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Relationship between safety data and biocontainment design in the environmental assessment of fermentation organisms – An FDA perspective

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SUMMARY

The Center for Veterinary Medicine requires strain/construct-specific data for recombinant fermentation organisms used in the production of animal drugs and feed additives. Fermentation plant biocontainment schemes are chosen based, in part, upon the ability of the organism to survive and persist in the environment and to transfer genetic information to indigenous organisms. Survival and persistence study methods may include one of the following ecosystems: activated sludge, mammalian gut, soil or river water. Gene transfer protocols can be incorporated into a persistence study. These studies are designed to show that the recombinant construct behaves similarly to the host in a representative ecosystem where the organism could be introduced inadvertently. The studies need to provide repeatable results and reflect current state-of-art design and methods. Data verification is conducted by FDA investigators during Good Laboratory Practice inspections. Biocontainment guidelines, such as those developed by the NIH Recombinant DNA Advisory Committee, set general biocontainment goals for large groupings of recombinant organisms. The FDA, as required under the National Environmental Policy Act, must base its decision making on verifiable scientific data specific to each application. Therefore, in addition to using these guidelines as benchmarks, sponsors are required to submit strain/construct-specific data to support the selection of an appropriate biocontainment level. Once additional well-controlled studies for a variety of constructs are available, broader generalizations as to biocontainment may be drawn.

INTRODUCTION

The potential for unanticipated adverse effects resulting from the release of genetically engineered microorganisms (GEMs) into the environment has been the source of much discussion since the development of recombinant DNA technology in the early 1970s. This paper will review various factors, biological and non biological, that should be considered prior to the widespread commercial use of GEMs in contained fermentations. Selected individual factors scrutinized by the Food and Drug Administration (FDA) for new animal drug applications involving recombinant DNA-derived fermentation microorganisms and how this information is used to determine a reasonable level of biocontainment will be addressed.

The FDA is responsible under the National Environmental Policy Act of 1969 (NEPA) for predicting the potential environmental impact of its actions and for disclosing to the public the assessment and evidence of how environmental factors were considered in the decision making [15]. The overall procedures for these activities are governed by the regulations of the President's Council on Environmental Quality (40 CFR 1500–1508). The CEQ regulations apply to all programs of the Federal government, except two, the registration of pesticides under the Federal, Insecticide, Fungicide and Rodenticide Act (FIFRA) and the review of chemicals under the Toxic Substances Control Act (TSCA).

The CEQ regulations allow FDA to prepare implementing procedures that: (i) identify environmental requirements for specific classes of actions; (ii) recommend classes of actions that do not individually or cumulatively present any potential for environmental impact (categorical exclusions); (iii) require applicants and petitioners to prepare environmental assessments (EAs); and (iv) provide technical guidance to assist preparers of environmental assessments [6].

Typically, a sponsor of a recombinant DNA-derived product must submit information similar to that which is required of non-recombinant products. The information is usually presented in an EA and must describe the environmental control and occupational exposure prevention measures that are part of the product manufacture. The EA must also contain information concerning potential impacts due to the use of the product, but this subject is outside the scope of this paper. FDA must be able to verify

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the accuracy and the appropriateness of the information contained in the EA. Upon acceptance of environmental documents prepared by applicants, the agency becomes responsible for the accuracy and objectivity of the EA. When the product is approved, the EA is released to the public.

In summary, NEPA establishes a framework for public oversight of FDA's environmental decision making. Since that decision making includes the potential impact of the manufacture of FDA-regulated products, biocontainment decisions for recombinant fermentation organisms are reviewed by the FDA and the public. Failure to consider appropriate information in those decisions is reviewable by the courts. What then, is the appropriate information used to formulate and support biocontainment decisions?

BIOLOGICAL CHARACTERISTICS

FDA considers individual animal drug products for approval. Therefore, it would appear logical to consider information specific to the biology of the individual production strain used to produce that product. However, it is not practical to attempt to completely characterize every biological aspect of the production strain. Which characteristics make the most sense to evaluate?

The overall environmental fate considerations associated with recombinant DNA-derived fermentation microorganisms that the Center for Veterinary Medicine (CVM) primarily considers are: (i) survivability and colonization potential of the recombinant organism in the environment; and (ii) the ability of the recombinant organism to transfer some or all parts of its genome to indigenous organisms in the environment, dependent or independent of its survival or colonization ability.

There are environmental effects considerations associated with the fate considerations. For example, a potential effect on the environment resulting from colonization would include the displacement of environmentally important microorganisms by recombinant microbes with consequential effects on microbe-mediated ecological processes (e.g., disruption of nutrient cycling, important symbiotic relationships, etc.). Another potential effect involved with an organism that displays any pathogenic characteristics could be the inadvertent release of and establishment of organisms pathogenic to humans, other animals or plants.

The discussion that follows uses as examples *E. coli* K-12 constructs with well-known plasmid expression systems. The characteristics we will discuss **should not** be thought of as unique to or specific hazards associated with *E. coli* strains. In fact, *E. coli* K-12 strains containing pBR322 expression systems incorporate many of the known safe biological characteristics that would be expected in a environmentally responsible construct. These basic biological characteristics may be used to evaluate entirely different bacterial constructs for use in commercial fermentations.

INFORMATION INDEPENDENT OF THE RECOMBINANT CONSTRUCT

The host, vector, and inserted genetic material in the recombinant organism and how that organism will be manipulated to produce indigenous proteins or their close derivatives need to be described in detail. The scientific literature must be searched for basic information concerning the GEM to provide: (i) the characteristics of the unaltered parental bacterium; (ii) the characteristics of the unaltered plasmid vector; and (iii) the presence of transposable elements within the parental bacterium and unaltered plasmid vector.

CHARACTERISTICS OF THE UNALTERED PARENTAL BACTERIUM

Let us now examine the previously mentioned biological characteristics individually, in a bit more detail, remaining within the boundary of *E. coli* K-12 batch fermentations. Within this fairly innocuous and relatively widely studied organism exists certain characteristics: pathogenicity, colonization ability, and gene transfer ability which can be identified as to being associated with a greater or lesser degree of potential risks to man and the environment.

Human and animal pathogenicity of the parent strain is an important initial characteristic that requires evaluation at the earliest stage in production strain development. Distinct types of *E. coli* are known to cause urinary tract infections, gastroenteritis, septicemia, wound infections and meningitis. Enteropathogenic, enteroinvasive, and enterotoxigenic *E. coli* are involved in *Salmonella*-type diarrhea, bacillary dysentery, and travelers diarrhea, respectively [2]. Mastitis can be caused by *E. coli* types as well. Piliation, which often contributes to adhesive tendencies, has been associated with virulence in *E. coli* and is a characteristic to be avoided in the selection of a suitable parent strain. In short, any characteristic that increases the potential pathogenicity of the parent strain increases the risk associated with using that strain.

Associated with pathogenicity is the colonization potential of the parent strain. Organisms that demonstrate the potential to establish and grow to sufficiently high cell density in a given environment can be associated with a greater potential to colonize humans and animals.

Colonization ability or survival potential is related to various characteristics of the organism. The competitive

ability (i.e., the ability to effectively compete with indigenous organisms for limiting nutrients, growth factor, etc.) nutritional requirements, resistance to environmental stress, and the ability to escape predation are all involved in colonization potential. Various experimental systems, such as microcosms or rodent gastrointestinal tracts can be used as model environments to assess colonization ability in a microorganism. The greater the potential to colonize an environment (human included) the greater the potential uncertainty associated with the commercial use of that strain.

The potential to transfer genetic material is an important character to examine in the parent strain. F plasmid conjugation (i.e., Hfr, F^+, and F′) is involved with the ability to transfer chromosomal and non-chromosomal genes to recipient F^- bacteria and must be clearly identified [3]. In addition, transformational and transductional transfer potential must also be evaluated for the parent strain. Any characteristic that increases the potential to transfer genetic material, especially the introduced novel gene, between related or unrelated microorganisms is undesirable in a commercial fermentation microorganism.

CHARACTERISTICS OF THE UNALTERED PLASMID VECTOR

Commonly used vectors for *E. coli* are the pBR322 and pBR325 plasmids [1]. Characteristics associated with the unaltered plasmid can be broadly evaluated in the same manner as for the parent bacteria strain. The ability to overcome natural barriers to plasmid transfer, the transfer efficiency of the vector (either conjugative or nonconjugative), the degree of sequence characterization of the plasmid, the inclusion or exclusion of potentially harmful sequences, the potential ability to transfer antibiotic resistance, and the predicted environmental stability of the plasmid all figure in an evaluation of the probable safety of a recombinant DNA protein production system [23]. Any characteristics that increase the chance of plasmid movement to other organisms are undesirable in a commercial fermentation microorganism.

Various factors exist that promote plasmid transfer between microorganisms. These factors include: (i) the ability to make effective contact between the donor and recipient cell; (ii) the donor cell's ability to transfer plasmid DNA to the recipient cell; (iii) the plasmid's ability to evade the defensive systems of the recipient cell (e.g., restriction enzymes); (iv) the plasmid's ability to replicate and segregate to daughter cells; and (v) the plasmid DNA's ability to express protein products (i.e., to be transcribed, translated, and phenotypically expressed) [23]. These barriers to movement should be evaluated when selecting the parent strain and plasmid vector.

The transfer efficiency of conjugative and nonconjugative plasmids must be taken into account prior to vector selection. Any characteristics that increase gene transfer efficiency are undesirable.

Large conjugative plasmids (60–120 kb) have several characteristics that can increase potential gene transfer and thereby the uncertainty associated with their use as vector/expression systems. Large conjugative plasmids often contain unidentified DNA, cryptic genes and transfer genes (Tra^+) [23]. They often have broad host ranges and can cross genus and species boundaries, particularly if they exhibit a lesser degree of replicon sequence specificity.

Small nonconjugative plasmids (1.5–15 kb) exhibit far less genetic transfer. They are maintained in the cell in low copy number, and lack the transfer genes (Tra^-) found in the conjugative plasmids [23]. However, a functioning mobilizing (Mob^+) system can overcome the lack of transfer genes (Tra^-) to transfer DNA to a new host [12,18]. Therefore, the selection of a plasmid that lacks transfer genes and contains a non-mobilizing system (Tra^- Mob^-) prevents potential mobilization by known helper plasmids.

TRANSPOSABLE ELEMENTS

The presence of transposable elements, associated with either the parent strain or the vector, needs to be determined as best possible. The type and number of transposable elements present (i.e., transposons or insertion sequences) and the factors controlling their movement and potential rearrangement need evaluation. These include the specificity of the target site, the molecular size of the element, the spectrum of transpositional host movement, the host range within gram negative bacteria, and the method of replication [23]. Generally, a greater number of elements present results in a greater potential for transfer.

USE OF PARENT STRAIN AND PLASMID INFORMATION IN SELECTING POTENTIAL PRODUCTION STRAINS

In light of these biological characteristics, it would not appear wise to use an *E. coli* strain for recombinant production possessing any known combination of pathogenicity, high colonization ability, and high genetic transfer ability. In the other extreme, known poor colonization ability, poor or no gene transfer ability, and poor or no pathogenic capabilities would provide evidence of a good choice for the parent of the recombinant organism to be constructed. The inclusion of drug resistance markers, if carried by the strain and used as a fermentation para-

meter, might facilitate colonization of the G-I tracts of fermentation hall workers, especially those receiving antibiotic therapy. This needs to be addressed, as most fermentation biocontainment levels are designed to minimize but not completely prevent, releases of organisms.

TIER TESTING: FATE AND EFFECTS TESTING

We approach the decisions involved with fate and effects testing using the tier testing regime. The results of each test in each tier (i.e., tier I – construct characterization, tier II – environmental introductions, tier III – environmental fate, and tier IV – environmental effects) are evaluated in order to determine the appropriate tests at the next higher tier, if necessary. For example, if after introduction, the organism survives or transfers genetic material to indigenous microorganisms, then there is a need to examine the potential for deleterious effects (i.e., environmental effects). This approach is analogous to reviewing environmental fate information to determine the need for environmental effects testing for xenobiotic chemicals.

STRAIN-SPECIFIC EXPERIMENTAL FATE DATA

Production strain/construct specific information, generated by laboratory experiments, in addition to the literature evaluation of biological characteristics of the GEM, should provide a scientific basis to support the drug sponsor's selection of a biocontainment system.

The initial data requested by CVM for recombinant production *E. coli* K-12 strains has been designed to address the two environmental fate concerns associated with GEMs mentioned previously. These include: (i) the survivability and colonization potential of the recombinant organism in the environment, and (ii) the ability of the recombinant organism to transfer some or all parts of its genome to indigenous organisms in the environment, dependent or independent of its survival or colonization ability.

ENVIRONMENTAL FATE EXPERIMENTAL DESIGNS

It is widely accepted that aquatic and terrestrial laboratory microcosms are useful for examining the fate and effects of introduced microorganisms as well as their survival and persistence in specific environments [22]. In 1989, the Committee on Scientific Evaluation of the Introduction of Genetically Modified Microorganisms and Plants into the Environment, of the National Research Council, published *Field Testing Genetically Modified Organisms: Framework for Decisions*. In Chapter 10, the Subcommittee on Microorganisms (i.e., Lenski (Chairman), Bottomley, Chakrabarty, Colwell, Farrand, Haselkorn, Milkman, Sequeira, and Tiedje) discussed the suitability of microcosms for testing microbial introductions and methods of monitoring GEMs in the environment.

CVM has supported animal drug sponsor use of microcosm-based experimental designs in order to test the survival, persistence and gene transfer potential of their recombinant DNA-derived *E. coli* K-12 fermentation production strains. These designs have included: (i) soil-containing microcosms [8,19,21]; (ii) water-containing microcosms [20]; (iii) activated sludge/wastewater-containing microcosms [9,10,14]; and (iv) the gastrointestinal (GI) tract of rodents [4,5,13]. We acknowledge the contributions of various firms for sharing much of their information concerning the use of these microcosms.

Environmental survival and persistence of the production *E. coli* strain should be determined in tandem with the unaltered parent strain as a reference. In some cases the homologous, plasmidless parent is used. The objective is to show that survival and persistence of the recombinant strain is not significantly different from the unaltered parent *E. coli* K-12 strain.

These studies start with the addition of a known quantity of viable *E. coli* into the test system and, at various time points thereafter, aerobically plating sample dilutions to estimate the viable growth in colony-forming units (CFUs) over time. The parent and recombinant strains are compared directly and die-off curves are plotted for each strain. These die-off curves are compared to representative data concerning the die-off of *E. coli* K-12 strains in similar settings in the scientific literature. So far, no significant differences have been observed between recombinants, their parents and representative K-12s.

Gene transfer studies can be initiated from survival and persistence microcosms by importing a gene detection protocol into the study. Once the recombinant *E. coli* CFUs have fallen below the limits of aerobic plate counting detection (i.e., usually less than 10^2 CFU/ml) in the survival and persistence study, the search for transfer to indigenous organisms in the microcosms can be attempted. Various combinations of gene amplification with recombinant-specific probes and hybridizations have been used to search for evidence of gene transfer [11,24,25]. So far, gene transfer from recombinant production strains to indigenous microbes has not been detected.

BIOLOGY INTERFACES ENGINEERING

This is where the greatest challenge lies for both the regulated industry and government reviewers. We need a better translation from biology into engineering and a means to confirm the quantitative accuracy of that translation. The scientific literature information concerning the

characteristics of the parental strain and the sponsor-generated experimental data specific to the recombinant production strain may together provide a way to make decisions concerning the appropriate level of biocontainment for the commercial fermentation facility. How do we make that translation?

Currently, we continue to have reservations concerning models that test designs used for comparing parental and recombinant microorganisms. The 'cup full of nature type' (i.e., constructed by adding nonsterile soils, waters, sediments, etc., to laboratory settings) microcosms lack the standardization and repeatability required for validation of a model system. The goals of a validated model would be: (i) to allow the prediction of potential hazards; (ii) to provide a valid comparison of one characteristic between the host and the GEM such that a conclusion of similarity between them can be drawn for important biological parameters; and (iii) to assist the design of biocontainment for the recombinant organism.

Strain-specific SOPs should be developed according to the known scientific information concerning the microorganism and the experimental data that have been generated. For example, the cultivation procedures from Master Seed through fermentation inoculation will require SOPs that reflect the known biological characteristics of the recombinant production strain. Validation of procedures and the development of SOPs for the inactivation procedures and all aspects of the fermentation process, including start-up, shake flask cultures, sample taking, preparation of stock solutions and media, etc., can be developed. In the event of catastrophic or minor fermentor releases of the recombinant microorganisms, spill and cleanup SOPs will need to be in place. Production strain-specific employee training programs for handling the recombinant microbes are also needed.

Strain-specific engineering decisions related to the biocontainment of the production strain may be made according to the known scientific information concerning the microorganism and the experimental data that have been generated. For example, will handling the recombinant microorganisms be done entirely in a closed system with exhaust gas filtering? What type of agitator and sample port seals will be necessary for the fermentors handling the recombinant microorganism? How are effluent streams to be inactivated prior to release? What type of protective clothing and equipment is necessary for the workers handling the recombinant strain? Do the contents of the entire fermentor need to be held prior to inactivation in the event of catastrophic fermenter failure? Should the fermentation hall carry restricted access and biohazard warning signs? All of these engineering decisions should be partly based upon what is known about the biology of the production strain. In addition, if this facility and fermentor are to be used for other fermentation products, how important is adaptability to the engineering?

These decisions cannot, of course, be made in vacuum. There are both guidance and constraint factors that interact with SOPs and engineering in the overall biocontainment decisions to be made. The National Institutes of Health's Recombinant Advisory Committee (NIH-RAC) published expert opinion guidance in May of 1986 for the safe handling of recombinant DNA microorganisms in laboratory settings [16]. These guidelines were expressly developed for controlled research environments. The Organisation For Economic Co-Operation and Development also prepared guidance in 1986 for cooperating member nations [17]. These guidelines were developed for research and commercial facilities. The two documents are similar in content and may be viewed as benchmarks for biocontainment decisions. Good Manufacturing Practice (GMP) regulations require the development of SOPs, record keeping and other documentation, and validation data supporting manufacturing procedures. Other constraints to be considered in the overall biocontainment decision include: (i) Federal, state, and local emissions requirements; (ii) water quality standards; (iii) workplace safety requirements (OSHA); (iv) hazard communication rules; (v) right to know impacts; and (vi) liability insurance costs.

AN EXAMPLE TRANSLATION OF BIOLOGY TO BIOCONTAINMENT

There is no clear-cut guidance for translating the scientific literature and the sponsor-generated production strain-specific data into specific biocontainment levels, replete with required SOPs and engineering.

Certain characteristics, such as frank pathogenicity and promiscuous gene transfer, should clearly be avoided. For example, if it was absolutely necessary to utilize an *E. coli* carrying a self-transmissible plasmid associated with enterotoxigenic properties (ENT plasmid), it would probably behoove the sponsor to incorporate a higher level of physical and biological containment (i.e., higher than an non-ENT carrying *E. coli*) at all stages of the fermentation process. This might be translated into maintaining a closed system at all stages of the fermentation and processing, HEPA-filtering fermentation exhaust air, and keeping a rigorous environmental monitoring regimen in place. This would be done to avoid transferring the undesirable phenotype to workers and to indigenous, fermentation hall-inhabiting microorganisms. Additionally, further complicating the matter, ENT plasmids often carry genes for colonization factors, which allow the bacteria to adhere to the intestinal wall, and also can carry genes for multiple antibiotic resistance [7]. How would these additional ENT plasmid characteristics be translated into biocontainment

if they were found to be present? Conversely, if the ENT plasmid was non-self-transmissible, which requires the activity of either transposons, conjugative plasmids or other plasmids to promote transfer into other strains, would a lesser degree of physical and biological containment be appropriate?

CLOSING REMARKS

At CVM, we are not all-knowing and all-seeing. There simply are no hard rules governing the biocontainment decisions to be made for commercial use of recombinant DNA-derived fermentation microorganisms. The testing CVM requests for its decision making continues to work towards developing the attributes of the best testing models in order to make better commercial and regulatory decisions.

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