

**REPORT OF AN INFORMAL CONSULTATION ON
THE GENETIC MANIPULATION OF LARVICIDAL
BACTERIA FOR DISEASE VECTOR CONTROL**

Cordoba, Spain, (6-8 September, 1996)



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**Report of the Informal Consultation on “*Genetic Manipulation
of Larvicidal Bacteria for Disease Vector Control*”**

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Report of the Informal Consultation on “ Genetic Manipulation of Larvicidal Bacteria for Disease Vector Control “

The meeting was opened by Dr Boris Dobrokhotov, Manager of the Molecular Entomology Committee (WHO/TDR), who welcomed the participants and explained the objectives of the consultation. He emphasized the need for an evaluation of the likely output of genetic manipulations of entomopathogenic bacteria for vector control. Dr Dobrokhotov suggested that the group make recommendations on the development of natural and altered entomopathogenic pathogens in vector control programmes, especially for the “control of malaria”.

Dr A. Klier, Chair of the Informal Consultation, provided the participants with a list of the limitations that currently preclude the more widespread use of entomopathogens in vector control programs. Though currently used in the Onchocerciasis Control Program, and in malaria control programs where larviciding is an effective strategy, Dr Klier emphasized the need for more efficacious and cost-effective products. Additionally, he noted the possibility, based on recent laboratory and field studies, for the development of resistance to bacteria used in existing products. Though these limitations exist, Dr Klier pointed out that current knowledge of the molecular biology of entomopathogenic bacteria along with recent discoveries of new larvicidal toxins presented numerous possibilities for genetic manipulation of these bacteria and toxins to provide improved bacteria-based products. Dr Klier emphasized that the mandate for the Informal Consultation was to have the participants critically review available information on entomopathogenic bacteria, focusing on knowledge of the molecular genetics of their larvicidal properties, with the aim being to make specific recommendations for research that could lead to improved entomopathogens for use in vector control programs.

1. Current use of bacterial larvicides in vector control programmes

Mosquitoes and blackflies are a significant threat to human health in many parts of the world due to their ability to transmit the causative agents of many important debilitating and fatal diseases. As vectors of malaria, filariasis, viral encephalitis, dengue and onchocerciasis, these insects threaten more than three billion people in both tropical and subtropical regions. Despite considerable national and international efforts to suppress these vector-borne diseases, they still obstruct improvement of health and socioeconomic development in many tropical countries. Moreover, prophylactic solutions based on inexpensive vaccines remain unavailable for protection against these diseases. Thus, at present chemotherapy and vector control remain the most important measures used to prevent or suppress important vector-borne diseases.

To a large extent, vector control programs still rely on the use of synthetic chemical insecticides. However, aside from their life-saving benefits, these chemicals often have other undesirable effects such as the development of resistance in target vector populations, adverse effects on many non-target organisms, and contamination of water and food supplies. For these reasons, the WHO has acted to facilitate the development of more environmentally compatible biological methods for vector control.

The discovery of the insecticidal bacteria, *Bacillus sphaericus* (*Bs*) and *Bacillus thuringiensis* subsp. *israelensis* (*Bti*), which are active against the larvae of numerous important mosquito and blackfly species, began a new chapter in the control of many disease vectors. The high toxicity of certain isolates of these bacteria, and their ability to be produced in large quantities via fermentation, led to their rapid development and commercialization. At present, several products based on *Bti*, and at least one product based on *Bs* are currently used in operational vector control programmes aimed at controlling the mosquito vectors of malaria, lymphatic filariasis, and dengue, and the blackfly vectors of onchocerciasis.

To meet the challenge of controlling vectors with different bionomics, and which breed in a great diversity of habitats, numerous formulations have been developed, largely by industry, for use under specific conditions. As a result, larvicides based primarily on *Bti*, but now also on *Bs*, are used to control vector mosquitoes and blackflies in West Africa, China, India, Brazil, and many other tropical countries. Moreover, the efficacy of these products has led to annual increases in their use to control nuisance mosquitoes and blackflies in the United States, Canada, Asia and Europe.

Though becoming more applied in operational programmes, bacterial larvicides are not widely used in vector control programmes in many countries owing to their high operational costs, low persistence or residual activity, poor stability of some of the toxins that are the active ingredients, and poor efficacy against some vector species. The latter limitations can require a high frequency of treatment and high rates of application, thereby resulting in high operational costs. Such high costs present significant obstacles to the more widespread use of bacterial larvicides in large-scale vector control programmes. Thus, there is a need for bacterial larvicides with better efficacy and cost-effectiveness. This can be achieved by using biotechnology to develop products of higher potency, better stability upon storage, and greater persistence after application in the field. New products with these characteristics will enhance the use of bacterial larvicides in vector control programmes.

2. State of the Art - Existing and new larvicidal strains and toxins

Mosquitocidal bacteria have been known since the early 1960's, when the first strains of *Bacillus sphaericus* (*Bs*) with larvicidal activity were discovered and evaluated in California. Though toxic to mosquito larvae, the first strains of *Bs* discovered were not sufficiently toxic to merit commercial development. Then in 1976, *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) was discovered in the Negev desert of Israel. This new subspecies of *Bt* was highly toxic to the larvae of many species of *Culex*, *Aedes*, and *Anopheles* mosquitoes, and was also highly toxic to blackfly larvae. As a result, it was quickly developed as a microbial larvicide for control of nuisance and vector mosquitoes and blackflies. By the mid-1980s, commercial larvicides based on *Bti* had become an important component of the Onchocerciasis Control Program in West Africa, and were finding common use in vector control programmes, including malaria control programmes, where larviciding was used as a major tactic.

The discovery of *Bti* stimulated a search for other larvicidal bacteria. As a result of these searches, many funded by the Special Programme for Research and Training in Tropical Diseases (TDR), additional isolates of larvicidal bacteria were discovered. These included many strains of *Bt* with properties similar to *Bti*, such as the PG-14 isolate of *Bt* subsp. *morrisoni*, as well as much more potent strains of *Bs*, such as 2362 isolated from an adult blackfly in Nigeria, and 2297 found in Sri Lanka. The 2362 isolate of *Bs* has a spectrum of activity restricted to mosquitoes. It is

particularly active against species of *Culex*, and owing to its high toxicity as well as its higher residual activity and apparent recycling ability, has recently been commercialized for use against certain filariasis vectors and nuisance species of this genus.

Studies of the toxicological properties of the most potent strains of *Bti* and *Bs* indicate that their toxicity is due primarily to a complex of proteins localized in the parasporal body produced during sporulation. In *Bti*, the major larvicidal proteins in the parasporal body are the Cry4A (125 kDa), Cry4B (135 kDa), Cry11A (68 kDa) and Cyt1A (27 kDa) toxins. In *Bs*, the major toxin is a binary toxin consisting of proteins 42 and 51 kDa.

In categorizing the types of larvicidal strains similar to *Bti* discovered to date based on the level of toxicity, three classes can be distinguished (Table 1). These can be briefly summarized as follows :

Class 1. Strains equivalent in toxicity to *Bti* which produce a parasporal body that has the same or a similar complement of endotoxins, but which belong to different serotypes.

Class 2. Strains nearly as toxic as *Bti*, but which produce a different complement of parasporal body endotoxins and belong to different serotypes or different species.

Class 3. Strains of only moderate to weak toxicity which produce a complement of toxins considerably different from those found in *Bti*.

Recent interest has focused on Class 2 strains because the isolates in this group, such as *Bt.* subsp. *jegathesan* (H28a, 28c) from Malaysia, and *Bt.* subsp. *medellin* (H30) from Columbia, produce a variety of novel toxins, some of which are known to be of high toxicity. An example is the Cry11B toxin of *Bt.* subsp. *jegathesan*, which is related to the Cry11A toxin of *Bti*, but which is approximately 10-fold more toxic. Another endotoxin of high toxicity which has not been well characterized yet is the MED 94 toxin of *Bt* subsp. *medellin*. Interestingly, Class 2 also includes the first anaerobic mosquitocidal bacterium, namely *Clostridium bifermentans* serovar *malaysia* (*Cbm*).

The proteins produced during sporulation by these strains are listed in Table 2. To date, only a few of the genes encoding these proteins have been cloned and sequenced. But as noted above, already some of these have been shown to be novel and of high toxicity in comparison to the known *Bti* and *Bs* toxins. In addition, to the *Bti* toxins, new *Bs* toxins have also been identified. These include a 100 kDa toxin, and smaller toxins in the 30-40 kDa range.

Although the results at this stage must be considered preliminary because most of the genes encoding these new toxins have not been cloned and sequenced, based on those studied to date, there appear to be at least four new kinds of proteins emerging from recent studies of *Bti* and *Bs* (Figure 1). These can be summarized as follows:

1. Those highly homologous to the known *Bti* Cry11A toxin, but which have higher toxicity. Examples include Cry11B and MED 94.
2. Those which share the same conserved blocks as the Cry10A *Bti* toxin, but which vary considerably from Cry10A in other regions. Examples include Cry16A, Cry17A and JEG65.
3. The *Bs* Mtx 100 kDa toxin.
4. The *Bs* Mtx2 and *Bs* Mtx3 toxins.

Table 1 : Mosquitocidal strains, activity and presence of *Bti* related polypeptides

Strain	Activity on :			Presence of <i>Bti</i> related polypeptides
	<i>A. aegypti</i>	<i>A. stephensi</i>	<i>C. pipiens</i>	
<i>Bacillus thuringiensis</i>				
<i>israelensis</i> 1884	+++	+++	+++	Cry4A, B, 11A, Cyt1A
<i>morrisoni</i> PG14	+++	+++	+++	Cry4A, B, 11A, Cyt1A
<i>canadensis</i> 11S2-1	+++	+++	+++	Cry4A, B, 11A, Cyt1A
<i>thompsoni</i> B175	+++	+++	+++	Cry4A, B, 11A, Cyt1A
<i>malaysiensis</i> IMR81.1	+++	+++	+++	Cry4A, B, 11A, Cyt1A
AAT028 K6	+++	+++	+++	Cry4A, B, 11A, Cyt1A
AAT021 B51	+++	+++	+++	Cry4A, B, 11A, Cyt1A
<i>medellin</i> 163-131	++	+++	++	Cyt1A
<i>jegathesan</i> 367	++	+++	++	-
<i>darmstadiensis</i> 73-E10-2	+/-	+	+	-
<i>kyushuensis</i> 74F6-18	-	+	+/-	-
<i>fukuokaensis</i>	-	+/-	-	-
<i>Clostridium bifermentans</i>				
<i>malaysia</i> CH18	++	+++	++	-

+++ indicate LC_{50} values similar to those found for *Bti* crystals.

++, +, +/- indicate LC_{50} values higher than those found for *Bti* crystals with ratios comprised between 2-10, 10-50, and 50-1,500 fold, respectively.

- indicates that crystals are not toxic for the species tested

Figure 1: Mosquitocidal bacterial proteins

Cry4A, 4B



Cry10A, Cry16A, Cry17A, JEG65



Cry11A, Cry11B, MED94



Cry2A



Cyt1Aa, Cyt1Ab



Cyt2A



100 aa

P51



P42



Mtx



Mtx2, Mtx3



Table 2 : Proteins produced during sporulation by
Bti, *Bsp*, *Btjeg*, *Btmed*, and *Cbm*

<i>Bti</i>	<i>Bsp</i>	<i>Btjeg</i>	<i>Btmed</i>	<i>Cbm</i>
135 kDa = Cry4B	51 kDa	80 kDa = Cry11B	94 kDa = MED94	68 kDa = Cry17A
125 kDa = Cry4A	42 kDa	72 kDa	68 kDa	66 kDa = Cry16A
72 kDa = Cry10A		70 kDa	30 kDa = Cyt1Ab	18 kDa
68 kDa = Cry11A		65 kDa = JEG65		16 kDa
28 kDa = Cyt1Aa		37 kDa		
		26 kDa		

Molecular weights indicated are as deduced from SDS-PAGE

1. Gene/endotoxin nomenclature used here is that which has been proposed to replace existing nomenclature. Cry4A and Cry4B are new names, respectively for CryIVA and CryIVB; Cry10A and Cry 11A are new names for ,respectively, CryIVC and CryIVD.
2. Cry10A occurs only in very minor amounts and may not contribute significantly to toxicity.

The discovery of these novel bacterial strains and toxins provides a significant resource for engineering improved bacterial larvicides, and well illustrates the point that it is highly probable that more such strains and toxins remain to be found in nature. Thus, it would seem to be highly beneficial to continue the search for more novel larvicidal bacterial strains and toxins, with particular emphasis on strains active against anopheline species. Among strains found to be active, specific procedures should be used to characterize any insecticidal proteins. These procedures would include determination of the peptide composition of parasporal inclusions by SDS-PAGE and immunoblotting using antibodies raised against known dipteran toxins. In addition, genes encoding proteins should be characterized using hybridization techniques and PCR analyses. The latter procedure may enable the identification of variants of known toxins as well as novel toxins. Identification of variant toxin proteins may prove useful as they could be more potent, and could provide alternative toxins for use in resistance management strategies. Indeed, the recent discovery of toxins such as Cry11B and MED 94, similar to Cry11A but with higher toxicity, show the potential utility of searching for and characterizing variant toxins.

Another approach to obtain new toxins would consist of the genetic modification of existing toxins through site-directed mutagenesis. Careful studies of the structural organization of toxin molecules may help to define regions and specific amino acids that could be modified to improve toxicity and/or the stability of these toxins.

Still another approach to developing new bacterial larvicides based on existing toxins would be to recombine genes, either wild-type or recombinant, to form new combinations. For example, recent studies indicate that the *Cyt1A* protein is moderately toxic to several mosquito species, synergizes the toxicity of *Cry4* and *Cry11A* proteins, and may also play an important role in delaying the development of resistance to *Bti*. Thus, adding the *Cyt1A* gene to *Bs* 2362 or 2297, might yield strains of *Bs* that are more efficacious, have a broader target spectrum, and are less likely to lead to the rapid development of resistance.

To assist with the identification of new bacterial isolates with potential for use as larvicides, it is recommended that the WHO Collaborating Centre at the Institute Pasteur, Paris, France, be maintained as the principal centre for identification and cataloguing new isolates of *Bs* and *Bt*.

3. New technologies for delivering toxins to target vectors

As noted above, existing bacterial larvicides have important advantages over chemical insecticides, especially with respect to environmental compatibility. However, they also suffer from disadvantages that are not the result of the endotoxins per se, but are due rather to the physiology of the producer organism, its interaction with the target insect, and its survival in the environment. In addition, there are disadvantages that are the direct result of certain national regulations, for example, the prohibition of preparations that contain viable spores by some countries. Thus, the development of more cost-effective and environmentally compatible larvicides through manipulation of toxin production and delivery systems should be a major goal of future research. Given recent advances in our knowledge of the molecular biology of toxin production, several ways are apparent for generating novel improved products by eliminating some or all of the deficiencies of current larvicidal bacteria. Examples of these advances and how they may be used to produce improved products are provided here.

Evidence is already available that the levels of toxin in larvicidal bacteria may be increased by the addition of heterologous regulatory sequences. For example, marked increases in *Cry1* toxin production were obtained by inserting an upstream sequence from the *cry3* gene upstream from the *cry1* gene. The insertion of such regulatory sequences upstream of larvicidal toxin genes may produce substantial increases in toxins used for mosquito and blackfly control.

Available evidence has also shown that the production levels of several proteins produced by *Bti* are increased substantially by the presence of accessory proteins such as ORF2 of the *cry2A* operon or the 20-kDa protein of *Bti*. These proteins may be acting as "chaperones" but may therefore enhance the stability/persistence of toxins in product formulations and in the environment. For example, the 20-kDa protein from *Bti* has been shown to protect the *Cyt1A* mosquitocidal toxin as well as truncated *Cry1C* proteins from proteolysis. Thus, it appears likely that the bacteria used to produce larvicidal endotoxins can be improved by "engineering" increased "chaperone" production, combining several chaperones in one host and transferring *Bt* "chaperones" into *Bs*.

High cytoplasmic protease activity is characteristic of sporulation, but may restrict the level of toxin production in natural hosts and cause toxin degradation during product storage. Thus, elimination of some or all of the protease production through genetic manipulation may improve toxin accumulation and product stability.

There is now strong evidence that the spore synergizes the activity of *Bt* toxins against some lepidopteran insects. At present, however, it is not clear whether the spore plays a significant role in the toxicity of *Bti* and *Bs* preparations. This should be investigated due to the requirement by some governments that viable spores be eliminated from larvicidal products and because elimination of the spore might have additional advantages. For example, freed from the requirement to produce the spore, the host cell may be capable of producing and accumulating higher levels of larvicidal toxins. Furthermore, the elimination of the spore may improve fermentation and formulation properties, and the conversion of the host cell to a capsule containing the toxins may improve toxin persistence. The utility of this approach depends on the demonstration that the spore is not required for maximum potency.

With the registration of recombinant products in mind, any new production host should preferably contain only DNA from the toxin producing organism. This can be achieved by integration of toxin genes, regulatory sequences, and accessory protein genes into the bacterial chromosome. This approach has the added benefits of increasing the stability of resident genes while restricting their mobility in the ecosystem.

It is well known that *Bacillus thuringiensis* and *Bacillus cereus* are very closely related, the former differing from the latter only by the presence of insecticidal parasporal proteins encoded by plasmids. This close relationship presents the possibility that some strains of *Bt* may produce *B. cereus* proteins known to be toxic to mammals. Recent evidence has confirmed that this is the case. Therefore, in the construction of new hosts for toxin production, the genes encoding proteins with mammalian toxicity should be deleted. *Bs* host cells should also be examined for the production of such toxins.

Current evidence suggest that *Bs* persists and/or recycles in the environment better than *Bti*. The genes responsible for these traits are not known and should be investigated.

Laboratory research with alternative hosts to *Bti* and *Bs* for the production of larvicidal toxins has suggested some new candidates which include cyanobacteria, protozoa, and other organisms. Further laboratory and simulated field studies might be considered to test the larvicidal potential of such alternative hosts.

4. Resistance to bacterial larvicides

Bti has been used in operational control programmes for mosquitoes and blackflies for more than fifteen years, and to date there have been no reports of resistance. However, resistance to the 2362 and 1593 strains of *Bs* has been demonstrated in *Culex pipiens* in both the field and laboratory. In total, there are now five cases of resistance to *Bs*, but only two of these are at a very high level, one being from the field, the other from laboratory experiments. In both cases, resistance was shown to be recessive and due to one major gene, but not the same gene. Studies of toxin binding to brush border membrane vesicles of resistant larvae from the highly resistant strains indicated that different mechanisms were involved in the resistance. In one case, binding of the toxin no longer occurred,

while in the other, binding occurred but there was no toxicity, demonstrating that the resistance involved processing of the toxin after binding.

The *Bs* and *Bti* proteins share no homology, and at present there is no evidence of cross-resistance between *Bs* and *Bti*. Cross-resistance between or among the different *Bs* strains, e.g., 2362/1593, 2297, IAB59, has not been completely investigated.

In light of the potential for the development of significant resistance to *Bs*-based products in the field, several types of studies should be conducted, among which are the following. The potential for cross-resistance between the primary binary toxin of *Bs* and the more recently discovered Mtx toxins should be evaluated. As noted above, the addition of the *CytIA* gene to *Bs* may improve the toxicity and target spectrum of *Bs* as well as delay the potential for resistance, and thus such a recombinant bacterium should be developed and evaluated. Standard resistance management techniques, such as rotation of larvicides, should be evaluated. Given the evidence that resistance might develop quickly to *Bs* in the field, it would be unwise to develop strains of surface-inhabiting organisms such as cyanobacteria to produce *Bs* toxins. Lastly, although no resistance has yet been observed in the field to *Bti*-based preparations, it would also seem unwise to develop and release recombinant surface-inhabiting microorganisms that produce one or more of the *Bti* endotoxins.

5. Regulatory constraints for the deliberate release of recombinant microorganisms

The problem of deliberate release of modified microorganisms into the environment was addressed. Several methods to develop environmentally compatible recombinant microorganisms are now available. As a result, the first agricultural *Bt* product, based on a recombinant *Bt* that contained no foreign DNA, was registered in the United States during 1996. However, it is imperative that requirements for such a release be developed after careful consideration of scientific studies that define the properties of the microorganism to be released, the site of the release, and the potential benefit for humans and the environment. Regardless of the microorganism involved, the following recommendations should be followed:

- avoid the presence of antibiotic resistance genes in the microorganism,
- eliminate unnecessary foreign DNA sequences,
- avoid the presence of unknown DNA sequences,
- reduce the possibility of horizontal gene transfer,
- limit the persistence of the recombinant microorganism in the environment.

In addition, support for projects dealing with the release of recombinant microorganisms must be dependent upon agreement to follow the guidelines established by governmental regulatory agencies, and must also include approval of release by these agencies.

6. Summary and Recommendations

Bacterial larvicides have been used with considerable success in nuisance insect and vector control programmes for almost two decades. However, current operational control costs for larvicides based on *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus* are high. Moreover, the development of resistance to *B. sphaericus* in field populations of *Culex quinquefasciatus* has raised concerns over the long-term utility of these agents. Thus, current operational costs and the potential for more widespread resistance demonstrate the need for improved bacterial larvicides. Research over the past several years has resulted in the discovery of new larvicidal bacteria and toxins, and advances in our understanding of the molecular biology of larvicidal toxin production and mode of action. Additionally, new recombinant DNA techniques and transformation systems provide excellent opportunities for genetic manipulation of these insecticidal proteins to construct improved bacterial larvicides. To take advantage of these opportunities, the following actions are recommended:

1. *New larvicidal toxins*

A wider range of new larvicidal toxins should be developed. This can be accomplished by:

- a) screening natural bacterial isolates for new toxins,
- b) improving existing toxins through genetic modification,
- c) improving knowledge regarding endotoxin mode of action.

2. *New combinations of toxins*

New combinations of toxins should be evaluated for improved efficacy and the capacity to avoid, delay or overcome the development of resistance. The following types of studies should be undertaken:

- a) binding studies on all toxin candidates to determine the degree of receptor uniqueness/redundancy,
- b) determination of toxin synergistic or antagonistic properties,
- c) studies aimed at elucidation of the mechanism of synergism,
- d) determination of the role of new and existing toxins and toxin combinations in delaying the development of resistance,
- e) determination of the role that the spore and cellular molecules exclusive of endotoxins play in toxicity.

3. *New and alternative host systems*

New and alternative host systems for toxin production should be evaluated for improved vector control efficacy. These studies should include the following:

- a) studies to optimize toxin production through:
 - i. determination of the role of regulatory DNA sequences,
 - ii. manipulation of molecular chaperones,
 - iii. chromosomal integration of toxin genes,
 - iv. elimination of bacterial proteases.

- b) improvement of environmental compatibility by :
 - I. elimination of the spore,
 - ii. elimination of *B. cereus* type toxins.
- c) study of alternative host systems that might lead to improved residual activity or recycling of endotoxin producing organisms.

4. *Optimization of Use*

In addition to studies that deal with the genetic manipulation of bacteria and insecticidal proteins, formulations and tactics that optimize use of bacterial larvicides should be improved. Therefore, industry in collaboration with vector control agencies should be encouraged to develop or improve the following:

- a) formulations with greater residual activity,
- b) methods for application in vector control programmes, especially for malaria,
- c) Methods for resistance management.

LIST OF PARTICIPANTS

- Dr Becker, Norbert** **German Mosquito Control Organization**
Ludwigstrasse 99
67165 Waldsee
Tel: +49 6236 418618
Fax: +49 6236 418622
- Dr Crickmore, Neil** **School of Biological Sciences, University of Sussex**
Brighton, U.K.
Tel: +44 1273 678917
E-mail: n.crickmore@sussex.ac.uk
- Dr Charles, Jean-François** **Unité des Bactéries Entomopathogènes, Institute Pasteur**
25 Rue du Dr Roux
75724 Paris Cedex 15, France
Tel: +33 1 40613182
Fax: +33 1 40613044
E-mail: jcharles@pasteur.fr
- Dr Davidson, Elizabeth W.** **Department of Zoology, Arizona State University,**
Tempe, AZ 85287-1501 USA
Tel: +1 602 9657560
Fax: +1 602 9657560
E-mail: e.davidson@ASU.edu
- Dr Delécluse, Armelle** **Unité des Bactéries Entomopathogènes, Institute Pasteur**
25 Rue du Dr. Roux
75724 Paris Cedex 15, France
Tel: +33 1 40613180
Fax: +33 1 40613044
E-mail: armdel@pasteur.fr
- Dr Dobrokhotov, Boris** **Molecular Entomology Committee, WHO/TDR**
1211 Geneva, Switzerland
Tel: + 41 22 7913816
E-mail: dobrokhotov@who.ch
- Dr Ellar, David** **Department of Biochemistry, Cambridge University**
Tennis Court Road
Cambridge, England, U.K.
Tel: +44 1223 333651
Fax: +44 1223 333345
E-mail: DJE@mole.bio.cam.ac.uk

- Dr Federici, Brian** **Department of Entomology, University of California**
Riverside, CA 92521 USA
Tel: +909-787 5006
Fax: +909-787 3086
E-mail: federici@ucrac.1.ucr.edu
- Dr Ganushkima, Ljudmila** **Martsinovsky Institute of Medical Parasitology and**
Tropical Medicine
Chief of Department of Medical Entomology
20, Ul. Pirogovshaya
119830 Moscow, Russia
E-mail: impitm@us1.gpmmt.msk.su
- Dr Ibarra, Jorge** **Cinvestav-IPN**
Apartado postal 629
36500 Irapuato, Gto.
Mexico
Tel: +52 462 51600
Fax: +52 462 45992
E-mail: jibarra@Irapuato.ira.cinvestav.mx
- Dr Klier, André** **Unité de Biochimie Microbienne**
Institute Pasteur
25 Rue du Dr Roux
75015 Paris, France
Tel: +33 1 45688811
Fax: +33 1 45688938
E-mail: aklier@pasteur.fr
- Dr Lecadet, Marguerite** **Unité des Bacteries Entomopathogènes**
Institute Pasteur
25 Rue du Dr Roux
75015 Paris, France
Tel: +33 1 45688227
Fax: +33 1 40613044
E-mail: lecadet@pasteur.fr
- Dr Margalit, Yoel** **Center for Biological Control**
Dept. of Life Sciences
Ben Gurion University of the Negev
Beer Sheva 84105, Israel
Tel: +972 7 6461340
Fax: +972 7 6472963
E-mail: yoelm@bgumail.bgu.ac.il

- Dr Orduz, Sergio** **Biotechnology and Biological Control Unit**
Corporation para Investigaciones Biológicas
Carrera 72A No 78B-141
Apartado Aereo 7378
Medellín, Colombia
Tel: +574 441 0855
Fax: +574 441 5514
E-mail: cibucb@medellin.cetcol.net.co
- Dr Poncet, Sandrine** **Unité de Biochimie Microbienne, Institute Pasteur**
25 Rue du Dr Roux
75724 Paris Cedex 15, France
Tel: + 33 1 45688848
Fax: +33 1 45688938
E-mail: sponcet@pasteur.fr
- Dr Santiago Alvarez, Cándido** **Catedra de Entomología Agrícola y Forestal**
Dpto. Ciencias y Recursos Agrícolas y Forestal
Universidad de Córdoba
Apartado 3048
Tel: +34 57 218475
Fax: + 34 57 298343
E-mail: crlsaalc@uco.es
- Dr Vilarinhos, Paulo** **Instituto de Saúde do Distrito Federal**
Sgan 601 Lotes "P" 1 "P"
Brasilia, D.F.70.830.010, Brazil
Tel: + 55 61 2264813
Fax: + 55 61 3219995
E-mail: vilapaul@cenargen.embrapa.br
- Dr Chungjatupornchai, Wipa** **Center for Molecular Genetics, Genetics Engineering**
Institute of Science and Technology for Research and
Development, Mahidol University, Salaya Campus
Nakornpathom 73170, Thailand
Tel: + 66 2 4419003 7 Ext. 1243, 1235
Fax: +66 2 4411013
E-mail: stwcj@st.mahidol.ac.th
- Dr Zaritsky, Ariel** **Ben Gurion University of the Negev**
Tel: +972 7 6461712
Fax: +972 7 6278951
E-mail: ariehz@bgumail.bgu.ac.il