Microbiome Hijacking Towards an Integrative Pest Management Pipeline

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1 Introduction

Most agricultural crops have well-known insect pests, which can seriously affect crop quantity and quality (Metcalf 1996; Oerke 2005; Oerke and Dehne 2004). The chemical pesticides used to combat these pests are usually nonspecific and therefore toxic to other beneficial insects, such as bees, or even to animals (Aktar et al. 2009). Developing specific pesticides for each type of pest would mean less harm to the environment and a lower load of chemicals on crops, which would also benefit consumers. In this work we propose such a method, based on the study and

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exploitation of the insect microbiome to specifically combat each insect pest. Just like the human microbiome plays an important role in our health, the insect's microbiome often contains bacteria vital to its survival. Identifying these vital bacteria for each pest paves the way for the study of specific inhibitors which target specific bacterial proteins. Targeting the bacteria affects the health of the insect host and thus its ability to infect the plant as a pest. We demonstrate this approach with a study of the olive fruit fly *Bactrocera oleae*, which affects the olive tree (*Olea europaea* L.) and harbors the endosymbiotic bacterium *Candidatus Erwinia dacicola*.

The olive tree (*Olea europaea* L.) is a major agricultural crop in the Mediterranean basin playing an important economical role in countries of this region. Its contribution to agriculture is recognized since antiquity (Kaniewski et al. 2012). Olive domestication occurred in the Middle East about 6000 years ago (Zohary and Spiegel-Roy 1975) and then spread across the Mediterranean basin (Damania 1995; Lavee 2013). 98% of the world's olive groves are cultivated around the Mediterranean basin, and the European Union is the greatest consumer. Greece is among the leading world producers in olive oil, being third in production globally, after Spain and Italy (Food and Agriculture Organization of the United Nations 2018). Olive cultivation in Greece corresponds to about 80% of the total tree cultivation, with approximately 150 million trees, covering 21% of the total agricultural land and producing 400,000 tons of olive oil yearly (Hellenic Republic – Ministry of Rural Development and Food 2018). Over the last 25 years, world consumption of olive oil has increased by one million tons (International Olive Council 2018). Virgin olive oil is characterized by its high nutritional values and is an integral part of the Mediterranean diet, a diet with a high content of bioactive substances such as vitamins, flavonoids, and polyphenols. Polyphenols have been demonstrated to have a positive effect against cardiovascular diseases and certain cancers (Andrikopoulos et al. 2002; Hertog et al. 1995; Kris-Etherton et al. 2002). Phenolic compounds also play an important role in the organoleptic characteristics of olive oil. The phenolic profile of olive trees, as well as the olive oil quality and composition, depends on many factors, such as genotype, tissue type, developmental stage, geographical origin, fruit maturity stage, harvesting method, olive storage, and oil extraction technique (Mitsopoulos et al. 2016; Tuck and Hayball 2002; Vinha et al. 2005). Another important factor affecting olive oil quality and composition is fruit health.

The most important enemy of the olive tree is *Bactrocera oleae*, the olive fruit fly, which poses a severe economic threat for commercial olive growers, as it can cause up to 30% reduction in the production of olive fruit (Neuenschwander and Michelakis 1978). Infection by *B. oleae* affects not only the quantity but also the quality of the olive fruit and olive oil, due to the increase of oleic acid, which is caused by the larvae's feces as well as the entry of secondary bacteria and fungi through the spot created by the insect during larvae deposition. Various methods of pest control are used, only some of which are environmentally friendly. The main method used is chemical insect control. Chemical substances such as dimethoate, pyrethroid, or spinosad are used to reduce the population of the pest, but the insect has developed resistance to these pesticides (Hsu et al. 2004; Margaritopoulos et al. 2008; Pavlidi et al. 2017; Skouras et al. 2007; Vontas et al. 2001; Vontas et al. 2002;

Vontas et al. 2011). The McPhail trap, which contains pheromones, ammonium salts, or hydrolyzed proteins and attracts the insect, is used to observe and estimate the size of the population, in order to plan the right amount and the correct timing of pesticide application (Economopoulos 1977). The biological control method is based on the action of other insect populations that are parasites, predators, or pathogens for the target insect, but it is not so widely used (Manikas and Tsiroyannis 1982; Navrozidis et al. 2000; Neuenschwander and Michelakis 1978). Furthermore, the sterile insect technique aims at the rapid reduction of the population through the release of sterile insects into the environment (Knipling 1955; Ras et al. 2017).

The larvae of the olive fruit fly grow and feed on the mesocarp of ripe and unripe olive fruits, which allows them to carry out several generations until the fruits ripen (Ben-Yosef et al. 2014, 2015). In contrast to the ripe fruit, the unripe olive fruit is an inhospitable environment in which the insect cannot survive, as oleuropein inhibits the growth of the larva. The metabolite oleuropein accumulates at high levels during the early stages of olive fruit ripening and decreases gradually as the olive fruit ripens (Soler-Rivas et al. 2000). Oleuropein has the ability to inactivate enzymes and reduce lysine digestion, thus inhibiting larvae growth (Ben-Yosef et al. 2015). However the larvae manage to survive in the hostile environment of the unripe olive fruit due to the presence of the endosymbiotic bacterium, *Candidatus Erwinia dacicola*, in the gut of the insect (Capuzzo et al. 2005; Estes et al. 2012). The mechanism of action of this bacterium is difficult to determine as Ca. *E. dacicola* remains uncultivated, but it is assumed that the bacterium can provide a source of dietary proteins or amino acids for the larvae. This may be achieved by the secretion of chemical substances by the bacterium which may facilitate the dissociation of oleuropein-protein complexes in the insect's gut. This way, proteins and lysine can be easily assimilated by the insect, similar to mechanisms seen in other insect-associated symbiotic fungi and bacteria (Ben-Yosef et al. 2014, 2015; Pavlidi et al. 2017).

Ca. *E. dacicola* is transmitted vertically from the female fruit fly to her offspring (Estes et al. 2012), although horizontal transmission of the bacterium between larvae living and feeding on the same olive fruit has also been hypothesized (Bigiotti et al. 2018; Viale 2014). The olive fruit fly is closely linked to symbiotic bacteria throughout its life stages (Ben-Yosef et al. 2010). Both larvae and adult flies have been morphologically adapted to host bacteria in their gut. The abundance of Ca. *E. dacicola* changes during olive fruit fly development (Behar et al. 2008). The larvae present a higher relative abundance of Ca. *E. dacicola* than the egg and the pupa (Estes et al. 2012). Furthermore, the ovipositing female has the highest relative abundance of Ca. *E. dacicola*, compared to males that have mated, virgin females and adults aged less than 12 hours (Estes et al. 2012). Ca. *E. dacicola* is one of the few examples of endosymbiotic bacteria, which switch from an intracellular to an extracellular existence during host-insect development. The bacterium is intracellular at the larval midgut, but it is extracellular at the front gut in the adult insect (Estes et al. 2012). Phylogenetic analysis of the Ca. *E. dacicola* demonstrates that this bacterium is closely related to various phytopathogenic and free-living *Erwinia* species, such as *E. amylovora*, *E. pyrifoliae*, *E. tracheiphila*, and *Pantoea stewartii*,

pathogens which persist primarily in association with their plant hosts and insect vectors with limited survival in the soil (Estes et al. 2012).

The genome of Ca. *E. dacicola* was recently sequenced (Blow et al. 2016). We used this information to select three proteins of interest, which have been extensively studied in other organisms for their role and mode of action. We created homology models for these three proteins from Ca. *E. dacicola* and used these models to deduce pharmacophore structures for potential small molecule inhibitors. This approach will allow us to screen chemical libraries for potential new inhibitors of vital functions of Ca. *E. dacicola*. Specifically, targeting Ca. *E. dacicola* in wild populations of *B. oleae* is expected to result in a reduction in the fruit fly's ability to infect unripe olives and will thus greatly reduce their potential as pests.

2 Methods

2.1 Identification of Template Structures and Sequence Alignment

The amino acid sequences were retrieved from the conceptual translation of the NCBI database (http://www.ncbi.nlm.nih.gov/). The blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify homologous structures by searching the Protein Data Bank (PDB). The multiple sequence alignment for homology modelling was performed using MOE (Dalkas et al. 2013; Vilar et al. 2008; Vlachakis et al. 2017).

2.2 Molecular Modelling

All calculations and visual constructions were performed using the Molecular Operating Environment (MOE) version 2013.08 software package developed by Chemical Computing Group (Montreal, Canada) (Vilar et al. 2008).

2.3 Homology Modelling

The homology modelling of the three proteins of interest was carried out using MOE (Vilar et al. 2008). The selection of template crystal structures for homology modelling was based on the primary sequence identity, similarity, and the crystal resolution (Nayeem et al. 2006; Papageorgiou et al. 2014; Papageorgiou et al. 2017; Papageorgiou et al. 2013). The MOE homology model method is separated into four main steps: (a) primary fragment geometry specification, (b) insertion and deletions task, (c) loop selection and side-chain packing, and (d) final model selection and refinement (Papageorgiou et al. 2014).

2.4 Molecular Electrostatic Potential

Molecular electrostatic potential surfaces were calculated by solving the nonlinear Poisson-Boltzmann equation (Vishnyakov et al. 2007), using the finite difference method as implemented in the MOE and PyMol (Mooers 2016; Vilar et al. 2008). The potential was calculated on solid points per side. Protein contact potential was calculated using Amber99 charges and default atomic radii (Chen and Pappu 2007).

2.5 Model Optimization and Molecular Dynamics

Energy minimization was done for all three models in MOE (Vilar et al. 2008), initially using the Amber99 force field implemented into the same package, up to a root mean square deviation (RMSd) gradient of 0.0001, in an effort to remove the geometrical strain (Sellis et al. 2009). The models were subsequently solvated with simple point charge (SPC) water using the truncated octahedron box extending to 7 Å from the model, and a set of molecular dynamic simulations was performed at 300 K and 1 atm with 2-second step size for a total of one hundred nanoseconds, using the NVT ensemble in a canonical environment (NVT stands for *n*umber of atoms, *v*olume, and *t*emperature that remain constant throughout the calculation). The results of the molecular dynamics simulation were collected into a database by MOE for further analysis (Loukatou et al. 2014; Vlachakis et al. 2014).

2.6 Model Evaluation

The models produced were initially evaluated within the MOE package by a residue packing quality function, which depends on the number of buried nonpolar side-chain groups and on hydrogen bonding. Moreover, the PROCHECK suite (Laskowski et al. 1996; Papageorgiou et al. 2017) was employed for further evaluation of the quality of the produced models. Finally, Verify3D (Von Grotthuss et al. 2003) was used to evaluate whether the three models are similar to known protein structures of their family.

2.7 Pharmacophore Elucidation

Computerized representations of hypothesized pharmacophores were analyzed in MOE, using the *pharmacophore query* feature (Vlachakis and Kossida 2013). A MOE pharmacophore query is a set of *query features* that are typically created from ligand *annotation points*. Annotation points are markers in space that show the

location and type of biologically important atoms and groups, such as hydrogen donors and acceptors, aromatic centers, projected positions of possible interaction partners or R-groups, charged groups, and bioisosteres (Vlachakis et al. 2013). The annotation points on a ligand are the potential locations of the features that will constitute the pharmacophore query. Annotation points relevant to the pharmacophore are converted into query features with the addition of an extra parameter: a nonzero radius that encodes the permissible variation in the pharmacophore query's geometry.

2.8 Conserved Motifs Exploration

For each protein of interest, homologs were identified by blastp, using default parameters. Organisms which gave good hits by blast for all three proteins were selected, and thus a dataset of 105 homologous sequences was downloaded for each of the three proteins. Multiple sequence alignments of the three datasets were performed using Matlab's progressive alignment methods (Papageorgiou et al. 2016). Conserved sequences motifs were identified from the multiple sequence alignments, and sequence logos were generated using Jalview software (Waterhouse et al. 2009).

3 Results and Discussion

Three proteins were chosen for analysis from Ca. *E. dacicola*, as they have been widely studied by our group and others previously: Helicase, Polymerase, and Protease-C (Vimal et al. 2018; Docherty et al. 2003). The blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify homologous structures by searching the Protein Data Bank (PDB). Multiple sequence alignment for homology modelling was performed using MOE (Vilar et al. 2008), and from these, the most closely related, and with best resolution, available structures were chosen as templates for homology modelling (template-PDB IDs – Helicase 4CEI, Polymerase 4GZY, Protease-C 1K7G). The three homology-based models were stereochemically and energetically evaluated and superposed to their templates (Fig. 1). As expected, the models retained the fold of their templates, but, more importantly, they shared similar physicochemical and kinetic profiles with the X-ray structures they are derived from.

Based on the homology modelling of the Ca. *E. dacicola* proteins, pharmacophore models were generated using MOE for each of the three proteins of interest (Fig. 2). A pharmacophoric feature characterizes a particular property and is not tied to a specific chemical structure. Different chemical groups may share the same property and also be represented by the same feature (Vlachakis et al. 2015; Chatzikonstantinou et al. 2017; Marinou et al. 2018). It is thus a mistake to name as pharmacophoric features, chemical functionalities such as guanidines or sulfonamides, or typical structural skeletons such as flavones or steroids. Once generated, a pharmacophore

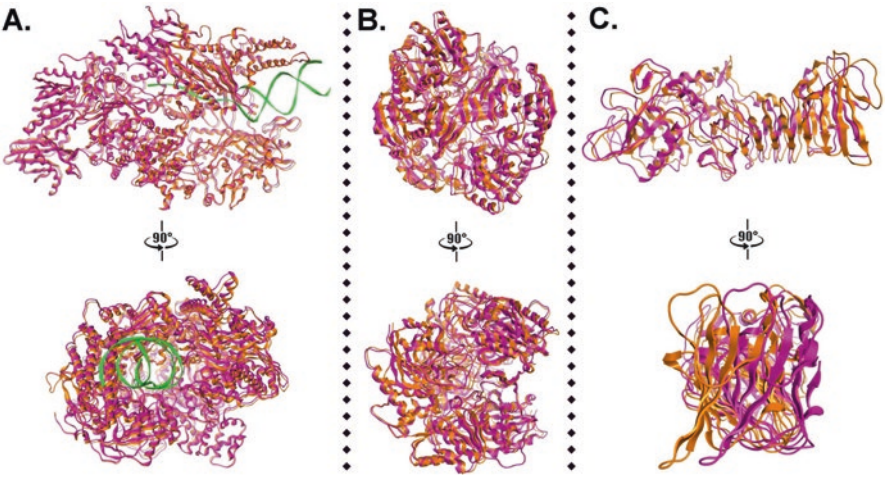

Fig. 1 Homology modelling. The homology models (orange colored) have been superposed to their templates (magenta colored) for each of the following targets: (**a**) Helicase, (**b**) Polymerase, and (**c**) Protease-C. The complexes are rotated by 90 degrees in the lower panel

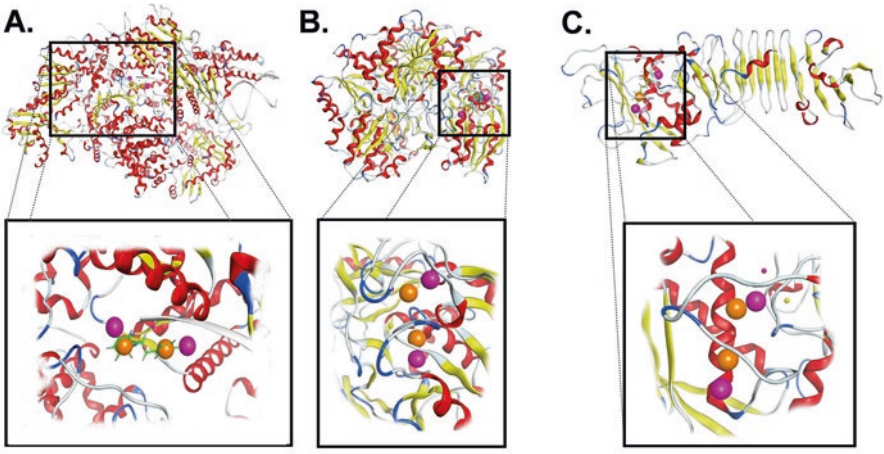

Fig. 2 Pharmacophore modelling. Pharmacophore models are given for each of the three target proteins: (**a**) Helicase, (**b**) Polymerase, and (**c**) Protease-C. The large magenta spheres represent electron accepting groups and the orange ones, aromatic moieties. The little spheres represent pharmacophoric features unique to the given protein. Yellow spheres represent sulfur groups capable of forming interactions of disulfide nature

query can be used to screen virtual compound libraries for novel ligands. Pharmacophore queries can also be used to filter conformer databases, e.g., output from molecular docking runs for biologically active conformations.

The main hindrance in in silico drug design and high-throughput virtual screening is the toxicity and nonspecific binding problem. This issue in the real world eventually escalates and results in resistance to the designed agents. Therefore, there is a dire

need for novel approaches and new strategies to be deployed. In this direction, the pharmacophore modelling refers to the generation of a pharmacophore hypothesis for the possible common binding interactions in a series of particular active sites (Vlachakis et al. 2015). In this study, the three different pharmacophore models were overlaid and reduced to their shared features so that common interactions were retained, in a so-called consensus pharmacophore. Such a *consensus pharmacophore* can be considered as the largest common denominator shared by a set of active molecules. As a result, we opt to eventually screen and discover compounds capable of interacting and inhibiting more than a single target. This novel stratagem is expected to act both in a parallel and serial mode. Blocking multiple targets cumulatively will be more destructive for the survival of the target organism (in this case, Ca. *E. dacicola*), and in case the bacterium establishes some form of full or partial resistance to one of our pharmacological targets, chances are that the other two targets will remain valid. This is based on the function of these three targets and the fact that they are involved in distinct and quite independent cellular pathways (i.e., inhibiting one of the three enzymes does not automatically block the other two).

The commonly reduced pharmacophoric features include two heavy aromatic regions (Fig. 2, orange color) and two hydroxy-like groups (Fig. 2, magenta color). The rest of the pharmacophore features shown in smaller spacefill spheres are unique for the given site and protein, so they were ignored given that they don't satisfy all three targets in this study. Electrostatic surfaces were drawn to be used as a filtering criterion for the screening process. It was found that all three sites share a mainly positively charged binding site (Fig. 3). Consequently, and based on both the electrostatic surface study and the pharmacophore modelling, the ideal compounds should be quite rigid and contain at least two aromatic rings as well as some -OH or -COOH groups. In addition, conserved motifs in each of the three proteins were analyzed by multiple sequence alignment to homologs identified by blast. Blastp was initially used to identify homologous sequences for the three Ca. *E. dacicola* proteins of interest in other organisms. The results were screened for organisms which gave significant hits to all three target proteins. Homologous sequences from 105 organisms were thus chosen and used in multiple sequence alignments. Conserved features were visualized with Jalview software (Supplementary Data 1, 2, and 3).

In conclusion, we present a novel approach for rational drug design against an important agricultural pest. On top of designing specific inhibitors for a certain protein of interest, based on the protein's structure, we combine the common characteristics of three protein active sites to design an inhibitor which targets all three. While this may mean we do not design the absolute best-fit inhibitor for each protein, the combined effect of a pesticide which has multiple targets means more efficient elimination of the pest and a huge reduction in the chance of developing resistance by chance mutations in three separate active sites. At the very least, this approach would greatly delay the development of any resistance and thus extend the effective life of the new compound. This approach will allow us to screen chemical libraries for potential new inhibitors of vital functions of Ca. *E. dacicola*. Specifically targeting Ca. *E. dacicola* in wild populations of *B. oleae* is expected to result in a reduction in the fruit fly's ability to infect unripe olives and will thus greatly reduce their potential

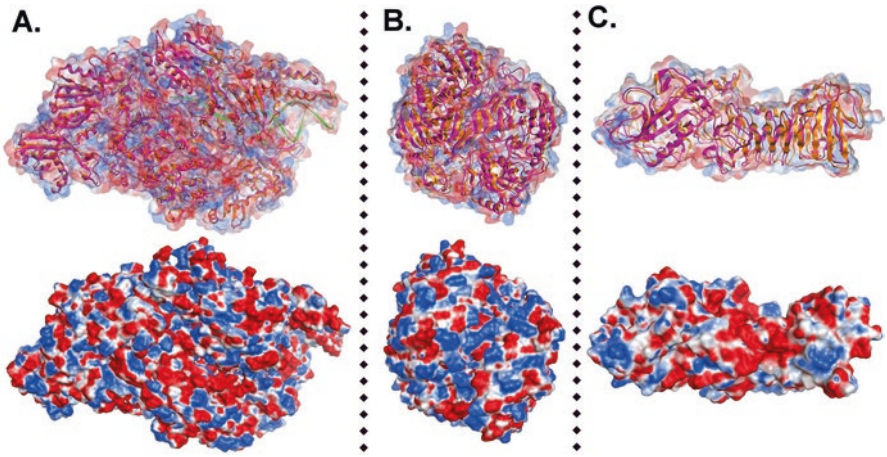

Fig. 3 Electrostatic surfaces. The electrostatic surface study for each of the following targets, (**a**) Helicase, (**b**) Polymerase, and (**c**) Protease-C, shows the area of positive charge in blue, area of negative charge in red, and neutral regions in white

as pests. Our methodology can be applied to other agricultural pests, reducing the chemical burden to the environment. With the recent availability of genomic data for ever-increasing numbers of pathogens and pests, as well as increasing knowledge of the role of the microbiome in the survival of an organism, this combinatorial approach to drug design is an important step forward.

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