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NEW FACTS AND TRENDS IN BACTERIOLOGICAL CONTROL OF MOSQUITOES

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As soon as Pasteur established his own Institute in Paris (1888), he looks also forward and his genious mind enterprised to create oversea Centers for protecting the health of other countries people, especially in tropical countries. That is the way how several other Pasteur Institutes have been created in the world, beginning with the Pasteur Institute of Saigon, the first one to be founded, depending on Dr Calmette then a young physician who was sent there by Pasteur in 1891. It was followed by about thirty other Institutes which still exist and are still devoted to the control of human diseases.

Major diseases in Tropical countries are, beside now the Sida, those ones transmitted by vectors, mainly insect vectors like mosquitoes and blackflies. Examples are malaria, yellow fever, dengues, encephalitis, lymphatic filariosis and onchocerchiasis (also called river blindness). In 1976, the WHO (World Health Organization) launched a large Programme of Research and Formation on the main Tropical diseases, in cooperation with the UNDP (United Nations Development Programme) and the World Bank. It included multiple aspects of reseārch and control such as diseases prevention by the control of vectors. Indeed in most cases it is the only way to fight the disease because of the lack of efficient vaccines or drugs. As a WHO Collaborating Center, my laboratory is involved in this Programme.

BIOLOGICAL CONTROL OF INSECT VECTORS

For insect vectors control, beside chemical insecticides, exist biological insecticides the main advantage of which being their safety for the non target organisms and environment, safety which derived from their specificity. Many biological agents are potential tools for controling insect vectors of tropical diseases. They can be found among bacteria, virus, fungi, protozoa, nematodes or other insects (parasites and predators). Even fishes have been used, against mosquito larvae. Among all these organisms, an outstanding role is given to bacteria belonging to the genus *Bacillus*, especially *B. thuringiensis* and *B. sphaericus*. The first one is commercialized since 1980 and used worldwide against mosquito and blackfly larvae, the second one is on the way.

Ten years ago, discovery (Goldberg & Margalit, 1977) and characterization (de Barjac, 1978) of the mosquito larvae killer *B.t.i. (Bacillus thuringiensis israelensis)* was a real breakthrough in the biological control of insects, and brought more to the fame of *B. thuringiensis* species than 30 years of application in agricultural and forest areas. The highest potency and specificity of *B.t.i.*, its striking speed of action, its consequence on general health care pushed forward the entire microbial control of insect vectors of human diseases.

THE TWO LINES OF RESEARCH

Two lines of research are prevalent. One consists in the search for new microorganisms isolated from natural breeding sites or samples of insects. The other one relies on genetic manipulation of already known microorganisms. This later approach got success in the cloning of genes coding for specific toxins, and expression of some of these genes into bacteria other than the parent ones, or into plants.

For instance, toxin-coding genes of *B.t.i.* (Plant Genetic System, 1987) or *B. sphaericus* (Tandeau de Marsac et al., 1987) have been cloned and expressed in cyanobacteria which serve as food for mosquito larvae. Once established in breeding sites of mosquitoes, such transgenic cyanobacteria could be a permanent source of toxins. Presently, there is a flourishment of genetic studies in many laboratories from Universities and Industries in

order to improve the potency of *B. thuringiensis* and *B. sphaericus*. Various goals are searched for: either an increase in the yield of toxins produced, or modification in the toxins secretion (like production of soluble components), or protection of the toxins against environmental degradation, or enlargement and change in spectrum of action. As everyone knows, release of such genetically transformed microorganisms in nature arise many problems.

THE ISOLATION OF WILD BACTERIAL STRAINS FROM NATURE

Coming back to the first research line, e. g. isolation of wild strains, great progress has also been made. Beside *B.t.i.*, several other *B.t. (B. thuringiensis)* strains have been proved larvicidal for mosquitoes. That infirms the so long-lasting theory of *B.t.* Lepidoptera specificity which prevailed till the *B.t.i.* H14 discovery. Classification of *B.t.* is now enlarged to 25 H serotypes (Table). *B.t.* strains such as PG14 belonging to serotype H8a8b or 73E10-2 belonging to serotype H10 are quite potent against mosquito larvae. (PG14 is as toxic as *B.t.i.*, but 73E10-2 is less toxic). A systematic screening of more than 1,000 *B.t.* isolates conducted in my laboratory showed that various *B.t.* strains belonging to serotypes H1, H3a3b, H9 and H10 are also very toxic to mosquito larvae (Jelusic & de Barjac, not published).

TABLE

Classification of *Bacillus thuringiensis* strains according to the H serotype

H Serotype	Biotype	Serovar
1	I	*thuringiensis* (Heimpel & Angus, 1958)
2	II	*finitimus* (id.)
3a	III 1	*alesti* (id.)
3a, 3b	III 2	*kurstaki* (de Barjac & Lemille, 1970)
4a, 4b	IV 1	*sotto* (Heimpel & Angus, 1958)
4a, 4b	IV 1'	*dendrolimus* (Talalaev, 1956)
4a, 4c	IV 2	*kenyae* (Bonnefoi & de Barjac, 1963)
5a, 5b	V 1	*galleriae* (Shvetsova, 1959)
5a, 5c	V 2	*canadensis* (de Barjac & Bonnefoi, 1972)
6	VI	*subtoxicus* (Heimpel & Angus, 1958)
6	VI'	*entomocidus* (id.)
7	VII	*aizawai* (Bonnefoi & de Barjac, 1963)
8a, 8b	VIII 1	*morrisoni* (id.)
8a, 8c	VIII 2	*ostriniae* (Ren Gaixin et al., 1975)
8b, 8d	VIII 3	*nigeriensis*
9	IX	*tolworthi* (Norris, 1964)
10	X	*darmstadiensis* (Krieg, de Barjac & Bonnefoi, 1968)
11a, 11b	XI 1	*toumanoffi* (Krieg, 1969)
11a, 11c	XI 2	*kyushuensis* (Ohba & Aizawa, 1979)
12	XII	*thompsoni* (de Barjac & Thompson, 1970)
13	XIII	*pakistani* (de Barjac, Cosmao, Shaik & Viviani, 1977)
14	XIV	*israelensis* (de Barjac, 1978)
15	XV	*dakota* (De Lucca, Simonson & Larson, 1979)
16	XVI	*indiana* (id.)
17	XVII	*tohokuensis* (Ohba, Aizawa & Shimizu, 1981)
18	XVIII	*kumamotoensis* (Ohba, Ono, Aizawa & Iwanami, 1981)
19	XIX	*tochigiensis* (id.)
20a, 20b	XX 1	*yunnanensis* (Wan-yu, Oi-fang, Xue-ping & You-wei, 1979)
20a, 20c	XX 2	*pondicheriensis* (de Barjac et al., 1987)
21	XXI	*colmeri* (De Lucca, Palmgren & de Barjac, 1984)
22	XXII	*shandongiensis* (Wang Ying et al., 1985)
23	XXIII	*japonensis* (Ohba & Aizawa, 1986)
24	XXIV	*neoleonensis* (Rodrigues et al.)
25	XXV	*coreanensis* (de Barjac et al.)

N. B.: a variety (non motile): *wuhanensis* (Hubei Inst. Microbiology, 1976).

Depending on the strains, specificity looks different. Some strains are more toxic on *Aedes* sp., other ones on *Anopheles* sp. or *Culex* sp. and they can be also toxic or not toxic to Lepidoptera like *Anagasta kuhniella* or *Spodoptera littoralis*. This specificity is a fascinating problem, probably related with multiplicity of the bacterial toxins and with specific receptors on target cells. That is a "key point" on which work should be focused, for making advances in microbial control of insects.

Bacillus sphaericus species harbors also numerous isolates with a high larvicidal power against mosquitoes. The most toxic strains belong to serotypes H5a5b, H6 and H25 (de Barjac et al., 1988). Classification of *B. sphaericus* contains now 48 H serotypes. H6 mosquitocidal strains have been discovered recently. Other serotypes like H1a, H2a2b or H26a26b are much less toxic.

COMPARISON BETWEEN *B.T.I.* AND *B. SPAERICUS*

Comparison between *B.t.i.*, other *B.t.* and *B. sphaericus* strains is interesting. If all these bacteria are mosquitocidal, their specificity, spectrum of action, mode of action, toxins and field efficacy are different.

Specificity — By decreasing order of susceptibility, the *B.t.i.* targets are *Aedes*, *Culex* and *Anopheles*. Moreover *B.t.i.* acts on blackfly larvae. PG14 isolate acts the same. As far as we know, the other *B.t.* are mainly toxic to *Aedes* larvae, but we know a blackfly toxicity for some H10 strains. *B. sphaericus* specificity is different. *B. sphaericus* strains are usually very toxic to *Culex* larvae, a little less on *Anopheles* and nearly not toxic on *Aedes* larvae. Again, in each toxic *B. sphaericus* serotype exist strains with different patterns of toxicity (Thiery & de Barjac, not published). *B. sphaericus* does not harm blackfly larvae.

Here we are back the key point above mentioned e. g. the specificity problem. Different approaches have been already used. For instance in my laboratory, Charles & Nicolas (1987) made a detailed study of the midgut proteases of 3 different mosquito spp. trying to detect a possible relation between the proteases of each species and the species relative susceptibility to *B.t.i.* and *B. sphaericus*. No relation has been found in that sense that every protease, even from not susceptible insects can split and activate the crystal protoxins (like also the trypsin). Another approach consisted in the search for a specific binding to the surface of susceptible - target cells, as suggested for *B. sphaericus* by E. Davidson (1988). Recent work on *B.t.* toxic to Lepidoptera by Hofmann et al. (1987) demonstrates clearly for the first time such a binding.

Mode of action — The mode of action of *B.t.i.* is believed to be similar to the general *B.t.* action, e. g. protoxins consisting in crystallized proteins called crystals which are activated into toxins by the gut proteases of the target larvae. Epithelial midgut cells should be the primary site of action, with a now well known cytopathology: dramatic swelling and bursting of epithelial cells, mitochondria alterations (Charles & de Barjac, 1983). The mechanism of action at the molecular level is unknown. Thomas & Ellar (1983) reported a binding of *B.t.i.* toxin to phospholipids of the cell membrane, thus disturbing the lipidic double strand by a "detergent-like" action. Afterwards, formation of small holes or "pores" in the cell cytoplasmic membrane could be generated by the *B.t.i.* toxic sub-unit 28 kDa. The main symptom of *B.t.i.* intoxication consists in larval paralysis before larval death which occurs very quickly (at high dosages less than one hour). Alkali-dissolved crystals are thousand times less toxic than native crystals, probably because of the special behaviour of mosquito larvae which are filter-feeding organisms (thus concentrating particles), and also possibly because of proteolytic degradation caused by solubilization. Nevertheless, such alkali-solubilized crystals show a significant cytotoxicity *in vitro* on insect tissue cultures. For instance, in my laboratory, Laurent & Charles (1984) showed with *B.t.i.* soluble crystals on *Aedes aegypti* cell lines an inhibition of cells growth as well as an inhibition of incorporation of ^{3}H-thymidine and ^{3}H-lencine.

Interpretation of *B. sphaericus* action on mosquitoe larvae has been suggested on the *B.t.i.* pattern, e. g. activation of crystal protoxins into toxins by the gut proteases. Cytopathology of the midgut epithelial cells has also been described, but the cell-swelling does not seem to occur and sub-cellular alterations look different. The sub-cellular mechanism of action is also unknown. *In vitro* cell toxicity has been

shown to be inhibited by various sugars like chitobiose and chitotriose. Hence suggestion has been made of carbohydrates receptors on the cell membrane surface.

Bacillus sphaericus intoxication like *B.t.i.* intoxication causes paralysis and, recently, selective action of *B. sphaericus* on nervous system and muscle has been reported. However, differences exist between *B.t.i.* and *B. sphaericus* symptomatology. *B. sphaericus* kills mosquitoe larvae slower than *B.t.i.* (even high dosage needs usually several hours). That is why biological titration of *B. sphaericus* is expressed in 48 h, instead of 24 h for *B.t.i.*

Toxins – *B.t.i.* is known as harboring different toxins as constitutive parts of its crystals: mainly 3 to 4 major proteins: a doublet around 130 kDa (or 135 and 128 kDa proteins), a 72 and a 28 kDa proteins. On *Aedes aegypti* larvae LC_{50} of native crystals is around 0,5 to 5 ng protein/ml. The 67 kDa protein has been found the most toxic *in vivo*, with an LC_{50} around 4 ng/ml (Chilcott & Ellar, 1988). In fact, each protein is mosquitocidal and supposed to contribute with each other for the final larvicidal power. In addition, the 28 kDa protein is cytolytic and hemolytic. The *in vivo* toxicity of the 28 kDa + 130 kDa doublet has been evaluated similar with the larvicidal power of solubilized *B.t.i.* crystals. *In vitro*, every protein unit of the crystals is toxic after activation by proteolytic enzymes like trypsin.

Genes coding for the 4 crystal proteins 135, 128, 72 and 28 kDa have been cloned from plasmid DNA and sequenced. An interesting feature is the partial homology of the *B.t.i.* 72 kDa crystal protein (cry D protein) with two other crystal proteins, the 66 kDa from *B.t. kurstaki* toxic to Lepidoptera and Diptera and the 73 kDa from *B.t. tenebionis* toxic to Coleoptera (Donovan et al., 1988).

The most toxic strains of *B. sphaericus* like strain 2362 or 2297 produce toxic crystals which contain 4 major proteins with respective kDa of 125, 110, 63 and 43 (Baumann et al., 1988). The 43 kDa protein has been first identified as the main mosquitocidal toxin (Baumann et al., 1985) the coding gene of which having been first cloned and sequenced in 1987 (Hindley & Berry). Now, both 43 and 63 kDa proteins are believed to be required for toxicity (Baumann et al., 1988) and sequence analysis of the two corresponding coding genes showed the concerned proteins have in fact MW of 41.9 kDa and 51.4 kDa. The 110 kDa protein seems to be another toxin which could have the 125 kDa protein as a precursor.

In fact, situation of *B. sphaericus* is not so clear, because of the lack of constancy of crystals. Among the most toxic strains known, only some of them produce regular and constant crystals, easy to see by phase-contrast microscopy (example strains 2362, 2297). Even more, mosquitocidal asporogenous and acrystalliferous mutants have been obtained. Their toxicity is lower than the wild strains toxicity, but it is quite significant. So, there must be a source of toxin other than crystals.

Field efficacy – A last difference between *B.t.i.* and *B. sphaericus* consists in their behaviour and persistence in field trials. On the contrary of *B.t.i.*, *B. sphaericus* acts well in polluted waters. On the other hand it can persist during one month or more, depending on the dosage. For instance in Cameroun remanence of *B. sphaericus* reached 3 months in sewage waters after a treatment with 10 g/m^2. It is a real advantage which explains the interest given to *B. sphaericus* and its soon coming commercialization. Reasons for such persistence are not well known. Recycling is often suggested, although probably not efficient in nature. Another hypothesis relies on a protective role of the exosporium towards the embedded crystal.

OTHER NEW POTENTIALITIES

Now there are some indications, or hints, pointing to bacteria other than *B.t.i.*, *B.t.* or *B. sphaericus* which are given a mosquitocidal power. As WHO Collaborating Centre for Entomopathogenic sporeforming bacteria, we receive each year from all parts of the world an average of 300 isolates or samples for identification, expertise and study. Recently, we got from Dr Singer (U.S.A.) several *B. alvei* strains which are pathogenic to mosquito larvae. They have less toxicity than *B.t.i.* or *B. sphaericus*, but nevertheless a real efficacy. Another example comes from Dr Cheong (Malaysia) who sent to us a mixture of *Clostridium* sp. and *Bacillus* sp. larvicidal to mosquitoes. It is too new to be judged but special physiology of such anaerobic bacteria could confer an advantage for further application in nature. Also, knowl-

edge of such new toxins, genetics and mode of action could permit further genetic manipulation.

Although significantly less toxic than the best known strains of *B.t.* and *B. sphaericus*, these newly isolated species point out the still unexploited potentialities existing in the bacterial world. It has always been my feeling that some elsewhere in nature exist bacteria other than *B.t.* and *B. sphaericus* which are able to kill insect pests and vectors of diseases. This field of research is open to everyone. First indications are there which anounce soon new discoveries. Let us hope that Brazil will contribute to these new developments and cooperate with us for its full achievment.

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