Carcinogenic Pesticide Control via Hijacking Endosymbiosis; The Paradigm of DSB-A from Wolbachia pipientis for the Management of Otiorhynchus singularis

THOMAS KOSTAROPOULOS1, LOUIS PAPAGEORGIOU1, SPYRIDON CHAMPERIS TSANIRAS2, DIMITRIOS VLACHAKIS1,3 and ELIAS ELIOPOULOS1

1Genetics Laboratory, Department of Biotechnology, Agricultural University of Athens, Athens, Greece; 2Department of Physiology, School of Medicine, University of Patras, Patras, Greece; 3Faculty of Natural & Mathematical Sciences, King’s College London, London, U.K.

Abstract. Background/Aim: Pesticides have little, if any, specificity, to the pathogen they target in most cases. Wide spectrum toxic chemicals are being used to remove pesticides and salvage crops and economies linked to agriculture. The burden on the environment, public health and economy is huge. Traditional pesticide control is based on administering heavy loads of highly toxic compounds and elements that essentially strip all life from the field. Those chemicals are a leading cause of increased cancer related deaths in countryside. Herein, the Trojan horse of endosymbiosis was used, in an effort to control pests using high specificity compounds in reduced quantities. Materials and Methods: Our pipeline has been applied on the case of Otiorhynchus singularis, which is a very widespread pest, whose impact is devastating on a repertoire of crops. To date, there is no specific pesticide nor agent to control it. The deployed strategy involves the inhibition of the key DSB-A enzyme of its endosymbiotic Wolbachia pipientis bacterial strain. Results: Our methodology, provides the means to design, test and identify highly specific pesticide control substances that minimize the impact of toxic chemicals on health, economy and the environment. Conclusion: All in all, in this study a radical computer-based pipeline is proposed that could be adopted under many other similar scenarios and pave the way for precision agriculture via optimized pest control.

Nowadays cancer has evolved to a worldwide plague; according to the latest statistics cancer is at the top of the list of causes of death worldwide (1, 2). It is a multi-step process (3) associated with genomic instability (4, 5) and caused by a variety of factors and substances, called carcinogens, including radiation, tobacco and alcohol, as well as lifestyle factors including an unhealthy diet, obesity and physical inaction (6-9). Likewise, chemicals of pesticides have been associated with increased incidence of cancer to those who are exposed to them directly or implicitly through their diet or their environment (10). The problem is more intense in agricultural regions where agricultural populations are exposed to an array of chemicals via the extensive use of pesticides and their residues in the ecosystem (11-13). Different studies argue that the substances of pesticides are related to the development of several types of cancer not only in adults but also in children (11, 14).

As it is easily perceivable, cancer-associated pesticides and pesticide residues is a serious health problem. There is a crucial need for the implementation of new strategies for the management of harvest pests by investing in the quality and not in the quantity of pesticides. This study intents to present a different approach concerning the management of Otiorhynchus singularis, a widespread pest of a variety of crops.

Members of genus otiorhynchus (Coleoptera: Curculionidae), commonly known as weevils, are polyphagous pests and they can be devastating for a wide variety of crops worldwide. It has been found that these pests infest approximately 150 plants species (15). Europe and the USA are the most common regions of infestations and it is estimated that there are over 1,000 species in Europe (15). Among them O. singularis (common name clay-colored weevil) is one of the most important in Europe. Adult weevils are nocturnal and they feed on leaves. This may not affect the vivacity of the plants, but concerning ornamental crops this may cause cosmetic damage and consequently reduce their market value (16, 17). Larvae orient
in the soil from July to May and they feed on the plants root system, which could be lethal especially for younger plants or recently transplanted cuttings (15, 18). Established crops are more resistant to O. singularis damage because their root system is fully grown (15, 19).

It is easily perceptible that the problem of weevil infestations has many aspects. First and foremost, there could be a total economic loss for the grower, especially for ornamental crops, horticultural crops and glasshouse crops. Consequently, this has an impact to local communities as agriculturists struggle to control weevil populations and their effect to production.

Previously, several strategies have been used to prevent a weevil infestation or manage an existing one (15, 20-22). Sticky bands on the stems of the plants have been implemented in small-scaled crops to restrict the movement of the adults (15, 20-23). Also, cultural practices such as crop rotation, crop remainder destruction and foliage thinning are recommended for the reduction of O. singularis population (15, 20-22). Regarding chemical control, the first attempts involved metal-containing pesticides which were later replaced by organochlorines (e.g. Aldrin) (15, 20-22). Current control depends on the use of organophosphates pesticides such as imidacloprid and chlorpyrifos (15, 20-22). Both larvicides and adulticides have been developed to control the clay-colored weevil. Larvicides aim the soil-born larvae of Otiorhynchus species and adulticides target the adults throughout the preoviposition periods (15).

Apart from the chemical treatment of the plants, biological agents are applied as an alternative control strategy. Formerly, entomogenous fungi were used to control the weevils and the most efficient infection appeared in the larval stage (15, 18, 20-22). Nowadays, the main biological control measure is entomopathogenic nematodes. The parasites infect all stages of the life cycle of the insect but generally their prime target is larvae (18).

All aforementioned measures and strategies for the control O. singularis have a diversity of shortcomings (24, 25). Cultural measures are often impractical to large-scale cultivations and fail to control weevils efficiently. The use of insecticides includes the risk of toxicity for plants, animals, humans, environmental impacts such as aquifer contamination and ecosystem destruction (15). Nematodes and fungi are eco-friendly and combine effectiveness and safety but their implementation cost is substantial (15). Therefore, there is an exigent need for new, effective and ecological control products against Otiorhynchus species.

Our approach is fixed onto the targeting the virulence – associated Dsb-A protein of Wolbachia pipiensis, which is an endosymbiont bacterium of Otiorhynchus singularis. Wolbachia infects mainly the reproductive tissues of its host and causes a series of phenotypical abnormalities such as feminization, parthenogenesis and male killing (26, 27). Dsb-A is a disulfide oxidoreductase that catalyzes the oxidative folding of disulfide bond containing proteins in the periplasm of many Gram-negative bacteria making them virulence factors (28). Targeting bacteria has many advantages in lab research environment as bacteria constitute a more versatile and easy to handle and manipulate lab model organism, when compared to insects. Furthermore, while little is known about Otiorhynchus, all necessary genetic, structural and molecular datasets are available for the Wolbachia pipiensis bacterium. Herein, a holistic genetic and structural study of the Dsb-A protein of Wolbachia pipiensis is presented, as a novel and specific proposed pharmacological target for the control and pest management of Otiorhynchus singularis.

### Materials and Methods

**Coordinates preparation.** The 3F4T entry of RCSB was used to obtain 3D coordinates (28, 29). This RCSB entry is the X-ray solved, crystal structure of Dsb-A of Wolbachia pipiensis and it is the full length, unbound form of Dsb-A. The resolution of the X-ray structure is 1.85 Å overall. All the important parts of the structure, including the catalytic site and the underlying layer, are very clear in their electron densities. The dimeric form of Dsb-A was used in all calculations in order to carry out this study.

**Sequence database search.** BLAST searches jointly with a mixture of key terms were employed in order to identify homologous Dsb-A protein sequences. The names and/or accession numbers of the characterized Dsb-A, including Ehrlichia sp. (28), Dictyocaulus viviparus, Wolbachia sp. and Anaplasm a phagocytophilum Dsb-A, were used to retrieve their corresponding amino acid sequences from UniProtKB (30). Consequently, these sequences were used as probes to search the non-redundant databases UniProtKB (31) and GenBank (32) by applying reciprocal BLASTp and tBLASTn. This process was reiterated until convergence.

**Phylogenetic analysis.** The retrieved Dsb-A peptide sequences were searched against the InterPro database to identify the boundaries of the catalytic nuclease domain (33). So as to optimize the sequence alignment, the predicted core nuclease domain was excised from the full-length protein and was used in our phylogenetic analysis. Subsequently, these trimmed sequences were progressive aligned using Matlab (34, 35) and the BLOSSUM family scoring matrices (36). The resulting multiple sequence alignment was then submitted to ProtTest3 (37) in order to determine the optimal model for protein evolution. Afterwards, using the Jukes-Cantor pairwise distance method (38) and UPGMA method (39) from the Matlab Bioinformatics toolbox (34), the phylogenetic tree was constructed. Bootstrap analysis (500 pseudo-replicates) was performed to test the robustness of the inferred tree. The phylogenetic tree was visualized with MEGA software circle representation option (40).

**Motif construction.** The phylogenetic tree that derived from the phylogenetic analyses was separated in sub-trees, in order to extract the most highly related protein sequences of the DSB-A protein of wolbachia pipiensis for the conserved motifs exploration (41). The full-length amino acid sequences of the closely related proteins were aligned using Matlab progressive alignment methods (34).
The evolutionary-conserved sequences motifs that were derived from the multiple sequence alignment were identified through the consensus sequence and logo graph where generated using Jalview software (42).

**Homology modelling.** MOE suite was used to carry out the homology modelling of the *Wolbachia* *pipientis* *Curculionidae* Dsb-A enzyme (NCBI: WP_052264650) (43). The crystal structure of the *Wolbachia* *pipientis* of *Drosophila melanogaster* was used as template (RCSB entry: 3F4T) (28). Subsequent energy minimization was performed using the Gromacs-implemented, Charmm27 forcefield and subsequently, models were structurally evaluated using the Procheck utility, as described previously (44, 45).

**Energy minimization.** In order to remove any residual geometrical strain energy minimizations were used in each molecular system, using the Charmm27 forcefield as it is implemented into the Gromacs suite, version 4.5.5 (46-50). All Gromacs-related simulations were performed through our previously developed graphical interface. At this stage, an implicit Generalized Born (GB) solvation was chosen, in an attempt to speed up the energy minimization process (51).

**Molecular dynamics simulations.** The Gromacs suite, version 4.5.5 (46, 48, 49, 52, 53) was employed to subject all molecular systems to unrestrained Molecular Dynamics simulations (MDs). MDS took place in a SPC water-solvated, periodic environment. Water molecules were added using the truncated octahedron box extending 7 Å from each atom. Molecular systems were neutralized with counter-ions as required. Canonical NVT environment conditions were applied at 300 K, 1 atm and a step size equal to 2 femtoseconds for a total 100 nanoseconds simulation time. An NVT

---

**Figure 1.** Dsb-A phylogenetic analysis. Phylogenetic tree of Dsb-A proteins. Colored shapes correspond to different bacteria genera. The length of the tree branches represents the evolutionary distance.
ensemble requires that the Number of atoms, Volume and Temperature remain constant throughout the simulation.

Pharmacophore elucidation. The structure-based pharmacophore module of MOE suite was used for the designing of the Dsb-A specific pharmacophore model for the purposes of this study. In this study, a reduced 3D pharmacophore model for the Dsb-A of Wolbachia pipientis was designed. Several different pharmacophore models for the same active site can be overlaid and reduced to their shared features so that common interactions are retained (41, 54-56). Such a consensus pharmacophore can be considered as the largest common denominator shared by a set of active molecules. Annotation points are markers in space that show the location and type of biologically important atoms and in vivo.  

Figure 2. Sequence motifs. Web logo representation of the conserved motifs of Dsb-A in the bacteria kingdom based on the alignment of the evolutionary study of Figure 1.

Figure 3. Sequence alignment between the Dsb-A Wolbachia pipientis of Otiorhynchus singularis and the Wolbachia pipientis from Drosophila melanogaster. All the structural motifs where marked in the alignment.
groups, such as hydrogen donors and acceptors, aromatic centers, projected positions of possible interaction partners or R-groups, charged groups, and bio-isosteres. Once generated, a pharmacophore 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.

Results and Discussion

At first, an extensive phylogenetic analysis of Dsb-A was performed. In total, approximately 1,000 homologous Dsb-A protein sequences in the genomes of bacteria were identified according to the NCBI taxonomy database (57). This
Table I. summarizes blast searches of all four identified conserved motifs across all species.

| Conserved Motif | Species | Description |
|-----------------|---------|-------------|
| >WP_091877102.1 disulfide bond formation protein DsbA | [Phyllobacterium sp. OV277] | hypothetical protein |
| >WP_046609979.1 | [Neorhizobium galegae] | hypothetical protein |
| >WP_046624228.1 | [Rhizobiales bacterium 63-7] | hypothetical protein |
| >WP_037080398.1 | [Rhizobium vignae] | bond formation protein DsbA |
| >WP_038542085.1 | [Rhizobiales bacterium CCH3-A5] | disulfide bond formation protein DsbA |
| >WP_062580069.1 | [Rhizobiales bacterium CF080] | disulfide bond formation protein DsbA |
| >WP_099057529.1 | [Rhizobiales bacterium Leaf306] | disulfide bond formation protein DsbA |

Continued...
indicates the extensive phylogenetic distribution of the Dsb-A across bacteria (Figure 1). According to Figure 1, Dsb-A sequences from our bacteria-target, Wolbachia pipientis, are placed to the top right of the phylogenetic tree. In addition, there are distinct areas in the tree that represent Ehrlichia sp., Methylobacterium sp., Aeromonas sp., Methylobacterium sp., etc. and the rest can be found in the tree. Evidently, the Dsb-A protein of Wolbachia pipientis is evolutionary ahead when compared to the Dsb-A strains from other bacteria genuses.

The multiple alignment of Dsb-A amino acid sequences provided us with a consensus sequence that led to the identification of four protein motifs, which are represented using sequence logos in Figure 2. These logos depict the invariant amino acid patches in the Dsb-A sequence. The taller letters indicate the most frequent residues at that location and the letters are ordered so the most frequent appears on the top. An interesting observation that emerged from the creation of the web logos is that the fourth conserved catalytic motif (Leu240 to Gly264) represents an a-helix in the secondary structure of the Dsb-A protein (Figures 2 and 3).

Homology modeling methodology was deployed for the establishment of the Wolbachia pipientis Dsb-A 3D model, using the homologous Dsb-A X-ray structure of wolbachia pipientis from Drosophila melanogaster (PDB entry: 3F4T) as a template (Figure 3). In absence of experimental data, comparative modeling based on a known 3D structure of a Dsb-A homologous protein is the only reliable method to capture the structural information. It is obvious from the superposition of the two structures (Figure 4 Right panel) that the model (represented in red colored ribbon), has retained the 3D structural arrangement of its template (green colored ribbon). The model that derived from the homology modeling study provided us with useful insights for the secondary and tertiary structure of the Wolbachia pipientis Dsb-A protein. The four invariant conserved motifs were fitted and highlighted on the Wolbachia pipientis Dsb-A model to reveal their strategic 3D conformational arrangement and their significance and suitability to be proposed as pharmacological targets (Figure 5, Table I). Motifs 3 and 4 are in the inner sides of two interacting a-helices, which establish numerous interactions. In silico mutagenesis to any of the conserved residues resulted in reduced interaction energy and an overall increase in the overall entropy of the system (28).

In an effort to identify structural conserved proteins with similar fold to Dsb-A from Wolbachia pipientis our model was used to design 3D annotation vectors in order to conduct a search into PDB using the implemented domain motif search (DMS), within the molecular operating environment (MOE) suite (58). Structure and protein fold, particularly in the proximity of the active site of enzymes, is often more conserved than a sequence at the one dimensional primary level (47, 58, 59). DMS is a comprehensive 3D vector search across the PDB database, for protein structures with full or partial 3D vector conformational layout to the input vector setup of the Dsb-A query model protein (Figure 6). The DMS study returned 35 proteins with similar fold to Dsb-A of Wolbachia pipientis indexed in the PDB database. Remarkably, although structure in very much conserved, sequence is not conserved at all, as in many cases sequence identity is less than 5% to the Dsb-A of Wolbachia pipientis input query sequence.

Using the Wolbachia pipientis Dsb-A model, MOE’s site finder was employed to detect plausible active sites and cavities in the 3D structure of the Dsb-A of Wolbachia pipientis as promising docking sites for potential inhibiting compounds (Figure 7). A rather large channel of hydrophobic
Figure 6. Structure motif search for the Dsb-A protein. Each structure is replaced by a set of secondary structure vectors in order to enable the search for matches through PDB. Pink vectors represent α-helices and cream vectors represent beta-sheets.
pockets that involve sidechain atoms were detected in the proximity of the conserved motifs that came out of the domain motif search that is described previously. Based on the aforementioned study, we proceeded with the designing of a custom-made and specific 3D pharmacophore model for the Wolbachia pipientis Dsb-A enzyme model. 3D pharmacophore design methods consider the 3D protein structures and the binding modes of receptors and inhibitors, so as to identify plausible sites for a particular interaction between the receptor and the ligand. Regarding the interactions involving the receptor protein and the ligand, the characteristic properties of the inhibitors and their impact on the protein’s active center are taken under consideration. The 3D pharmacophore for Dsb-A enzyme model was established using structural information from the catalytic region of the protein, taking also under consideration all steric and electronic features that are essential to ensure optimal non-covalent interactions with the protein. The 3D Pharmacophore model of the Wolbachia pipientis Dsb-A enzyme revealed a series of pharmacophoric annotation points (PAP) that included several hydrophobic and lone pair regions (Figure 8). The designed 3D pharmacophore model can be an invaluable tool for the high throughput virtual screening of compound libraries with thousands of entries, towards the identification of promising inhibiting agents for the Wolbachia pipientis Dsb-A enzyme with high specificity.

There are many advantages in indirectly controlling an insect via its endosymbiotic bacteria. Firstly, genetically and structurally wise, much more is known about the bacteria rather than the insect itself. Consequently, much more effective and specific agents can be custom designed using state of the art evolutionary and pharmacophore elucidation tools. Moreover, although very hard and impractical to cultivate and biologically evaluate the effectiveness of a series of compounds on the insect itself, it is very easy, cost and time efficient to cultivate the bacteria model in a laboratory. Finally, the toxic load of the compound that will be released in the field is going to be drastically reduced, as
permeability and final inhibition of a bacterial enzyme can be achieved at much lower concentrations, compared to the amount required to kill the insect.

**Conclusion**

All in all, in this study a radical computer-based pipeline is proposed, that could be adopted under many other similar scenarios and pave the way for precision agriculture via optimized pest control. A structural and phylogenetic analysis of the Dsb-A protein of *Wolbachia pipientis* was accomplished through the exploitation of all available biochemical and molecular data and with the use of the essential *in silico* means. The conserved catalytic motif (Leu^{240} to Gly^{264}) that came out of the multiple alignment of Dsb-A amino acid sequences represents an a-helix in the secondary structure of the Dsb-A protein and constitutes a possible locus for future targeting and inhibition of the Dsb-A protein of *Wolbachia pipientis*. Thus, this work discloses insights for the management of *Otiornychus singularis*.

Supplementary data can be downloaded here: [http://www.geneticslab.gr/papers/DSBsupplMat.zip](http://www.geneticslab.gr/papers/DSBsupplMat.zip)

**References**

1 Plummer M, de Martel C, Vignat J, Ferlay J, Bray F and Franceschi S: Global burden of cancers attributable to infections in 2012: A synthetic analysis. *Lancet Glob Health* 4(9): e609-616, 2016.
17 Hirsch J, Sprick P and Reineke A: Molecular identification of larval stages of *otiorrhynchus* (coleoptera: Curculionidae) species based on polymerase chain reaction-restriction fragment length polymorphism analysis. J Econ Entomol 103(3): 898-907, 2010.

18 Yates L, Spafford H and Learmonth S: Apple weevil (*otiorrhynchus cribricollis*) management and monitoring in pink lady™ apple orchards of south-west australia. Plant Protection Quarterly 22(4): 155-159, 2007.

19 Hirsch J, Strohmeier S, Pfannkuchen M and Reineke A: Assessment of bacterial endosymbiont diversity in *otiorrhynchus* spp. (coleoptera: Curculionidae) larvae using a multitag 454 pyrosequencing approach. BMC Microbiol 12(Suppl 1): S6, 2012.

20 Evenhuis HH: Bionomics and control of the black vine weevil *otiorrhynchus* sulcatus. Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent 43: 607-611, 1978.

21 Evenhuis HH: Control of the black vine weevil *otiorrhynchus* sulcatus (coleoptera, curculionidae). Mededelingen van de Faculteit Landbouwwetenschappen, Rijksuniversiteit Gent 47(2): 675-678, 1982.

22 Wilson M, Nitzsche P and Shearer PW: Entomopathogenic nematodes to control black vine weevil (coleoptera:Curculionidae) on strawberry. J Econ Entomol 92(3): 651-657, 1999.

23 Reding ME and Persad AB: Systemic insecticides for control of black vine weevil (coleoptera: Curculionidae) in container- and field-grown nursery crops. J Econ Entomol 102(3): 927-933, 2009.

24 Shah FA, Ansari MA, Prasad M and Butt TM: Evaluation of black vine weevil (*otiorrhynchus* sulcatus) control strategies using metahizium anisopliae with sublethal doses of insecticides in disparate horticultural growing media. Biological Control 40(2): 246-252, 2007.

25 Lola-Luz T, Downes M and Dunne R: Control of black vine weevil larvae *otiorrhynchus sulcatus* (fabricius) (coleoptera: Curculionidae) in grow bags outdoors with nematodes. Agricultural and Forest Entomology 7(2): 121-126, 2005.

26 Walden PM, Halli MA, Archbold JK, Lindahl F, Fairlie DP, Inaba K and Martin JL: The alpha-proteobacteria *wolbachia pipientis* protein disulfide machinery has a regulatory mechanism absent in gamma-proteobacteria. PLoS One 8(11): e81440, 2013.

27 Smith RP, Paxman JJ, Scanlon MJ and Heras B: Targeting bacterial dsb proteins for the development of anti-virulence agents. Molecules 21(7), 2016.

28 Kurz M, Iturbe-Ormaetxe I, Jarrott R, Shouldice SR, Wouters MA, Frei P, Glockshuber R, O'Neill SL, Heras B and Martin JL: Structural and functional characterization of the oxidoreductase alpha-dsbA from *wolbachia pipientis*. Antioxid Redox Signal 17(7): 1485-1500, 2009.

29 Westbrook J, Feng Z, Chen L, Yang H and Berman HM: The protein data bank and structural genomics. Nucleic Acids Res 31(1): 489-491, 2003.

30 Kurth F, Rimmer K, Premkumar L, Mohanty B, Duprez W, Halli MA, Shouldice SR, Heras B, Fairlie DP, Scanlon MJ and Martin JL: Comparative sequence, structure and redox analyses of klebsiella pneumoniae dsb show that anti-virulence target dsb enzymes fall into distinct classes. PLoS One 8(11): e80210, 2013.

31 The UniProt C: Uniprot: The universal protein knowledgebase. Nucleic Acids Res 45(D1): D158-D169, 2017.

32 Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J and Sayers EW: Genbank. Nucleic Acids Res 45(D1): D37-D42, 2017.
