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► To cite this version:

Bruno Lapied, Cedric Pennetier, Véronique Apaire-Marchais, Patricia Licznar, Vincent Corbel. Innovative applications for insect viruses: towards insecticide sensitization. Trends in Biotechnology, 2009, 27 (4), pp.190-198. 10.1016/j.tibtech.2008.12.005 . hal-03248998

HAL Id: hal-03248998

<https://hal.science/hal-03248998>

Submitted on 10 Jun 2021

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Where do we stand in innovative vector control strategies?

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Abstract

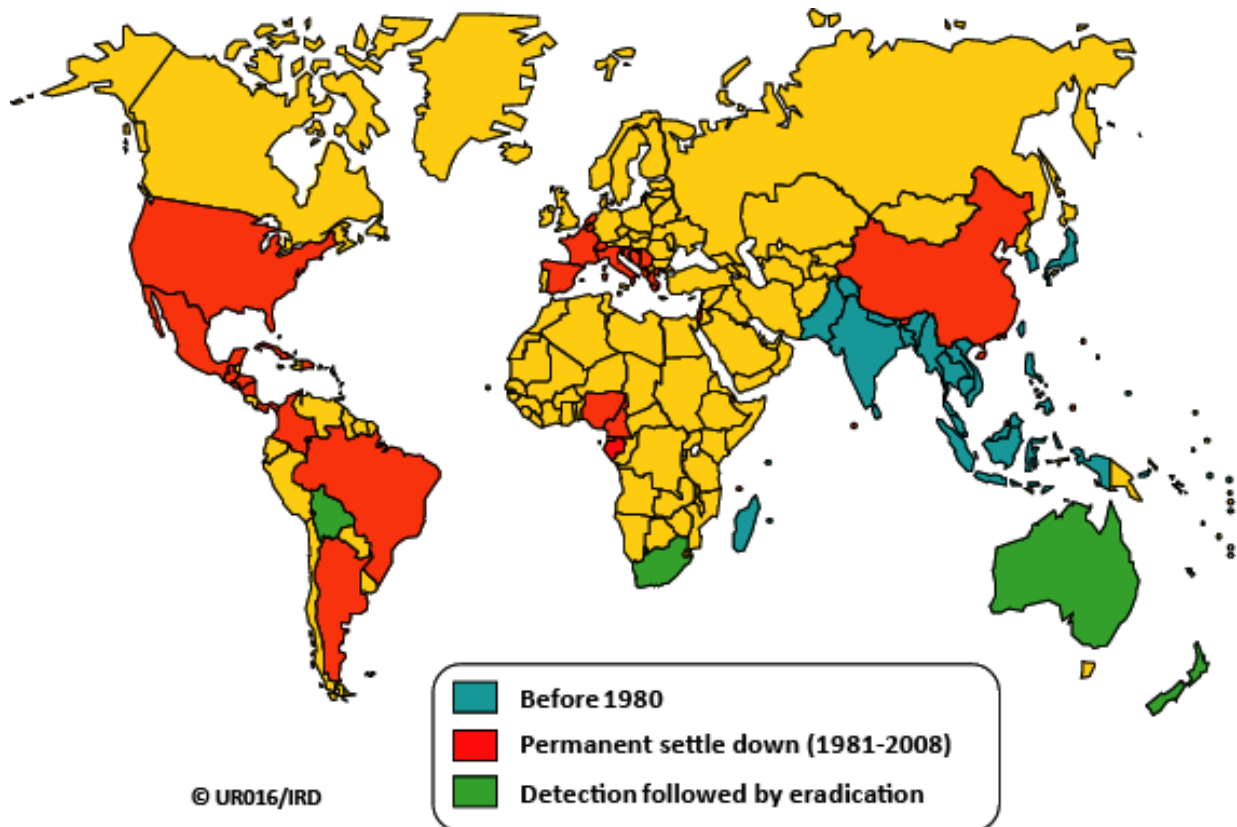
The effective management of emerging insect-borne disease is dependent on the use of safe and efficacious chemical insecticides. Given the inherent ability of insects to develop resistance, it is essential to propose innovative strategies since insecticides remain the most important element of integrated approaches to vector control. Recently, identified intracellular phosphorylation and dephosphorylation of membrane receptors and ion channels targeted by insecticides, have been described as a new process to increase the sensitivity of insecticides. Consequently, an efficient method consists to infect host insects by microencapsulated-recombinant virus expressing specific protein phosphatase and/or kinase, known to regulate specific insecticide-sensitive targets. This attractive strategy leading to obtain more sensitive insect, will help to reduce doses of insecticides while increasing efficacy of treatments.

Introduction

Emergence / resurgence of vector-borne diseases

The last 25 years of the 20th century has seen drastic changes of ecologic, climatic, demographic and economic determinants that promote the emergence and resurgence of vector-borne diseases which account for around 17% of the estimated global burden of infectious diseases [1]. While some newly recognized insect transmitted diseases have emerged, e.g. Lyme diseases or West Nile encephalitis [2], the biggest problem comes from the resurgence of well known diseases that are once effectively controlled, e.g. malaria, leishmaniasis, Rift valley fever, dengue and chikungunya. In Europe, several arthropod-borne diseases have recently emerged, i.e. the tick-borne encephalitis transmitted by *Ixodes sp.* [3], the Bluetongue virus transmitted by *Culicoides* species [4], the West Nile and Chikungunya viruses transmitted by the mosquitoes *Culex* and *Aedes*, respectively [5,6]. Climate changes partly explain this trend as it contributes to increase the average exposure of human populations to vector-borne diseases by changing the geographical distribution of conditions that are suitable for the vectors and disease pathogens [7]. Establishment of the Asian tiger mosquito *Aedes albopictus* in several European countries as well as the steadily increase of imported cases of *Aedes*-borne viruses such as dengue and chikungunya [5] raise the question of risk of tropical diseases becoming now established and spreading in temperate regions (Box 1).

Box 1. Emergence and spreading of Asian Tiger mosquito vector



Recently, several vector-borne, parasitic or zoonotic diseases have (re)-emerged and spread in the northern hemisphere with major health, ecological, socio-economical and political consequences. Among them, the establishment and spread of the Asian tiger mosquito *Aedes albopictus* is of great concern [41]. *Aedes albopictus*, originating from Asia, has been first described by Skuse in 1894, and has recently spread to all continents [5]. In the USA, the species have been imported at the beginning of the 1980's from South-East Asia and/or, more probably from the northern region of Japan. It was first recorded in Europe in Albania in 1979. Then, *Ae. albopictus* was introduced in Italy through shipments of used tyres from USA. It has quickly spread across the country, since the first record of a breeding population in the outskirts of Padova (Veneto region) in 1991, showing a great ability to adapt to different ecological situations. In Rome, *Ae. albopictus* has encountered particularly favourable

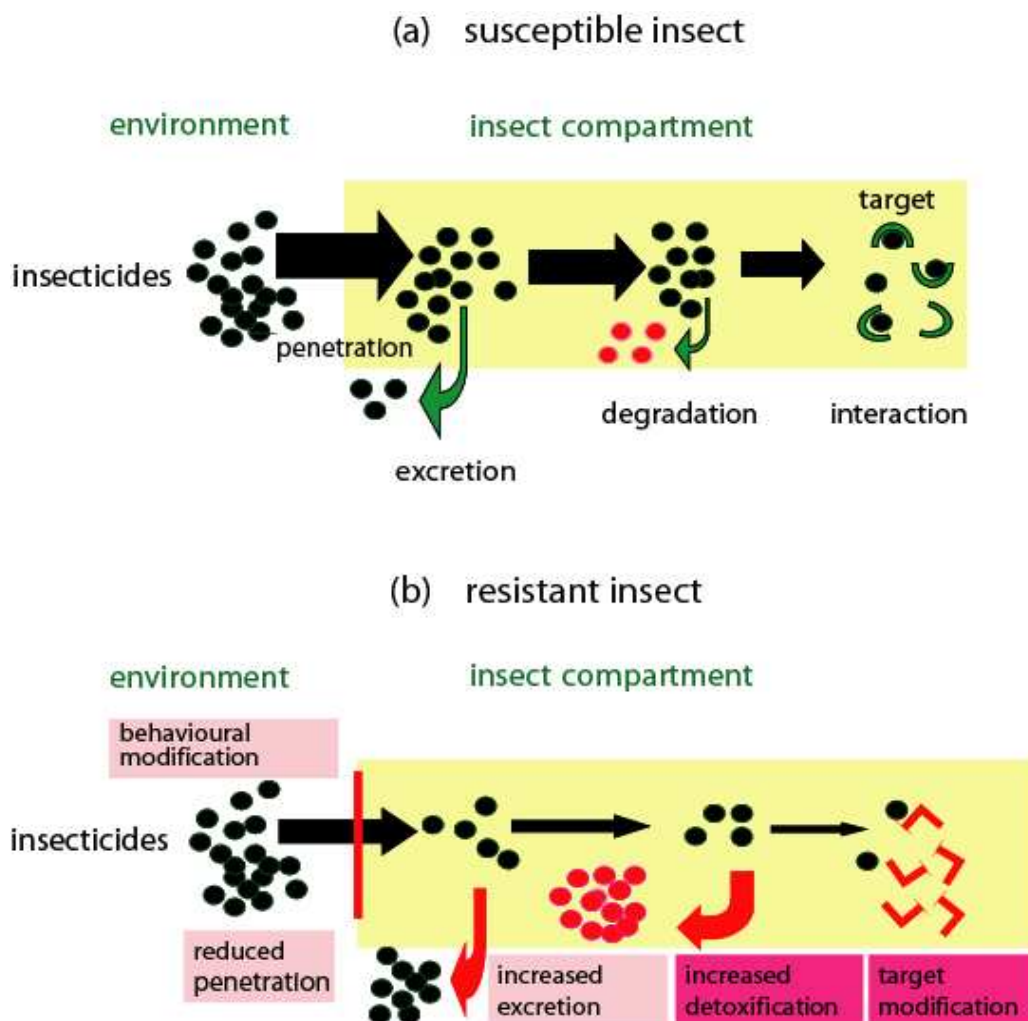
conditions. Since the first record in the capital city in the 1997, the species have colonised the entire metropolitan area, despite efforts to block its spread. It is now present in all European countries around the Mediterranean Sea, particularly abundant in Italy and is becoming a major nuisance in South-East France, close to the Italian boarder since 2005 [5]. Based on genetic algorithm (GARP) modelling system, Benedict and colleagues [42] recently predict a global invasion of *Ae. Albopictus* throughout the world, especially in Central American and sub-Saharan African countries which show high numbers of potential suitable niches for this species. A major outbreak of chikungunya has occurred in the Indian ocean between 2004 and 2007, causing more than 3 million cases in Kenya, the West Indian Ocean Islands, India, Indonesia. In 2007, a limited chikungunya outbreak has also been recorded in Italy with 205 human cases reported [6], which show evidence that northern hemisphere is also at risk for arthropod-borne diseases [43].

Insecticide resistance mechanisms

Despite advances in therapeutic and vaccinal researches, the control of vector borne diseases is still dependent to a large extent on vector control. However, force is to note that few vector control strategies are, to date, implemented with sustainable success. Failures are generally attribute to lack of knowledge on insect vector bio-ecology and vector-pathogen-human interactions as well as unsuited vector control strategy in partly due to the development of resistance to an increasing number of insecticides [8]. Indeed, due to the widespread use of chemicals for agricultural practises and/or domestic hygiene since the 60s, insecticide resistance has developed in most of insect vectors of diseases including mosquitoes [9]. Resistance mechanisms can be divided into two groups, metabolic (alterations in the levels or activities of detoxification proteins) and target site (non-silent point mutations within

structural receptor genes) (Box 2, Table 1). Increased expression of the genes encoding the three major xenobiotic metabolizing enzymes (the cytochrome P450 monooxygenases, glutathione transferases (GST) and carboxy/cholinesterases (CCE)) is the most common cause of insecticide resistance in insects. Genomic analysis of detoxification genes using detox-chip microarrays in the mosquitoes *Anopheles gambiae* [10] and *Aedes aegypti* [11] reveals an abundance of genes belonging to CYP P450s (e.g. CYP4, CYP9, CYP6, CYP12), CCE and GST families that are over-expressed in resistant mosquitoes compared with their susceptible counterparts. However, higher gene expression in insecticide-resistant colonies does not necessarily guarantee relevance to insecticide resistance. Up to now, only CYP6 P450s genes are clearly involved in cellular mechanisms known to metabolize DDT and pyrethroid insecticides in dipterans [12,13] and lepidopterans [14]. Further experimental validation including enzyme characterization and RNAi interference are necessary to conclusively incriminate candidate genes in insecticide metabolism and resistance. In other hand, point mutations in receptor genes e.g. the well known Knock-down resistant mutations (*kdr*), insensitive acetylcholinesterases (*Ace*) and GABA receptor (*Rdl*) changes confer cross-resistance to all chemical classes acting on the same target sites [9]. Alone or in combination, these two groups of mechanisms confer resistance, sometimes at an extremely high level, to all available insecticide classes including the organochlorates, the organophosphates, the carbamates and the pyrethroids.

Box 2. Schematic representation of the diversity of insecticide resistance mechanisms in insects.



To be effective, an insecticide have to enter in contact with an insect, penetrate into its body, move to its target and then interact with it **(a)**. Any event/mechanism that blocks one of these events can lead to resistance **(b)**. The first mechanism is avoidance of the insecticide, which can be genetically determined or acquired by a learning process after previous contact with the toxic chemical. Insects can avoid eating toxic plants as soon as they are able to detect them visually, olfactory or via contact. Contact avoidance can involve different behaviours. In many cases, genetically determined oviposition behaviour prevents females from laying eggs on unsuitable breeding sites. Insects can also escape to an insecticide-free site by shifting their resting behaviours, or by exploiting the host in different conditions [44]. After a sufficient

contact (or an ingestion), the insecticide can be readily excreted or metabolized before excretion. Detoxification metabolism involves a variety of enzymes that could be induced after contact with the insecticide [44,45]. Metabolic resistance often results from the overproduction of 'detoxification enzymes' that can metabolize insecticides. This mechanism could be associated with phenotypic plasticity, as the production of detoxification enzymes is usually induced by the presence of insecticides in the environment of the insect. However, resistance to insecticide might also be the consequence of specific mutations in genes encoding enzymes, enhancing their catalytic activity toward plant toxins [44].

Finally, mutation in the target of the insecticide can reduce or eliminate its deleterious effects. In public health, the main target site mutations reported and that are alarmingly spreading among different mosquito vector species are the *Kdr* (mutations on sodium channel gene) and *Ace1^R* (mutations on the acetylcholinesterase gene) [46,47].

Table 1. Insect resistance mechanisms to the main insecticide families used in Public health

Insecticides	Molecular target	Resistance mechanisms		References
		target site	enzymatic mechanisms	
Pyrethroids, type I	sodium channel	Kdr and super Kdr mutations	monooxygenases + esterases	[9,48,49]
Pyrethroids, Type II	sodium channel	Kdr mutations	monooxygenases + esterases	[9,48,49]
Organochlorates	sodium channel	Kdr mutations	GS-transferases + monooxygenases	[9,48,49]
N-alkyl-amides	sodium channel	no resistance reported against insect of public health importance		
Organophosphates	acetylcholinesterase	Ace1 ^R mutation	esterases + GS-transferases + monooxygenases	[9,46,50-54]
Carbamates	acetylcholinesterase	Ace1 ^R mutation		[9,46,50-54]
Neonicotinoids	nicotinic acetylcholine receptor	not reported	monooxygenases	[55,56]
Spinosad	nicotinic acetylcholine receptor	not reported		
Cyclodienes, Lindane, Bicyclic phosphates	GABA receptor	Rdl mutation	GS-transferases	[49,57]
Phenylpyrazoles	GABA receptor	Rdl mutation	GS-transferases	[49,57]
Avermectines	GABA receptor	undiscribed	monooxygenases + esterases	[49,57]
Insect Growth Regulators	Ecdysone agonist/disruptor or inhibitor of ATP synthase, chitin biosynthesis or lipid synthesis	no resistance reported against insect of public health importance		
Bacillus thuringiensis var. israelensis	microbial disruptors of insect midgut membranes	reported against Culex pipiens s.l. but non discribed		[58]

Current strategies for vector control

The arsenal of safe and effective insecticides has dwindled considerably due to environmental and toxicological considerations. Economic factors have also limited investment into research and development of new compounds and their applications for control of public health pests and vectors. For these reasons, the management of vector populations and the strategies used to slow down the evolution of pesticide resistance are currently based on an optimal use of existing compounds that are acting on different target sites [15,16]. In public health, appropriate rotations of different insecticides have allowed to reduce the pressure of selection on black flies larvae and have maintained an effective and sustainable control of the river blindness burden in West Africa for more than 30 years. In mosquito control, the use of i) “two-in-one” combination of different chemicals such as insecticides (pyrethroids, organophosphates or carbamates) with repellents (e.g., DEET or Icaridin) applied to bednets [17-19] or ii) the use of systematic rotations and mosaics for Insecticide Residual Spraying [15] have shown promising results for malaria vector control and offer a potential tool for resistance management. However, some questions remain concerning the residual activity of these “cocktails” (e.g. insecticides may have different decay rate), their cost-effectiveness and their potential toxicity for humans. In the same context, genetic control of insect pests is an old concept that has been the subject of a renewed interest in public health since the last 10 years [20]. In theory, genetic control of vectors has many advantages compared to classical insecticide use. In this case, no chemical are used and compounds are not released in the

environment. Furthermore, this genetic strategy is often specific for species. Two main approaches are currently under development: i) the release of genetically modified vectors which are unable to transmit diseases (e.g. malaria or dengue) to humans and ii) the sterile insect technique (SIT). Unfortunately, few studies have shown evidence for effective and sustainable control of mosquitoes using this latter technique [21]. If the use of transgenic mosquitoes appears promising for controlling vectors of human diseases [22], there are also several constraints in routine including: identification of genes of interest, mosquito transformation, gene expression, mosquito production, development of genetic drive mechanisms, fitness of modified mosquitoes, mosquito diversity at population and species levels, and last but not least risk management and acceptability by populations.

Virus as innovative vector control strategies

Viruses as bioinsecticides

Among insect pathogenic viruses, baculoviruses and densoviruses are those that offer real potential as biological control agents of insects. Densoviruses have been isolated in seven different insect orders to date including *Diptera*. They are small DNA viruses that belong to the family *Parvoviridae*, non-enveloped viruses and relatively stable in the environment. Despite their high virulence and infectivity for natural hosts, densoviruses are not known to infect mammalian cells. Many insect hosts of densoviruses, particularly mosquitoes or cockroaches, have medical or economic importance, but few of densoviruses have yet been tested in biological control [23,24].

Viruses that have been researched with the greatest biological potential are baculoviruses. Indeed baculoviruses are pathogenic for insect predominantly of the orders *Lepidoptera*, *Hymenoptera* and *Diptera*. The family *Baculoviridae* comprises

two genera based on occlusion body morphology: the *Nucleopolyhedrovirus* (NPV) and the *Granulovirus* (GV). The NPVs produce large occlusion bodies (also called polyhedra or occluded virus or OV) containing many nucleocapsids (multiple NPV or MNPV) surrounded by a matrix composed mainly of polyhedrin whereas GVs produce small granular occlusion bodies (granules) that normally contain a single virion surrounded by the granulin structural protein (only found in the *Lepidoptera*). The OVs are ingested by insects, dissolved by the alkaline pH of the midgut and they release occlusion derived viruses (ODVs) which enter midgut cells and initiate viral replication. Budded virus (BVs) are produced during an early stage of infection. They are responsible for the systemic spread of the virus within an infected insect. Late gene expression (polyhedrin and p10) leads to production of another phenotype named occluded virus (OV) which is involved in spread of the virus from insect to insect and in survey of viruses in environment

Baculoviruses are usual vectors for the expression of a variety of recombinant proteins in insect larvae or insect cells. Since they are highly selective for insect species and do not replicate in vertebrates and in plants, they are biopesticides particularly attractive. Natural baculoviruses (both NPVs and GVs) have been registered, and successfully used against Lepidopteran pests, especially for the protection of soybean and forests in South and North America [25-27]. Despite their potential, use of viruses as bioinsecticides has been limited by their slow speed of kill (days to weeks between virus application and insect dead). During this time, the insects can still cause significant damage and the cost of such damage limits the commercial use of virus insecticides. Because natural viruses are imperfect insecticides, they are often genetically modified. Several approaches have been used for producing genetically enhanced virus insecticides with reduced kill time.

Very few recombinant densoviruses have been constructed for biological control. The major limitation of these viruses as expression vectors is the small size of their genome that is imposed by the geometry of the particle. Moreover all of the viral gene products are necessary for viability. So the size of the inserted DNA foreign sequence is limited. However, recombinant genomes can be replicated and packaged into virions in the presence of helper virus making transducing particles [23]. Recently, the first densovirus (AgDNV) has been isolated in *Anopheles gambiae*. Using a two-plasmid helper-transducer system, recombinant AgDNV was able to transduce expression of a foreign gene [28]. The production of recombinant densoviruses would lead to genetically modified mosquitoes and raise the same questions and constraints as for SIT, particularly fitness of modified mosquitoes, mosquito diversity population and acceptance of citizens.

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) has the greatest potential for development of recombinant baculoviruses as bioinsecticide. It has the widest known host specificity among baculoviruses, infecting more than 30 species of Lepidopteran insects [29]. Moreover insects such as mosquito and cells of non permissive insect cell line have been found to incorporate baculovirus [30]. Many advances lead to generate a large number of recombinant baculoviruses that show variable improvements in speed of kill, in comparison to the wild type parent baculovirus. The insecticidal activity of wild-type baculoviruses is frequently improved by inserting a foreign gene that exhibit insecticidal activity, classically under strong polyhedrin or p10 promoters from the very late phase of infection. Recombinant baculoviruses have been constructed to express insect-selective toxins (from scorpions, mites, spiders, sea anemones, *Bacillus thuringiensis*), hormones (diuretic hormone, neuropeptid hormone, juvenile hormone esterase (JHE) and eclosion

hormone (EH)), or enzymes (proteases) They have been shown to improve significantly insecticidal efficacy and trials have demonstrated that recombinant baculoviruses are competitive with fast-acting chemical insecticides [25,26,31,32]. Recombinant baculovirus insecticides have been tested in field in the United States, in the United Kingdom and China. However, none have been registered for commercial use in these countries [33].

Because of the development of resistance and adaptation mechanisms to chemical insecticides by insect borne-disease vectors, we must explore modern and innovative approaches to control mosquitoes. Baculovirus system of expression of foreign genes has many advantages over other systems: production of very high levels of foreign genes, expression of more than one gene, accommodation of large pieces of foreign DNA, propagation in insect cell lines. Application of this strategy to increase mosquito sensitivity to chemicals is a novel modern alternative method to decrease chemical pesticide rates.

Modification of insecticide targets using recombinant insect virus to increase mosquito sensitivity to chemicals

It is well known that voltage-dependent sodium channels that play fundamental role in the electrical activity of excitable cells, together with acetylcholine receptors and GABA receptors that are crucial elements of excitatory and inhibitory synaptic transmissions and acetylcholinesterase involved in the hydrolysis of acetylcholine within the synaptic cleft are one of the most important targets for neuroactive insecticides [34,35]. However, their important use in the last 20 years has led to the

development of resistance in many insect species particularly those of medical importance (see Box 2). In this context, new insect-control compounds that are active against resistant pest strains have been developed. But some studies also report the emergence of insect insensitivity strains to these new compounds. Very recently, the intracellular regulation of plasma membrane receptors and/or ion channels targeted by insecticides have been described as a new process to affect the sensitivity of insecticides [36]. Evidence that the posttranscriptional modifications such as RNA editing on insect GABA receptors, which induce a decrease in fipronil sensitivity, has been reported [37]. Furthermore, an initial search for patterns of conserved amino acid residues associated with phosphorylation sites in voltage-dependent sodium channel alpha subunits and in nicotinic acetylcholine receptor subunits shows that they possess potential phosphorylation sites for PKA, PKC, CaM-Kinase and PKG. In the last case for instance, it has been clearly demonstrated in insect neurons, that elevation of intracellular cAMP concentration via

Figure 1 near here

a calcium-CaM-sensitive adenylate cyclase, modulates the functional properties of the nicotinic acetylcholine receptor by activating PKA. This phosphorylation, which play important role in modulating nicotinic acetylcholine receptor opening, affects the mode of action and efficiency of neonicotinoide insecticides [38]. In the same way, an increase in intracellular calcium concentration results in the formation of the complex calcium-CaM which activates the CaM-Kinase. The overexpression of the latter affects inactivation properties of the insect voltage-dependent sodium channels which thereby increases by 1000-fold the sensitivity of sodium channels to oxadiazine insecticides (personal data). From these results, it appears that identified intracellular regulatory

mechanisms of insect targets have fundamental consequences for the sensitivity of targets for insecticides. This opens an exciting research area to use better effective insecticide. Based on these findings, it is possible to propose such strategy (activation and/or overexpression of phosphatase and kinase) to increase mosquito sensitivity to chemical insecticides.

To reach this objective, an original strategy consists of construction of recombinant baculoviruses expressing enzymes to modify insecticide targets. Recombinant baculoviruses are traditionally generated by replacing the polyhedrin gene with a foreign gene through homologous recombination. A rapid and efficient method (available commercially from Invitrogen) is based on the production of recombinant virus by site specific transposition in *Escherichia coli*. There have been extensive molecular studies performed with this engineering on NPV due to the availability of a variety of permissive cells [30]. Recently, the same approach has been performed on GV [39].

This tool is a very attractive strategy to obtain recombinant AcMNPV (Figure 1): gene of interest (kinase or phosphatase) is inserted into a donor vector downstream from the polyhedrin promoter flanked by the left and right arms of Tn7. Then, transformation is performed in *E. coli* DH10Bac cells carrying a baculovirus shuttle vector (AcMNPV bacmid) which contains a mini-F replicon, an antibiotic resistance marker, and the attachment site for the bacterial transposon Tn7 (mini-*att*Tn7), all inserted into the polyhedrin locus of AcMNPV. The mini-Tn7 element on the donor plasmid transposes to the target bacmid in *E. coli* when Tn7 transposition functions are provided by a helper plasmid. Recombinant bacmid DNA is used to transfect insect cells. The polyhedrin protein is not essential for virus replication because the

budded virus is the infectious morphotype. With this rapid method, pure stocks of recombinant virus are obtained within 7 to 10 days.

Then our original aim is to infect mosquito neuron cells with these recombinant viruses in order to overexpress kinase or phosphatase *in vitro*. Then, recombinant baculoviruses lead to the overexpression of enzymes involved in intracellular regulatory pathway known to affect plasma membrane protein target. Using recombinant baculoviruses associated with an insecticide will allow a shift to the left of the concentration response curve. Finally, this strategy leads to both reduction of doses of insecticides and increase the efficacy of treatments.

Appropriate formulation method for recombinant baculovirus: microencapsulation

Pesticide microcapsule formulations are used to reduce mammalian toxicity and extend activity, to control evaporation, to protect pesticide from rapid environmental degradation, to reduce leaching and to reduce pesticide levels in the environment. Microencapsulation is a technology that allows sensitive compounds to be physically enveloped into a protective matrix also named polymeric encapsulating agent such as for instance Eudragit®, polyacrylates, cyclic acrylate, ethylcellulose used alone or in combination, in order to protect these compounds from adverse reactions. Although microencapsulation is an emerging sector, less is known about microencapsulated insecticidal pathogen for application to vector control strategies. However, toxicity enhancement via successful delivery insecticidal pathogen (fungal, bacterial or viral) is one of the most exciting alternative ways of insecticide delivery research. In the case of vector control strategies, use of recombinant baculoviruses as bioinsecticide may

consist of spreading microencapsulated-recombinant viruses in aquatic breeding sites (Figure 2). Formulations that microencapsulate the virus to a matrix (e.g., lignin-encapsulation and derivatives), have previously been used to minimize loss of activity due to, particularly, UV radiation [40]. However, because viruses need to be ingested to cause infection of the host, the appropriate formulation to control the release of the active virus with an optimal efficiency must be designed and evaluated. The first challenge will be to develop appropriate formulation that encapsulate the virus. This involves i) suitable active ingredients of polymeric materials that do not dilute or dissolve in water, rain and heavy dew, ii) polymeric material-based adjuvant, iii) size of formulated inoculum particles, since relatively small granule (around 400 μm) used to encapsulate insect pathogens can only be ingested by larger insects, iv)

Figure 2 near here

types of ions (e.g., Mg^{2+}) into formulations that would be more effective regardless of the water quality, v) volume of microcapsule and virus dosage, vi) time of exposure. Infection of larvae and adult mosquitoes occurs following ingestion of microencapsulated virus-contaminated water. In the context of recombinant baculoviruses, as the gene of interest disrupts polyhedrin gene, recombinant baculoviruses will not produce polyhedrin matrix involved in survey of viruses in the environment. Thus, microencapsulation will protect recombinant-virus expression vectors allowing extended activity once ingested. Another challenge will be to obtain sufficient quantities of functional proteins from recombinant-virus in infected insect organisms. Various approaches to increase production of properly processed functional proteins have been proposed. A number of studies have already

documented enhanced protein production following co-transfection with baculoviruses expressing chaperone proteins, which are known to aid in the folding of newly synthesized proteins. However, irrespective of the protein being produced, a major advantage of the recombinant baculovirus-insect cell expression system is the ease scale-up from the laboratory to a large-scale production system.

Concluding remarks

Innovative, safe and cost-effective measures are urgently needed to ensure an effective management of vectors (and the diseases they transmitted) and to delay the selection of resistance genes that shorten the useful life of conventionally used pesticides. These diseases are in expansion throughout the world and changes of geographical distribution of vectors raise the risk of establishment and spreading of these tropical diseases in northern hemisphere.

Knowledge of the regulatory intracellular pathways in insect shows that the phosphorylation/dephosphorylation processes strongly increase sensitivity of insect ion channels and receptors targeted by insecticides. These novel data allow us to consider development of new strategies to control the mosquito vectors. Recombinant viruses overexpressing kinase and/or phosphatase provide an attractive tool for controlling mosquito-borne diseases. Even if recombinant AcMnPV is today the best candidate as bioinsecticide, discovery and genetic manipulation of an *Aedes*-specific baculovirus should be the ideal tool. Indeed, the best virus candidate has to be host-specific with a limited persistence in environment. This implies to microencapsulate recombinant virus to protect the virus against environmental conditions without risk for the environment. The use of these bioinsecticides will allow an increase of sensitivity

of mosquitoes and a reduction of the rate of chemical pesticide that are known to have negative environmental, human health and ecological effects.

Acknowledgements

The authors would like to thank Pierre Olivier Peccoz for technical assistance. This work was financially supported by the Agence Nationale pour la Recherche (ANR, programme REAC SEST06 030 01).

Figure legend

Fig. 1: Overall procedure of modification of insecticide targets using recombinant insect virus. **(a)** Recombinant bacmids are constructed by transposing a mini-Tn7 from a recombinant plasmid containing the gene of interest to the mini-*att*Tn7 attachment site on the bacmid with Tn-7 transposition functions provided in *trans* by a helper plasmid. **(b)** Purified bacmid DNA was transfected into insect cells. **(c)** Recombinant viruses were obtained and amplified to produce a virus stock. **(d)** Mosquito neurons were infected with recombinant baculoviruses in order to overexpress enzymes to modify insecticide targets such as receptor or ion channel. **(e)** In mosquito neuron, recombinant baculoviruses associated with an insecticide allow a shift to the left of the concentration response curve.

Fig. 2: Microencapsulated recombinant viruses as innovative vector control strategy.

(a) untreated area: a given insecticide dose is applied to kill larvae, nymphs or adult mosquitoes. **(b)** pretreated area with microencapsulated recombinant virus: a lower insecticide dose is enough to kill contaminated organisms.

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Figure 1

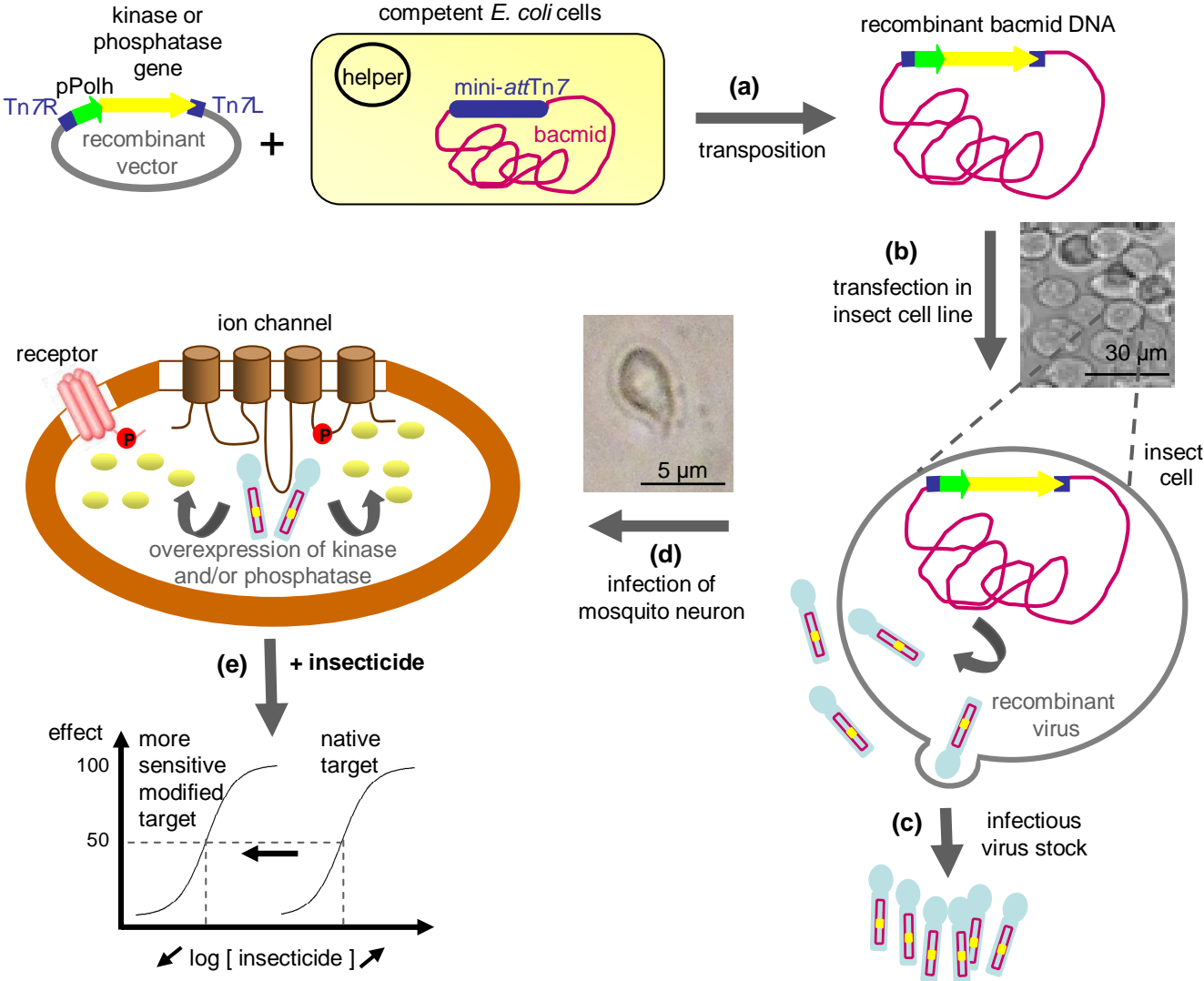


Figure 2

