes (9 cm) with a scalpel, and then suspended and agitated (vortex, 2 min.) in a sterile solution (distilled water and 0.01% Tween). A Neubauer chamber was used to obtain a concentration of 1×10^8 con/ml. The viability of the conidia was assessed by inoculating the suspensions in Petri dishes containing PDA (39 g/l, DIFCO®) and YE (1%, MERC®) and counting the numbers of germinated and ungerminated conidia (ALVES, 1998; FERNANDES et al., 2009).

Disk diffusion test in artificial solid medium

Ten microliters of fungal suspension at a concentration of 1×10^8 con/ml, from the three isolates (CG 138, CG 228 and ESALQ 986) were spread evenly with a sterile swab in Petri dishes containing PDA (39 g/l) and YE (1%). Similarly to the method used by Hunt (1986) and Urbanek et al. (2012), three filter paper disks (6 mm in diameter) were autoclaved (120 °C for 20 min) and dried in a Pasteur oven (100 °C), and then were immersed in the macerate of the larvae. After the procedure, the disks were placed in dishes cultured with the fungal suspension. The same procedure was carried out with the mucus samples. These dishes (three dishes/isolate) were incubated for three days at 25 ± 1 °C and 70-80% RH. The fungal inhibition zone was verified on the second and third days after the start of the experiment. The assays were carried out twice to obtain more reliable results.

Results

Similarity analyses based on the 16S rRNA sequence (~1,450 bp), on a representative strain, revealed that these isolates belonging to the genus *Stenotrophomonas* were more closely related to the *S. maltophilia* strain K279a (accession number AM743169.1). Phylogenetic analysis based on the 16S rRNA gene of a representative isolate was compared with the most closely related bacterial strains retrieved from the database, and these confirmed the result (Figure 1).

The macroscopic characteristics of the bacterial colony were: regular borders, brightness, light brown color, smoothness and convex and mucoid appearance (Figure 2a). According to the microscopic characteristics, the bacterium was Gram-negative, with a bacillary straight or slightly curved form. The catalase test was positive. Bacillary bacteria and bacterial ultrastructures such as a long polar flagellum and microfibers were also viewed when the larval surface was observed using SEM (Figure 2b).

The disk diffusion test (Figure 2c) showed bacterial growth on all the filter paper disks. The bacteria grown on the disks had the same macro characteristics as observed in the BHI Petri dish colonies (Figure 2a). An inhibition zone was formed around the disk in the dishes cultured with the three fungal isolates (Figure 2c). Disks immersed in larval mucus or immersed in macerated larvae all presented the inhibition zone. The conidial suspensions used in the present study had 100% viability.

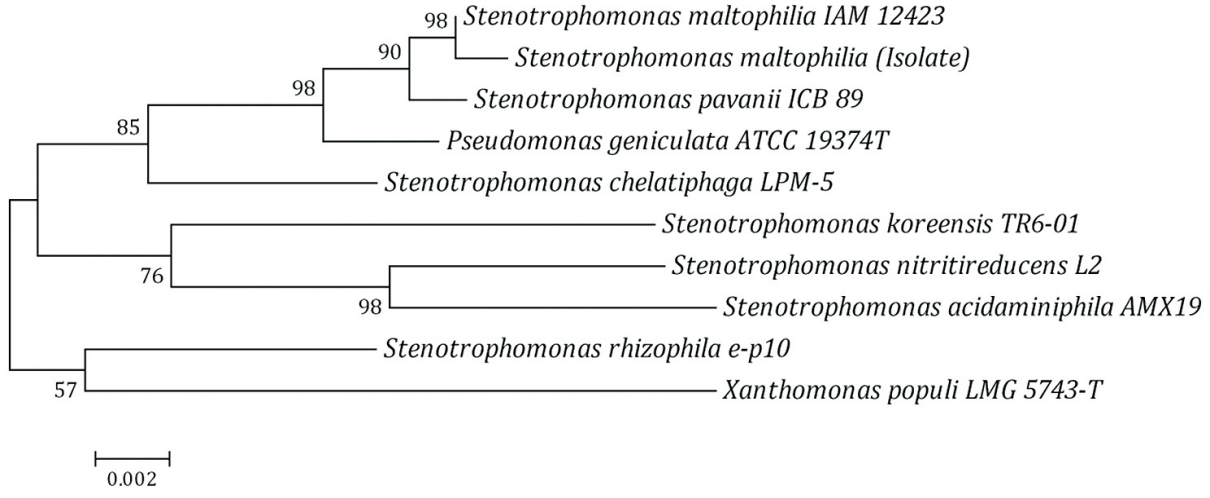

Figure 1. Neighbor-joining phylogenetic tree based on partial 16S rRNA gene sequences from the Brazilian isolate and reference strains of *Stenotrophomonas* and other related bacteria. The database accession numbers are indicated after the bacterial names. Bootstrap analyses were performed with 1000 cycles. The dendrogram was constructed using the MEGA4 program, and the clustering was carried out using the neighbor-joining method. Bootstrap values of 450% are indicated at nodes. Scale bar = 0.002 substitutions per nucleotide position.

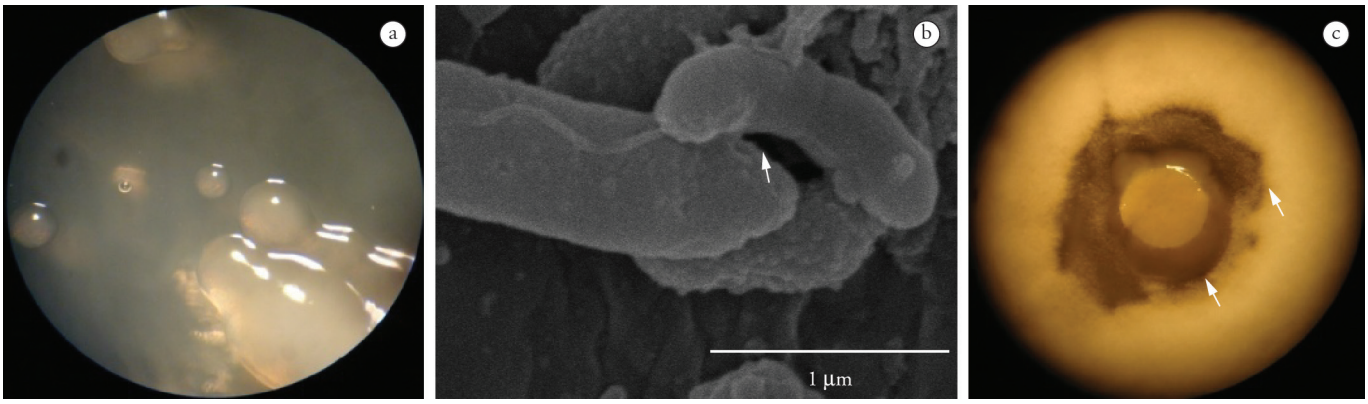

Figure 2. *Stenotrophomonas maltophilia* present in mucus and macerate and on *Stomoxys calcitrans* larvae. (a) Bacterial colony in brain and heart infused agar (BHI), showing macroscopic characteristics; (b) scanning electron microscopy (SEM) (16,000 ×) showing ultrastructural characteristics (arrow); (c) bacterial growth and inhibition of *Beauveria bassiana s.l.* growth (arrow).

Discussion

Molecular biology-based techniques provide rapid and precise bacterial identification, while traditional techniques based on morphological and physiological characteristics are inconclusive (VIDEIRA et al., 2004). For this reason, PCR was used to identify the bacterium. Furthermore, SEM revealed bacterial ultrastructures (Figure 1) similar to those described by others in identifying *S. maltophilia* (OLIVEIRA-GARCIA et al., 2002).

According to Konemman et al. (2001) and Almeida et al. (2005), *S. maltophilia* can be obtained from different sources. Oliveira et al. (2000) identified it in the microbiota from the digestive tract of female *Lutzomyia longipalpis* flies and Chansang et al. (2010) isolated the same species on the surface of *Siphunculina funicola* flies. However, there are no reports of this species as part of the *S. calcitrans* larval microbiota, and therefore the present report can be considered to be its first description in this host.

The metabolic versatility of *S. maltophilia* (RYAN et al., 2009) allows it to colonize human urine (KONEMMAN et al., 2001), canine urine (KRALOVA-KOVARIKOVA et al., 2012), crocodile kidney (septicemia) (HARRIS; ROGERS, 2001) and buffalo liver (PETRIDOU et al., 2010). However, there is no description of its presence in the urine or feces of cattle, horses or other production animals. Accordingly, the presence of *S. maltophilia* in plants, soil and water (ALMEIDA et al., 2005), and in urine and fecal materials, may be one of the reasons that makes it part of the microbiota of *S. calcitrans* larvae, since the immature stages of this fly develop in moist contaminated environments comprised of a mixture of urine, feces, soil and decaying plant (BITTENCOURT; CASTRO, 2004; BITTENCOURT, 2012).

In the literature, these microorganisms' ability to produce substances that inhibit growth and affect competing agents has been reported (DILLON; DILLON, 2004). Some metabolites synthesized by *S. maltophilia*, such as pyrrolnitrin and maltophilia, allow this bacterium to survive in polymicrobial niches (KERR, 1996; ALMEIDA et al., 2005). Kerr (1996) reported that ten *S. maltophilia* strains inhibited the growth of *Candida krusei*, *C. keyfr*, *C. guilliermondii*, *C. tropicalis*, *C. lusitaniae*, *C. parapsilosis*, *C. pseudotropicalis*, *C. albicans*, *C. glabrata* and *Aspergillus fumigatus*. However, this activity was not demonstrated in other bacteria such as *E. coli* (NCTC 10418), *Staphylococcus aureus* (NCTC 6571) and *Haemophilus influenzae* (NCTC 11931).

Ryan et al. (2009) reported that *S. maltophilia* can be used for biological control of phytopathogenic fungi such as *Fusarium graminearum* and *Verticilium dahliae*. The disk diffusion test (Figure 1c) aimed to demonstrate that the bacterium naturally obtained from the mucus and macerate of *S. calcitrans* larvae has antifungal activity against three *B. bassiana s.l.* isolates. This result suggests that the larval microbiota/microenvironment is a factor that can affect the use of *B. bassiana s.l.* fungus for biological control of *S. calcitrans* larvae.

S. maltophilia was identified in *S. calcitrans* larvae and larval mucus. This bacterium has antifungal activity against *B. bassiana s.l.* CG 138, CG 228 and ESALQ 986 isolates. Knowledge of the microbiota of *S. calcitrans* larvae is of considerable importance for integrated pest management in which entomopathogenic fungi are applied, since the substances produced by these bacteria can disrupt them. Accordingly, studies are required in order to select tolerant and effective fungal isolates for biological control of the immature stages of *S. calcitrans*.

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