Genetic Toxicity Assessment of Microbial Pesticides: Needs and Recommended Approaches

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Introduction and Objective

Effective use of pesticides is an essential element of economical food production, and the use of agents with a high selectivity for pests and minimal toxic potential for humans and farm animals is the key to safe and effective use in pest control strategies (Montesinos, 2003). Microbial pesticides offer the advantages of higher selectivity and lower toxicity than conventional chemical pesticides, but evaluation of their safety may require modification of the traditional approaches used for chemical agents or may require unique tests for safety hazards that are specific to microbial agents. Thus, consideration must be given to the unique biological characteristics of microbial pesticides, and safety testing must be designed to address both general and specific potential hazards. Assessment of genotoxic potential poses special considerations, as the nature of the various test organisms employed in relation to the microbial pesticide must be taken into account.

General guidelines for assessment of the genotoxic potential of microbial pesticides have been implemented by the U.S. Environmental Protection Agency, Canada, and the European Community (USEPA\(^a\); Health Canada, 2001; EEC, 2001). These guidelines address the factors to be considered and testing approaches to be used to evaluate the potential for genetic damage from exposure to microbial pesticides. The Organization for Economic Co-operation and Development (OECD) has issued Guidance for Registration Requirements for Microbial Pesticides that is an important step in harmonizing registration requirements for microbial pesticides (OECD, 2003), and the Working Party on Chemicals, Pesticides and Biotechnology and the Chemicals Committee of the Environment Directorate have been working jointly to finalize OECD recommendations. It is noteworthy that the EPA, Canadian, and EU guidances on genetic toxicity testing are similar, but not identical, and this is recognized in the OECD Guidance for Registration Requirements for Microbial Pesticides of May 23, 2003. Extension of the OECD guidance to include specific protocols for the testing of microbial pesticides that are accepted worldwide is very important.

The purpose of this report is to provide comment and recommendations on the Commission Directive 2001/36/EC of the European Communities (“the Directive”; EEC, 2001) and needs for further harmonization of testing strategies and testing protocols for assessment of the genotoxic potential of microbial pesticides through the Organization for Economic Cooperation and Development (OECD)\(^b\).

Safety Concerns

The Directive appropriately notes the need to differentiate data requirements for microbial pesticides from those for conventional chemical pesticides. This is due to differences

\(^a\) EPA Toxicology Data Requirements for Microbial Pesticides:
http://www.epa.gov/pesticides/biopesticides/regtools/guidelines/40cfr158_740c.htm

\(^b\) In providing this assessment, Dr. MacGregor has used his best efforts to assure the accuracy of information and his best judgment in making recommendations, but the OECD assumes responsibility for any actions or decisions based on this information.
in the characteristics of microbial agents from conventional chemicals. These characteristics may either increase or decrease concern for potential hazards of specific microbial agents relative to those for chemical agents. Depending on the nature of the biological agent, there may be concerns over potential infectivity, exchange of DNA from the microbe into mammalian species, or the potential for exo- or endotoxins to exert biological effects. Exo- or endotoxins can be appropriately tested in standard tests designed to detect genotoxic effects of small MW chemicals that can readily enter cells and interact with cellular macromolecules, but non-infective organisms that do not produce specific toxins are unlikely to be genotoxic either to mammalian species or in conventional mutagenicity test systems. It is thus critical to have a flexible approach to safety evaluation that takes into account the known biological properties of the specific microbial agent under consideration, and to customize the safety testing strategy to address those concerns that are most likely to occur based on knowledge of the characteristics of the specific organism.

Testing Requirements and Protocol Design

Recognition of the differences between biotechnology-derived products or organisms vs. chemicals has led certain authoritative bodies to conclude that the types of genotoxicity studies routinely conducted for chemical products are not applicable to these types of products and are therefore not needed for product registration. This is the position of the pharmaceutical community, which states in its guidance for safety evaluation of biotechnology-derived pharmaceutical products (ICH, 1998):

“The range and type of genotoxicity studies routinely conducted for pharmaceuticals are not applicable to biotechnology-derived pharmaceuticals and therefore are not needed. Moreover, the administration of large quantities of peptides/proteins may yield uninterpretable results. It is not expected that these substances would interact directly with DNA or other chromosomal material ……” Further, this guidance states that “The use of standard genotoxicity studies for assessing the genotoxic potential of process contaminants is not considered appropriate. If performed for this purpose, however, the rationale should be provided.”

A similar conclusion was reached by a task force of the German-speaking section of the European Environmental Mutagen Society, the Gesellschaft für Umweltmutationsforschung (Goecke et al., 1999), which addressed the needs for genotoxicity testing of biotechnology-derived products recognized however that certain specific proteins could potentially modify DNA metabolism or cellular growth. This group concluded that conventional genotoxicity tests may be appropriate under certain limited circumstances and that appropriate specific testing needs to be considered on a case-by-case basis. This group conducted a survey of approximately 30 companies that produce biological products and also reviewed the open literature to determine approaches that had been used to evaluate the safety of proteins and other biological products. References are provided to other existing guidelines for safety evaluation of gene therapy products, vaccines, and plasmid DNA vaccines that may have relevance to certain biotechnology products or organisms. A “decision tree” was developed to assist in the evaluation of appropriate testing needs based on the characteristics of specific products. Development of an analogous decision tree for the testing of microbial pesticides would be useful, and could be undertaken by a recognized group of international experts, as is discussed further below.
In those cases in which the conventional genotoxicity tests applied to small-molecule chemicals are appropriate for the evaluation of microbial products or lysates of microbes, careful consideration needs to be given to appropriate protocols that avoid uninterpretable or misleading results. Some examples of factors that could confound standard tests, when they are applied to the analysis of microorganisms or products derived from microorganisms, are provision of nutrients by lysates (e.g., histidine, which would allow growth of the auxotrophic tester strains in the Ames Salmonella assay), growth factors that may produce abnormal growth, growth inhibition, or DNA synthesis (e.g., erythropoietin which causes micronuclei in bone marrow via induction of abnormal cell proliferation; lectins that may stimulate DNA synthesis in \textit{in vitro} mammalian cell tests), enzymatic activity that could mimic endogenous activity in the test organism (e.g., kinase or phosphokinase activity in the \textit{tk}^{+/−} or \textit{hprt} assays), or intracellular molecules with nuclease or proteolytic activity from \textit{in vitro} lysates that would not normally have access to mammalian cells \textit{in vivo}. Careful consideration to specific test design is needed to ensure that appropriate modifications of standard test protocols are made when necessary to avoid uninterpretable, false positive, or false negative results.

**Commission Directive 2001/36/EC**

The following are my personal thoughts and comments on the EC Commission Directive 2001/36/EC on the use of micro-organism as plant protection products, with a focus on possible further refinement of specific guidelines under the OECD process. As discussed below, there is precedent for utilization of international expert groups in the development of such specific requirements, using either existing or specifically constituted expert groups, and this approach may be appropriate in this case to ensure the best input from global experts as well as harmonization of new guidance with existing guidances and regulations in individual countries or regions.

The focus in the Directive on flexibility in determining the need for testing and formulation of test strategies, the need to address the presence of metabolites with potential adverse health effects, and the need to address potential toxicities, pathogenicity, infectivity of the organism itself, and the need to discuss the potential for insertional mutagenesis by viruses are reasonable guidelines. The requirement to test purified or partially purified constituents from organisms known to produce exotoxins is also reasonable. Such testing should include any necessary test modifications suggested by the nature of the exotoxin expected (e.g., whether it is a small molecule, a peptide, an enzymatically active protein, etc.).

The Directive appears to require \textit{in vitro} testing for mutagenicity in all cases, unlike the EPA or Canadian guidelines, including bacterial mutagenesis and mammalian cell clastogenicity and mutagenicity tests. The presumptive intent is to screen for unsuspected constituents with the potential to induce genetic damage. Although these tests are reasonably inexpensive and the objective is not unreasonable, the conduct of these tests on whole cell lysates is not necessarily straightforward. It would be useful to convene panels of experts with 1) in-depth knowledge of these core tests and 2) knowledge of the secondary metabolism of the classes of microorganisms used to produce microbial pesticides. These panels could develop guidelines for the extent of
testing, if any, required for different classes and species of microorganisms, based on general levels of concern related to their relationship to analogous known microorganisms, their potential for toxin production, and the anticipated level and route of human and livestock exposure, and could also develop standardized protocols for different types of representative organisms (bacteria, fungi, viruses) so that the need to customize protocols to avoid the potentially confounding factors discussed above could be minimized. This could include, for example, a standardized method for histidine removal for the Ames reverse mutation test, standardized methods for production of lysates of bacteria and fungi for testing, preliminary test methods for determining mitogenic potential of lysates or extracts, etc. Although these examples come immediately to mind, there are undoubtedly others that would occur to experts with in-depth hands-on experience with various test methods. Although the initial expense of developing such expert consensus recommendations is not trivial, it would probably be small compared to that required to independently refine more general guidelines in individual countries or regions and the resulting harmonized approach would avoid needless repetition of testing by companies to satisfy differing regional requirements.

The Tier II testing for potentially genotoxic agents proposed in the Directive is more problematic, and requires further input by qualified experts. The recommendation to automatically use the in vivo UDS test or the mouse spot test when in vitro mutagenic activity is observed should be modified. To my knowledge, the mouse spot test has not been used in a regulatory setting and is not routinely available in those contract laboratories that perform regulatory testing. The UDS test is appropriate if a bulky DNA adduct is expected and if the expected target tissue is one in which the assay can be conducted, such as liver or skin. However, if the available information suggests that mutagenesis results from reactive oxygen species or DNA strand breakage, the UDS assay is not appropriate because it is very insensitive to damage repaired by short-patch repair. In this latter case, a Comet assay, which detects DNA strand breakage or labile sites, in an appropriate tissue would be more appropriate. In cases in which the expected target tissue in vivo has not been shown to be applicable to UDS assay, or in which the mechanism of mutagenesis may be obscure, transgenic mutagenesis models such as the lacI, gpt, or lacZ mutagenesis test system may be a more appropriate choice. Recommendations for the appropriate use of several of these in vivo models have been made by international working groups of experts (e.g., Hartmann et al., 2003; Tice et al., 2000 (Comet assay); and Tybaud et al., 2003; Heddle et al., 2000 (transgenic mutagenesis assays)) and such important information about the state of international consensus should be included. In contrast, to my knowledge there has been no international consensus on appropriate protocols or usage of the mammalian spot test which was recommended in the Directive.

With regard to the in vivo germ cell tests, the need for case-by-case consideration is appropriate. Most experts would probably not agree that the dominant lethal test should currently be considered to be a “suitable” test, as it is generally regarded as insensitive and variable in response. The use of newer tests such as transgenic mutagenesis models for germ cell mutations or tests for DNA damage in germ cells would be considered more appropriate by most investigators, when such testing is indicated (which would be relatively rare). A discussion of these tests and their current state of validation and of consensus on appropriate protocols should be included.
Clear guidelines are needed that define when *in vivo* testing may be necessary, and the appropriate test methods to employ. For each test system anticipated to be used, there is a need for the development of protocols that include guidelines on appropriate dose routes, dosage forms, dose levels, and methods to be employed. As discussed below, it is highly desirable that these guidelines should be developed through an international consensus process in order to avoid controversy over results or the need for unnecessary repetition of experiments in different regions.

**Previous Experience with Complex Organic and Biological Samples**

Many toxic, mutagenic, and carcinogenic secondary metabolites have been identified in microorganisms, particularly fungal species. Recognition of the potential health consequences of human exposure to such sources has resulted in much attention, beginning the early 1960s (Rodericks et al., 1977b), and a wide variety of biologically active molecules of diverse chemical structure, including carcinogens and mutagens, have been identified using various methods of isolation and bioassay (e.g., see Steyn, 1977; Enomoto and Saito, 1972; Tazima, 1982; Rodericks et al., 1977a; Schrader et al., 2001; Sakai et al., 1992; Lakshmi et al., 2003). However, although specific products under consideration as microbial pesticides have been tested (e.g., Genthner et al., 1998) it does not appear that a general method of screening fungi or other microorganisms for mutagenic activity has been developed. Although this has not been researched thoroughly, telephone contacts to a limited number of well-known scientists in these fields and a cursory literature search failed to identify reference to an established general testing scheme. The lack of reference to such a scheme in the EC Directive (2001), or the Health Canada (2001), EPA, or OECD Guidances (2003), support this conclusion.

In addition to fungi, complex organic materials reported to have been evaluated for mutagenic or anti-mutagenic activity include plants (Clark, 1982; Kaur et al., 2005), foods (Knudsen, 1986; Kaur et al., 2005), botanicals used as medicinals or cosmetics (Göggelmann and Schimmer, 1986; Cosmetic Ingredient Review Expert Panel, 2004), infected plant materials (Yahiaoui et al., 1994), and soil and water (Watanabe et al., 2005; Maruoka and Yamanaka, 1982). Experience with these materials points to those experimental factors that require consideration when testing complex materials, and provides some examples of modifications of conventional testing methods that have allowed these methods to be used successfully to test complex organic and biological materials. For example, preparation of samples for chemical analysis has been addressed for mycotoxins (e.g., Boenke, 1995; Scott and Trucksess, 1977; Stoloff, 1977) and extensive analytical mycotoxin surveys have been conducted (see e.g., Rodericks and Stoloff, 1977), and this experience will certainly be of value in identifying appropriate methods for preparation of samples suitable for mutagenicity analysis. Methods that have been applied to address the types of problems associated with mutagenicity analysis of complex organic samples include extraction procedures, the use of resins or binding substrates to isolate and concentrate active materials (Maruoka and Yamanaka, 1982; Scott and Trucksess, 1977), inclusion of enzymatic preparations that release conjugated forms of mutagenic constituents (Tamura et al., 1980), and destruction of nutrient constituents known to interfere with the assay.)
Thus, there are numerous examples in the existing literature of methods used to evaluate complex mixtures of organic materials for mutagenic and/or anti-mutagenic activity. Many of the problems commonly encountered with the mutagenicity assessment of complex organic and biological substrates are expected to apply to testing of the classes of microbes used as pesticides. These problems include 1) potential interference from toxic components, 2) the presence of nutrients that may interfere with certain assays (especially those based on mutation of genes required for synthesis or utilization of nutrients required for growth) (Nylund and Einisto, 1992), 3) the occurrence of potentially active constituents as bound or complexed forms (e.g., as glycosides or other conjugates, or bound to macromolecular constituents) (Tamura et al., 1980), or 4) physical interference due to inactive bulk constituents and/or precipitates. Other potential problems include biological activity of the test mixture, such as growth stimulatory or inhibitory constituents (such as lectins or other polysaccharide constituents that may stimulate lymphocyte proliferation, or agents with specific growth regulatory effects in vivo (Yajima et al., 1993). In each of these cases, methods have been successfully modified to allow reliable bioassays to be conducted. Although these modifications are necessarily specific to the organism upon which a particular assay is based and have generally been applied to a specific type of analyte, consideration of these analytical issues and their solutions would be a useful preliminary step in the identification of potential problems and potential solutions required for implementation of a generally applicable analytical strategy for assessment of the mutagenic potential of microbial pesticides.

Often, interference is evident from test results. For example, in the conventional Ames assay in which exposure occurs in top agar, the presence of histidine in the test sample is evident from excessive growth of the background lawn and should not lead to “false positive” conclusions as long as careful observations are made. The problem of histidine in samples for analysis using the Ames assay is well recognized, and methods of recognizing and avoiding this problem have been described (Aeschbacher et al., 1983; Busch and Bryan, 1987; Nylund and Einisto, 1992; Salmeen and Durisin, 1981). Likewise, excessive growth stimulation of the target cells in the in vivo micronucleus assay would result in an increased reticulocyte/erythrocyte ratio and in in vitro cell assays would lead to increased proliferation indices or altered growth kinetics. Since the nature of potential interference and appropriate indices for assuring that such interference has not occurred are dependent on the specific organism and testing protocol used, it is important that consensus guidelines on appropriate protocols and criteria for a valid assay are developed for suitable standard testing strategies by experts knowledgeable about the experimental variables involved.

Development of Harmonized International Testing Approaches and Methodologies

As markets and products have become more global, international harmonization of required testing approaches and testing guidelines has become essential. In the area of safety testing, a number of organizations have played a significant role in facilitating the development of harmonized guidelines. These organizations have often either worked with the OECD, or have made their conclusions and recommendations available to the OECD, thereby extending the resources and activities of the OECD itself. The precedents established by these organizations
provide a framework to consider in the present case, and the organizations themselves could potentially be engaged to assist in the development of harmonized guidelines.

The OECD is a key consensus-building body because its membership includes most industrialized countries and they have agreed by treaty to accept testing approaches and protocols that have been approved via the OECD consensus process. Thus, the OECD Guidance and its extension to more detailed protocol guidelines are very important.

Notable activities that provided a basis for harmonization of general genetic toxicology testing methods include the U.S. EPA GeneTox Program (Auletta et al., 1991) and the International Workshops on Genetic Toxicology Testing (IWGT) (Kirkland et al., 1994, 2000, 2003). Each of these groups brought together recognized experts in specific testing methods from around the world to review the existing literature, share their practical experience, and develop consensus recommendations on appropriate testing methods, interpretation of results, and/or testing strategies. The EPA GeneTox Program was supported by the EPA, and included representatives of OECD so that consensus recommendations could move quickly through the OECD process. The IWGT was originally an “ad hoc” group but has now been formalized under the International Association of Environmental Mutagen Societies (IAEMS). There is a major meeting to address selected issues in conjunction with each of the International Conferences on Environmental Mutagens, which is organized by the IAEMS every four years, and working groups meet additionally as necessary.

Given the magnitude and importance of the implementation of harmonized international regulatory guidelines for genotoxicity testing of microbial pesticides, it would seem prudent that interested parties work to engage those organizations that can support focused collaborative international expert workshops to develop the necessary strategy and protocols. This might include development of an appropriate decision tree for selection of test methods, analogous to that described by Goecke et al. (1999) for biotechnology products, as well as development of appropriate testing protocols for the different types of microbial pesticides. This could be undertaken with joint international support from government bodies, industry trade groups, and non-government organizations, with consideration to engaging those experienced groups that have already contributed to the development of genetic toxicology testing methods for conventional pesticides.

**Critical Elements of Testing Strategy and Recommendations for Development of Consensus**

The characteristics of both the biological test systems employed and the specific biological matrix being tested need to be considered in order to assure that testing is conducted in a manner that allows appropriate exposure of the test organism and avoids interference by non-mutagenic constituents in the test material that could modify the normal response of the test organism. Both considerations require specialized technical knowledge, and since a general testing scheme will need to utilize several different specialized *in vivo* and *in vitro* testing methodologies to evaluate several different types of organisms and/or products, development of an appropriate approach that can be applied uniformly among global regulatory agencies will
require a team of international experts with appropriate technical knowledge. The OECD and/or the IAEMS/IWGT\textsuperscript{c} organizations would be appropriate to identify appropriate experts and to develop recommended approaches that would be adopted by global regulatory bodies.

The factors that need to be addressed in order to develop an appropriate consensus approach include definition of the tests required for specific classes of pesticide products and exposure scenarios, appropriate sample preparation and/or fractionation schemes, and appropriate protocols for testing in each individual bioassay. Appropriate positive and negative control conditions, specific positive control chemicals, and methods for demonstrating a lack of modification of the control responses by constituents present in the test fractions, will need to be defined. These factors are summarized below in Tables 1-3.

Conclusions

- The general approach proposed in the EC Directive is reasonable, but details of appropriate test selection and specific guidelines have not been addressed sufficiently

- Certain of the tests recommended in the EC Directive are not the most appropriate and newer or alternative methods are available that are more appropriate for regulatory application

- Existing testing guidelines for chemical pesticides may not be directly applicable to testing microbial products, and specific guidelines for each test system and type of microbial pesticide to be evaluated, modified as necessary to avoid interference by constituents in the test samples, are needed

- A more comprehensive discussion, with references, of the available testing methodology and appropriate protocols, should be developed

- A “decision tree”, analogous to that developed for the testing of biotechnology products by the GUM section of the European EMS, would be useful as a guide to appropriate testing strategies and methodologies

- Recommendations would best be developed by existing or specifically constituted working groups of international experts with experience in genetic toxicology assessment, testing of biotechnology and cellular products, knowledge of microbial secondary metabolism, and with specific experience in the use of the test systems to be proposed for use

- International harmonization of guidelines is highly desirable, and an international process that includes representatives from those countries and regions with existing guidelines should be used to ensure consistency of guidelines worldwide

- OECD guidelines based on the above are highly desirable as a means to assure harmonization of appropriate regulatory testing approaches throughout the world

\textsuperscript{c} International Association of Environmental Mutagen Societies/International Workshops on Genetic Toxicology
Table 1. Testing Strategy: Needs for consensus

- Products to be tested (incl. criteria for concern and extent of testing, such as relationship to species known to produce genotoxicants)
  - Fungal products
  - Bacterial products
  - Viral products (including integration into mammalian genome)

- Extent of testing
  - Specific tests
    - “Core” screening tests
    - Extended testing
  - Factors triggering extended testing
    - Exposure determinants (extent and route of human exposure)
    - Testing outcomes (e.g., positive in initial screen, or level at which positive)
    - Relationship to microbial species known to produce genotoxicants
    - Other (SAR, class experience, etc.)
  - Factors influencing selection of follow-up tests
    - Nature of original response and/or mechanism
      (e.g., bulky adducts vs. strand breaks, mutation vs. chromosomal aberration)
    - Knowledge of mechanisms (e.g., involvement of reactive oxygen or nitrogen species, pool imbalance, spindle disruption, etc.)
    - Criteria for in vivo measurements (route of human exposure, tissues with greatest concentrations, metabolic activation requirements, etc.)

- Risk assessment criteria
  - Criteria for interpretation and for significant vs. insignificant or low risk
  - Relevance to humans
### Table 2. Sample Preparation and Testing Protocols: Needs for Consensus

- Methods of lysis of intact cells (e.g., physical disruption, detergents, solvents, etc.)
- Extraction and/or separation methods (e.g., solvents, columns, adsorbents, filtration, etc.)
  - For liquid culture (e.g., exotoxins)
  - For whole cells (intra-cellular constituents)
  - For cellular products
  - Release from bound forms (e.g., conjugates and macromolecular binding)
- Removal of interfering substances
  - Toxic constituents (toxic to test organism)
  - Protein, fat, carbohydrates
  - Precipitates
  - Nutrients, growth factors or inhibitors (e.g., histidine in Ames test, lectins in lymphocyte chromosomal aberration test, inflammatory agents in *in vivo* tests, etc.)
  - Enzymatic activity (e.g., kinase or phosphoribosyltranseferse activity in *tk*+/− or *hprt* assays)
- Protocols specific to each test system and class of microbial pesticide
  - Positive and negative controls
    - Appropriate positive control chemicals
    - Procedure to demonstrate lack of modification of response by inactive cellular constituents
Table 3. Test Systems to Consider, and Definition of Circumstances in which Appropriate

- **In vitro** gene mutation tests
  - Ames test (*his*<sup>−</sup> reversion in bacteria)
  - Mouse lymphoma *tk*<sup>−/−</sup> forward mutation assay
  - Chinese hamster *hpkt* assay
- **In vitro** chromosomal aberration assays
  - Chinese hamster CHO or V79 assays
  - Human lymphocyte assay
  - *In vitro* micronucleus assays
  - Other
- **In vitro** DNA damage assays
  - Comet assay
  - Hepatocyte UDS assay
  - Microbial *rec*<sup>−/−</sup> or SOS assays
- **In vivo** chromosomal aberration assays
  - Erythrocyte micronucleus and bone marrow chromosomal aberration assays
  - Lymphocyte chromosomal aberration and micronucleus assays
  - Micronucleus or chromosomal aberration assays in other tissues
- **In vivo** gene mutation assays
  - Lymphocyte *hpkt* or *tk*<sup>−/−</sup> assays
  - Transgenic mutation assays (*lacI, lacZ, gpt/spi*)
- **In vivo** DNA damage assays
  - Comet assay
  - UDS assay
  - DNA adduct assays
  - Alkaline elution assay
- **In vivo** germ cell assays
  - Transgenic mutation assays in germ cells
  - Spermatogonial cytogenetics
  - Sperm assays, incl. aneuploidy
  - Dominant lethal and heritable translocation assays
  - Mouse spot and specific locus tests
- Human assays
References

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