Determining Genes Involved in Bacteria-Bacteria Killing in Hawaiian Bobtail Squid Reproductive Symbionts

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Introduction

- Symbiotic bacteria must compete for colonization space in their host organism. The outcome of these competitions shape microbiome communities, which can impact host health and disease. **Although bacteria have evolved many competitive mechanisms, the associated genes and their regulation are often unknown.**
- We use the Hawaiian bobtail squid, *Euprymna scolopes* (**Fig. 1A**), as a model to investigate intrabacterial competition during host colonization[1]. Previous research has shown that the different bacterial taxa in the accessory nidamental gland (ANG, **Fig. 1C-D**) segregate into different tubules (**Fig. 1E**), which could be due to intrabacterial competition.
- To investigate competition between ANG bacteria, we studied ***Leisingera* sp. ANG-M7**, which has been found to inhibit ***Leisingera* sp. ANG-DT** *in vitro,* but the competitive mechanism is unknown.

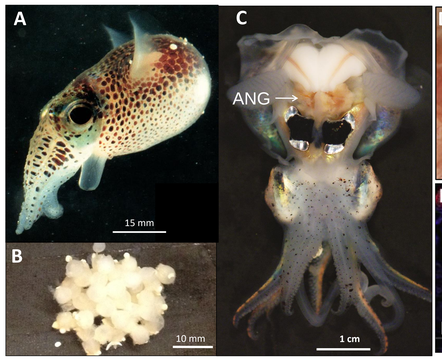


Figure 1. *Euprymna scolopes* female reproductive symbiosis. *E. scolopes* squid **(A)** lay eggs **(B)** that develop for a month on coral rubble. Ventral dissection of a female **(C)** shows the accessory nidamental gland (ANG), which contains a bacterial community in many tubules **(D)**. These bacteria are deposited into the eggs and protect against fungal infections[3]. Fluorescence in situ hybridization of ANG cross sections **(E)** has shown that different groups of bacteria, such as the *Verrucomicrobia* (blue) and *Alphaproteobacteria* (red), colonize different tubules[2].

Objectives

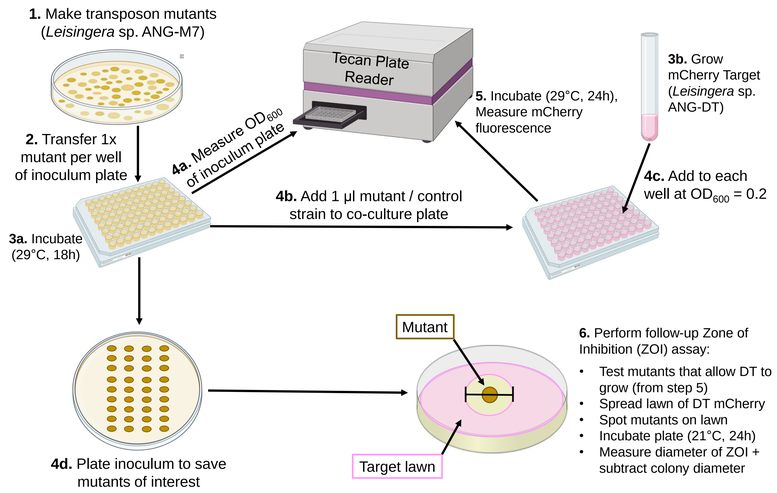
- Use transposon mutagenesis to knock out random genes in "killer" strain, *Leisingera* sp. ANG-M7.
- Screen for loss of killing phenotype in ANG-M7 mutants using liquid co-culture and zone of inhibition assays.
- Sequence "no-kill" mutants to determine which ANG-M7 genes are necessary for killing *Leisingera* sp. ANG-DT.

Methods

Table 1. Strains and plasmids used for co-incubation assays

Strains Used	Antimicrobial activity	Plasmids Used	Plasmid Reference
***Leisingera* sp. ANG-M7**	Killer	pJLS71 (Kan^R)	4
		pEVS170 (Erm^R, Mini Tn5 transposon)	5
***Leisingera* sp. ANG-DT**	Target	pGS002 (Gen^R, mCherry)	6
		pAMS006 (Cm^R, GFP)	This study
***Leisingera* sp. ANG1**	Non-killer control	pJLS71 (Kan^R)	4

96-well co-culture screen of ANG-M7 transposon mutants:



Identification of "Non-killer" ANG-M7 Mutants in Zone of Inhibition (ZOI) Assay

Table 2. Number of ANG-M7 transposon mutants that have passed each step of the screen to identify non-killer mutants

Step in Screen	Number of Mutants
Total mutants screened in 96-well co-culture assay	5,469
Mutants screened in ZOI assay (Passed 96-well screen, allowed ≥70% ANG-DT growth)	277
Mutants passed ZOI screen (Had smaller or no zone of inhibition compared to ANG-M7 wild type)	34
Mutants with transposon location sequenced to date	14

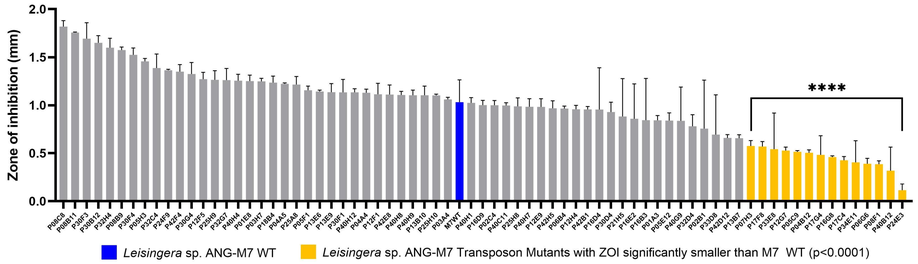


Figure 2. Average diameter of ZOI for the ANG-M7 mutants. The blue bar represents the ZOI diameter of ANG-M7 wild type (WT). The yellow bars represent transposon mutants that had a significantly smaller ZOI compared to ANG-M7 WT based on the results of unpaired t-tests ($p < 0.0001$). Results are from three trials. Gray bars represent transposon mutants that were not selected for further testing.

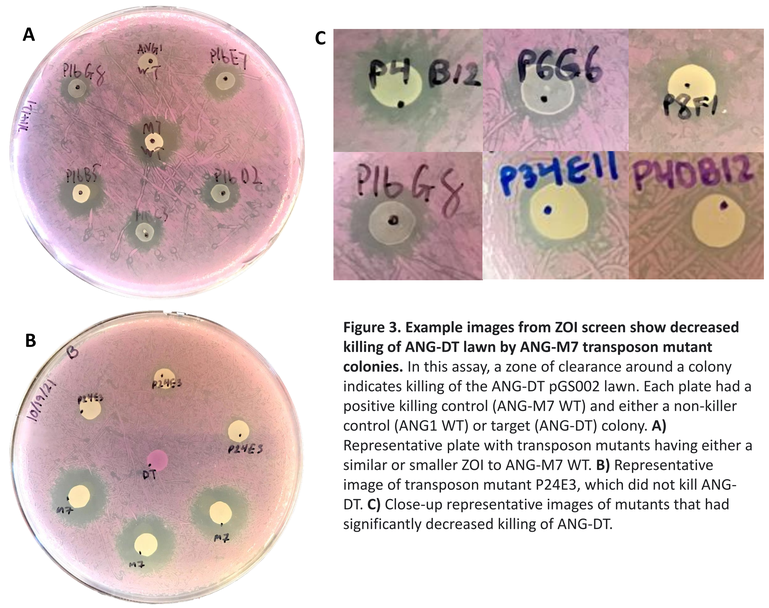


Figure 3. Example images from ZOI screen show decreased killing of ANG-DT lawn by ANG-M7 transposon mutant colonies. In this assay, a zone of clearance around a colony indicates killing of the ANG-DT pGS002 lawn. Each plate had a positive killing control (ANG-M7 WT) and either a non-killer control (ANG1 WT) or target (ANG-DT) colony. **A)** Representative plate with transposon mutants having either a similar or smaller ZOI to ANG-M7 WT. **B)** Representative image of transposon mutant P24E3, which did not kill ANG-DT. **C)** Close-up representative images of mutants that had significantly decreased killing of ANG-DT.

Liquid Coincubations of Mutants & ANG-DT

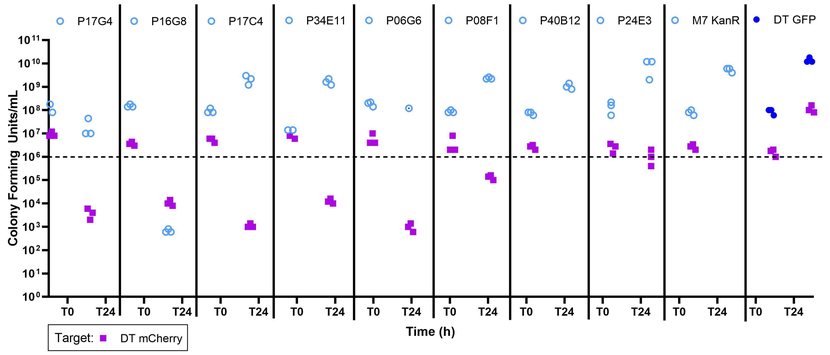


Figure 4. Liquid coincubations in larger volumes than initial 96-well screen show that some ANG-M7 mutants that have decreased killing in ZOI assay can still inhibit growth of ANG-DT. ANG-DT and transposon mutants were incubated for 24 hours at 29°C, 220 rpm. Colony forming units (CFUs) were measured at 0 and 24 hours and can be equated to the number of cells/mL. The dotted line is a reference point for one million CFUs/mL. Transposon mutants are represented by the open light blue circles, ANG-DT by the purple squares. M7 KanR is the positive control for killing (DT was not detected at 24 hours). DT GFP is the negative killing control.

Identification of Mutated Genes

Table 3. DNA sequencing identified the gene disrupted by random transposon insertion in the ANG-M7 genome

Mutant	Mutated Gene
P24E3	Adenosylhomocysteinase, *ahcY*
P16G8	Intergenic region (before rRNA)
P12G7	23S rRNA large subunit
P33E8	Intergenic region after a guanine deaminase and before a long chain fatty acid coA ligase
P33D8	Carbamoyl-phosphate synthase small chain (EC 6.3.5.5) gene (*carA* or *pyrA*)
P2B1	FAD-binding oxidoreductase
P40G9	Hydroxymethylpyrimidine phosphate synthase, *thiC*
P1A3	Large subunit rRNA gene

P24E3 is the only mutant found that does not kill ANG-DT in ZOI or liquid co-culture assay.

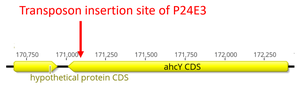


Figure 5. Transposon insertion site of P24E3. DNA sequencing revealed that the P24E3 mutant has a disruption in the adenosylhomocysteinase, *ahcY*, gene. This enzyme catalyzes the following reaction: S-adenosyl-L-homocysteine + H_2O < = > Adensoine + L-homocysteine. Homocysteine is a non-proteinogenic homolog of cysteine.

Conclusions

- This is the first study to introduce genetic tools (fluorescent plasmids and transposon mutagenesis) into bacterial isolates from the *E. scolopes* reproductive system symbiosis.
- A decrease in target strain fluorescence (ANG-DT) in initial screen does not always correlate to a mutant having a decreased zone of inhibition on plates. Only 5% (14/277) of mutants that passed the initial liquid screen also had a significantly smaller ZOI compared to ANG-M7 WT. This could be due to differences in gene expression between growth in liquid vs. agar surfaces.
- A small or no ZOI indicates that there has been a mutation in a gene important to the "killing" behavior of ANG-M7 bacteria. DNA sequencing has allowed us to identify the gene disrupted by random transposon mutagenesis.
- P24E3 mutant showed almost no ZOI and did not kill in liquid coculture. Sequencing results located the mutation in adenosylhomocysteinase (*ahcY*). This same gene mutation has been found in an *Agrobacterium radiobacter* K84 transposon mutant, which has been linked to production of an antibiotic[7].

Future Directions

- *Leisingera* sp. ANG-M7 has a genome size of 4.6 Mb[8], so at least 9,200 mutants must be screened to assume we have screened a mutation in every gene at least twice. More screening will be necessary to confirm genes involved in ANG-M7's antimicrobial pathway.
- To gain a better understanding of ANG-M7's antimicrobial pathway, more sequencing of identified non-killer mutants is necessary. The mutation in adenosylhomocysteinase is linked to decreased methionine production in *A. radiobacter* K84[7] which may be a precursor for antibiotic production, thus future research will determine if methionine will restore the killing phenotype in P24E3.
- ANG-M7 mutants will also be tested against a more diverse range of target bacteria to determine if the killing mechanism has a broad spectrum of activity.

Acknowledgements

This research is supported by NIH NIGMS grant R35 GM137886. A. Suria was supported by the UNC SPIRE IRACDA program. Special thanks to all members of the Septer lab for their assistance in troubleshooting this research and providing helpful feedback.

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