CASE STUDY No. VI

BIOREMEDIATION AND BIOSENSING USING BACTERIA

Overview

This case study examines the federal regulatory process with respect to a bacterium that was genetically modified to detect the presence of, and degrade, hazardous wastes derived from petroleum. Genes from several other organisms were introduced into the transgenic bacterium. The primary regulatory statute involved is the Toxic Substances Control Act (TSCA), 15 U.S.C. §§ 2601-2692, administered by the United States Environmental Protection Agency (EPA).

1. Proposed Organism and Use

This case study examines the decision to allow the field testing of a genetically engineered bacterium designed to detect and degrade hazardous chemical wastes. These wastes, derived from crude or refined petroleum products, are found in many hazardous waste sites, and present a serious public health risk. The recombinant bacterium, with genes introduced from several other organisms, was developed by the Department of Energy (DOE) and the University of Tennessee. The genus *Pseudomonas,* on which the recombinant bacterium is based, is known for its broad nutritional versatility. This versatility enables pseudomonads to use many naturally-occurring and synthetic wastes as sources of carbon and energy, thus reducing organic wastes to less toxic metabolites (Silver, et al., 1990). This genus was, therefore, a logical candidate to enhance for biodegradative and biosensor applications.

The recombinant bacterium *Pseudomonas fluorescens Strain HK44* was released in 1996 for the detection and biodegradation of polycyclic aromatic hydrocarbons (PAHs) in soil. This small-scale field test represented the first environmental release of a recombinant microorganism for bioremediation in the U.S. *Pseudomonas fluorescens Strain HK44*, containing the recombinant plasmid pUTK21, was released to large partially enclosed containers referred to as lysimeters. These lysimeters are located at the Department of Energy's (DOE's) Y-12 site at the Oak Ridge National Laboratories near Oak Ridge, Tennessee.

Pseudomonas fluorescens Strain HK44 was intended to both degrade PAHs, and to serve as a biosensor which produces visible light in the presence of bioavailable PAHs. PAHs are found in crude and refined petroleum oil products, and consist of two or more benzene rings fused together with at least two common carbons. PAHs are present in higher concentrations in heavier petroleum hydrocarbon blends and particularly in certain fuel oils, coal tars, wood-treating chemicals, creosote, soot, and refinery wastes. These compounds have limited water solubility, and adhere strongly to subsurface materials. PAHs are a concern due to their potential to cause adverse human and ecological effects, and are present in the soils and sediments of many hazardous waste sites across the U.S. Substances containing PAHs are recognized, for example, as skin carcinogens in humans and animals. Biosensors such as HK44 offer a less expensive and more rapid way to monitor PAH concentrations in soils, sediments, and groundwater at hazardous waste sites, as opposed to traditional chemical detection

methodologies. Such monitoring is useful to assess initial concentrations of contaminants, determine contaminant movements off-site, and assess the progress of clean-up activities associated with a site. Further, biosensors may provide a way to assess what fraction of PAHs at a site are actually bioavailable for uptake by humans or wildlife.

Bioremediation of organic wastes such as PAHs and other contaminants has become a broadly accepted remediation technology in the U.S. and elsewhere: the technology can often be applied in a cost-effective manner, and it employs naturally-occurring bacteria and other microorganisms which often utilize organic wastes as carbon sources for growth. In addition to bioremediation, the use of genetically engineered microorganisms as alternative means of detecting hazardous wastes is the subject of research at present. Bacteria such as HK44 may offer a way to detect wastes in a less expensive way than by chemical means (gas chromatography, mass spectroscopy, electrophoresis, etc.) (Rogers and Gerlach, 1999). Strain HK44 cells which produce light (due to introduced light-producing genes) can be either applied directly to the soil, or placed in small photomultiplier probes; the organisms then produce light in amounts relative to the concentrations of contaminants in polluted soil or groundwater.

Strain HK44 was identified by EPA as a *P. fluorescens* Biovar II, and was fully characterized in terms of the taxonomy of the recipient bacterium and its introduced DNA on plasmid pUTK21. The recipient strain, and the donor strain for the plasmid pKA1 on which the recombinant plasmid pUTK21 is based, were both identified as members of the species *P. fluorescens*. Additional DNA used in constructing HK44 came from the bacteria *Photobacterium fischeri* and *Escherichia coli.* Details on the construction of HK44, identity of taxa and DNA used, and explanation of how HK44 functions as a PAH degrader and biosensor, can be found in **Appendix 1**.

2. Relevant Regulatory Agencies, Regulatory Authority, and Legal measures

Pre-release approval for Strain HK44 was obtained under the Toxic Substances Control Act (TSCA) authority of the U.S. Environmental Protection Agency (EPA), after submission to EPA of a Pre-Manufacture Notification (PMN). The PMN was prepared by the University of Tennessee and contained the bulk of the safety information reviewed. This information was also considered by the U.S. Department of Agriculture Animal and Plant Health Inspection Service (USDA/APHIS) as part of its review under the Plant Pest Act, by DOE safety coordinators involved with the field test, and by DOE under the National Environmental Policy Act (NEPA), 42 U.S.C. §§ 4321-4370e. The initial release of 10^{14} cells and subsequent survival of Strain HK44 were monitored over a two-year period by Oak Ridge National Laboratory (ORNL) and University of Tennessee under an EPA Consent Order issued under TSCA. A summary of the risk assessment for the release of Strain HK44 can be found in Sayre (1997), while the results of the field test can be found primarily in Ripp, et al. (2000). Details of these processes are described below.

TSCA applies to microorganisms for uses not specifically excluded by Section 3 of the statute (e.g., pesticides which are covered under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), 7 U.S.C. §§ 136-136y; and drugs addressed under the Federal Food, Drug, and Cosmetics Act (FFDCA), 21 U.S.C. 301 et seq.) Under Section 5 of TSCA (15 U.S.C.

§ 2604), EPA conducts premanufacture reviews of "new" microorganisms, as well as traditional chemicals, which are manufactured or imported into the U.S. Such reviews apply to "intergeneric" microorganisms, irrespective of the process by which they were created. The TSCA Section 5 authority extends broadly to a number of different types of microorganisms. According to the TSCA regulations, "*microorganism* means an organism classified, using the five-kingdom classification system of Whittaker in the kingdoms of Monera (or Procaryotae), Protista, Fungi, and the Chlorophyta and the Rhodophyta of the Plantae, and a virus or virus-like particle."(40 CFR725.3). If there is reason to believe a microorganism might be a plant pest, APHIS also has authority to review the applications.[1]

EPA has defined intergeneric microorganisms as those microorganisms resulting from the deliberate combination of genetic material originally isolated from organisms classified in different genera: for example, a *Pseudomonas sp*. bacterium, with DNA from an *Escherichia sp*. bacterium, would be considered intergeneric (40 CFR 725.3). Examples of commercial uses of microorganisms subject to TSCA include specialty chemical and enzyme production, bioremediation, biosensors of environmental contaminants, biofertilizers, ore mining, oil recovery, and biomass conversion.

The Office of Pollution Prevention and Toxics (OPPT) issued final TSCA biotechnology regulations in 1997 that describe both the various biotechnology submissions, and exemptions (62 Fed. Reg. 17, 190 (April 11, 1997)). These regulations created a reporting vehicle specifically designed for microorganisms: the Microbial Commercial Activity Notice (MCAN) (40 CFR 725.3 and 725, Subpart D). Persons intending to use intergeneric microorganisms for commercial purposes in the U.S. must submit an MCAN to EPA at least 90 days before such use. EPA has 90 days to review the submission in order to determine whether action is necessary to protect human health or the environment. The rules also address intergeneric microorganisms used in research and development (R&D) for commercial purposes and create a vehicle for reporting on testing of new microorganisms in the environment -- the TSCA Experimental Release Application or TERA (40 CFR 725.3 and 725, Subpart E). A TERA must be submitted to EPA at least 60 days prior to initiating such field trials. The TERA is designed, in recognition of the needs of researchers, to provide a high measure of flexibility and a shorter review period (60 days).

In addition to these types of submissions under TSCA, certain intergeneric microorganisms are exempt from the requirement to submit an MCAN if the manufacturer meets criteria defining eligible microorganisms, introduced DNA, and containment practices. This exemption is most applicable to the manufacture of specialty and commodity chemicals, particularly industrial enzymes. Intergeneric microorganisms used for R&D in contained structures are exempt from EPA reporting requirements, if researchers maintain records demonstrating eligibility. Researchers are exempt from this record-keeping requirement when

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1 Some examples of transgenic fungi for which APHIS has prepared environmental assessments include: *Cephalosporium gramineum* (APHIS number 96-127-02r), a pathogen of wheat, which was engineered with a marker gene to gain a better understanding of the biology of the fungus and the mechanism of its infection process, and *Fusarium moniliforme*(98-355-01), genetically engineered to not produce fumonisin toxins and to be resistant to the antibiotic hygromycin B.

the researcher or institution is in mandatory compliance with the National Institutes of Health (NIH) "Guidelines for Research Involving Recombinant DNA Molecules" (59 FR 34496, July 5, 1994). Those researchers voluntarily following the NIH Guidelines can, by documenting their use of the NIH Guidelines, satisfy EPA's requirements for testing in contained structures. Alternatively, researchers can take the exemption by documenting that they meet eligibility criteria identified by EPA, including oversight of the research by a technically qualified individual, and containment and inactivation of microorganisms used. Certain intergeneric microorganisms in R&D field testing are also exempt due to prior experience with their release. For example, testing on ten acres or less involving *Bradyrhizobium japonicum* and *Rhizobium meliloti* is exempt when the criteria specified by these rules are met. These criteria address the inclusion of only specific introduced genetic material (that which is poorly mobilizable), and specify restrictions on exposure to the rhizobia during field testing.

When considering intergeneric microorganisms under Section 5 of TSCA and the implementing regulations at 40 CFR Part 725, EPA reviews the microorganisms for their potential to cause unreasonable risks to human health and the environment (15 U.S.C. § 2604(a)). TSCA does not expressly define unreasonable risk"; however it does provide a list of factors in section 6 to consider when making that determination (15 U.S.C. § 2605(c)(i), see also § 2604(b)(4)(A)(ii)). These factors make it clear that during its review, EPA is required to consider both the extent to which risks would be avoided by regulation and the burden imposed by that regulation. If EPA identifies any unreasonable risks, it is required to take action to prevent the unreasonable risks before the microorganism can be manufactured or imported either for research and development, or on a commercial scale (15 U.S.C. § 2604(f), see also 40 CFR Part 725). With a few exceptions established in the regulations, this would include situations prior to a release to the environment. The TSCA review is considered the functional equivalent of a NEPA review because it encompasses all foreseeable hazards/risks, whether to human health or the environment.

This case study focuses on the premarket approval process for an intergeneric bacterium proposed for use in bioremediation and biosensing under Section 5 of TSCA and its accompanying regulations. However it should be noted that other microorganisms- intergeneric and naturally occurring- can be addressed under other sections of TSCA if there exists a need and these products are already in commerce. If a microorganism subject to TSCA is not intergeneric (e.g., intrageneric or naturally occurring), and concerns are raised regarding its safety through TSCA § 8(e) notifications, TSCA FYI notifications, or other means, EPA has authority to address these issues under Sections 4, 5, 6, 7, and 8 of TSCA (15 U.S.C. §§ 2603-2607). EPA has also publicly asserted jurisdiction over other living organisms, such as certain plants intended for the cleanup of wastes. When EPA proposed the rule for intergeneric microorganisms, it said, "EPA is reserving the authority under TSCA to screen transgenic plants and animals in the future as needed" (EPA, 1994a). This was confirmed in a letter to participants in the current regulatory assessment from EPA dated December 22, 2000.

EPA regulations at 40 CFR 725.3 define microorganisms to include organisms in the kingdoms Monera, Protista, and Fungi, the Chlorophyta and the Rhodophyta of the Plantae, and viruses and virus-like particles. Genetically engineered, or naturally occurring, microorganisms from all these kingdoms (including fungi and algae) would be regulated under these regulations

if the organism were intergeneric, or if EPA had, by rule, designated their use to be a Asignificant new use." Moreover, if necessary, EPA could rely on its authority under TSCA sections 4, 6, 7, or 8 to regulate such organisms, either genetically engineered or naturally occurring. Research and development for commercial purposes are those activities that are funded directly, in whole or in part, by a commercial entity regardless of who is actually conducting the research; or which will obtain for the researcher an immediate or eventual commercial advantage.

EPA did not finalize its TSCA Part 725 biotechnology regulations until 1997 (EPA, 1997). Therefore, the 1995 OPPT review of Strain HK44 did not follow these regulations. However, the substantive nature of the OPPT review process was available in the 1994 proposed TSCA biotechnology rule (EPA, 1994a), and the TSCA portion of the Office of Science and Technolgy Policy (OSTP) "Coordinated Framework for the Regulation of Biotechnology; Announcement of Policy and Notice for Public Comment" (51 Fed. Reg. 23313-23338.). EPA had also provided guidance on preparation and submission of premanufacture notices for microorganisms under TSCA in its "Points to Consider" document (EPA, 1984). Finally, EPA had available its longstanding procedure for reviewing premanufacture notifications under 40 CFR Part 720 to guide its consideration of Strain HK44. Therefore, the risk assessment and review done on Strain HK44 under TSCA in 1995 was equivalent to what would have been done under the 1997 final TSCA regulations.

Under Section 5 of TSCA, EPA had 90 days in which to review the PMN submission for Strain HK44. If the 90 days passed without action by EPA, the submitter would have been free to manufacture or import Strain HK44 without controls. However, the review period can be extended under TSCA section 5(c) for good cause; it may also be suspended voluntarily by the mutual consent of EPA and the PMN submitter. During the review period, EPA may take action under TSCA section 5(e) or 5(f) to prohibit or limit the production, processing, distributing in commerce, use, and disposal of new chemical substances that raise health or environmental concerns.

For Strain HK44, most of the information EPA reviewed to make its regulatory decision was supplied by the submitter, who had been guided by the EPA "Points to Consider" document. The specific details of the Strain HK44 safety review are discussed below. In addition to consideration of the potential risks of the organism, the potential benefits of its use were also factored into the regulatory decision. EPA concluded that there was sufficient uncertainty about the risks of the field test that regulatory controls were appropriate at the Y-12 site, and that there were outstanding issues which needed to be addressed before HK44 was marketed commercially. The mechanism EPA used to impose controls was a consent order under section 5(e) of TSCA, as described below.

In summary, EPA regulates intergeneric microorganisms under TSCA for any uses not excluded under Section 3 of the Act. EPA's role during this review is to identify and prevent any unreasonable risk of injury to health and the environment from manufacture, processing, use or disposal of the microorganism. EPA works with other agencies, as appropriate, in its review of these intergeneric microorganisms to ensure that all relevant issues are considered. For example, EPA will review an intergeneric microorganism used as a pesticide intermediate under

TSCA, while it will review the pesticide itself under FIFRA. EPA will also refer manufacturers of intergeneric microorganisms to other agencies when review is appropriate under multiple statutes. In addition, EPA may defer regulatory oversight altogether, when the requirements of TSCA section 9 are met. EPA's regulation of intergeneric microorganisms extends from research and development for commercial purposes to commercial manufacture and use.

A review of microorganisms is conducted under USDA/APHIS regulations, in addition to that done under TSCA, if the microorganism (in this case *Pseudomonas fluorescens*) is regarded as a possible plant pest. The USDA review was integrated into the TSCA risk assessment for Strain HK44. As a general matter, most microorganisms being investigated for use in bioremediation are not plant pests. USDA/APHIS has regulations and a procedure for determining if a microorganism is regulated. APHIS regulations (7 CFR 340.2) lists groups of microorganisms which are or contain plant pests. Any organism belonging to any taxa contained within any listed genera or taxa is only considered to be a plant pest if the organism "can directly or indirectly injure, or cause disease, or damage in any plants or parts thereof, or any processed, manufactured, or other products of plants." A particular unlisted species within a listed genus would be deemed a plant pest for purposes of 7 CFR 340.2, if the scientific literature refers to the organism as a cause of direct or indirect injury, disease, or damage to any plants, plant parts or products of plants. If there is any question concerning the plant pest status of an organism belonging to any listed genera or taxa, the person proposing to introduce the organism in question should consult with APHIS to determine if the organism is subject to regulation. If APHIS determines that the microorganism is a plant pest or has the potential to be a plant pest, the organism introduction (importation, interstate movement, and field release) would be regulated as described in the herbicide tolerant soybean case study.

3. Hazard Identification, Risk Assessment, and Regulatory Review of Product

Overview of Risk Assessment Process under TSCA

The information submitted to EPA by University of Tennessee in conjunction with the Department of Energy was that specified in the EPA's "Points to Consider in the Preparation and Submission of TSCA Premanufacture Notices (PMNs) for Microorganisms" (EPA, 1994). An updated version of TSCA "Points to Consider" is available at www.epa.gov/opptintr/biotech. Using this information, the EPA's Office of Pollution Prevention and Toxics conducted a full risk assessment under TSCA based on the PMN submission, and additional information received from the submitters at EPA's request prior to the field test. This information is publicly available in the EPA Docket in Washington, D.C., and a summary of the risk assessment is also in the literature (Sayre, 1997).

The risk assessment process used to evaluate the proposed ORNL field test included detailed analyses of potential human health and ecological hazards, likely exposure scenarios, and taxonomic and construct analyses. The risk assessment addressed the risks posed at the sites of production (fermentation site at ORNL), and use (the Y-12 field site) of the HK44 microorganism. These conclusions are in the EPA's risk assessment (Broder, 1995) which provided the basis for the TSCA 5(e) Consent Order, and approval for the field test itself. The full range of scientific assessments used to reach a conclusion on risks during the EPA review of

the proposed field test, and the justifications for statements and issues noted in the Consent Order, are listed in Appendix 2. In addition to these reviews used for the risk assessment, an economic analysis was done to determine the cost of complying with TSCA and the product's benefits. The following sections under item 3 summarize the findings of the EPA risk assessment, and the regulatory conclusions reached.

Organism Characteristics

The identification of major microbial taxa and introduced DNA form a fundamental basis for assessing the potential hazards posed by a new microorganism, as well as for determining aspects of genetic stability and transfer of DNA associated with Strain HK44. The taxonomy of the recipient bacterium and identification of the introduced DNA used in constructing Strain HK44 have already been described in section 1 of this case study and its associated appendix (Appendix 1).

Review of Health and Ecological effects of Strain HK44 due to its Placement in the Pseudomonas fluorescens Biovar II Taxon

The health and ecological impacts of *P. fluorescens* have been reviewed in separate reports by Syracuse Research Corporation for EPA (1995) and by McClung (1995), respectively. Pathogenicity and toxicity information on the species *P. fluorescens* (Ballows, et al., 1991) indicates that clinical cases have been documented for this species include emphysema, urinary tract infections, postoperative infection, pelvic inflammatory disease and fatal transfusion reactions due to contaminated blood. Palleroni (1984) notes that *P. fluorescens* is not prevalent in clinical laboratories and hospitals; its ability to grow at refrigerator temperatures can lead to contamination of clinical samples, but it may not be able to grow at body temperature. *P. fluorescens* is a complex species with some members being innocuous or beneficial, and other being potentially harmful. For example, although some strains of *P. fluorescens* have beneficial effects in that they inhibit the growth of some microbial plant pathogens, some strains within *P. fluorescens* Biovar II also causes soft rot of onions (Wright and Hale, 1992), alfalfa (Turner and Van Alfen, 1983), broccoli (Canaday et al., 1991), lettuce (Miller, 1980), and other plants. Further, some strains can cause blight of cucumbers (Ohta, et. al., 1976), and have been associated with opportunistic pathogenicity in fish which are under stressed conditions (Bullock, 1964). A letter from USDA to Dr. Gary Sayler at University of Tennessee (USDA, 1996) placed the parental strains of Strain HK44 into the *Pseudomonadaceae RNA Group I Biotype D* and concluded that Strain HK44 is not a plant pathogen. Therefore, the strain was not considered a regulated article as set forth in 7 CFR Part 340. Further details from USDA supporting this conclusion, and the letter sent to USDA by Dr. Gary Sayler with information used by USDA to make its conclusions, were not available.

Environmental and human health pathogenicity were addressed in part by data requested by EPA: growth curve information (Sayler, 1995c) showed that Strain HK44 does not increase in numbers at 37^{o}C, so it was considered to be unlikely to grow at mammalian body temperatures. This finding indicated that it was unlikely that HK44 posed any human health or other mammalian toxicity/pathogenicity issues.

Review of Health and Ecological Effects of Strain HK44 due the introduced pUTK21 DNA, and the role of Gene Transfer

A concern with the introduced DNA was identified in the EPA health and ecological reviews (SRA, 1995; and McClung, 1995) is the presence of the tetracycline resistance gene. Risk issues arise when there is potential for an antibiotic resistance gene to spread (from an introduced microorganism) to microbial pathogens that are controlled (in clinical, agricultural, or veterinary settings) by the antibiotic against which the resistance is active (Neu, 1992). Expert panels convened by both the EPA (EPA, 1989) and by Health Canada (1995) found that tetracycline resistance is among the least desirable resistance markers to include in microorganisms released to the environment. This finding was made based on clinical and veterinary use of major antibiotics, and on the transmissibility of the replicons carrying the resistances. The presence of this gene imbedded in two transposons carried by a conjugative plasmid increases the potential for it to spread to other taxa.

Health and ecological effects could also result from PAH breakdown products produced by Strain HK44 due to the introduced DNA. Also, such partially biodegraded PAH products could cause concern if produced by pseudomonads other than Strain HK44 following the transfer of degradative genes from Strain HK44 to these bacteria in the environment. Although available gene transfer data did not indicate this to be a likely scenario, the possibility remains that such a gene transfer could occur due to the nature of the genetic construct (for further details on these points, please see Appendix 3). If such degradative products were produced, any associated toxicity of these products could be offset by further degradation of these potentially toxic metabolites by other soil bacteria.

Further insight into the generation of byproducts resulting from catabolic degradation of xenobiotic wastes present in soils can be gained when the specific soils to be tested are considered, along with potential gene transfer mechanisms. The interactions of Strain HK44 with specific contaminants in the soils to be added to the lysimeters were undetermined since the nature of the contaminated soils to be treated in the lysimeters had not been decided. Following completion of the EPA risk assessment, an uncontaminated, nonsterile, loamy soil was selected and spiked with napthalene, phenanthrene, and anthracene. This soil was placed in the lysimeters for treatment with Strain HK44. Therefore, no toxic metabolites were expected from these three PAHs after degradation with HK44. If the *nah/sal* pathway was transferred to another microorganism that does not have the ability to degrade salicylate and its analogs, these intermediates would be present in the soils. However, degradation of these intermediates by other microorganisms present in the soils was thought to be likely. Microorganisms may generate toxic waste metabolites that are more water soluble and, therefore, have increased mobility as compared with the parent waste. However, this factor played little role in assessing overall risk of PAH metabolites due to the degree of physical containment provided by the lysimeter design.

Other contaminants could undergo partial degradation if present in test soils. However, none of these contaminants were present in the soils in the lysimeters or at the Y-12 site. Dibenzofuran, in the presence of strains carrying NAH7, is converted to a dead-end product 4-[2'-(3'-hydroxy)benzofuranyl]-2-keto-3-butenoic acid (Selifonov, et al., 1991). Napthalene-

related compounds can be converted by pseudomonad dioxygenases to oxygenated products that on steric grounds would not be anticipated. Chapman (1978) cited data indicating that pseudomonads which initially convert napthalene to 1,2-*cis*-dihydrodiol encounter a related napthalene waste -- 1,5-dimethyl-napthalene -- it is a methyl substituent which is oxygenated to the primary alcohol and then converted to 1-methyl-5-napthoic acid with neither of the aromatic rings being oxidized. Chapman notes other studies in which acenapthene is converted by a napthalene-grown pseudomonad to 1-acenapthenol and then to 1-acenapthenone. The stability and toxicity of these compounds was not noted. All issues noted regarding toxic metabolites, while not of great concern for the proposed field test at ORNL, were noted as needing re-evaluation should Strain HK44 be used at other sites (with differing contaminants, containment, and soils).

Production, Application, Monitoring, and Disposal Characterization

Production of the cells for use in lysimeters was done using fermentors at the Oak Ridge National Laboratories adjacent to the Y-12 field test site. These fermentors had standard containment and spill mitigation procedures and equipment in place. All spent samples, and solid and liquid wastes, were autoclaved and/or chemically treated to eliminate viable cells. Air releases from fermentor off-gassing were to be vented through at least one filter. Descriptions of production, application, and disposal characterization of the ORNL field release are detailed in PMN PMN P95-1601 (University of Tennessee, 1995).

The cells were delivered from the fermentors to the Y-12 field site in secured carboys, and then released to contained lysimeters on the two-acre Y-12 site. This site is moderately sloped, has uncontaminated soils and sediments, is close to electrical power, and is reasonably secure.

The four lysimeters were originally designed for use in uranium leaching experiments connected with the disposal of uranium wastes generated by the Y-12 nuclear plant. The microorganisms were released to four 8-foot diameter by 10-foot deep lysimeters which are arrayed in a circle around a central core. Each lysimeter consists of a vertical 1/8 inch thick corrugated steel pipe which is fitted with a steel lid, rests on a concrete apron, and has a leachate collection system that empties into a 55-gallon drum. The core is large enough to allow researchers to enter and gather samples, store monitoring equipment, etc. The treatments were as follows: one lysimeter had PAH-contaminated soils only, one lysimeter had Strain HK44 in uncontaminated soils, and two lysimeters had both PAHs and Strain HK44 added.

Lysimeters were loaded with soil in a layered fashion, placing the soil containing HK44 cells between layers of clean soil. A Huntington loam soil with 1.3% organic carbon was used to prepare both clean, and PAH- and HK44-amended soil layers. Approximately 23 cm^3 of soil received the following final pre-testing concentrations of PAHs: 1,000 mg/kg napthalene, 100 mg/kg anthracene, and 100 mg/kg phenanthrene. Due to approximately 90% loss of PAHs prior to lysimeter loadings, on day 135 after test initiation additional anthracene and napthalene were added in 833 L Exxon Univolt 60 transformer oil was added via irrigation tubes directly above

contaminated soils to re-establish approximate concentrations of 1,000 mg/kg napthalene and 100 mg/kg anthracene.

The 92-cm deep treatment zone of soil was sprayed with HK44 inoculum suspended in a saline solution, at the rate of approximately 4 L of cells (containing approximately 10^{11} bacteria) per 10 cm lift of soil. A 19-liter garden sprayer with an extended nozzle was used for the application. The application of the microorganism took approximately 12 h due to the volume of soil that was sprayed. Nutrients and air were added to the lysimeters as appropriate.

Strain HK44 can be detected by several techniques including a bioluminescence MPN procedure (detection limit approximately 10 cfu/g), agar plates with tetracycline and salicylate (detection limit 10^2 - 10^3 cfu/g), and by using *nah* and *sal* probes. Air monitoring during the application was done using selective media in either gravity plates or in one-stage Anderson air samplers. Details of the monitoring procedures can be found in the PMN submission, and in Ford, et al. (1999). Representatives of the EPA Regional office for Tennessee, and EPA Headquarters staff were on site during the release, as well as State and DOE safety officials.

All instruments, equipment, soils, and other samples were sanitized. In order to show the efficacy of hypochlorite inactivation, University of Tennessee provided data (as part of the PMN submission) that showed colonies on plates cultured with yeast extract/peptone/glucose were unable to form colonies after treatment with 1 - 2% hypochlorite.

Only skilled workers familiar with microbiological techniques were involved in the field test, and no workers with open cuts or sores were allowed on site. Gloves, and respirators to protect against organic vapors, were worn on advice from DOE on-site safety personnel.

Engineering and Exposure Assessments

Exposure assessment information included the exposures possible as a result of (1) the ORNL fermentation system that produced the HK44 cells needed for the field test, and (2) the field test itself. Information on the field test design, detection limits for Strain HK44, gene transfer information on Tn*4431* and pUTK21, proposals for worker protective gear, and sensitivity of Strain HK44 to hypochlorite. These items were already discussed in Sections 1.2. and 1.3.3. All of these issues, with the exception of gene transfer, were examined in the EPA engineering (Radian, 1995) and exposure (US EPA, 1995) assessments. For more detail on the overall process for EPA engineering assessments, please see Sayre, et al. (1994).

In addition to the information above, data on Strain HK44 and its ability to establish in a variety of nonsterile microcosms containing contaminated soils and sediments was provided to EPA (Sayler, 1995c). Contaminants added to the soil in these studies included napthalene alone, diesel fuel, and a mixture of PAHs and other organic contaminants. In napthalene-contaminated microcosms, the population of Strain HK44 increased over time with simultaneous degradation of napthalene. After introduction at approximately 10^4 cells/g, concentrations reached 10^7 cfu/g at Day 10, then declined to 10^4 - 10^5 cfu/g at Day 17 (with 4% of napthalene remaining) and less than 10^3 cfu/g after 6 months. The declining concentration of Strain HK44 with decreasing PAH

concentrations is considered beneficial from a risk assessment standpoint since the microorganisms should decline in a similar fashion during field application.

Risk Assessment, and Regulatory, Conclusions for Strain HK44

The EPA concluded that the release of Strain HK44 at the ORNL Y-12 site did not pose an unreasonable risk to human health or the environment, as long as the release was conducted in accordance with the TSCA Section 5(e) Consent Order.

In the March 27, 1996 Consent Order, EPA and the submitter agreed to certain conditions that would govern the field testing of Strain HK44. Failure to comply with these conditions would have been a violation of TSCA section 15, and subjected the submitter to penalties (15 USC 2614 and 2615). Specifically, the Consent Order provided that Strain HK44 was only to be used at the Oak Ridge Y-12 site. Introduction of the Strain by means of a pesticide applicator that minimized spray drift of Strain HK44 was required. Sanitization of soils and other contaminated samples, equipment, and instrumentation was required. Such sanitization was considered effective when there was no colony forming units at the limit of detection, considered to by 10 cfu/gram of soil or liter of water. Routine monitoring in the area around the lysimeters was requested, particularly during periods when aerosol generation is more likely (such as during introduction of Strain HK44 into the contaminated soils, and the soil's subsequent introduction into the lysimeters).

In accordance with the Consent Order, quarterly reports on the status of the experiment generated for University of Tennessee and the Department of Energy were forwarded to EPA. Reports included operation evaluation of the lysimeters, operation evaluation of the monitoring equipment, analysis of data from sampling and monitoring, sampling schedule, and environmental safety and health evaluation (including accidents and injuries). Separate records of the progress of the field test were kept by the University of Tennessee for several topics including production volume, standard operating procedures, sampling information, routine monitoring activities for detection of Strain HK44, and effectiveness of sanitization techniques.

The Consent Order also identified three issues that required resolution prior to allowing the use of the same microorganism at any other site or under less stringent containment conditions. These issues would also be relevant to full-scale commercialization of Strain HK44 if the intent were to release it the many hazardous waste sites that contain PAHs. Since transfer of the tetracycline resistance to microbial pathogens could be a potential concern, data on the frequency of transfer of pUTK21 and Tn4431 should be examined. Second, the presence of persistent toxic metabolites may need to be addressed prior to commercialization. Finally, plant and animal pathogenicity concerns may need to be addressed in more detail prior to commercialization.

In addition to the EPA and USDA reviews, DOE conducted a NEPA review based on a checklist form supplied by the researchers conducting the field test. The DOE concluded that this field test qualified for a categorical exclusion from further NEPA review and consideration under 10 CFR 1021, Subpart D, Appendix B (La Grone, 1994). The exclusion under Appendix B, item B3.10 applies to small-scale research and development projects and small-scale pilot

projects conducted (for generally less than two years) to verify a concept before demonstration actions, performed in an existing structure not requiring major modification.

The application of Strain HK44 to waste sites on a commercial scale will likely require regulatory coordination with other legal mandates. For example, on a Superfund site, there is generally an EPA Remedial Project Manager (RPM) in charge of the selection of the remedy, or remediation technology, used to clean up a site. The RPM also has nine criteria, as laid out under Superfund laws, used to direct the remedy selection process. One of the criteria to consider is the extent to which the remedy provides overall protection of human health and the environment. The TSCA review of HK44 provides considerations for application at the Y-12 site, but also provides considerations for concerns at other sites. For more on the Superfund risk assessment process, see Rock and Sayre (1999). The information in the TSCA risk assessment and consent order could be passed on for consideration by RPMs. Similar coordination with site managers could occur for other non-Superfund sites where RCRA (Resource Conservation and Recovery Act) and/or State considerations dominate site decisions.

Results from the Y-12 Field Test in Open Literature

Two recent articles in peer-reviewed journals, based on results from the 1996 Y-12 field test, provide insight into the performance and containment of Strain HK44. Ripp, et al. (2000) found that Strain HK44 was capable of real-time monitoring of bioavailable PAHs in soils. Sayler later noted that the detection limits for napthalene are in the low ppm range, and that the luminescence intensity was well correlated with napthalene concentrations (Sayler, 2000). Perhaps most importantly, Ripp et al. (2000) also noted that the study showed that it is possible to establish a recombinant bioremediation microorganism in a soil ecosystem over a prolonged period. Other findings by Ford, et al (1999) showed that although 10^{14} cells of Strain HK44 were spray-applied during the field test, selective agar plates and Anderson samplers only detected HK44 cells in a few cases. HK44 colonies were only found on 36 of 260 exposed plates, only 2 plates had more than 46 colonies, and no plates outside the 4-m range (from where the cells were applied) detected viable HK44 bacteria.

4. Information and Data

As already noted, the information submitted to EPA by University of Tennessee in conjunction with the Department of Energy was that specified in the EPA's "Points to Consider" in the preparation and submission of TSCA premanufacture notices (PMNs) for microorganisms (EPA, 1994).

Information requirements are tailored in the Points to Consider guidance document to the particular type of biotechnology application: for example, different information requests are made for fermentation applications, as opposed to field tests. This information, submitted by the manufacturer is then reviewed by EPA's Office of Pollution Prevention and Toxics, with the resulting EPA risk assessment documents generated on which decisions are based (see Appendix 1 for example of risk assessment documents generated). Often, as in the case with the Strain HK44, additional data and information are requested. This can lead to extensions of the EPA review period. Outside literature, academicians, experts in other Agencies, and others are often

consulted by the Agency in making its decision. In some cases, risk issues are brought to EPA Federal Advisory Committee Act committees (FACAs) for consideration. These committees consist largely of academicians. EPA has the legal authority and technical capacity to require or generate all data considered necessary.

5 and 6. Mitigation and other management considerations, and monitoring

TSCA provides EPA with the authority to require any practical measure - including preventing commercialization - to prevent unreasonable risk. In the case of HK44, EPA's OPPT addressed concerns with the field test through the issuance of the TSCA 5(e) consent order. The conditions of this Order required monitoring, restricted use of the bacterium to one site, and mandated record-keeping and reporting to the Agency as detailed in Section 6 above. The Consent Order also identified issues for broader use of the bacterium at other sites for future consideration. For other biotechnology products reviewed under TSCA, additional conditions on production, distribution, marketing, use and disposal are or might be prescribed, including Significant New Use Rules (SNURs) to restrict additional uses of a microbial product (15 U.S.C. § 2604(a)(2)).

Prior to any further field tests, or commercialization, issues such as those raised in the risk assessment for the release at the Y-12 site would have to be considered.

7. Enforcement and compliance

EPA has the legal authority to enforce TSCA, and has an enforcement/inspection program for TSCA in place that currently includes biotechnology products (15 U.S.C. § 2610 and §§ 2614-2616).

8. Public involvement and transparency

All rulemakings concerning TSCA Biotechnology were conducted with public notice and comment pursuant to Administrative Procedures Act. EPA also has had public meetings and consulted with Agency/Government workgroups when developing its current biotechnology regulations in 40 CFR part 725. In some cases, the Agency consults its technical FACA committees on individual biotechnology product risks, although that was not deemed necessary for the review of Strain HK44.

Under TSCA sanitized versions of all notices received, rules/consent orders issued, and certain support documents (all of which have TSCA Confidential Business Information removed) are placed in a public docket for anyone to view. For more on TSCA confidentiality issues see 15 U.S.C. § 2613 and 40 CFR 725, Subpart C. The Agency also informs State and EPA Regional officials when field tests subject to TSCA are proposed in their area. Notification of TSCA reviews such as that of Strain HK44 are announced publicly in the Federal Register. The EPA's OPPT also has regular teleconferences to update all EPA's Regions on biotechnology activities. In the case of Strain HK44, both the State of Tennessee and EPA's Region IV Office were notified in advance of the field test by EPA Headquarters in Washington, D.C. EPA Headquarters and Regional representatives were present during the release of Strain HK44.

Other regulatory bodies involved in the HK44 release included the Department of Energy that sponsored development of the microorganism, and the USDA that conducted a review regarding the plant pest status of Strain HK44 under the Federal Plant Pest Act (FPPA), 7 U.S.C. §§ 150aa-150jj. Much of the information regarding the OPPT TSCA biotechnology program is available on the EPA website (www.epa.gov/opptintr/biotech).

Appendix 1: Characterization of the Recombinant Bacterium

Organism identification under TSCA consists of verifying the identity of the inserted DNA, site(s) of insertion, and the taxa used as major donors and recipients to construct the final GEOP. Strain HK44 consists of the recipient strain *P. fluorescens Strain 18H* which contains the 16kb plasmid pUTK21.

The recipient *Pseudomonas fluorescens Strain 18H* is an obligate aerobe which was originally isolated from a contaminated Manufactured Gas Plant soil, is resistant to ampicillin, and is able to degrade salicylate but not napthalene. Taxonomic identification of *Strain 18H* as a *Pseudomonas fluorescens Biovar II* was confirmed by Segal (1995).). The recipient Strain 18H which served as the recipient had an index of 0.394 for *P. chlororaphis* and on of 0.319 for *P. fluorescens* Biovar II. Strain 18H appears to be a transitional species between *P. chlororaphis* and *P. fluorescens* Biovar II. The DSM-German National Collection of Type Cultures accepted both strains as *P. fluorescens*. The Strain HK44 also produces the green fluorescing compound typical of the species (as opposed to the green pigment characteristic of *P. chlororaphis*), and therefore EPA agreed with the identification of Strain HK44 as a *P. fluorescens* Biovar II. Later Strain 18H was definitively identified as a *P. fluorescens* by 16srRNA analysis. According to the PMN submitter, the *ortho*-degradative pathway for salicylate degradation is chromosomally located, and there are two cryptic native plasmids in this strain that are also present in Strain HK44 (personal communication, EPA and PMN submitter).

Plasmid pUTK21 is derived from three sources: (1) the entire plasmid, except for the Tn4431 insert, comes from plasmid pKA1 found in a second Manufacture Gas Plant soil isolate *Pseudomonas fluorescens Strain 5R; (2)* Tn 4431 is comprised of genes from the bacterium *Vibrio fischeri Strain MJ-1* (now considered a *Photobacterium*) , which was isolated from the light organ of the fish *Monocentris japonicus* (Engebrecht, et al., 1983); Tn4431 also contains genes from (3) Tn5 and (4) *E. coli* Strain D1021 (Orskov & Orskov, 1973.

Verification of the final construct was provided in part by reference to King, et al. (1990) that documents development of Strain HK44, and by other data in the PMN submission (Sayler, 1995). EPA guidance includes a request for a detailed flow diagram that identifies all introduced DNA, vectors, and taxa used to develop the subject GEOP (see the OPPT APoints to Consider@ guidance document). The construction of pUTK21 was well described, and only a limited number of sequences were unidentified according to Sayre (1995), primarily nondegradative genes associated with the pKA1 backbone of plasmid pUTK21. Plasmid pKA1 is approximately 101 kb, and shares extensive homology with the well-described plasmid NAH7 in the 25 kb region which encodes the *nah* and *sal* pathways to the extent that these two pathways in pUTK21 and NAH7 can be considered homologous (Sanseverino, et al., 1993). However, NAH7 is only 83 kb (Yen and Serdar, 1988), and Herbes, et al. (1978) found significant differences in the restriction patterns of pKA1 as compared to NAH7 in the nondegradative portions of the plasmid.). Further details of the characterization and verification of pUTK21 can be found in Sayre (1995) and Tou (1995).

According to Sayre (1995) and King et.al (1990), Strain HK44 functions in the following manner. In the presence of napthalene, or the regulatory inducer salicylate, the pUTK21 bioluminescent reporter plasmid *lux* cassette produced visible light. The construct is intended to function as an indicator of bioavailable PAHs in soils so that the ability to degrade the PAHs using the microorganism can be assessed. For napthalene, the *nah* pathway present on pUTK21 degrades napthalene to salicylate, which then serves as an inducer for further napthalene degradation. Salicylate itself would normally then be degraded by the *sal* pathway genes present on pUTK21, except that the first gene in that pathway B *nahG* which encodes a salicylate hydrolase B has been inactivated by the insertion of transposon Tn4431 which contains the *lux* genes that lead to light production. According to the Premanufacture Notice (PMN) P95-1601 (Sayler, 1995a) submitted to EPA, translation stop codons in the two insertion sequences located on either end of the transposon prevent translation in all three reading frames. Therefore, there is no fusion protein resulting from *nahG* and *lux* genes, and there is no translation of the *sal* genes downstream of transposon Tn4431. The salicylate is, however, degraded further by an *ortho*-degradative pathway located on the chromosome of Strain HK44.

Appendix 2: EPA Risk Assessment Review Documents for Approval of Strain HK44 Release at the Y-12 Site

OPPT Risk Assessment Reports prepared for Biotechnology Submissions, with specific citations to Reports for This Bioremediation Field Release

Name of Report	Focus of Report
Taxonomy Report (Segal, 1995; 3 pp.)	Identifies genus and species of recipient microorganisms. May address donor microorganisms also
Chemistry Report (Tou, 1995; 9 pp.)	Identifies genetic manipulations made to construct intergeneric microorganism. May include a flow diagram for construction process and final construct illustration

Construct Analysis (Sayre, 1995; 22 pp.)	Identifies hazard and gene transfer issues associated with introduced DNA used to construct the intergeneric microorganism. Identifies any inserted DNA whose function is uncertain. May include a flow diagram for construction process and final construct illustration.
Ecological Hazard Assessment (McClung, 1995; 16 pp.)	Identifies potential environmental impacts of the recombinant microorganism and its products on environmental receptors such as aquatic and terrestrial vertebrates, invertebrates, and plants
Human Health Assessment (SRA Technologies, 1995; 12 pp.)	Identifies potential impacts of the recombinant microorganism and its products on human health. Pathogenic and toxic effects are considered.

Engineering Report (Radian, 1995; 23 pp.)	Identifies releases of microorganisms and their products to environmental media and estimates worker exposure to the subject microorganisms
Exposure Assessment (U.S. EPA, 1995)	Identifies concentrations of microorganisms in receiving air, water and soil
Risk Assessment (Broder, 1995; 15 pp.)	Balances hazard and exposure concerns to arrive at an overall determination for the field test

Appendix 3: Ability of Strain HK44 to Degrade PAHs, and Genetic Stability and Transfer of Introduced DNA Sequences

There was some uncertainty identified in the EPA construct analysis (Sayre, 1995) with regard to the substrate range of the degradative enzymes expressed by pUTK21, based on the lack of information on the full substrate range of NAH7. It is known that pUTK210-encoded enzymes are able to degrade the three-ring PAHs anthracene and phenanthrene to salicylate intermediates (Sanseverino, et al., 1993), which should be further degraded by the chromosomal *ortho*-degradative pathway of Strain HK44. Sayler (1995b) noted that compounds from napthalene to high molecular weight aromatics could be degraded by these enzymes, but not heavy tar residues. Hydroxylated and carboxylated intermediates could be expected from higher molecular weight PAHs, but other microbial populations present in soils are likely to further degrade these compounds. The issue of toxic metabolites that are generated from Strain HK44 is moot since pUTK21 would likely produce no different metabolites than pseudomonads which naturally bear pKA1, and the related NAH7, plasmids.

There is, however, a concern for salicylate-like metabolites if the *nah/sal* pathway of pUTK21 is transferred to other bacteria which may not bear the *sal* operon (Sayre, 1995). In this case, metabolites structurally analogous to salicylate may be produced in pseudomonads in the environment which receive the *nah/sal* pathway from HK44 via gene transfer. *P. fluorescens* Strain 5RL (donor of pUTK21 and original host for pKA1) does not have the *ortho*-degradative pathway and accumulated 1-hydroxy-2-napthoic acid and 2-hydroxy-3-napthoic acid from degradation of phenanthrene and anthracene, respectively (Menn, et al., 1993). Other pseudomonads in the environment which acquire the degradative genes from pUTK21 may also be unable to degrade salicylate analogs. Again, such intermediates are likely to be further degraded by other bacteria in the environment, since pseudomonads containing NAH7, or plasmids similar to pKA1 could mineralize all three compounds (Sanseverino, et al., 1993). Transfer of the *nah/sal* pathway could occur by conjugation since plasmid HK44 is a fully conjugative single copy plasmid (Sayler, 1995a). Transfer of the *nah/sal* pathway could also occur through mobilization of Tn4655-like transposon which may bracket the *nah/sal* pathway (as it does in NAH7). This assisted transposition could be aided by the Tn*1721* sequences present in Tn*4431*. Other gene transfer mechanisms are also theoretically possible.

Data relevant to assessing the stability of Strain HK44 and its ability to transfer pUTK21 and Tn*4431* were noted in Sayler (1995a). In nonselective chemostat experiments with Strain 18H, the Strain HK44 population experienced a 99% loss of the pUTK21 plasmid: 39 generations and a dilution rate of 0.086/hr resulted in $1.58X10^4$ cfu/ml that retained the plasmid. The approximately $1X10^4$ cfu/ml concentration was maintained for another 23 generations. Plasmid preparations showed that Tn*4431* remained stable in pUTK21, and all samples of isolates which lost pUTK21 were Tet^S showing that the transposon did not insert into the chromosome.

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