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On the bright side of a forest pest-the metabolic potential of bark beetles' bacterial associates

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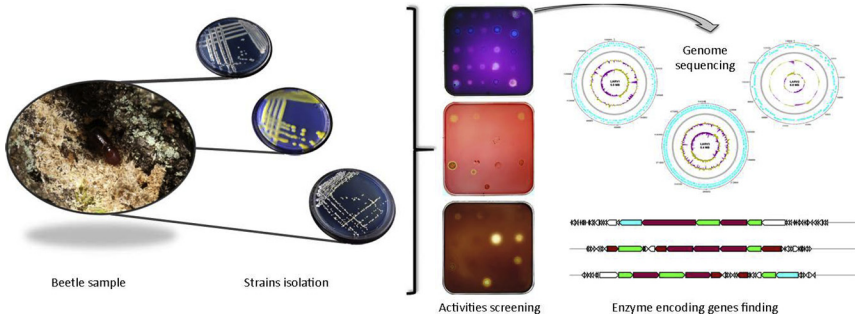
HIGHLIGHTS

- A total of 58 bacterial strains from bark beetles were isolated and identified.
- These bacteria are able to degrade plant cell compounds and colorants.
- Genes encoding lignocellulolytic enzymes are present in the genomes of 3 strains.
- Bark beetles are new sources of bacteria with biotechnological potential.
- These results will help further studies regarding beetle and bacterial ecology

GRAPHICAL ABSTRACT



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ABSTRACT

Bark beetles reproduce and overwinter under the bark of trees, and are associated with bacteria that may influence the fitness of their hosts. As regard the aim of this study was to test the metabolic potential of bacterial strains, isolated from the bark beetle species *Cryphalus piceae, Ips typographus* and *Pityophthorus pityophthorus* and collected in the Czech Republic from fir, spruce and pine trees, respectively, to degrade plant cell compounds. The bacterial strains were identified as belonging to the genera *Curtobacterium*, *Erwinia*, *Pantoea*, *Pseudomonas, Rahnella, Staphylococcus,* and *Yersinia*. Several activities related to the degradation of lignocellulosic materials, such as cellulose, xylan and starch, were found. Moreover, the genomes of three of these strains were sequenced and analyzed, and the presence of the enzymatic machinery required for biomass hydrolysis was discovered. This finding supports the idea that bacteria aid in the provision of nutrients to the beetle from the hydrolysis of tree compounds, results that are relevant for studying the ecological implication of bacterial strains in the bark beetle life cycle. In addition, the activities found in association with the bacterial strains could be useful in biotechnological processes, such as the production of biofuels from biomass, colorant degradation, in the textile industry and for wastewater treatments. Furthermore, the gene sequences of the lignocellulolytic enzymes found within the genomes serve as a basis for future studies regarding the potential application of these bacteria, and their metabolic machinery, in processes such as biomass hydrolysis and bioremediation.

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1. Introduction

Bark beetles are a large group of insects belonging to the family *Curculionidae*, subfamily *Scolytinae*. They are mostly known as a causative agent of worldwide woodland devastation owing to their ability to severely attack trees, which serve as their natural habitat and nutrient source (Fig. S1, Supplementary material).

These beetles live in association with fungal and bacterial species, and although the interactions between beetle and fungi have been studied in-depth over the years, the role of bacteria in bark beetle ecology has only recently been considered (García-Fraile, 2018). Under tree bark, the bark beetles feed on tree tissues. The complex polysaccharides, which form these tree tissues, constitute the main carbon sources available to bark beetles, but are difficult for the insects to digest. Some studies have proposed that bacterial symbionts aid beetles in the digestion of plant cells, based on the capability of several bacteria to degrade plant compounds. To date, actinobacterial strains isolated from the *Dendroctonus valens* gut were found to hydrolyze cellulose (Morales-Jiménez et al., 2009; Morales-Jiménez et al., 2012). Also, bacterial strains isolated from *Dendroctonus rhizophagus* and identified as *Stenotrophomonas maltophilia*, *Kocuria marina*, *Ponticoccus gilvus, Pseudomonas azotoformans* and *Arthrobacter* sp. (Morales-Jiménez et al., 2012) were found to hydrolyze this polymer. Bacteria belonging to the genera *Pseudomonas, Brevundimonas, Serratia, Pseudoxanthomonas, Methylobacterium, Sphingomonas, Bacillus,* and *Paenibacillus* obtained from *Dendroctonus armandi* larvae (Hu et al., 2014) and the novel species *Pseudomonas coleopterorum* isolated from the bark beetle *Hylexinus fraxinii* (Menéndez et al., 2015) have also been found to have this activity. Moreover, Briones-Roblero and collaborators (Briones-Roblero et al., 2016) showed the ability of *Pseudomonas* strains isolated from *D. rhizophagus* to produce amylases and *Arthrobacter* and *Pseudomonas* strains isolated from the same beetle to produce xylanases. However, the capacity to hydrolyze plant cell compounds has not been reported in bacterial strains from other bark beetle species different from those four mentioned above.

Cellulose and hemicelluloses are the main polymers constituting such plant tissues. Also, starch is an important plant cell compound, and is the most abundant carbon storage compound. If bacterial strains associated to bark beetles are implicated in their nutrition, it could be expected that some of these bacteria have the enzymatic machinery necessary for the degradation of these plant compounds. Complete hydrolysis of cellulose requires the synergistic effect of diverse enzymes, which are broadly known as cellulases (Sadhu and Maiti, 2013). Xylan is the most abundant component of hemicelluloses. Its complete hydrolysis involves several enzymes, which are known as xylanases. Hydrolysis of starch, the most abundant polysaccharide, produced by plants as water insoluble granules, after cellulose, is performed by the enzymes called amylases (Antranikian, 1992).

Apart from their role in the production of sugars, that is to say, energy for the organisms that ingest these plant tissues, all of these lignocellulolytic enzymes can be engineered and have diverse applications in different biotechnological processes. These processes include: (i) cellulases used in the production of biofuels (bioethanol and biomethane) (Gupta et al., 2012), in pulp and paper factories, in the textile and detergent industries and in the animal feed and food industries (Sukumaran et al., 2005); (ii) xylanases used to clarify juice, biobleaching - the deinking of recycled paper - and the saccharification of hemicelluloses to xylose sugars, among others (Soni and Kango, 2013); and (iii) amylases with applications in a large number of industrial processes such as food, fermentation and pharmaceutical industries (De Souza and de Oliveira-Magalhaes, 2010).

Thus, the aims of this study were to isolate bacterial strains from three bark beetle species, *Cryphalus piceae, Ips typographus* and *Pityophthorus pityophthorus,* and to analyze their metabolic ability to hydrolyze tree tissues. The results of this study provide a better understanding of the potential role of bacterial strains in the life cycle of bark beetles. Moreover, this study opens up the possible use of bark beetles as a new source of enzymes which may have potential applications in bio-based industries.

2. Materials and methods

2.1. Bacterial isolation

Adult bark beetles of *Cryphalus piceae* and *Pityophthorus pityophthorus* and larvae from *Ips typographus* were obtained from the branches of *Abies alba*, *Pinus sylvestris,* and *Picea abies* trees, respectively. *A. alba* and *P. abies* branches were collected in Rohozná u Přelouče and *P. sylvestris* branches were collected in Kunratice forest, both locations within the Czech Republic. In the lab, under aseptic conditions, the bark was removed searching for the insects. *A. alba* and *P. sylvestris* contained adult specimens of *C. piceae* and *P. pityophthorus,* respectively. Branches of *P. sylvestris* contained just few larval individuals of *I. typographus*. Several specimens of each type were outer-disinfected by immersion in ethanol for 2 min and washed several times with sterile distilled water. Inside an anaerobic workstation, *C. piceae* and *P. pityophthorus* adults were sorted in 5 groups of 3 indivudals per each beetle species (30 individuals in total) The only six *I. typographus* larvae found in *P. abies* branches were pooled together. Individuals of each pool were crushed in 500 μl of sterile water into 1,5 ml sterile plastic tubes. From these suspensions, serial dilutions were made and aliquots were plated by spread plate method on Tryptic Soy Agar (TSA, Sigma) medium.

Samples were incubated in Leonard jars with AnaeroGen™ sachets (OXOID™, Thermo Scientific™) at 24 °C. Bacteria were recovered after 7 days and pure cultures were obtained picking up all colonies with different morphology. Cells from each isolate were stored in 20% glycerol at −80 °C for long-term preservation. Growth under aerobic conditions was also tested by incubating the plates in aerobiosis.

2.2. DNA extraction and bacterial identification

For strains identification, bacterial lysates were obtained by picking colonies into tubes containing 50 μl of Lysis buffer (0,2 g of NaOH and 0,25 g of SDS in 100 ml of sterile distilled water) and incubating the tubes for 10 min at 95 °C. After that, tubes were centrifuged at 8.000*g* for 5 min. Finally, 10 μl of the supernatant, containing genomic DNA, were transferred to a clean tube containing 90 μl of sterile distilled water. Then, the 16S rRNA gene was amplified as described in (Rivas et al., 2007).

PCR products were visualized on a 1% agarose gel after electrophoresis, and the bands corresponding to the 16S rRNA gene were excised with sterile scalpels and processed using the QIAquick™ Gel Extraction Kit (QIAGEN), following manufacturer's instructions. For 16S rRNA sequencing, the purified PCR products were sequenced at Macrogen (Seoul, Korea). The sequence fragments obtained with both primers were aligned using the ClustalW software (Thompson et al., 1994) and the resulting sequences were compared with those from the type strains of all of the bacterial species described using the EzTaxon-e on-line server (Kim et al., 2012).

Genomic DNA for genome sequencing was obtained with the kit Quick-DNA Fungal/Bacterial MiniPrep (Zymo Research), following the instructions given by the manufacturer.

2.3. Screening of enzymatic activities and colorants degradation

Medium TSA containing 1% of CMC (carboxymethyl cellulose), xylan or starch was prepared in order to detect cellulolytic, xylanolytic or amylolytic activities, respectively, as previously reported elsewhere (see for instance: Mateos et al., 1992; García-Fraile et al., 2007; Saleem et al., 2008).