

GUEST-FELLOWSHIP FINAL REPORT

How to make a little worm pump like a big worm

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ABSTRACT

Infections with parasitic helminths expose serious health threats to humans and animals alike. Prevention of disease is dependent on the effective treatment using anthelmintics. Unfortunately, anthelmintic resistance (AR) has evolved in many helminth species during the past decades and meanwhile poses a major constraint to established worm control approaches. This project aimed to improve our understanding of the molecular mechanisms by which helminths, particularly the potentially deadly horse parasite *Parascaris* sp., become capable of withstanding drug treatment. To this end, *Parascaris* P-glycoproteins (Pgp), belonging to an important group of mediators of anthelmintic resistance, were introduced into the model organism *Caenorhabditis elegans* using the CRISPR/Cas9 technology. The resulting transgenic lines will subsequently be analyzed to functionally elucidate the role of putatively AR-associated *Parascaris* Pgp sequence polymorphisms.

1- Introduction

Helminths are one of the most important and prevalent groups of infectious organisms occurring in major parts of the human population as well as in essentially all animals. Depending on the infection intensity, host immune status and constitution as well as helminth species infections may not or only mildly affect the health of the infected individual. However, often helminthoses are associated with severe clinical disease sometimes leading to the host's death. Accordingly, it is a key medical and veterinary objective to prevent the potential human and animal hosts from symptoms caused by helminth infections. Noteworthy, in contrast to other groups of infectious organisms such as viruses or bacteria, for helminths, with only very few exceptions, vaccination is not (yet) a practical option. Instead, a broad range of

highly effective and well tolerable anthelmintic compounds have been developed starting approximately 60 years ago. Among these, the macrocyclic lactones class (MLs) covers the broadest spectrum of helminth species of all anthelmintics and being also the most often used in both humans and animals. Inevitably, their intensive use has over the decades, similar to the bacteria concerning antibiotics, led to the selection of drug resistant helminth populations. The ultimate aim of the project, into which the experiments reported here are embedded, is to improve our understanding of the molecular mechanisms of how helminths exert resistance against MLs. Based on previous work, particularly the P-glycoprotein (Pgp) mediated efflux of MLs through helminth membranes appears of particular interest in this context. P-glycoproteins represent ATP-binding cassette transmembrane pumps, which were found to be associated with decreased drug susceptibility

not only in helminths but also in e.g. insects, fungi, bacteria or cancer cells. Herein, we chose to study the Pgps of the horse helminth *Parascaris univalens*. This intestinal worm mainly leads to severe infections in foals, sometimes causing a complete obstruction of the small intestine if present in larger numbers due to its size of up to 50 cm. Since to date there are no genetic or experimental tools available to specifically modify and examine putative drug-resistance associated genetic changes in *Parascaris* we decided to instead study these by introducing the *Parascaris* Pgp-genes of interest into the model helminth *Caenorhabditis elegans*. The latter is a tiny (1 mm) free living nematode for which a wide range of genetic tools are available. We successfully used this model species in past to study the activity of *Parascaris*-Pgps by extra-chromosomally (over-)expressing them in *C. elegans* Pgp mutant lines (Janssen et al. 2015).

Recently, we were able to demonstrate that the extra-chromosomal expression of *Parascaris* Pgp genes driven by different tissue specific *C. elegans* promoters leads to significant and tissue-specific reductions of susceptibility to MLs (Gerhard et al. in preparation). Following the observation of specific *PunPgp11.1* SNPs only found in phenotypically ML-resistant *Parascaris* populations (Janssen et al. 2013a), we were interested to introduce *Parascaris* Pgp11.1 coding sequences with and without the respective SNPs into the above mentioned tm0333 line.

In a further step by the works performed during this Le Studium guest-fellowship period we aimed to establish *C. elegans* lines transformed by *Parascaris* Pgps with putatively ML-resistance associated single-nucleotide-polymorphisms (SNP) using the recently developed CRISPR/Cas9 technology. This gene replacement strategy has previously been successfully applied at INRAE and was herein supervised by Dr. Abdallah Harmache.

2- Experimental details

To study the function of *Parascaris univalens* Pgp 11.1 gene sequences, we aimed at their heterologous expression in a *C. elegans* mutant

lacking functional Pgp11 (line tm0333). In contrast to our previous studies based on overexpression, we now wanted to conduct chromosomal insertion of the gene into a single, putatively neutral genetic locus. Employing the CRISPR/Cas9 technology and a respective promoter it was aimed to express the Pgps specifically in the intestinal epithelium. To this end (i.e. to obtain proof that the approach works in our hands), we initially transformed tm0333 worms with a construct driving the expression of mCherry, a red fluorescent protein, under an intestinal promoter. A single guide RNA (sgRNA) was generated to allow the cutting of the *C. elegans* chromosome at the selected insertion site. The sgRNA was incubated together with Cas9 protein to form Cas9 ribonucleoproteins (RNPs) and microinjected into the gonads of young tm0333 worms together with a *PunPgp11.1* containing plasmid with flanking homology arms leading to the insertion at the aimed locus by taking advantage of the natural homology-mediated repair system present in all cells. To be able to select transgenic worms, we also included an excisable antibiotic resistance gene as well as a gene leading to the so-called roller phenotype. The integration was subsequently confirmed by PCR-based amplification of the DNA isolated from transformed *C. elegans* lines using a primer binding upstream of the insertion position together with a second primer positioned within the inserted sequence followed by sequencing of the respective PCR products.

3- Results and discussion

A total of five different CRISPR/Cas9 experiments was performed employing two *Parascaris* Pgp11.1 constructs without the ML-resistance associated SNPs and two with these. Furthermore, one transformation with the plasmid encoding for mCherry was conducted. All experiments resulted in worms exhibiting the roller phenotype and showing the expected antibiotic resistance. Offspring of worms transformed using the mCherry-containing construct driving the expected intestinal expression was confirmed using fluorescence microscopy (Fig. 1).

For the confirmation of successful integration in the correct genetic locus a range of PCR primer pairs was designed and tested in several of the obtained *C. elegans* lines. Finally, one of the primer pairs resulted in the generation of fragments which were confirmed by Sanger sequencing to represent the inserted mCherry encoding construct. Unfortunately, due to the Corona crisis inflicted closing of the laboratory on the 17th of March 2020, the respective analyses for the lines obtained from the CRISPR/Cas9 experiments using the Pgp11.1 constructs could not yet be concluded. However, all lines were cryopreserved and will be revived as soon as possible following the end of the current shut-down.

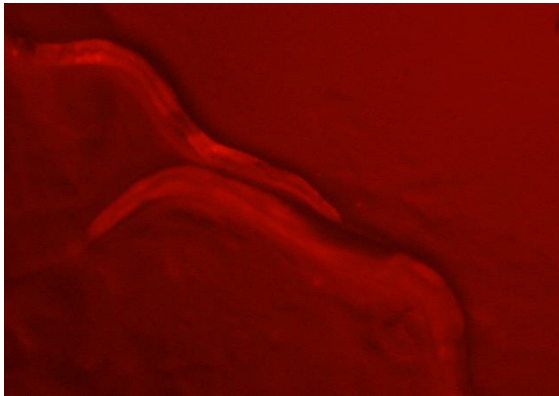


Figure 1. Two tm0333 worms showing intestinal red fluorescence based on expression of mCherry and exhibiting the roller phenotype.

Due to the integration of genes of interest as a single copy in a putatively neutral genetic locus, we expect to obtain an expression which does not exhibit over-expression associated artefacts (e.g. toxicity, nonspecific integration of the expression plasmid leading to unrelated phenotype or mosaic expression of the expression plasmid). This is expected to render best possible comparability concerning the expression of the different *PunPgp11.1* sequence variants used for transformation and their contribution for ML susceptibility.

4- Conclusion

The integration of the mCherry reporter gene and its expression under the intestinal promoter demonstrates that the CRISPR/Cas9 mediated

transformation of the tm0333 line worked in our hands. This gives rise to the hope that this was also achieved with the *Parascaris* Pgp11.1 constructs. Following the awaited respective confirmation of this, the different lines will be used for the analysis of their ML susceptibility in established larval development tests (Janssen et al. 2013b).

5- Perspectives of future collaborations with the host laboratory

We expect to continue our very fruitful collaboration on the functional analysis of *Parascaris* Pgp11.1 sequence modifications in the context of ML-resistance in the near future. Within the planned succession of a soon ending research project funded by the Deutsche Forschungsgemeinschaft, we agreed to also include collaborative research components e.g. on the generation of additional *Parascaris* Pgp-expressing *C. elegans* lines and their characterization using latest state of the art microscopic and molecular approaches (e.g. light sheet microscopy, single cell sequencing). Noteworthy, during this guest-fellowship the first scientific conference of a new research consortium entitled Ascarid Research and Training Initiative (ARTI) was successfully planned and considerable funding obtained by Le Studium and industry (e.g. Boehringer Ingelheim, Germany).

6- Acknowledgements

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7- References

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