Investigating the ability of *Asaia* sp. to act as a paratransgenic vehicle in the fight against malaria

Graduate Program:
Molecular Biology
and Biomedicine

Supervisor:
Christos Louis
University of Crete,
Department of Biology,
Greece

Elvira Mitraka
September 2010

Table of Contents

Thanks

So many people to thank for this one.

First of all of course I'd like to thank Prof. Christos Louis for giving me this opportunity and for showing me an aspect of biology I hadn't really thought much about before. Great thanks also to Inga Siden-Kiamos for it was she who showed me most of the things I know now. Then, of course, everybody else in the lab, Lefteris, Elena and Rhiannon, without them this undertaking wouldn't have been that much fun. And not to forget the people I first worked with when I came to the lab as an undergraduate student, Pantelis and Manolis.

This project couldn't have been completed without the help of Prof. Fotis Kafatos and George Christophides and everybody else in the lab at Imperial College in London and I am very grateful for their assistance.

I would be amiss if I didn't mention John Vontas and his lab – especially Vaggelio - at the Biology Department of the University of Crete, since they let me borrow their space and lend me their time.

And now to thank everybody that wasn't "directly" involved with this project, my parents, my sister, Chris, George, Emma, Aphroditi, Elpi and Manos. And special thanks to Niki, Rania and Semeli for putting up with me.

Thank you all and hopefully I'll mention in you again in 3-4 years from now…..

Περίληψη

Το ζητούμενο της εργασίας αυτής ήταν να πραγματοποιηθεί μια μελέτη πάνω σε έναν πιθανό φορέα για την μέθοδο της παρα-διαγονικής παρέμβασης στον κώνωπα, με απώτερο σκοπό την αντιμετώπιση της ελονοσίας. Βασισμένοι σε προηγούμενα πειραματικά δεδομένα, όπου έχει αποδειχθεί ότι το βακτήριο *Asaia* sp. έχει την ικανότητα να εδραιωθεί στην μικρο-χλωρίδα του μεσεντέρου του *Anopheles stephensi* και του *Aedes aegypti* (Favia et al., 2007) προχωρήσαμε στην μελέτη της δυνατότητας της εδραίωσης του *Asaia* στο *Anopheles gambiae*, ο οποίος είναι και ο κύριος φορέας της ελονοσίας στην Αφρική.

Συγκεκριμένα χρησιμοποίηθηκε το στελέχο *Ngousso* το οποίο είναι ανήκει καθαρά στην Μ μορφή. Προνύμφες του μολύνθηκαν με το βακτήριο ένα μετασχηματισμένο με το πλασμίδιο *Asaia* το οποίο του προσδίδει τη δυνατότητα να εκπέμπει πράσινο φως υπό φθορίζοντα φωτισμό. Η μόλυνση ήταν επιτυχής και οι αποικίες στο έντερο της προνύμφης ήταν ευδιάκριτες. Το ίδιο φαινόμενο παρατηρήθηκε και στο μεσέντερο του ενήλικου ατόμου.

Μία επιπλέον παρατήρηση ήταν η διαφορά σε ρυθμό ανάπτυξης μεταξύ μολυσμένων και μη μολυσμένων προνυμφών. Οι μολυσμένες με *Asaia* προνύμφες παρουσίασαν μια γρηγορότερη – κατά 1 εώς 2 μέρες – ανάπτυξη, διανύοντας τα τέσσερα προνυμφικά στάδια και φτάνοντας στο στάδιο της νύμφης και του ενηλίκου με ταχύτερο ρυθμό από ότι οι μη μολυσμένες προνύμφες. Στα ενήλικα άτομα δεν παρατηρήθηκε κάποια αναπτυξιακή διαφορά.

Τέλος έγινε μια συλλογή RNA από προνύμφες σε όλα τα στάδια – μολυσμένες και μη - με απώτερο σκοπό να πραγματοποιηθεί μία ανάλυση με microarrays περαιτέρω ανάλυση των αποτελεσμάτων και πιθανή συσχέτιση γονιδίων που ήδη είναι γνωστό πως επηρεάζονται από το *Plasmodium.*

Το *Asaia* sp. δείχνει να παραμένει σταθερό τόσο στα προνυμφικά στάδια όσο και στο ενήλικο στάδιο του κώνωπα. Πολλά ερωτήματα, όπως πιθανές ποινές σχετικά με την ύπαρξης ενός μετασχηματισμένου βακτηρίου στον οργανισμό, παραμένουν ακόμα αναπάντητα. Εάν όμως τα πρώτα αυτά δείγματα θετικών αποτελεσμάτων συνεχίσουν να υφίστανται, τότε το *Asaia* θα μπορούσε να παίξει τον ρόλο του παρα-διαγονικού φορέα κατά της ελονοσίας.

Abstract

The goal of this undertaking was to conduct a research on the topic of a possible vector for a paratransgenic approach in the mosquito, so as to combat malaria. Based on results from other laboratories, where it has been proven that the bacterium *Asaia* sp. has the ability to colonize the micro-flora of the midgut lumen of *Anopheles stephensi* and *Aedes aegypti* (Favia et al., 2007), we moved on to investigating the ability of *Asaia* to colonize *Anopheles gambiae*, which is the major vector of malaria in sub-Saharan Africa.

Due to the speciation event that is happening in the *Anopheles* complex, it was decided that a specific strain of *Anopheles gambiae* was going to be used, namely the *Ngousso* strain, which is M form only. The advantage of this is that the results will be as genetically "clean" as possible.

Larvae were infected with a transformed strain of the bacterium, with a plasmid containing a GFP cassette for easy detection under fluorescent light. The infection was successful and colonies in the gut of the larvae were clearly visible. The same observation was made for the midgut of the adult mosquito.

Another, unexpected, observation was a difference in the developmental rate between infected and non-infected larvae. Infected larvae showed a faster advance in development, ranging from 1 to 2 days, traversing the four developmental stages of larvae and arriving at the pupal stage faster than their non-infected counterparts. No difference in development was observed for adult mosquitoes.

Finally, RNA was extracted and collected from all the different larval stages, both for infected and non-infected, so as to conduct an expression profiling analysis through microarrays for possible correlation of genes which are already known to be affected by the *Plasmodium* parasite.

Asaia sp. seems to remain stable in both larval stages and in the adult stage of the mosquito. Questions regarding any potential fitness costs regarding the presence of the transformed bacteria in the mosquito need to be answered, but if any of those early indications prove to be facts, *Asaia* could fill the role of a paratransgenic vehicle in the fight against malaria.

Introduction

Insect borne diseases cause significant human morbidity and mortality. Current control and preventive methods against vector-borne diseases rely mainly on insecticides. The emergence of insecticide resistance in many disease vectors highlights the necessity to develop new strategies to control these insects. One of the major transmitted diseases is malaria. Each year, there are approximately 350–500 million cases of malaria, killing between one and three million people, the majority of whom are young children in sub-Saharan Africa (Snow et al., 2005). Malaria is naturally transmitted by the bite of a female Anopheles mosquito. When a mosquito bites an infected person, a small amount of blood is taken, which contains malaria parasites. These develop within the mosquito, and about three weeks later, when the mosquito takes its next blood meal, the parasites are injected with the mosquito's saliva into the person being bitten. After a period of between two weeks and several months spent in the liver (depending on the parasite species), the malaria parasites start to multiply within red blood cells, causing symptoms that include fever, and headache. In severe cases the disease worsens leading to hallucinations, coma, and death. Despite intensive efforts in the last few decades, an effective vaccine has not yet been developed (Marquardt et al., 2004).

More recently, a great deal of attention has been devoted to the development of a novel approach, based on the manipulation of bacterial symbionts inhabiting the midgut lumen of the vector, in order to generate symbionts producing anti-plasmodial effector molecules inside mosquitoes (Riehle et al., 2005). Indeed, the midgut hosts the oocyst, the most vulnerable stage of Plasmodium development, which represents a bottleneck of the malaria cycle since only few oocysts develop inside a single mosquito (Ghosh et al., 2000).

Genetic manipulation of bacteria is simpler and faster than genetic manipulation of mosquitoes; bacteria are much easier to introduce into mosquito populations and can be produced easily and cheaply in large quantities. Furthermore, the introduction of modified bacteria into a mosquito population will bypass genetic barriers of reproductively isolated mosquito populations which often occur in endemic malaria. However, there are requirements that must be met for this approach to work for a given vector/parasite combination (Damiani et al., 2010). Six characteristics have

been identified that are necessary for a successful paratransgenic control strategy (Beard et al., 2002):

1. Symbiotic relationship between bacteria and vector
2. Symbiotic bacteria can be cultured and genetically engineered
3. Genetically engineered bacteria should be stable
4. Fitness of the engineered bacteria should not be compromised
5. An effective anti-parasite molecule should be produced by the engineered bacteria
6. An efficient means of distributing the bacteria must be devised

It was first demonstrated by the transformation of the bacterial symbiont, *Rhodococcus rhodnii*, of the kissing bug, *Rhodnius prolixius,* which is a vector of Chagas disease (Durvasula et al., 1997). Chagas disease is a tropical parasitic disease caused by the protozoan *Trypanosoma cruzi*. *T. cruzi* is ingested by *R. prolixus* when the bug consumes a blood meal from an infected vertebrate host. The parasite develops within the midgut and is passed to the next vertebrate host with the feces when the bug defecates during blood feeding. The parasite can enter the vertebrate host when rubbed into the bite, through a wound or mucous membranes. *T. cruzi* is ideally suited to paratransgenic control because *R. prolixus* requires the bacterial symbiont, *R. rhodnii*, in the gut lumen for survival (Baines, 1956; Harrington, 1960). The bacterial symbionts are acquired by first- or second-instar nymphs through ingestion of *R. prolixus* fecal material containing the symbiotic bacteria. Nymphs that do not obtain the bacteria fail to reach adulthood and they usually die after the second instar molt. This absolute requirement for the bacteria, as well as the bacteria's close proximity to the parasite in the midgut lumen, made it an attractive target for genetic modification.

The effector molecule chosen to be expressed and secreted by the symbiotic bacteria was Cecropin A, a 38 amino acid antimicrobial peptide. Cecropin A attacks microbes by inserting itself into cell membranes and forming pores, resulting in cell lysis and death. This peptide efficiently lyses *T. cruzi* cells without affecting the *R. prolixus* host or the symbiotic bacteria (Durvasula et al., 1997).

Recently, symbiotic bacteria from *Anopheles stephensi* were isolated, and one of those was *Asaia* sp (Favia et al., 2007). *Asaia* is a recently described genus consisting of two members, i.e., *A. bogorensis* and *A. siamensis*. This genus is included in the acetic acid bacterial lineage and is phylogenetically closely related to the genera *Acetobacter, Gluconobacter, Acidomonas*, and *Gluconacetobacter*. The natural habitats of *Asaia* spp. have been reported to be in flowers of the orchid tree, plumbago and fermented glutinous rice. Among acetic acid bacteria, the genus *Asaia* differentiates because it does not (or weakly) oxidize ethanol to acetic acid (Yamada et al., 2000).

Asaia has been found stably associated with larvae and adults of *An. stephensi*, dominating the microbiota of the mosquito. PCR analysis showed that *Asaia* DNA is present in eggs, pupae, and different larval stages, as well as in various mosquito organs, including gut, salivary glands, ovaries, and testes (Favia et al., 2007).

The bacteria were successfully transformed with GFP-expressing plasmids and used to re-infect adult mosquitoes through sugar or blood meals, which contained the transformed bacteria. Transformed *Asaia* were found in mosquito organs which are sites for pathogen development, such as midgut and salivary glands, as well as in male and female reproductive tracts although with less efficiency. Vertical and venereal transmission of *Asaia* has been demonstrated by crossing males fed with the GFP-tagged *Asaia* with normal females of *Anopheles stephensi* in the laboratory. After mating, fluorescent bacteria can be detected in the spermatheca and in the terminal portions of the gastrointestinal tract, thus indicating the transmission of the bacterium along with sperm. Furthermore, the vertical transmission of the bacterium to the progeny has also been observed. Progeny resulting from matings between females and males fed with and without GFP-tagged *Asaia* respectively were observed to be colonized by fluorescent cells (vertical or maternal transmission) (Favia et al., 2007).

Asaia exhibits all the required ecological characteristics making it the best candidate, available to date, for the development of a paratransgenic approach for manipulation of mosquito vector competence. Key features of *Asaia* are: (i) dominance within the mosquito-associated microflora; (ii) cultivability in cell-free media; (iii) ease of transformation with foreign DNA and (iv) wide distribution in the larvae and adult

mosquito body, as revealed by transmission electron microscopy, and in situ-hybridization experiments (Favia et al., 2008).

All of the above renders *Asaia* a potentially very useful tool in the fight against malaria.

Materials and Methods

Mosquitoes

Anopheles gambiae mosquitoes from the *Ngousso* strain were obtained from the insectary of Imperial College in London. The strain in London was established through collection at a research facility in Cameroun. The mosquitoes were reared under standard conditions in the insectary at IMBB: 16/8 light/dark cycle, RT=28^{o}C, humidity=80%. There was a slight difference in the rearing conditions of the larvae in the insectary at the Department of Biology of the University of Crete, were the experiments on the treated and non-treated specimen were conducted. The conditions there were: 16/8 light/dark cycle, RT=27 ^{o}C, humidity=60%.

After mosquito eggs were laid they were transferred into a pan containing water and left to rest for 48 hours. Splitting of the large number of 1st stage larvae into different pans containing 50-60 individuals followed. Ground up cat food was sprinkled on the water surface as a food source, three days later additional cat food was added. On day 8 the first pupae appeared which were collected and placed in a water filled cup that was transferred into a mosquito cage. A cotton ball soaked with 10% glucose was placed atop the cage. The soaked cotton ball is the food source for the emerged adult mosquitoes. The collection of pupae was continued for 3 days. Ten days after emergence the cage containing the adult mosquitoes is starved, e.g. the soaked cotton ball was removed from the cage for 4 hours, after which an anesthetized mouse was placed on top of the cage in order for the females to obtain their blood meal. The blood meal lasted for 30 minutes. Two days after the blood meal a water filled cup was placed inside the cage so the females could lay their eggs.

Growing of *Asaia* sp. and colonization of the larval gut

Asaia sp. SF2.1(Gfp) was grown 24h at 30°C in GLY medium. GLY medium comprises of 25 g·liter^{-1} glycerol, 10 g·liter^{-1} yeast extract, pH 5. The glycerol stock was prepared by growing an *Asaia* culture as described above and diluted 1:20 in 25ml of GLY. It was incubated until early log phase (OD_{600}=0.6-0.8). Centrifugation at 1500rpm for 10min at 21^{o}C followed. It was washed twice with 3ml 1xPBS, after that pellet was resuspended in 5ml of 20% v/v sterile glycerol. Another centrifugation at the same conditions followed and the pellet was resuspended in 0.5ml of 20% v/v

sterile glycerol stock. The final solution was aliquoted in eppendorf tubes 50µl each and stored at -20^{o}C.

Asaia sp. were grown in 3ml GLY medium, 50µg/ml of kanamycin was added, together with 15µl of *Asaia* from glycerol stock. It was incubated until it reached OD_{600}=1.2-1.5. Centrifugation at 1500rpm for 10min at 21^{o}C followed. It was washed twice with 3ml 1xPBS. Each larvae pan was filled with 400ml of distilled water and 40 first stage larvae were released in it. The PBS containing the *Asaia* was added into the water along with a small amount of ground up cat food.

Larvae dissection and observation under fluorescent microscope

After 48, 72 and 96 hours 10 treated larvae were removed from the pan and washed twice with 1xPBS to remove any material carried over from the water. They were placed individually on a glass slide and the peritrophic matrix was removed using dissection forceps. The peritrophic matrix was placed on a microscope slide, a small amount of Vectashield from Vector Labs was added and it was observed under fluorescent light, using an Nikon E800 fluorescent microscope.

RNA extraction

RNA extraction was performed using the Qiagen RNeasy kit together with the RNase-Free DNase Set from the same company. The protocol used was: Purification of Total RNA from Animal Tissues.

cDNA creation and RT-PCR conditions

cDNA was created using the ThermoScript RT-PCR System by Invitrogen and using 2µl of RNA.

RT-PCR was performed using the Phusion Hot Start High-Fidelity DNA Polymerase by Finnzymes. 1µl of cDNA and 1µl of S7 primers from a stock solution of 25pmol/µl were used. The nucleotide sequence is as follows:
S7A: GGCGATCATCATCTACGT
S7B: GTAGCTGCTGCAAACTTCGG

Initial denaturation at 98^{o}C for 30 sec was followed by 33 cycles of denaturation at 98^{o}C for 15 sec, annealing at 60^{o}C for 15 sec and extension at 72^{o}C for 2 min. A final extension at 72^{o}C for 10 min was the last step before holding at 4^{o}C.

Results and Discussion

Ngousso strain

It was decided that a specific strain of *Anopheles gambiae* was going to be used, namely the *Ngousso* strain, which is monomorphic for the M molecular form and which was recently established from mosquitoes caught in Cameroun. The advantage of this is that any results obtained could directly be compared to other data previously obtained at Imperial College since the strain is also routinely used by the Christophides lab. The *Ngousso* strain was thus first introduced into the insectary of the IMBB and the insectary of the Department of Biology at the University of Crete; it was acclimated to the local conditions, which to some extent differ from those at the London laboratory. It was readily and successfully established using standard culture conditions. Several generations have now passed and the strain remains extremely stable.

Colonization of mosquito larvae with *Asaia* sp.

The bacterium used to infect the mosquito larvae was *Asaia* sp., strain SF2.1, which had been stably transformed with the pHM2 plasmid, thus having a kanamycin resistance phenotype. In addition a GFP cassette is present in that plasmid for easy detection under fluorescent light (Favia et al., 2007). This particular strain of *Asaia* has been shown previously to colonize *Aedes aegypti, Anopheles stephensi* as well as *Scaphoideus titanus*, and it is therefore an ideal microorganism for potential paratransgenesis uses.

We first wanted test whether symbiosis was true for *Anopheles gambiae* as well, in particular on the *Ngousso* strain. To this end newly hatched 1st stage larvae were infected through feeding with the above mentioned bacterial strain and allowed to reach the pupal stage. The larvae were dissected and observed under a fluorescent microscope.

After 72 hours of growth colonies of *Asaia* were clearly visible in the larval guts (Fig. 1). The colonies were present only in the peritrophic matrix and not in the actual gut of the larvae. Infection was maintained for the whole duration of the larval stages. There didn't seem to be any loss in bacteria numbers (Fig. 2).

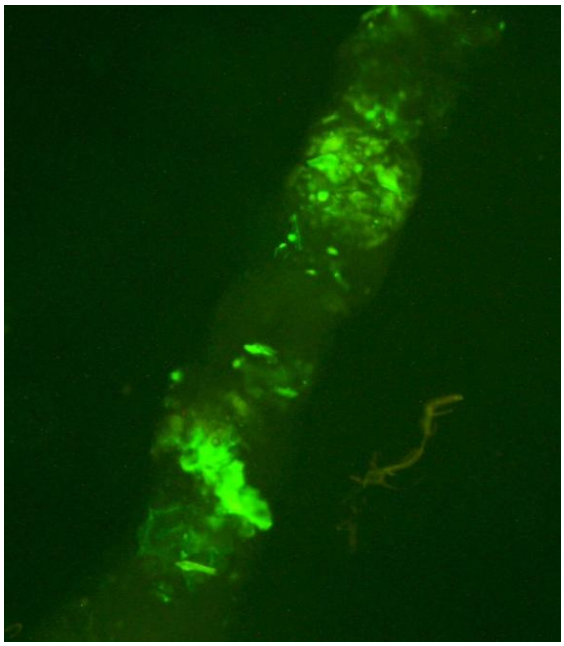

Fig. 1: Peritrophic matrix of an *Anopheles gambiae* larvae 72 hours after infection with *Asaia*.

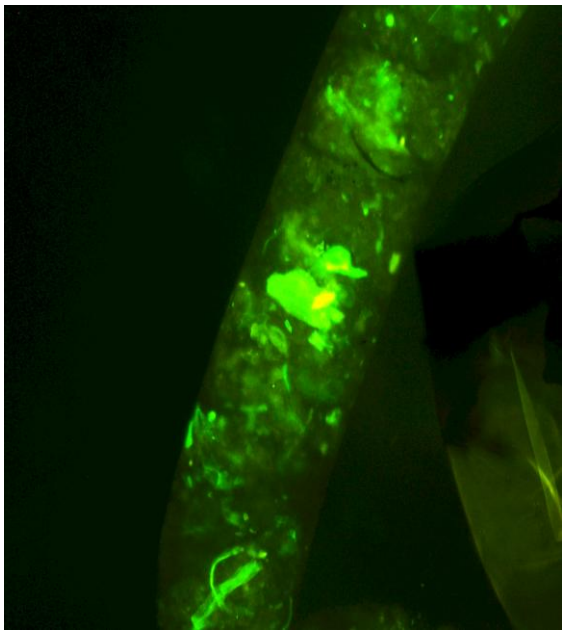

Fig. 2: Peritrophic matrix of an *Anopheles gambiae* larvae 96 hours after infection with *Asaia*.

The above pictures make it obvious that *Asaia* is able to stably colonize the larval gut of *Anopheles gambiae*. The ability of the bacteria to be established in such an early developmental stage of the mosquito is important, because it is easier to manipulate the larval stages than the adult mosquito. If *Asaia* turns out to be a viable paratransgenic vehicle, its introduction in the larval stages will also greatly reduce costs - monetary and environmental -, since a different approach, rather than rearing

thousands of infected adult mosquitoes and releasing them into the environment, will be possible.

Colonization of adult mosquito guts with *Asaia* sp.

In order to expand on those findings, some larvae were allowed to reach the adult stage. Through dissection of the midgut it was shown that the bacteria continued to be present even after the transformation from pupae to adult (Fig. 3). Also, the infected adults kept the infection stable for the whole duration of their lifecycle. *Asaia* colonies were clearly visible from the emergence of the adults up until the 27th day of their lifecycle (Fig. 4), after which they started to perish.

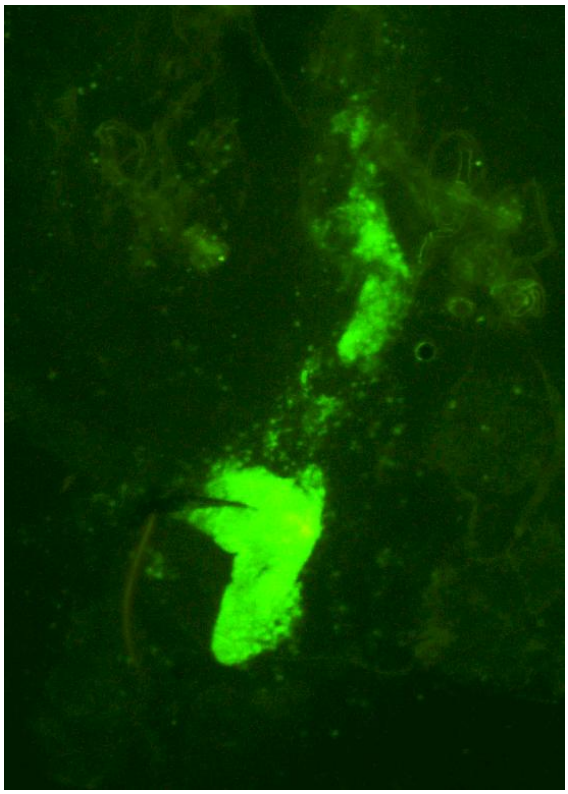

Fig. 3: Midgut of an infected adult mosquito, 5 days after emergence from the pupal stage.

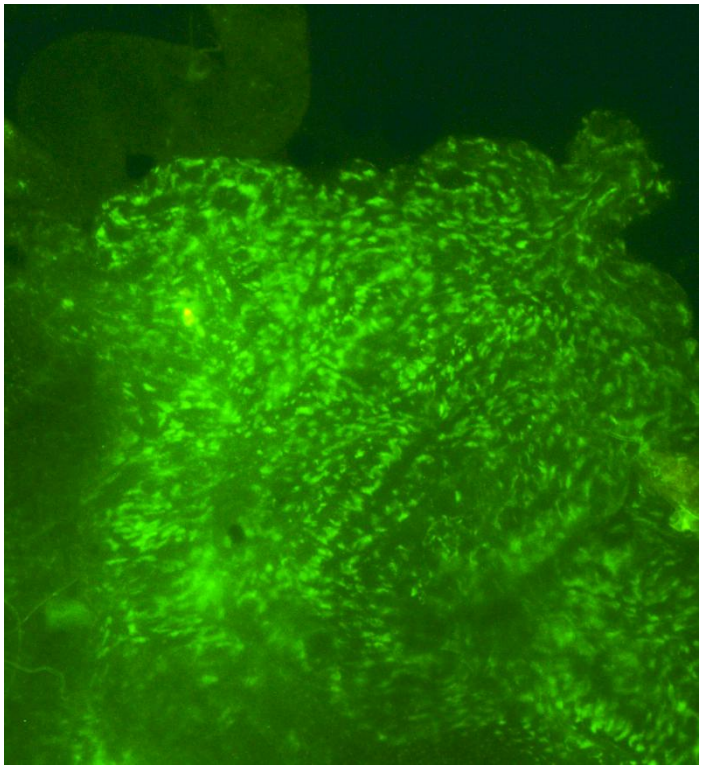

Fig. 4: Detail of the midgut of an adult infected mosquito, 24 days after emergence from the pupal stage.

This shows clearly that *Asaia* is easily transferred through vertical transmission rendering it a potentially valuable candidate for a paratransgenic approach, since after the initial infection is made there is no further need to manipulate the disease vector even more, thus potentially tampering with the delicate balance of the host/parasite relationship between the mosquito and *Plasmodium*.

Taken together with the fact that colonization is stable when introduced in the larval stages, it – again – proves the ability and value of *Asaia* as a potential paratransgenic vehicle.

Side effects from colonization with *Asaia* sp.

Apart from the above another potentially noteworthy observation was made. The infected larvae showed a significantly faster growth and development through the different larval stages. After 48 hours of infection with *Asaia* the infected larvae showed a notable size difference - it was clearly visible to the naked eye - to their non-infected counterparts. The difference in size was maintained throughout the four larval stages (Fig. 5, Fig. 6).

Fig. 5: 72 hours old larvae. The one on the left is infected with *Asaia*, the one on the right is untreated.

Fig. 6: 96 hours old larvae. The left one is untreated, the right one is infected with *Asaia*.

In addition to showing a significant difference in size, the infected larvae also had a more rapid development, reaching the different larval stages at a faster pace than their non-infected counterparts (Table 1).

	Infected	Uninfected
24 hours	1st instar	1st instar
48 hours	2nd instar	1st instar
72 hours	3rd instar	2nd instar
96 hours	4th instar	2nd instar
5 days	4th instar/Pupae	3rd instar
6 days	Pupae/Adults	3rd instar
7 days	Pupae/Adults	4th instar
8 days	Adults	Pupae
9 days	Adults	Pupae/Adults

Table 1: Comparison of development between treated and untreated mosquito larvae.

The rapid growth and development of the infected larvae could be due to the bacteria being a better food source than the normal food the larvae are given. Or it could be due to the fact that the bacteria growing inside the peritrophic matrix are releasing products that are speeding up those processes. Experiments to distinguish if *Asaia* is playing an active role or just providing better nutrition are currently underway.

Since infected larvae show such an advanced development the next logical step was to investigate if the same is true for the adult stages. To this end, bacteria treated

larvae were allowed to survive past the pupal stage and their lifespan compared to untreated ones.

Adult infected *Anopheles gambiae* showed no difference in their lifecycle and duration of their lifespan when compared to their uninfected counterparts. Their size was the same and both groups survived for the same time period (27-30 days).

The above findings combined lead us to the tentative conclusion that whatever role *Asaia* sp. plays, it does not affect adult mosquito life cycle. Further analysis is needed to confirm that indication. This, of course, seems to point to that the bacteria truly are a better food source for the larvae and that their colonization of the peritrophic matrix doesn't play a deeper role in the developmental cycle. Early results of experiments where dead bacteria were given to the larvae proved to be inconclusive.

That the colonization of *Asaia* in the adult midgut doesn't seem to have any effect on the developmental rate of the adult *Anopheles gambiae* could be attributed to the fact that: a) adult mosquitoes don't feed on bacteria, they feed on sugar and the females also need a blood meal to lay their eggs and b) different genes are active during the larval stages than in the adult stage. Those gene products could be targeted by the products of the bacterial colonies in the peritrophic matrix while the organism is in the larval stages.

To this end, as will be mentioned below, a microarray analysis will be conducted, in order to identify the possible up- or down-regulation of genes involved in the developmental process of the mosquito.

RNA extraction and collection

After establishing the fact that *Asaia* can stably colonize the gut of the larvae of *Anopheles gambiae* the next step was to extract RNA from all the larval stages of both treated and untreated specimen. Newly hatched larvae were infected with the bacteria and after 24 hours 10 individuals were selected and their RNA extracted. This was repeated every 24 hours until the pupal stage was reached. The same procedure was repeated for the untreated larvae. In order to confirm the presence of RNA, cDNA was created and then amplified through RT-PCR using S7 primers (Fig. 7). Those are primers for ribosomal tandem repeats that give a product with a

molecular weight of about 200kb, while they give a bigger product when applied on genomic DNA (300 kb). Thus it was used as a means of control.

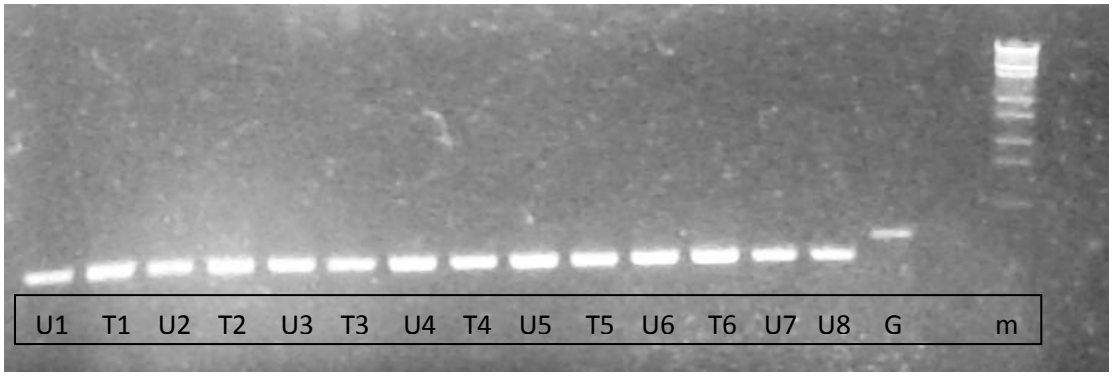

Fig. 7: Agarose gel from PCR product of amplified cDNA created by the extracted RNA from the mosquiro larval stages. U=untreated, T=treated, G=genomic DNA, m=marker, numbers correspond to the day of RNA extraction after the passing of the 24 hours of treatment.

It is clearly shown in the gel that the RNA extraction was a success and no contamination can be detected. The extraction was repeated 3 times so as to collect enough material for further experiments. The next step in that direction is an expression profiling analysis through microarrays. By comparing the two different groups expression levels of genes that are already known to be affected by the malaria parasite or to be part of the immune response against *Plasmodium*, will become clear. Even novel genes, which affect or are affected by *Plasmodium* could be identified and thus become available for further studies.

Conclusion

The ultimate "goal" is the identification of a bacterium that could be symbiotic to mosquito vectors and could then be used as a paratransgenesis vehicle. From the results of previous work (Favia et al,. 2007 and Favia et al., 2009) and the results of the above described experiments, in addition to unpublished data from the lab of Prof. Christophides, the potential of *Asaia* sp., to fill that role is made clearly visible. It fits the first three of the aforementioned criteria that are necessary for a successful paratransgenic control strategy (Beard et al., 2002) and further experiments will investigate if the other three can be met as well, although there are early indications that this is the case (personal communication).

Based on the results reported here, mosquitoes of the genus *Anopheles* could represent another environmental niche for *Asaia* where it lives at a particularly high density in the gut. This acetic acid bacterium could be taken up by mosquitoes from their environment, i.e., from water during the larval stages or from flowers during the first sugar meals as an adult.

Although it is still early to say with any kind of surety, there are many factors that point to the fact that *Asaia* is a stable symbiont of *Anopheles gambiae*. Further, more detailed analysis, of the workings of the bacteria inside the mosquito, both in its larval and adult stages, are needed and are currently underway.

Asaia shows a stable association with larval and adult mosquitoes as witnessed by its presence in all the samples that have been analyzed, which belonged to different generations. Questions regarding any potential fitness costs regarding the presence of the transformed bacteria in the mosquito need to be answered, but if any of those early indications prove to be facts, *Asaia* could fill the role of a paratransgenic vehicle in the fight against malaria.

References

Aksoy, S., Weiss, B., Attardo, G. (2008) “Paratransgenesis applied for control of tsetse transmitted sleeping sickness”. Adv Exp Med Biol 627: 35-48.

Baines, S., (1956). “The role of the symbiotic bacteria in the nutrition of *Rhodnius prolixus* (Hemiptera)”. J. Exp. Biol. 33, 533–541

Beard, C.B., Cordon-Rosales, C., Durvasula, R.V., (2002). “Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission”. Annu. Rev. Entomol. 47, 123–141.

Bourtzis, K. (2008) “*Wolbachia*-based technologies for insect pest population control”. Adv Exp Med Biol 627: 104-13.

Christophides, G.K. (2005) “Transgenic mosquitoes and malaria transmission”. Cell Microbiol. 7 (3): 325-33.

Coutinho-Abreu, I.V., Zhu, K.Y., Ramalho-Ortigao, M. (2010) “Transgenesis and paratransgenesis to control insect-borne diseases: current status and future challenges”. Parasitol Int 59(1): 1-8.

Crotti, E., Damiani, C., Pajoro, M., Gonella, E., Rizzi, A., Ricci, I., Negri, I., Scuppa, P., Rossi, P., Ballarini, P., Raddadi, N., Marzorati, M., Sacchi, L., Clementi, E., Genchi, M., Mandrioli, M., Bandi, C., Favia, G., Alma, A., Daffonchio, D. (2009) “Asaia, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders”. Environ Microbiol 11 (12): 3252-64.

Dale, C., Moran, N.A., (2006) “Molecular interactions between bacterial symbionts and their hosts”. Cell 126 (3): 453-65.

Damiani, C., Ricchi, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., Capone, A., Ulissi, U., Epis, S., Genchi, M., Sagnon, N., Faye, I., Kang. A., Chouaia, B., Whitehorn, C., Moussa, G.W., Mandrioli, M., Esposito, F., Sacchi, L., Bandi, C., Daffonchio, D.,

Favia, G., (2010). “Mosquito-Bacteria Symbiosis: The Case of *Anopheles gambiae* and *Asaia*”. Microb. Ecol. (ahead of print).

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Aksoy, S., Merrifield, R.B., et al., (1997) “Prevention of insect-borne disease: an approach using transgenic symbiotic Bacteria”. Proc Natl Acad Sci USA. 94 (7): 3274–8.

Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E., Marzorati, M., et al. (2007) “Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector”. Proc Natl Acad Sci USA 104: 9047–9051.

Favia, G., Ricci I, Marzorati, M., Negri, I., Alma, A., Sacchi, L., Bandi, C., Daffonchio, D. (2008) “Bacteria of the genus *Asaia*: a potential paratransgenic weapon against malaria”. Adv Exp Med Biol 627:49–59

Ghosh, A., Edwards, M.J., Jacobs-Lorena, M., (2000). “The journey of the malaria parasite in the mosquito: hopes for the new century”. Parasitol. Today 16, 196–201.

Greenwood, B. (2009) “Can malaria be eliminated?”. Trans R Soc Trop Med Hyg 103 Suppl 1:S2-5

Harrington, J.S., (1960). “Studies on *Rhodnius prolixus*: growth and development of normal and sterile bugs, and the symbiotic relationship”. Parasitology 50, 279–286.

Irvin, N., Hoddle, M.S., O'Brochta, D.A., Carey, B., Atkinson, P.W. (2004) “Assessing fitness costs for transgenic Aedes aegypti expressing the GFP marker and transposase genes”. Proc Natl Acad Sci USA 101 (3): 891-6

Ito, J., Ghosh, A., Moreira, L.A., Wimmer, E.A., Jacobs-Lorena M. (2002) “Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite”. Nature 417 (6887): 452-5.

Li, C., Marrelli, M.T., Yan, G., Jacobs-Lorena, M. (2008) “Fitness of transgenic *Anopheles stephensi* mosquitoes expressing the SM1 peptide under the control of a vitellogenin promoter”. J Hered 99 (3): 275-82.

Lycett, G.J., Kafatos, F.C. (2002) “Anti-malarial mosquitoes?”. Nature ;417 (6887): 387-8.

Marrelli, M.T., Moreira, C.K., Kelly, D., Alphey, L., Jacobs-Lorena, M. (2006) “Mosquito transgenesis: what is the fitness cost?” Trends Parasitol. 22 (5): 197-202.

Marquardt, W.H. (2004). “Biology of disease vectors”. Academic Press, 2nd edition.

Moreira, L.A., J. Ito, A. Ghosh, M. Devenport, H. Zieler et al. (2002) “Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes”. J. Biol. Chem. 277: 40839 - 40843.

Moreira, L.A., Wang, J., Collins, F.H., Jacobs-Lorena, M. (2004) “Fitness of Anopheline Mosquitoes Expressing Transgenes That Inhibit Plasmodium Development”. Genetics 166 (3): 1337-41.

O’ Brochta, D.A., Handler, A.M. (2008) “Perspectives on the state of insect transgenics”. Adv Exp Med Biol 627: 1-18.

Ren, X., Hoiczyk, E., Rasgon, J.L. (2008) “Viral paratransgenesis in the malaria vector *Anopheles gambiae*”. PLoS Pathog 4 (8): e1000135.

Riehle, M.A. and Jacobs-Lorena, M. (2005). “Using bacteria to express and display anti-parasite molecules in mosquitoes: current and future strategies”. Insect Biochem Mol Biol. 35 (7): 699-707

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y., Hay, S.I. (2005). "The global distribution of clinical episodes of Plasmodium falciparum malaria". Nature 434 (7030): 214–7.

Yamada, Y., Katsura, K., Kawasaki, H., Widyastuti, Y., Saono, S., Seki, T., et al. (2000). “*Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the *alfa-Proteobacteria*”. Int J Syst Evol Microbiol 50: 823–829.

Zieler, H., Keister, D.B., Dvorak, J.A., Ribeiro, J.M. (2001) “A snake venom phospholipase A(2) blocks malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface”. J Exp Biol. 204 (23):4157-67.