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Characterization of bacteria strains collected from Crete as biopesticides with larvicide properties against *Culex pipiens molestus*

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Background

Vector-borne diseases transmitted by mosquitoes has led to millions of infections and over 700.000 deaths annually. Therefore, control of the vectors and pests has been primarily done using synthetic pesticides. Although useful over several decades, continuous synthetic pesticide use has brought different concerns due to risk of human and animal health, negative effects on beneficial insects, long half-life in the environment, and pesticide resistance. The European Commission has provided the "Green Deal" to reduce the use of synthetic pesticides by 50% until 2030 (EU, 2022). There is a current need for other alternatives that are both effective at controlling vectors and pests while encouraging sustainable strategies for the environment.

An alternative to vector control could be done using biopesticides. Biopesticides are defined by the United States Environmental Protection Agency (EPA) as natural substances capable of controlling pests. A type of biopesticides can be produced by microorganisms (microbial entomopathogens) (EPA, 2023).

Goals

The goals of this study were (1) to evaluate the insecticidal activity of 40 bacteria isolates collected from different sources from Crete (Greece) against *Culex pipiens molestus* and (2) identify the genus/species of the top isolates based on genome sequencing.

Methods

1. Preparation of bacterial powders and larvicide bioassays

To produce semi-formulated bacterial bioinsecticides for assay, a heat-inactivated method was chosen to prioritize candidate isolates that produce stable, thermally-tolerant insecticidal compounds (Fig. 1A). Bacterial cultures were collected using a sterile pipette tip and inoculated in 5 mL of LB broth and incubated for 72 hours at 28 ± 1° C and 150 rpm in a shaking incubator. Samples were then incubated in a water-bath for one hour at 75± 1°C . Successful inactivation was confirmed by plating the isolates into LB agar before and after heat-treatment. Sterile LB broth was used as a negative control for all experiments. Pellets were prepared in 6-well plates (Sigma-Aldrich, Cat. # CC302) whereby 3 mL of heat-inactivated bacterial culture was mixed with 3 mL of 4% agar (Merck Group, Cat. # 05040-1KG), and 100 mg of ground fish powder was used as an attractant (Tropical Fish Food PRODAC International, Cittadella, Italy). Plates were then incubated at 27 ± 1°C at 100 rpm for 30 min to homogenize and solidify the preparations. From this, three circular pellets of the final product were excised using the blunt end of a sterile pipette tip.

For each isolate, the screening bioassays were done by adding 10 3rd-4th instar larvae into plastic cups containing 100 mL of distilled H_2O and three bacterial pellets. Bioassays were replicated tree times (*n* = 90 larvae per treatment). Mortalities were recorded every 24 h for seven days.

2. Identification if the toxin(s) are attached to the cell membrane or secreted in the supernatant

A separation method was used to determine whether the insecticidal factor are either secreted, attached into the bacterial cell membrane or both (Fig. 2A). For each isolate, individual colonies were inoculated into 500 mL LB broth and incubated at 150 rpm and 27°C for five days. The inoculates were then centrifuged at 3500 rpm and 4°C for 30 mins. After centrifugation, the supernatants were stored in 50 mL Falcon tubes and the pellets were combined. The separated pellets and supernatants were heat-inactivated in a water bath at 75 ± 1°C for one hour. Bacterial precipitates and supernatants were desiccated inside an oven at 45 ± 1°C for 24 hours (precipitates) and four days (supernatants). The desiccates were then crushed into powder and used to prepare the pellets as previously described.

Equivalent amounts of pellet and supernatant were prepared for each strain. The amounts of pellet/supernatant were mixed with 8% agar 100 mg of ground fish powder. The pellet preparations were done in 1.5 mL Eppendorf tubes. Bioassays were done as mentioned in the previous section.

4. Identification of the four bacteria isolates using phylogenetics and overall nucleotide identities (ANI)

The genomes of the top four isolates were sent for long-read sequencing using Oxford Nanopore Technologies. The identification of the bacterial species was done using two different metrics (Fig. 3). First, we used OrthoANI to calculate the average nucleotide identity (ANI) between orthologous sequences. There is a consensus in literature that a genome achieving ANI of 95% or higher is confidently classified at the species. Second, phylogenetic trees were built based on maximum likelihood using 81 core genes based on the Up-to-date Bacterial Core Genes (UBCG2) pipeline. For each gene, a multiple sequence alignment was created using MAFFT. The individual gene alignments served two purposes; 1) to concatenate and build a highly resolved maximum likelihood tree using FastTree, and 2) to build individual gene trees and to calculate the Gene Support Index (GSI) that was later applied into the branches of the highly resolved tree.

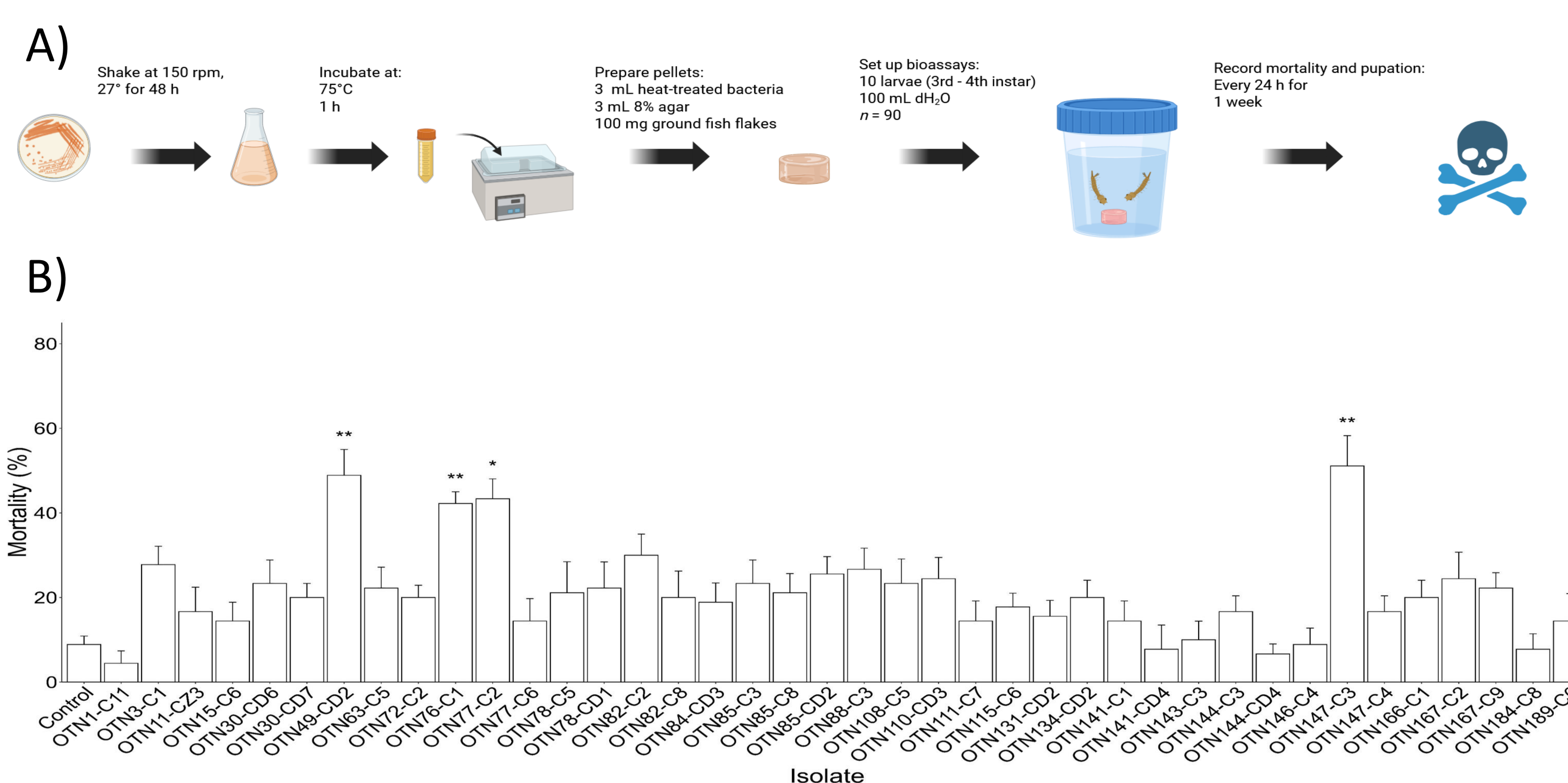

Figure 1. Screening bioassays of 40 bacterial isolates using heat-inactivation preparations. A). Diagram of the methodology. B) Bioassays using four biopesticide isolates against (A) larvae and (B) adults of *Cx. pipiens molestus*. Mortalities correspond to day 7 post-exposure. Bars with significant differences compared to the controls are marked with asterisks (*** p values < 0.001 based on the Dunn's test).

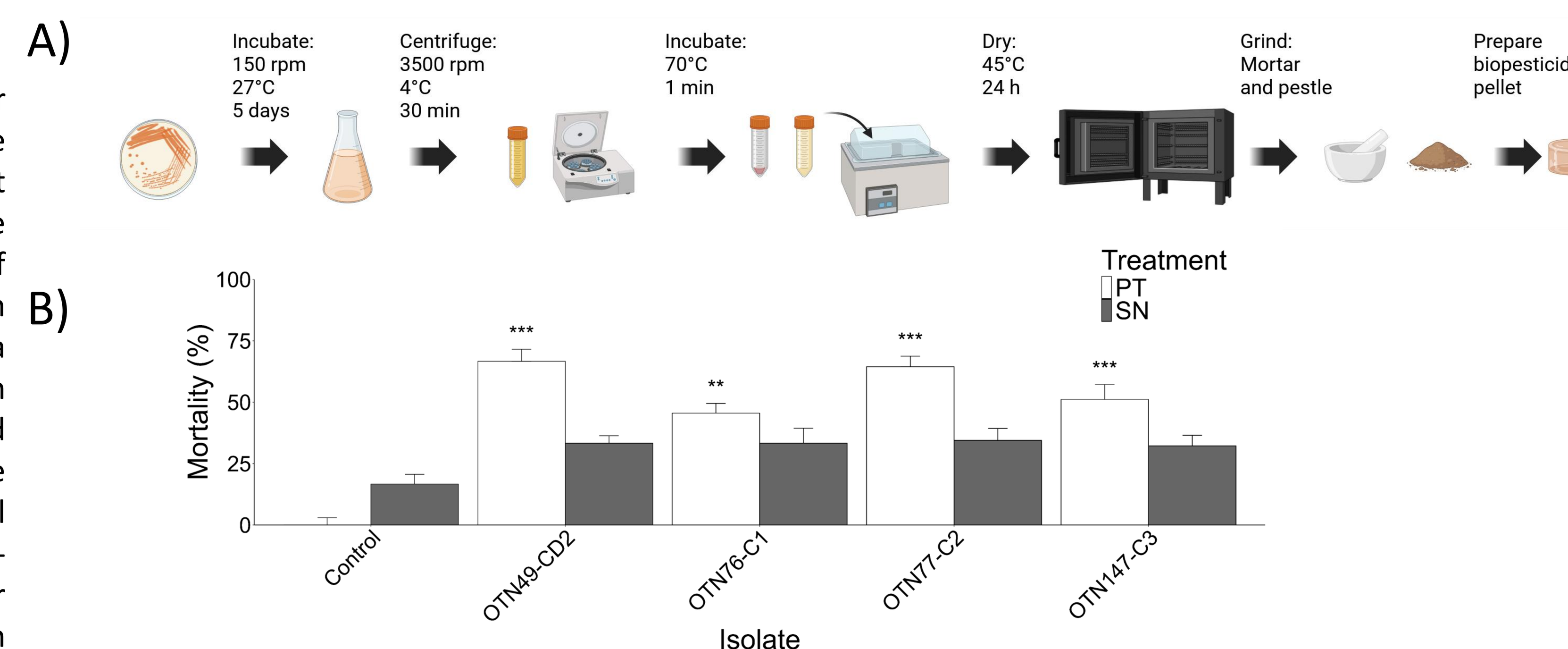

Figure 2. Mortality of the top four bioinsecticide isolates using different preparations as active ingredient (cell-precipitates and supernatants): A) diagram of the methodology and B) bioassay results at day 7 post exposure. Bars with significant differences compared to the controls are marked with asterisks (** p values < 0.01, *** p values .001 based on the Dunn's test). Pellet preparations are referred as "PT" and "SN" which correspond to cell-precipitate and supernatant, respectively.

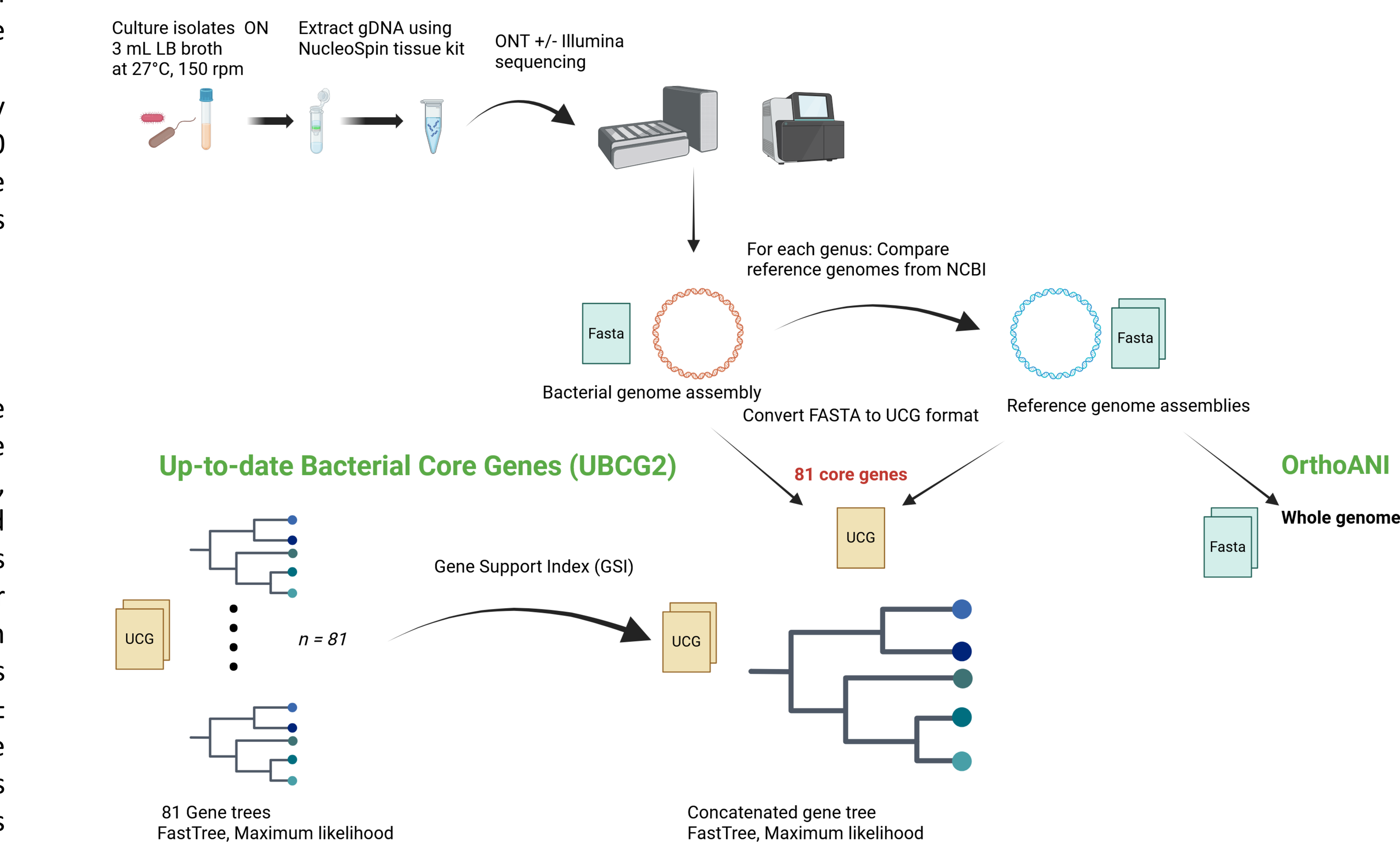

Figure 3. Methodology of the bacterial genome sequencing using ONT and Identification of the four bacteria isolates using phylogenetics and nucleotide identities (ANI). UCG = files containing the 81 cores genes from each genome.

Conclusions

There are four bacterial bioinsecticides with promising heat-stable activities. Two of these isolates belong to novel species: OTN76-C1 (*Microbacterium* sp.) and OTN147-C3 (*Oerskovia* sp.). The other two isolates belong to known species: OTN49-CD2 (*Pseudoxanthomonas danubii*) and OTN77-C2 (*Pseudomonas danubii*). Future preparations will be focused on cell-phase preparations (faster preparation of insecticidal pellets).

Future steps will be focused on the identification of the metabolites using organic solvent extractions and bioassay-guided fractionations

Results

1). Screening of bioinsecticide bacteria using heat-treated preparations

Larvae mortalities tested with different bacteria isolates did not follow a normal distribution (Fig. 1B) (Shapiro-Wilcox test, W = 0.913, p value < 0.001). Differences in mortalities across treatments were significant (Kruskal-Wallis, χ^2 = 119.1, df = 40, p-value < 0.001). There were four isolates with higher mortalities compared to the controls (8.89%); OTN147-C3 (51.1%, Dunn's test, Z = -4.25, p value = 0.008), OTN49-CD2 (48.8%, Z = -4.32, p value = 0.006), OTN77-C2 (43.3%, Z = -4.10, p value = 0.0167), and OTN76-C1 (42.2%, Z = -4.23, p value = 0.009). The mortalities of larvae exposed to the other 36 isolates did not differ to the control (mortalities ranged between 4.44 to 30.0%).

2). Identification of the source of the toxin(s)

The source of the larvicidal activities were determined for the four isolates that produced highest mortality at day 7 (Fig. 2B). There were significant differences in the mortalities across preparations and isolates (Kruskal-Wallis, X^2 = 55.1, df = 9, p-value < 0.001). The four bacterial cell precipitates resulted in significantly higher mortality of larvae compared to the controls (16.6%) : OTN49-CD2 (66.6%, Z = -5.54, p value < 0.001), OTN76-C1 (45.5%, Z = -3.93, p value = 0.0018), OTN77-C2 (64.4%, Z = -5.47, p value < 0.001), and OTN147-C3 (51%, Z = -4.47, p value < 0.001). Regarding the supernatant preparations, there were no significant differences between the five isolates and the controls.

3). Identification of bacteria

The bacterial genus/species were identified based on (1) orthologous average nucleotide identity and (2) phylogenetic analysis (Table 1). Although there was an agreement between the two approaches regarding the closest relative species, the results suggest that two isolates (OTN76-C1, and OTN147-C3) correspond to new species. The rationale to declare the discovery of new species was based on the ANI threshold lower than 95% or low GSI scores. For instance, the isolate OTN76-C1 shared the highest identity to *Microbacterium abyssi* (83.68% ANI) and the relationship to the same species was also observed in the phylogenetic tree with high support (GSI = 81/81). The isolate OTN147-C3 shared the highest identity to *Oerskovia rustica* (95.85% ANI) and the relationship to the same species was also observed in the phylogenetic tree, but with a low support (GSI = 41/81). The isolates OTN49-CD2 and OTN147-C3 belong to known species. For example, the isolate OTN49-CD2 shared the highest identity to *Pseudoxanthomonas kaohsiungensis* (97.4% ANI) and the relationship to the same species was also observed in the phylogenetic tree with high support (GSI = 81/81). Likewise, OTN77-C2 shared the highest identity to *Pseudomonas danubii* (98.90% ANI) and the relationship to the same species was also observed in the phylogenetic tree with high support (GSI = 81/81).

Table 1. Identification of the four bioinsecticide bacteria to genus/species level based on overall average nucleotide identity (ANI) and phylogenetic relationships using UBCG2 . GSI = Gene Support Index.

Isolate	Closest species	ANI (%)	UBCG2 species	# GSI*	Consensus genus/species
OTN49-CD2	*Pseudoxanthomonas kaohsiungensis*	97.4	*Pseudoxanthomonas kaohsiungensis*	81/81	*Pseudoxanthomonas kaohsiungensis*
OTN76-C1	*Microbacterium abyssi*	83.7	*Microbacterium abyssi*	81/81	***Microbacterium***
OTN77-C2	*Pseudomonas danubii*	98.9	*Pseudomonas danubii*	81/81	*Pseudomonas danubii*
OTN147-C3	*Oerskovia rustica*	95.9	*Oerskovia rustica*	41/81	***Oerskovia***

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