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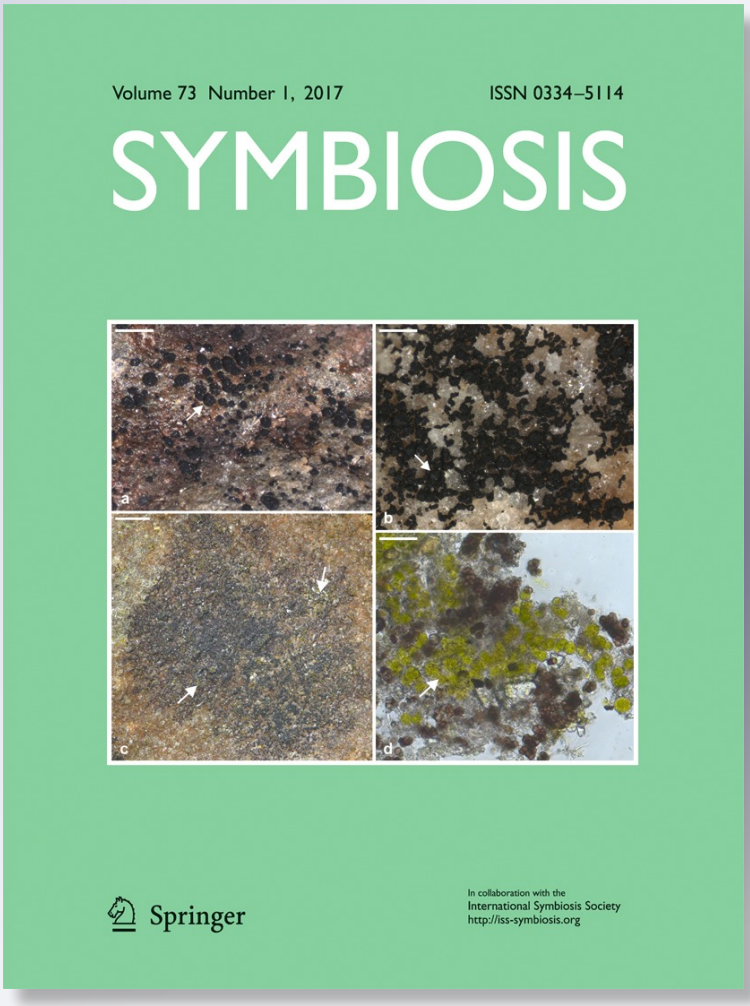

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Isolation of *Serratia marcescens* involved in chitin degradation in the bulb mite *Rhizoglyphus robini*

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Abstract There is an increasing awareness of the importance of the microbiome of arthropods to understand their host's biology. In the bulb mite, *Rhizoglyphus robini*, associated bacteria have been found to be involved in its chitinolytic abilities. The bulb mite, a plant pest feeding on below-ground parts of mostly Liliaceae crops, prefers fungus-infested plants. Moreover its fitness is higher when feeding on a fungal food source than when feeding on non-infected plants. In this study we isolated a chitinolytic bacterium from mite homogenate and identified it molecularly as *Serratia marcescens (Bizio 1823)*, which is a model organism for chitin degradation. Precise identification of the bacterium can be important for the development of biological control programs of the mite as well as for further studies investigating *Serratia marcescens* and its chitinolytic machinery.

Keywords Chitinolytic activity · Biological control · Primary pest · Secondary pest · *Serratia marcescens* · *Rhizoglyphus robini* · Mite holobiont · arthropod microbiome

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1 Introduction

Exploration of the microbiome associated with many arthropods has proven to be very important to understanding their biology (Zindel et al. 2011, 2013; Six 2013; Engel and Moran 2013; Bouchon et al. 2016). Among the many functions microbes can have in their hosts' biology, nutritional provision is of high importance (Dillon and Dillon 2004; Chen et al. 2016). Arthropods may engage in obligate or facultative interactions with microbes and rely on bacterial symbionts for the uptake of essential amino acids, vitamins, and digestive enzymes, as well as for the metabolism of carbohydrates, fatty acids and nitrogen processing (Grunwald et al. 2010; Guenduez and Douglas 2009; Snyder et al. 2010). For omnivorous arthropods, bacterial associates may be important to help them extract nutrients from variable sources (Feldhaar and Gross 2009; Sabree et al. 2009). Overall, arthropods with highly divergent life strategies, from sap or blood feeders to omnivorous species, live in close association with microorganisms.

Rhizoglyphus robini lives in the soil, and on the bulbs and tubers of its host plants (mainly ornamentals and crops such as lily, onion and garlic) and is therefore commonly referred to as the bulb mite. Field observations show that *R. robini* is almost exclusively found on plants which suffer from fungal infections (Hanuny et al. 2008). Morover, it has been shown to choose fungus-infected over uninfected hosts in preference tests (Ofek et al. 2014; Hanuny et al. 2008; Okabe and Amano 1991) and to be attracted to alcohols extracted from cultures of *Fusarium oxysporum* (Okabe and Amano 1990). In addition, *R. robini* exhibits high fecundity on fungal food sources (Ofek et al. 2014; Zindel et al. 2013; Hanuny et al. 2008; Okabe and Oconnor 2001). Even the survival of *R. robini* on wet filter paper alone was explained by the mite's ability to derive nutrients from fungal mycelia developing on the cellulosic matrix (Wooddy and Fashing 1993). In a

previous study we could already demonstrate very important aspects of the *R. robini*- holobiont (Zindel et al. 2013): We showed that the *R. robini* microbiome contains several bacterial genera known to contain chitin degraders. Also we confirmed that the mite's fecundity (number of eggs) is indeed higher on a fungal- than a non-fungal food sources. Last but maybe most importantly we demonstrated chitinolytic activity of mite homogenate through the production of a clear zone, a "halo", around a droplet of homogenate in a chitin layer. Slightly touching this clear zone with a pipette tip, growing it overnight, and re-applying it to a chitin-covered Petri-Dish resulted again in halo-formation, thus confirming bacterial origin of the chitinases (Zindel et al. 2013).

Rhizoglyphus robini is probably one of few pest species (Zindel et al. 2013) for which a thorough description of the microbiome has been achieved. However, only the precise identity of the most important associated bacteria can be used in the development or improvement of biological control programs of the mite. Here, a combined approach of selective media cultivation methods and DNA identification (sequencing of an approximately 1400 bp fragment of the 16S rRNA gene) was used to isolate and identify the bacteria responsible for the chitin degradation.

2 Materials and methods

Mite origin and rearing The mites used in this study stemmed from a field population originally collected on Lily (*Lilium candidum*) and kept in the laboratory since 2005. Mites were reared in Petri dishes containing wet filter paper (Whatman, cellulose, grade 4) and non-sterilized, shell-less, coarsely ground peanuts, and kept in a closed box in the dark. Petri dishes were stacked on a rack standing in soapy water to prevent escape of individual mites. Cultures were maintained by transferring 10 to 30 individual mites to a new Petri dish every 2 weeks approximately. Tap water and peanuts were added every 14 days.

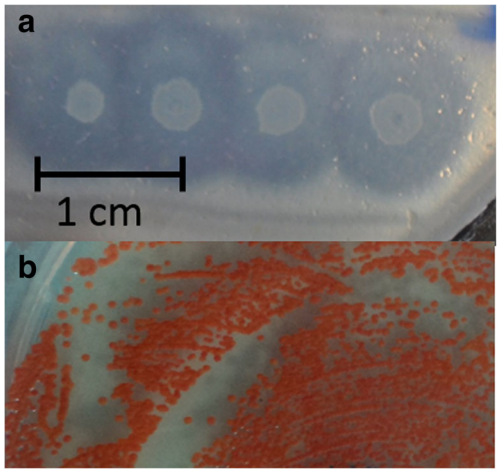

Fig. 1 Demonstration of chitin degradation (haloes) around 1 μl-droplets of mite homogenate after 12 h of incubation on chitin covered minimal medium (a) and bacterial *S. marcescens* colonies, picked from halo centers, plated on LB Agar after 12 h of incubation in liquid chitin medium (b)

Selective cultivation Crabshell chitin (Sigma) was washed as described in the literature (McBride and Braun 2004; Reichenbach and The Prokaryotes, 2006), spread in a thin layer (2 ml per plate) and allowed to dry on minimal medium containing 50 mg filter-sterilized nystatin solution per liter of medium, according to Faramarzi et al. (2009). Mites were starved and surface-sterilized by rinsing them with 95% ethanol and washing them for two minutes in a 1% bleach solution. Sterility was confirmed by rolling the mites on the surface of Lysogeny broth (LB) agar plates. The plates were inspected 48 h later for the development of bacteria or fungi. Mite homogenates were prepared by grinding approximately 100 individual adult mites in 150 μl sterile saline solution. Of the homogenate, 4 × 1 μl (per replicate) were pipetted in droplets of 0.5 μl on chitin agar medium (Fig. 1). Sterile saline solution (0.85% NaCl) served as negative control. Plates were incubated at 30 °C and inspected after 12, 24 and 36 h for the presence of haloes. Bacteria were picked from the center and edge of the haloes with a autoclaved wooden toothpick and transferred to liquid chitin medium, which was, after a night of incubation at 30 °C on a shaker, plated on

Table 1 Primers used in this study

Primers	Annealing temp (°C)	Reference
Eub9_27: 5′-GAGTTTGATCCTGGCTCAG-3‘	60–55*	Brosius et al. 1978
Eub1542: 5′-AGAAAGGAGGTGATCCAGCC-3‘	60–55*	Brosius et al. 1978
27F: 5′-AGAGTTTGATCMTGGCTCAG-3′	54.5	Lane, 1991
907R: 5′-CCGTCAATTCMTTTGAGTTT-3′	50	Muyzer et al. 1993
341F: 5′-GCCTACGGGAGGCAGCAG-3′	62	Muyzer et al. 1993
1513R: 5′-ACGGYTACCTTGTTACGACTT-3′	54.5	Russell et al. 2009

bp base pairs, °*C* degree Celsius

* touchdown PCR program

non-selective LB Agar and incubated over night at 28 °C. Colonies were visually checked for morphological consistency before three identical looking colonies per plate were used in colony-PCR with the primers Eub9_27 and Eub1542 (Table 1) for preliminary assessment of diversity. PCR was performed in a total volume of 50 μl containing: 2 μl template DNA, 39.75 μl water, 5 μl 10 x buffer, 1 μl 10 μM of each primer, 1 μl 1 mM dNTP's and 0.25 μl Taq polymerase at 5 U/μl. Touchdown PCR cycling conditions were: 4.30 min at 94 °C, then 10 cycles of 94 °C for 30 s, 60 °C–55 °C for 30 s, 72 °C for 1 min 30 s, decreasing annealing temperature by −0.5 °C with each cycle, followed by another 20 cycles with a constant annealing temperature of 55 °C and a final elongation of 10 min at 72 °C.

Clone culture Preliminary identification of 20 bacterial colonies through comparison of a 600 bp fragment in BLAST (as described above) confirmed the presence of just one bacterial genus: *Serratia*. Five colonies from 5 different plates were selected to be cultured. Prior to extraction, clones were picked with a pipette tip, which was dropped in 10 ml of liquid LB, and incubated for 6 h at 28 °C on a shaker. DNA was extracted using GenElute™ bacterial Genomic DNA-Kit (Sigma-Aldrich) following the manufacturer's instructions. Of four out of five clones, a 1440 bp fragment of the 16S rRNA gene was assembled from fragments sequenced using the primers 27F, 341F, 907F, 1543F, Eub9_27 and Eub1542 (Table 1).

Sequencing PCR products were cleaned either by filtration (Millipore) or the PCR product band was cut from the gel, frozen and the DNA was later extracted by compressing the agarose piece between two layers of Parafilm® (Sigma). Sequencing was carried out on an automated ABI3130xl Genetic Analyzer machine using ABI BigDye version 3.1 Terminator Sequencing chemistry. Chromatogram output quality was checked by eye and corresponding sequences (forward and reverse) were aligned. The different fragments of the 16S rRNA gene were assembled in Sequencher 4.9 (Gene Codes, Ann Arbor, USA). Assembled sequences are deposited in GenBank (JX872281- JX872284).

Sequences were queried using the online sequence databases; GenBank (National Center for Biotechnology Information (NCBI), using nucleotide Basic Local Alignment Search Tool (BLAST) and Sepsi-Test™ -BLAST, a tool designed to identify bacteria, yeast and fungi. We accepted positive identification to the species-level obtaining at least 99% "Max ident" at a coverage of 100% in BLAST and Sepsi-Test.

3 Results

Mite homogenate (0.5 μl to 1 μl) applied to chitin-covered minimal-medium resulted in haloes with 0.5 cm radius around a central colony within 12 h and a complete digestion of the chitin layer after 48 h (Fig. 1a). Colonies were picked, and incubated after 12 h in liquid medium overnight. Colonies growing after plating liquid culture looked identical (shape, approximate size) apart from the color where they displayed a range from light red to dark red (Fig. 1b). The five colonies, c1 to c5, chosen for further sequencing represent a color range: c1 – lightest red; c2 – light red; c3 – dark red; c4 – red; c5 – red. All sequencing but with the primer 341F gave high quality results and initial BLAST queries indicated the isolation of a single species of the genus *Serratia*. The colony C4 probably contained a contamination and could not be used for further analysis.

The four assembled 1440 bp sequences were identified as a *Serratia marcescens* strain, based on their 16S rRNA gene sequences that are at least 99% identical to previously characterized *Serratia marcescens*, and are most similar to *Serratia marcescens* subspecies *sakuensis* (AB061685) (99.4–99.8% identity in Sepsi-Test).

4 Discussion

Based on the 16S rRNA gene sequences we identify the chitinolytic bacteria isolated from the bulb mite *Rhizoglyphus robini* as *Serratia marcescens*. The genus *Serratia* contains many chitin degraders. *Serratia marcescens* is the most common among them and has the best studied chitinolytic machinery known to date. In addition to three chitinases (ChiA, ChiB and ChiC) it also produces a chitobiase and a putative chitin-binding protein (Brurberg et al. 2001). Already in 1988, Ordentlich and colleagues could demonstrate that the bacterium can grow solely on fungal mycelium as a food source (Ordentlich et al. 1988).

Serratia spp. can be found in a variety of environments and are not seldom found associated with arthropods, as pathogens (*S. marcescens*, *S. liquefaciens* in a wide variety of animals), symbionts (*S. symbiotica* in aphids, *S. marcescens* in the red palm weevil *Rhynchophorus ferrugineus* and *Serratia* spp. in the fruit fly *Bactrocera dorsalis*), or with unknown status (Grimont and Grimont 1978; Wang et al. 2011, 2014; Zimmermann et al. 2016; Scracia et al. 2016). They have been reported from diseased and dead individuals, but have also been isolated from healthy organisms, as secondary and maybe even primary symbionts. The facultative symbiont *Serratia symbiotica* is vertically transmitted and provides defense against environmental heat-stress to its host, the aphid *Acyrtosiphon pisum* (Montllor et al. 2002). *Serratia symbiotica* is phylogenetically clearly identified as a *Serratia*, but has a strongly reduced genome size as many symbionts compared to their free-living sister species (Burke and Moran 2011). The genome size is being even more reduced in a *S. symbiotica* strain infecting the aphid *Cinara cedri*, where it is believed to be in the transition of becoming a primary symbiont (Lamelas et al. 2011). Several *Serratia* species can be pathogenic to higher animals and humans and are often involved in hospital-acquired infections

(HAI) of the human urinary tract and general wound infections (Mahlen 2011).

Three different chitinolytic *Serratia* species have previously been isolated from the mycophagous mites *Tyrophagus putrescentiae* and *Archegozetes longisetus* (Astigmata, Acarinae): *S.liquefaciens*, *S. rubidea* and *S. marcescens* (Smrz and Catska 2010). The authors mention that mites and bacteria form very efficient collaborations to reduce fungal cover, whereas the bacteria or the mite alone have little influence on an advanced fungal infestation. However, Hover et al. (2016) showed that some *Serratia sp.* strains had the means to move along fungal hyphae and kill them eventually without the help of an arthropod host. These observations are very valuable in the context of biological control of pathogenic fungi by chitinolytic organisms. Chitinolytic *Serratia* species have also by other studies been suggested as a control agent of fungi (Brurberg et al. 2001, Hover et al. 2016). However, in the case of the *Serratia* - host being a pest itself, a use of this system might be difficult.

The most similar sequence published stems from *Serratia marcescens* subspecies *sakuensis.* The subspecies *sakuensis* has been proposed after characterization of an endospore-forming isolate from activated sludge in a waste water treatment tank in Japan. Our sequences are more similar (on average 0.2%) to the *sakuensis* than to the *marcescens* subspecies (AJ233431). *Serratia marcescens sakuensis* is supposedly the first known endospore-forming *Serratia*. Endospores are bacterial cells resistant to unsuitable conditions which enable the bacterium to survive in a dormant state for long periods of time. In our case the capability to form endospores may be a strategy developed by the bacterium to survive long time periods outside of the host, without a food source, while spreading to new host individuals. Microscopy for endospore confirmation and other characterization tests (cell morphology, use of various carbon sources, biochemical tests, mass spectrometry or microarray assays) should be conducted in order to taxonomically assign the species found in *R. robini* to a subspecies (Ajithkumar et al. 2003 among others). Further sequence analysis, such as of the intergenic spacer region between 16S rRNA gene and 23S RNA gene, could also lead to a higher resolution of the taxonomic status.

In a previous article we described the bacterial community associated with *R. robini*. The genus *Serratia* was among the three dominant genera along with *Myroides* and *Alcaligenes* (Zindel et al. 2013). A total of 22 OTU's (operational taxonomic unit) assigned to *Serratia* were found, differing in at least 3% of the sequence, suggesting several *Serratia* species to be present (unpublished). In the present study we probably only identified one species. Other chitin-degrading *Serratia* species and chitin-degrading bacteria in general could have been missed in this study due to unsatisfied growth requirements other than carbon-source in this specific protocol. It is estimated that up to 99% of soil organisms are not detectable by conventional cultivation methods (Hugenholtz et al. 1998; Rondon et al. 2000). This percentage could be even higher in microorganisms adapted to the special environment of an organism's inside.

In conclusion we can say that the identification of *Serratia marcescens* as one of the main chitin-degrading bacteria in *Rhizoglyphus robini* may not be surprising, because of its known chitinolytic qualities and previous findings of representatives of the genus in mycophagous mites. The information is nevertheless very interesting and important in many respects, of which we will mention two: 1) It is an important step in the characterization of a pest organism to identify the associated bacterial community and identify its impacts on the host's biology. We can now assume that the *R. robini* – host plant interaction includes two more players – the fungus and *S. marcescens*. For example, the elimination of either of the two could be an approach worth testing to decrease mite damage on crop plants. 2) *Serratia marcescens* is a model organism for chitin degradation and has, due to its efficiency, been discussed for fungal control. Smrz and Catska (2010) raise the idea of using chitinolytic bacteria in association with fungivorous mites in a combined approach of biological control of fungi - the mites being responsible for locomotion (grazing), whereas the bacteria supply the enzymes destroying the pest fungus. In our case, mite and bacteria are already assembled, althogh the pest/pathogen status of neither of the two, *R. robini* nor *S. marcescens,* is yet fully understood and more research would be needed.

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