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REPORT OF AN INFORMAL CONSULTATION ON THE ROLE OF MOLECULAR BIOLOGY AND GENETIC ENGINEERING IN THE DEVELOPMENT OF BIOCONTROL OF DISEASE VECTORS

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1. INTRODUCTION

The Scientific Working Group on the Biological Control of Vectors, UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR), held an Informal Consultation on the Role of Molecular Biology and Genetic Engineering in the Development of Biocontrol of Disease Vectors. The meeting was held in Geneva, Switzerland, from 4 to 6 March 1987.

The meeting was opened by Dr Tore Godal, Director of the Special Programme, who outlined the current use of biological control agents in vector control and asked the participants to consider how this could be improved through the application of genetic engineering techniques. Dr R. Slooff, Director of the WHO Division of Vector Biology and Control, spoke of the place of biological control in integrated vector control and of the possibilities that genetic engineering might have in vector control in the future.

Dr P. Lüthy was nominated Chairman of the meeting and Dr H.C. Chapman agreed to act as Rapporteur.

The aims of the Consultation were: (1) to provide advice on the scientific prospects that genetic engineering might improve the application of microbial biological control agents in vector control; (2) to indicate steps that might be taken for these prospects to be realized.

2. RESTRAINTS IN THE USE OF EXISTING MICROBIAL CONTROL AGENTS IN THE FIELD

The microbial agents currently available for the control of the larvae of mosquitos and blackflies are *Bacillus thuringiensis* H-14 and *Bacillus sphaericus*. The use of these bacteria for practical vector control has both advantages and drawbacks. The main advantage is the production of stable spore-crystal mixtures, which are readily formulated for use in conventional application equipment and which are remarkably safe for man and other non-target fauna. However, these bacteria rarely induce epizootics and the commercial preparations are rapidly inactivated after application, so only short-term protection from the vector population is obtained from a single treatment. Currently, there is an enhanced interest in the use of these bacteria because some mosquito larvae are now resistant to many commonly used chemical pesticides and also because the potential for manipulating the cloned genes of the toxins provides the opportunity of improving the larvicidal efficacy of these toxins.

Among these bio-agents, *B. thuringiensis* H-14 is effective against all species of *Aedes*, *Anopheles*, *Culex* and, to some extent, *Mansonia*, the main mosquito vectors of tropical diseases, and also against vector species of *Simulium*. However, this biocontrol agent in its present formulation tends to disappear quickly from the feeding zone of vector mosquito larvae, particularly anophelines. It appears to be unable to propagate itself effectively in the biotope, and thus there is need to apply it repeatedly to be operationally effective. There is a dearth of information on the biology and ecology of this bio-agent and on its reproduction in nature. It is used operationally against *Simulium* since only short exposures in specific formulation are required.

B. sphaericus is still in the development stage and is effective against mosquitos, but not against *Simulium*. Among the mosquitos, it is effective particularly against species of *Culex* and generally less effective against *Anopheles*, *Mansonia* and *Aedes*. It is yet to be confirmed how long *B. sphaericus* remains effective in the biotope. If it is proven that it is able to propagate in the biotope, this would reduce the rate of application. There is also a dearth of information on the biology and ecology of *B. sphaericus*. There is some evidence that UV rays affect its efficacy in clear water.

With regard to efficacy, the technical material from which bacterial larvicides are formulated is comparable in vector toxicity to many organochlorine and organophosphate insecticides. For example, the LC_{50} of powders made by drying fermentation slurries of B. thuringiensis H-14 and B. sphaericus is usually in the range of 0.1-0.2 mg/d (fourth-instar larvae of Culex quinquefasciatus). When applied in the field, these powders applied at rates of 100-400 g/hectare yield mortality levels above 90% for third and fourth instars of many mosquito species. These figures take on added significance because the actual amount of larvicidal toxin in these preparations is only a fraction of the material applied and thus high application rates are needed. In B. thuringiensis, the toxin(s) are estimated to represent 20-25% of the cell dry weight, and in B. sphaericus the toxin is thought to represent only 5% of the dry weight. Thus, because these toxins are proteins, improvement of the toxic potential, using the tools of genetic engineering, particularly in the case of B. sphaericus, is a realistic possibility.

The meeting concentrated on the information available on these two bacteria, with emphasis on their protein toxins and the genes which encode these toxins. It also discussed possible ways in which knowledge and technology can be used to develop more efficacious bacterial larvicides, and identified problems which must be resolved to achieve this objective.

3. BASIC METHODOLOGY OF GENETIC ENGINEERING

It is well established that DNA contains the information for all proteins produced in a cell. During the past 10 years, genetic engineering has brought a revolution in the study of gene structure and function. It is now possible to isolate genes and to determine their nucleotide sequence. This includes both the coding region (from which the protein sequence can be determined) and the regulatory region, which determines the expression. It is also possible to modify the coding region by directed mutagenesis or by gene fusion. This allows a detailed study of the structure-function relationship of a protein. The expression can also be influenced by modifying the regulatory region. Finally, it is possible to transfer and express genes into new hosts. In this way desired traits can be exchanged between unrelated organisms.

The basis of this new technology, which is most typically referred to as recombinant DNA technology or genetic engineering, rests on our ability to cleave DNA at specific sites using restriction enzymes, and then transfer, select and study genes of interest and their protein products in pure form in, or as obtained from, other hosts. Until now, the most commonly used alternate host is the bacterium Escherichia coli, although a variety of other hosts including yeasts, algae, and the cells of higher animals and plants can now be used to express and study foreign proteins.

With regard to microbial control agents for vectors, the development of recombinant DNA technology has provided the opportunity to study and improve the efficacy of microbial larvicides, particularly those based on the bacteria B. thuringiensis H-14 and B. sphaericus, whose insecticidal properties are due to the highly selective protein toxins they produce.

4. STATE OF GENETIC ENGINEERING OF B. THURINGIENSIS H-14

The groups of microorganisms pathogenic to insects are varied and diverse. The major species of bacteria which infect and kill insects are spore-forming bacilli, among which are found two major groups: B. thuringiensis and B. sphaericus. A variety of strains exist within each group; these strains differ in a number of characteristics, including the structure of the toxins, their mode of action and their targets. B. thuringiensis H-14 has been extensively studied due to its high toxicity to mosquito and blackfly

larvae, which are vectors of widespread tropical diseases, such as malaria, filariasis and onchocerciasis.

4.1 Biochemical and Toxicological Characteristics of *B. thuringiensis* H-14 and Its Parasporal Body

Numerous studies have documented the high toxicity of *B. thuringiensis* H-14 to mosquitos and blackflies. This strain does not produce detectable levels of beta-exotoxin, and thus its toxicity is due primarily to the protein inclusions present in the parasporal body.

The parasporal body of *B. thuringiensis* H-14 is spherical, 0.7-1.2 µm in diameter and composed of at least three different types of inclusions. These inclusions differ from each other in size, shape and electron density, and each is enveloped in one or more layers of a mesh-like envelope, which also surrounds the entire parasporal body. The largest inclusion is typically rounded to angular, and the least electron-dense of the three inclusion types. It is estimated that this inclusion represents from 30-50% of the protein within the parasporal body, and based on the relative abundance of major parasporal body proteins detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), this inclusion is thought to contain the cytolytic protein which has a relative molecular mass of 28 000 (M_r 28*). The two other inclusion types readily observed within the parasporal body of *B. thuringiensis* H-14 are a moderately dense polyhedral inclusion, which often appears bar-shaped in transverse sections, and a dense, rounded to spherical inclusion. Based on the relative abundance of parasporal body proteins observed by SDS-PAGE, these inclusions are thought to contain proteins of M_r 65 and M_r 135, respectively. With regard to the most dense inclusion type, it has been shown that two inclusions of this type can occur within the parasporal body. This is an interesting observation because there is some evidence that the parasporal body of *B. thuringiensis* H-14 actually contains two high molecular-weight proteins, with masses of M_r 126 and 135, and thus each of these may be present in a separate inclusion.

It is already clear that the intact parasporal body of *B. thuringiensis* H-14 is composed of a series of proteins ranging from M_r 28 to around M_r 135, as determined by SDS-PAGE and column chromatography. The number of proteins reported has varied and is dependent largely on the method of parasporal body preparation and the degree of degradation prior to analysis. If on the basis of relative abundance only major proteins are considered, the parasporal body contains only three or four. Allowing for some variation in size estimates for the same protein by different investigators, the major proteins are those of M_r 28, 65 and 130. In several studies the latter protein has been resolved by SDS-PAGE into two with sizes of about M_r 126 and 136, respectively. The M_r 28 protein has recently been shown through analysis of the gene encoding this protein to consist of 249 amino acids with a mass of M_r 27,340.

In addition to these proteins, several minor proteins with sizes of about M_r 25, 36-40, 53 and 67 have also been observed when intact parasporal bodies are analysed by SDS-PAGE. If parasporal bodies are solubilized in alkali, these minor proteins increase in amount concomitantly with a decrease in the amounts of the major proteins. Thus, most of the minor proteins are thought to result from cleavage of the major proteins by alkaline proteases associated with the parasporal body. A similar proteolysis most likely occurs in the insect gut and, as in other strains of *B. thuringiensis*,

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* Throughout this document, relative molecular mass (M_r) is expressed in thousands.

is probably important in activating toxins in the parasporal body. At this point, there is evidence in many of the studies cited above that the M_r 25 protein results from cleavage of the M_r 28 protein, the M_r 35 to 40 proteins from the M_r 65 protein, and the M_r 53 and 67 proteins from the two proteins of around M_r 130.

Although there is general agreement that the toxicity of B. thuringiensis H-14 is due to the parasporal body, there is considerable disagreement as to which of the parasporal body proteins is essential for larvicidal activity. Since it was demonstrated in 1983 that the alkali-solubilized parasporal body was larvicidal and cytolytic in vectors, there has been a trend to assign larvicidal activity to only one protein, thereby excluding others from such a role. For example, several early and a few more recent studies have reported that the M_r 25-28 protein is the larvicidal toxin and is also the protein responsible for the broad cytolytic activity of B. thuringiensis H-14. In other studies it has been reported that the M_r 65 protein or the M_r 130 protein or even a protein of around M_r 31-35 is the larvicidal toxin. The broad cytolytic activity of the M_r 25-28 protein has been documented in many of these studies, and the controversy at present is over the degree to which this protein and each of the others within the parasporal body is larvicidal. A detailed discussion of the data regarding the properties of each protein is not appropriate here, but the following summary derived from the studies cited above and the data provided in Table 1, may provide some insight into which proteins contribute to the parasporal body's toxicity.

TABLE 1. TOXICITY OF DIFFERENT BACILLUS THURINGIENSIS PARASPORAL BODY PROTEINS TO MOSQUITO LARVAE

PROTEIN	LC_{50} (ng/ml)		INSTAR[a]
	INTACT	SOLUBILIZED	
M_r 25-28	125	-	3rd
	-	12,500	1st
	-	18,500[b]	3rd
	-	>25,000	3rd
	>1,000[c]	>10,000	3rd
M_r 30-35		220	1st
	-	6,680	3rd
M_r 65	43	-	1st
	600[d]	-	3rd
	-	180	4th
	-	400	1st
	-	720	3rd
M_r 128,135	40[c]	350	3rd

[a] Various species

[b] Estimated from original data

[c] Bound to latex beads

[d] Precipitated

When assayed against first or second instars of Aedes aegypti, the LC_{50} for the intact parasporal body is generally in the range of 0.05 to 2 ng/ml. Against third or fourth instars, the LC_{50} is four- to five-fold this amount. Once it is solubilized the toxicity of the parasporal body decreases by ten-fold or more. Because mosquito larvae feed on particulate matter, this loss of toxicity is thought to be due to slower rates of toxin ingestion. The latter creates problems in assessing the toxicity of purified proteins because typically they are assayed in a soluble form. This problem can be partially overcome by precipitating the protein or attaching it to latex beads. In any case, the toxicity of most proteins is significantly less than that of the intact parasporal body and, importantly, also much less than that of the solubilized parasporal body. Typically, the LC_{50} values reported for purified solubilized proteins range from several hundred to well over 1000 ng/ml, indicating these proteins are from 20 to more than 100 times less toxic than the solubilized parasporal body.

A similar loss in toxicity is found when values for purified proteins bound to latex beads or precipitated are compared with values for intact parasporal bodies. Thus, it appears that on an individual basis, none of the proteins, even in particulate form, will prove to be as toxic as the intact parasporal body. The above studies do, however, provide evidence that the major proteins differ in toxicity to larvae, and at this point, the data indicate that the M_r 65 and 130 proteins are more toxic than the M_r 28 protein.

These studies imply that the high toxicity of the parasporal body is possibly due to a synergistic interaction of two or more proteins. Several recent studies have provided data which support such an interaction. For example, it has been reported that the toxicity of the M_r 28, 65 or 130 proteins tested alone was very low in comparison to mixtures of the M_r 28 and 65, or 28 and 130, or 28, 65 and 130 proteins. Additionally, in a different study it was shown that the bar-shaped polyhedral inclusion, which contains a M_r 65 protein, was much less toxic than the intact parasporal body, but that contamination with small amounts of the M_r 28 protein significantly increased this inclusion's toxicity.

In summary, data currently available indicate that the toxicity of B. thuringiensis H-14 is due to one or more proteins in the parasporal body, although the specific toxicity of each of the four major proteins has not been determined accurately. Additionally, there is some evidence that the high toxicity characteristic of the parasporal body may not be due to a single protein, but rather to an interaction, perhaps synergistic, of two or more proteins.

4.2 Mode of Action of the Toxin(s)

At present, little is known about the sequence of events that lead to the death of mosquito or blackfly larvae treated with B. thuringiensis H-14. The M_r 28 protein is known to be very hydrophobic, and has been shown to be cytolytic for a wide range of invertebrate and vertebrate cells in vitro. Recent preliminary X-ray crystallographic analysis of this protein indicates a structure consistent with a helical motif, suggesting it can pass into or through membranes. Nothing at all, however, is known about the role of the M_r 65 or 130 proteins in toxicity. At the histological level, shortly after ingestion of the parasporal body, the midgut loses its osmotic integrity. The epithelial cells swell and eventually lyse, causing severe damage to the basement membrane. Intoxicated larvae become paralysed and die shortly thereafter. Although the sequence of pathological changes that lead to death has been described, virtually nothing is known about the mode of action of the toxins at the molecular level.

4.3 Genetic Engineering of the Larvicidal Proteins of B. thuringiensis H-14

To date, there has been relatively little genetic engineering of B. thuringiensis H-14 in the sense of using recombinant DNA techniques to improve toxicity. However, several different laboratories have cloned and sequenced the genes encoding the M_r 28 toxin and the two toxins of around M_r 130. Additionally, one of the latter proteins has been engineered into a cyanobacterium.

Strains of B. thuringiensis H-14 harbour several resident plasmids, the size of which range from 4.5 to 180 kilobase pairs (kb). From plasmid curing experiments and by transfer of plasmids between strains, it has been shown that the genetic determinants of the crystal proteins are located on a 112 kb plasmid.

Several groups have reported the cloning and the sequencing of the gene encoding the M_r 28 protein. In some cases, it was shown that the E. coli clones harbouring the recombinant plasmid show an haemolytic activity and have a slight toxicity to mosquito larvae. The corresponding insert DNA were introduced into B. subtilis competent cells using shuttle vectors. B. subtilis clones harbouring the recombinant plasmid produced haemolytic activity only in the sporulation phase. The synthesis of the toxin is dramatically decreased in mutants blocked at early stages of sporulation. The toxin is deposited as refractile inclusions when it is produced in sufficient amounts. The inclusions were purified and consisted solely of the M_r 28 polypeptides. It has been shown that only the native insoluble inclusions are toxic to A. aegypti larvae. Solubilized extracts are not toxic. Analysis of the hydropathy plot of the M_r 28 protein showed that it consists of high hydrophobic regions interrupted by short hydrophylic stretches.

The M_r 130 polypeptide is resolved by SDS-PAGE into two bands suggesting the existence of two genes. One of these genes has been cloned by ligating DNA fragments of the 112 kb plasmid with the plasmid pBc 16 and transformed into a plasmidless strain of B. megaterium. The resulting recombinant strain produced irregular phase refractile crystalline inclusions during sporulation and its toxicity to mosquito larvae was similar to that of the original B. thuringiensis strain. It was further demonstrated that the recombinant plasmid induced the synthesis of a M_r 130 polypeptide which reacted with antibodies directed against the B. thuringiensis H-14 crystal proteins.

Another research group has cloned two genes encoding the M_r 130 polypeptide by screening with specific antibodies an E. coli colony bank for the production of the toxin. Two kinds of plasmids were obtained. One (pR8) is partially homologous to the DNA fragment described above and induces the synthesis of a M_r 130 polypeptide with the same toxic characteristics. The second one (pR7), which has a nonidentical restriction map in the 5' part of the gene, also encodes a toxic M_r 130 polypeptide. The sequence of the first gene has been determined and the putative protein sequence showed some homology with the M_r 130 protein of B. thuringiensis kurstaki in the C-terminal half. Near the first copy of the M_r 130 protein gene is located another gene encoding a putative polypeptide of M_r 72, which is slightly toxic to A. aegypti larvae. Subcloning of the two parts of the pR8 plasmid suggests a participation of the putative polypeptide in the toxicity of the E. coli (pR8). However, it appears that the gene is not the gene encoding the M_r 65 polypeptide. Cloning of the latter gene is in progress in several laboratories.

As an example, the structural maps of the plasmid pCB4, pRX7 and pRX8 harbouring inserts encoding for the M_r 28 protein, and part or the entire M_r 130 proteins are shown in Figure 1.

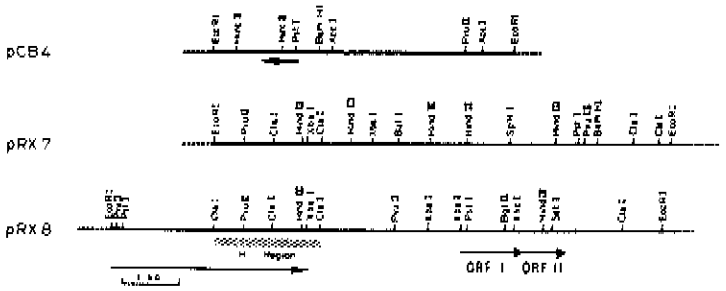

Fig. 1. Restriction maps of the recombinant plasmids containing toxin genes from the B. thuringiensis H-14 112 kb plasmid: pCB4 containing the M_r 28 protein gene; pRX8 containing the larvicidal toxin gene; pRX7 homologous to part of the larvicidal toxin gene. The H region indicates the region of homology between pRX7 and pRX8. Dotted regions represent part of the vectors used.

From Bourgouin, C., Klier, A. and Rapport, G. Molec. Gen. Genet., 205: 390-397 (1986).

Another potentially useful approach for a more efficient application of larvicidal toxins is the introduction and expression of the toxin gene in cyanobacteria. This approach may have advantages which require more investigation. Because many algae live in the upper layers of aquatic habitats, the target insect larvae may be more effectively reached.

Certain cyanobacteria will propagate and therefore persist longer in the desired environment than Bacillus species currently being developed.

A transformation system for the cyanobacterium Synechocystis 6308 has been developed. Foreign DNA can be stably integrated in the chromosome using a non-replicative vector. This vector consists of an origin of replication for propagation in E. coli and fragments of chromosomal DNA of Synechocystis in which a kanamycin (km) resistance monitor gene is inserted. In the Synechocystis transformants, the km resistance gene has been inserted in the chromosome by homologous recombination.

A gene encoding a M_r 130 endotoxin has been isolated from B. thuringiensis H-14. The recombinant protein, produced in E. coli, shows toxicity against A. aegypti comparable to that of the intact inclusion body of B. thuringiensis H-14.

This gene, under control of the P_L promotor of phage lambda, was cloned into the vector described above. Synechocystis cells transformed with this chimeric construct were recovered. The presence of the toxin gene in the chromosome was confirmed by Southern blotting. Western blotting with an antiserum against the toxin showed that the M_r 130 protein is expressed in transformed cells. These cells produced some mortality among A. aegypti larvae within 48 hours, whereas the same amount of non-transformed Synechocystis cells were completely nontoxic.

The low expression level of the toxin is very likely due to the poor activity of the phage lambda P_L promoter in the cyanobacterial cells. An improved toxicity can be expected when strong promoters of Synechocystis are used. Currently, constructs with the strong promoter of the ribulose biphosphate carboxylase operon of Synechocystis are being tested.

Taken together, the above results show the feasibility of the expression of a B. thuringiensis H-14 toxin in cyanobacterial cells.

5. BIOCHEMICAL AND TOXICOLOGICAL CHARACTERISTICS OF B. SPHAERICUS

B. sphaericus is a spore-forming bacterium which, at the beginning of sporulation, produces a parasporal protein toxic to larvae of many mosquito vectors of major diseases. In some aspects it complements B. thuringiensis H-14, being especially active against Culex and having the added advantage of longer persistence in polluted water.

In some strains the toxin is synthesized as an initial peptide of M_r 125 (strains 1593, 1691, 2297 and 2362). In other strains (e.g. 1593) the toxin is composed of a M_r 38 protein which can reversibly aggregate to molecular forms of a mass larger than M_r 200. Subsequently in the course of sporulation this peptide is degraded primarily to proteins of M_r 110, 63 and 43 (strains 2297 and 2362). The M_r 110 and 43 peptides are toxic to C. pipiens larvae, while the M_r 63 peptide is not toxic to this species.

When tested using tissue-cultured cells of C. quinquefasciatus, it was found that the M_r 43 -- but not the M_r 110 -- protein was toxic. These results indicate that in some strains of B. sphaericus the M_r 125 and 110 proteins are protoxins which in the course of sporulation become activated to a M_r 43 toxin.

Crystals purified from a 48-hour culture of B. sphaericus 2362 consist mostly of M_r 43, 63 and 110 peptides. Ingestion of these crystals by larvae of C. pipiens, A. aegypti and A. gambiae results in rapid degradation of the M_r 110 and 63 proteins, as well as the conversion of the M_r 43 peptide to a M_r 40 protein. Larval gut proteases from these species convert the M_r 43 to a 40 peptide, a process which results in a 54-fold decrease in the LC_{50} of the protein for tissue-cultured cells of C. quinquefasciatus. These results indicate that in B. sphaericus there are two sequential pathways of toxin activation: a conversion of M_r 125 to 43 proteins during sporulation and a conversion of M_r 43 to 40 proteins in the larval gut.

Tissue-cultured cells of C. quinquefasciatus, A. gambiae and A. dorsalis were sensitive to the M_r 40 toxin, with an approximate LC_{50} of 1, 7 and 13 µg/ml, respectively. The effect of the toxin was reduced by preincubation with a number of different amino sugars.

Although there was specificity in this effect, the results suggest that differences in toxicity could not be explained only in terms of qualitative differences in the receptor sites. It is possible that these differences are reflected in the structure of a subsequent target site on which the toxin acts.

6. GENETIC ENGINEERING OF B. SPHAERICUS TOXIN

Two laboratories have cloned the B. sphaericus larvicide. Using the bifunctional vector pHV33, a 3.5 kb HIND III fragment was cloned into E. coli and B. subtilis. Both strains became toxic for the larvae of C. pipiens. The gene for the larvicidal toxin was ligated into pvc 303, a shuttle vector suitable for cloning in E. coli and the cyanobacterium, Anacystis nidulans R2. The protein was expressed in the cyanobacterium which had an LC_{50} for larvae of C. pipiens comparable to that of the recombinant E. coli and B. subtilis clone. The cloning of the gene into the cyanobacterium may be of considerable use in maintaining the larvicide in aquatic environments.

Similarly, using the vector λgt11, the gene was cloned into E. coli and subsequently the 3.5 kb HIND III fragment was subcloned using PGEM-3-BLUE. The resulting clones made a M_r 43 and 63 peptide and were toxic to the larvae of C. pipiens. They reacted in Western immunoblots with antiserum to the M_r 63 and 43 proteins from the crystal of B. sphaericus 2362.

In both these studies, probes derived from the cloned genes hybridized only with DNA from the highly toxigenic strains of B. sphaericus.

Progress has been made in the development of procedures helpful for genetic manipulation of this organism. Techniques were developed for DNA transfer by protoplast transformation, now allowing the introduction of different plasmid vehicles carrying antibiotic markers necessary for gene cloning. A certain number of plasmid vectors for cloning in B. sphaericus 1593 were also constructed.

7. GENETIC ENGINEERING OF OTHER B. THURINGIENSIS STRAINS

The delta-endotoxins of lepidoptera-specific strains of B. thuringiensis are currently the most intensively studied microbial insecticides. When tested on a series of lepidopteran species, crystal proteins from different strains show distinct insecticidal spectra.

Immunoassays with polyclonal and monoclonal antisera indicate that multiple, structurally distinct polypeptides of M_r 130-140 can be present in single strains. These proteins are proteolytically converted into active toxins of M_r 55-70 by the larval midgut proteases.

In order to investigate the properties of individual crystal proteins, several genes were cloned, sequenced and expressed in E. coli. The recombinant toxin "bt2" is an example of this. A gene encoding a M_r 130 crystal protein was cloned from B. thuringiensis berliner 1715 and expressed in E. coli. The recombinant protein (bt2) is a potent toxin against several lepidopteran species. The bt2 protoxin is processed by larval midgut proteases into an active M_r 60 toxin. The M_r 60 tryptic fragment is as toxic against larvae of Pieris brassicae and Manduca sexta as is intact bt2. Genetic mapping in combination with amino acid sequencing was used to localize the minimal toxic fragment between amino acids 29 and 607 of the protoxin.

The antigenic determinants of four monoclonal antibodies recognizing the M_r 60 toxin were mapped using fragments of the toxin produced in E. coli. One of these monoclonal antibodies recognizes a topographical determinant on the active toxin molecule. The reactivity of this monoclonal antibody with several truncated derivatives of bt2 indicates that the M_r 60 toxin has a very specific 3D conformation which is disturbed by the removal of a few amino acids at the C- or N-terminus. The three other monoclonal antibodies recognize linear determinants at the N-terminus of the toxin and clearly inhibit the toxicity in vivo and in vitro. This indicates that the N-terminal sequence is involved in the toxic action. Monoclonal antibodies are also used to map the toxin domain that is involved in the binding of the toxin to midgut membranes and insect cells in vitro.

From study of the different cloned genes the following picture emerges. The active toxin is always localized in the N-terminal half of the protoxin molecule. The amino acid sequence of the N-terminus of the active toxin is almost completely identical for all published sequences. In this part of the molecule two stretches of hydrophobic amino acids can be distinguished, which might be implicated in an interaction with the membrane of the target cells. In the second half of the toxin molecule, much more heterogeneity is observed.

Mode of action

In investigating the mode of action of these toxins, several steps can be distinguished, each of which can play a role in the high specificity of the delta endotoxins:

a) Solubilisation of the protoxin in the insect midgut: some lepidopteran species are much more susceptible to the solubilized crystals of some B. thuringiensis strains than to the intact crystals. Other strains that are more active against these species produce crystals that are more easily dissolved in the larval midgut.

b) Proteolytic degradation of the protoxin into an active toxin: the toxicity of some crystal proteins against certain lepidopteran larvae is greatly increased after in vitro proteolytic activation.

c) Binding of the activated toxin to a receptor: there are several indications that the toxin interacts with glycoproteins of the midgut cell membranes.

d) Disturbance of the osmotic balance, swelling and lysis of the midgut epithelial cells: several propositions have recently been made to explain these effects, but no clear evidence has yet been given for a link between a biochemical action of the toxin and the observed cytological effects.

Expression of B. thuringiensis has also been achieved in other organisms, such as the prokaryotes, E. coli, B. subtilis and Pseudomonas fluorescens, and in eukaryotes with the expression of modified B. thuringiensis genes in transgenic plants.

Starting from bt2 (a cloned toxin gene of B. thuringiensis berliner, see above), modified toxin genes have been constructed: truncated genes encoding the active toxin and gene fusions containing 5' fragments of bt2 fused to the neomycin phosphotransferase (NPTII) gene (neo) of transposon 5. The latter encodes stable fusion proteins which are equally toxic as the original bt2 and which exhibit specific NPTII enzyme activity, comparable to the intact NPTII protein. Chimeric genes consisting of these modified toxin genes, flanked by the regulatory sequences of plant genes, have been transferred to tobacco plants using Agrobacterium vectors. Transgenic plants were obtained by expressing active toxins in their tissue. The expression levels were sufficiently high to substantially protect the plants from feeding damage caused by larvae of M. sexta. The insect resistance trait was stably inherited.

8. ASSESSMENT OF RISKS OF DELIBERATE RELEASE INTO THE ENVIRONMENT OF MICRO-ORGANISMS CONTAINING RECOMBINANT DNA AND INTENDED FOR VECTOR CONTROL

8.1 Regulations and Policies for Testing and Evaluation of Genetically Engineered Microbial Pesticides

Precedents for the testing and risk assessment of both naturally occurring and genetically altered microbial pest control agents have been set forth by international and national bodies. These include the WHO memorandum "Mammalian safety of microbial agents for vector control"[1] and the US Environmental Protection Agency's testing guidelines for registering microbial

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1 Bulletin of the World Health Organization, 59 (6): 857-863 (1981).

pesticides[1], which both set forth a tiered testing scheme for microbial pesticides with regard to evaluation of risk and regulation for safe use. To provide for regulation of genetically engineered microbial pesticides, the US Government has also published an "Interim Policy"[1], setting forth data and information requirements for review prior to any small-scale environmental release. This policy was later contained in the "Office of Science and Technology Policy (OSTP) Coordinated Framework for Regulation of Biotechnology"[1]. The principles for evaluation set forth in these documents are consistent with the "Recombinant DNA Safety Considerations" issued in 1986 by OECD.

8.2 Risk Factors of Genetically Engineered Microbial Pesticides

In testing and evaluating for risk associated with these pesticides, it is possible to identify a set of general factors which may contribute to risk. Because each product and its pattern of use is unique, these risk factors must be considered on a case-by-case basis respecting the regulatory and testing precedents for microbial pesticides identified above.

a) Because of their inherent pesticidal nature, these organisms are intended to have biological activity. They may control pests by a variety of modes of action: lethality; pathogenicity; competitiveness or displacement; deterrence of feeding; inhibition of growth; or adverse reproductive effects. Furthermore, the organisms are designed for environmental application; they may be applied to food crops, forests, rangeland or, for vector control, close to homes. Therefore, exposure to non-target organisms and humans may be widespread.

b) Unlike chemical pesticides, microbes are living entities which may survive and replicate. Once released into the environment, absolute containment is not possible. Thus, prior to any release of genetically engineered microbes, their capacity to flourish and find new ecological niches or to compete successfully against other microorganisms in the environment must be carefully evaluated. Any assessment of environmental impact must take into consideration expected levels of the organisms in the environment but, in addition, must include reliable predictions of the effect of unusual environmental conditions and their influence on localized increases or blooms in the microbial population.

c) Any testing scheme for characterizing genetically engineered microbes or for screening their effects on or exposure to non-target organisms provides data for characterizing risk. However, placing an engineered organism into a new habitat can lead to unforeseen consequences. Thus, the ecological scenario for any proposed application of engineered organisms must be carefully evaluated, using approaches from a variety of scientific disciplines.

d) Certain types of engineered vectors have a high probability of genetic transfer. For example, transposons with broad host-range spectra are highly moveable chromosomal genetic elements which are able to transfer

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1 "Pesticide Assessment Guidelines, Subdivision M, Biorational Pesticides", USEPA 1983; "Data Requirements for Pesticide Registration", Title 40, Code of Federal Regulations, Part 158; "Microbial Pesticides; Interim Policy on Small-Scale Field Testing", Federal Register, Vol. 49, No. 202, 17 October 1984, pp. 40659-40661; "Microbial Products Subject to the Federal Insecticide, Fungicide and Rodenticide Act", Federal Register, Vol. 51, No. 123, 26 June 1986. Copies of these documents may be obtained from: Mr Fred Betz or Ms Millie Vannoy, TS769C, 401 M Street S.W., Washington, DC 20460, USA.

readily from one organism to another. This can lead to two problems: inserted genes might be expressed in other species of microorganisms in the environment and, in addition, multiple copies of the gene might possibly be produced in an individual species of microorganism, thereby increasing the activity. It is desirable to eliminate these kinds of genetic segments from the vectors used for genetic engineering.

e) Antibiotic resistance may be a significant problem in the environment. Therefore, it is desirable when possible in research and development to use antibiotics which are not important in the treatment of human or animal disease.

f) Certain genetically engineered microbial pesticides may have enhanced efficacy against the target pest or may incorporate two toxins in one organism. Even though the host range of the non-engineered organism and its effect on beneficial insects may be known, it is worthwhile to re-evaluate the effects of the engineered microorganism by screening pathogenicity and infectivity in non-target and beneficial insects.

g) The genetically engineered microbial pesticide, by virtue of a unique combination of traits, may be able to persist in the environment in new niches in sufficient numbers to cause adverse effects on non-target species.

h) Increased environmental persistence may be a problem under certain circumstances. For example, the B. thuringiensis H-14 toxin gene can be inserted into a long-lasting or readily reproducing recipient or host, such as blue-green algae. This can be desirable for enhancing the useful lifetime and availability of the B. thuringiensis toxin to mosquito larvae, but may also lead to unforeseen problems, especially if the engineered strain is not narrowly focused in its target pest specificity.

i) With widespread use, after an engineered microbial agent passes all safety screens, the target pest may develop resistance to microbial toxins to the extent that they persist for long periods or continuously in the environment at high levels.

9. RECOMMENDATIONS

Studies carried out over the last few years have demonstrated that the larvicidal properties of Bacillus thuringiensis H-14 and B. sphaericus are due to highly specific insecticidal proteins produced by these bacteria. The advent of modern biochemical techniques and recombinant DNA technology offer the potential to make considerable improvements in the efficacy of these larvicides and to develop novel methods for increasing their residual toxicity. The achievement of these objectives will be dependent upon an increased knowledge of the molecular biology of these and related protein toxins and the genes that encode them. Listed below is a series of objectives and recommendations for studies aimed at increasing knowledge of these proteins and developing more efficacious larvicides that are safe for the environment.

9.1 Objective I: Improvement of the Toxicity of B. thuringiensis H-14 and B. sphaericus

Recommendations:

a) The contribution that each major parasporal body protein, and any primary products of proteolytic cleavage, makes to toxicity should be determined. Specific toxicities per unit weight of protein should be

determined as well as the toxicity of different proteins in combination. Standardized bioassays for precise determinations of toxicity should be developed to facilitate comparison of results obtained from different laboratories.

b) The genes encoding the toxic proteins should be cloned and sequenced.

c) Recombinant strains with improved toxicity should be genetically engineered using information derived from the above studies. This engineering will require the development of improved systems for genetic transformation between bacterial strains and species. Increasing toxin production per cell should receive special emphasis.

d) Attempts should be made to engineer bacterial strains capable of combining expressions of the toxins of both B. thuringiensis H-14 and B. sphaericus. This could include the development of fusion proteins that combine the toxins of these two bacteria.

e) Studies should be undertaken to identify the receptors for toxic proteins on and within cells and to determine the mode(s) of action at the molecular level.

f) Attempts should be made to increase the vector host range of the B. thuringiensis H-14 and the B. sphaericus toxins through directed mutagenesis.

g) Attempts should be made to engineer strains of B. sphaericus that will grow on media rich in carbohydrates, as opposed to media rich in amino acids currently required, thereby reducing production costs.

h) The feasibility and efficacy of engineering the toxins of B. thuringiensis H-14 and B. sphaericus in alternative prokaryotic and eukaryotic microorganisms should be critically investigated, especially in organisms which have simple nutritional requirements.

i) The genetic stability of engineered strains should be determined and optimized. Engineering approaches should attempt to immobilize transferred genes. Mechanisms for self-containment or self-destruction of engineered organisms should be developed, especially for those which might potentially present a risk to the environment.

9.2 Objective II. Enhancement of the Residual Activity of Larvicidal Bacterial Toxins

Recommendations:

Studies should be undertaken to determine the feasibility of enhancing the propagation of larvicidal bacterial toxins in vector habitats to increase the availability of these toxins to target vectors. The studies should be directed towards the following objectives:

a) investigation and selection of microorganisms in vector habitats which might be engineered to express toxins;

b) determination of the ecology, population dynamics and nutritional requirements of these microorganisms to assess feasibility and cost-effectiveness of using them to increase persistence;

c) development of more information regarding the ecology of wild-type strains of B. thuringiensis H-14 and B. sphaericus;

d) development of transformation systems for the engineering of alternative organisms suitable for use as persistent larvicides.

9.3 Objective III. Development of Methods to Assess and Reduce the Risk of Introducing Genetically Engineered Larvicidal Organisms into the Environment

Recommendations:

a) In addition to the existing guidelines for safety testing of microbial agents for vector control, standards and methods for a stepwise approach to testing the efficacy and safety of genetically engineered micro-organisms from the laboratory to the field should be developed. Testing beyond the laboratory should move sequentially to (i) simulated microcosms, (ii) containment greenhouses or growth chambers, (iii) restricted small-scale field evaluations.

b) The engineered product should be precisely defined and selected with emphasis on desirable traits to allow for adequate assessment of ecological impact.

c) Quality control of engineered strains should be well developed. In microorganisms developed for environmental application, the potential for genetic drift should be eliminated or minimized to the extent technically feasible. Transferred genetic elements should be immobilized, and, as noted above, mechanisms for self-containment or self-destruction should be engineered into recombinant microbial pesticides.

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