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Environmental assessment of recombinant DNA fermentations

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SUMMARY

This issue of the *Journal of Industrial Microbiology* contains a compilation of papers presented at the 1992 National Meeting of the Society for Industrial Microbiology in two symposia entitled 'Environmental Assessment of Recombinant DNA Fermentations'. It focuses on three areas of particular interest to industry using *Escherichia coli* K-12 strains to make recombinant proteins: (i) the current regulatory environment; (ii) plant design; (iii) results from five different companies all of whom are using or planning to use recombinant *E. coli* in commercial fermentations. The results from all five companies pursuing the questions of environmental fate and the potential for gene transfer in different studies reached the same conclusions. That is, recombinant *E. coli* K-12 strains and their plasmidless hosts were unable to survive in any environmental microcosm tested. Additionally, there was absolutely no evidence of gene transfer despite the use of highly sensitive techniques to measure such an event. It seems reasonable to conclude that *E. coli* K-12 strains with recombinant, non-conjugating, poorly mobilizable plasmids do not represent environmental hazards in the event of an accidental release of such microorganisms into the environment.

INTRODUCTION

The use of genetically modified microorganism to produce proteins or other products of agricultural or pharmaceutical use promises to provide mankind with a plethora of advantages. The workhorse of this technology has been *Escherichia coli* K-12, a Gram-negative microorganism about which we know more physiology, biochemistry, and genetics than for any other microorganism. The K-12 strain, which was originally isolated in 1922 from the feces of a diphtheria patient at Stanford Medical School [1], has been maintained under laboratory conditions for the past seven decades. As is characteristic of most microbes isolated from man, continual sub-culturing in vitro results in the selection of a strain that is attenuated; that is, it has lost its ability to grow efficiently in its natural habitat, namely the intestinal tract of warm-blooded vertebrates [4,9]. Nevertheless, the advent of recombinant DNA technology raised concerns over the safety of genetically engineered *E. coli* K-12 strains. In the 1970s, extensive research was conducted with *E. coli* K-12 strains because of a fear that the genetic manipulation of this microorganism could generate viable microbial strains with unknown, and therefore potentially deleterious, effects upon the environment. While DNA exchange between microbial species was not unusual in natural environments [6], recombinant DNA allowed exchange of genetic material across boundaries that presumably are not normally crossed in nature. Concerns, particularly of the unknown, caused a slow down and re-evaluation of recombinant DNA studies. Consequently, *E. coli* K-12 strains were constructed which contained mutations [5] to ensure the destruction of these strains in the event that these strains accidentally escaped from the laboratory. As more and more data were accumulated over the last two decades, it became clear that *E. coli* K-12 strains were not the environmental threat originally feared. Most of these studies, however, were conducted at laboratory scale with respect to volumes and numbers of bacteria, and employed typical laboratory strains of *E. coli* K-12. It was not until the 1980s that *E. coli* went commercial. Suddenly, the prospect of 10^{17} or more recombinant *E. coli* K-12 being inadvertently released into the environment during some type of 'catastrophic' industrial accident ignited interest in the ability of *E. coli* K-12 to survive in the environment and to transfer its recombinant-DNA to members of the indigenous microbial microcosms. While previous studies in academic laboratories indicated that the answer to both of those questions was a resounding NO, there was a nagging concern to the regulatory agencies: Are commercially relevant recombinant *E. coli* K-12 strains grown to high cell densities in fermenters different from their laboratory cousins? Regulatory agencies needed to be assured that commercially used recombinant *E. coli* K-12 strains were indeed like the laboratory strains studied in the 1970s. It is the intent of this series of articles to detail the results from several different laboratories which used different approaches to study the fate of commercially relevant recombinant *E. coli* K-12 strains.

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This issue of the *Journal of Industrial Microbiology* contains a compilation of papers presented at the 1992 National Meeting of the Society for Industrial Microbiology in two symposia entitled 'Environmental Assessment of Recombinant DNA Fermentations'. The objective of publishing this material in one volume are two-fold. First, it compiles the experiences of the five companies studying the fate of their respective *Escherichia coli* K-12 hosts in various microcosms. Most of these studies were generally not accepted for publication in other scientific journals for essentially one reason: namely, the reviewers felt that there was nothing new in these results since the scientific community had accepted the fact that *E. coli* K-12 would not survive in the environment and would not transfer its DNA to other indigenous microorganisms. Nevertheless, industry was unable to show the regulatory agencies published studies indicating that commercial *E. coli* K-12 strains were unable to survive or were unable to transfer their DNA to indigenous microbes. It was critical for both industry and the regulatory agencies to have these data peer reviewed so that the fate of commercially relevant strains of *E. coli* could be documented. This brings me to the second objective which is to propose that these results coupled with the extensive data base on laboratory strains of *E. coli* presents a picture that fermentations with recombinant *E. coli* K-12 strains do not present any environmental hazards. Therefore, subsequent in-depth studies on the fate of commercially relevant strains of *E. coli* K-12, such as those presented in this issue, are not really necessary. It is our intent to provide both industry and regulatory agencies with overwhelming data on which to judge the risk of such commercial operations.

RESULTS AND DISCUSSION

Regulatory issues

The concerns of the regulatory agencies are presented in the first two articles in this issue. In the first paper, Jones and Matheson from the Center for Veterinary Medicine at the Food and Drug Administration propose guidelines for industries to consider when developing commercial recombinant fermentations. It describes the concerns of the agency in this area given their responsibilities to govern products that are destined for man either directly or indirectly through the food chain.

The second paper by Van Houten and Fleming looks more at the global biosafety issues relating to biotechnology in general. It presents the latest concerns of both the US and the European Economic Community in this area. They describe a relatively new classification called Good Industrial Large-Scale Practices (GILSP or GLSP) which represents the least stringent conditions for manufacturing. Additionally the criteria used to judge the commercial organism are enumerated. By these criteria all of the *E. coli* K-12 host vectors described in this issue would be grown in a facility conforming to GLSP regulations.

Plant design

Miller and Bergmann describe how the biology and regulatory interests are transformed into a facility. The approach of this presentation is that companies would build to accommodate Biosafety Level 2 – Large Scale (BL2-LS). This is two levels up form GLSP but can be rationalized by saying that the *E. coli* K-12 host may not always be used in the production facility. By putting in the required safeguards for BL2-LS at the front end you save on capital and have a more adaptable plant design.

In vitro studies

The fate of recombinant *E. coli* in environmental microcosms is addressed in articles by Bogosian et al. and Heitkamp et al. In the first instance, *E. coli* strain W3100G containing pBGH1, a pBR322-based plasmid carrying the gene for bovine somatotropin (BST), was examined in Missouri river water. Previously it was reported that a marked version of the commercial host vector for BST did not survive in laboratory microcosms containing Missouri river water [3]. In the current report the authors used the highly sensitive polymerase chain reaction to look for gene transfer from the recombinant *E. coli* to indigenous microbes. Even with this technique no evidence of transfer was found. This may not be surprising given the requirements needed to transfer a pBR322 based plasmid from an F^- strain such as W3110G [2].

Heitkamp et al. describe the fate of a spontaneous nalidixic acid-resistant derivative of W3110G transformed with pBGH1 in semi-continuous activated sludge (SCAS) units. These microcosms contained sewage from a commercial sewage treatment plant in the St. Louis area. The theme described previously in the literature and recently reviewed [2] was repeated here. The recombinant *E. coli* K-12 strain could not establish itself in the microbial sewage community despite attempts to favor such an event.

In vitro studies were also conducted by scientists at Pitman-Moore, using the *E. coli* K-12 strain HB101 with and without a plasmid encoding porcine somatotropin [7], and at American Cyanamid, using *E. coli* K12 strains 4300 and HB101 with and without plasmid encoding BST. Both groups presented their data at the meeting but their manuscripts are not included in this Journal. The Pitman-Moore study [7] used a laboratory waste treatment facility containing either synthetic or authentic sewage. There results

were essentially the same as that reported by Kane et al. (Kane, J.F., P.E. Jung, M.D. Hale, M.A. Heitkamp, and G. Bogosian. 1990. Fate of Recombinant *Escherichia coli* in Non-sterile Water, Soil, and Sewage. Abstr. Annual Meeting of the American Society of Microbiology) and Heitkamp et al. (this issue and Kane, J.F., P.E. Jung, M.A. Heitkamp, M. Bianchini, M.D. Hale, D.B. Weber, and G. Bogosian. 1993. Fate of recombinant *Escherichia coli* K-12 strain in sewage. Abstr. Annual Meeting of the American Society of Microbiology). There host with or without the plasmid did not survive longer than 77 hours in this system. Hybridization tests for the presence of the plasmid in indigenous microbes were also negative. Although the number of indigenous microorganisms tested were relatively small, these results do imply a lack of transfer of the plasmid from the recombinant *E. coli* to a significant part of the indigenous population.

The presentation by Smolin et al. from American Cyanamid covered several microcosms. These authors kindly provided me with a transcript of their presentation in order to include the results of their study with those reported here. Their study objectives were to assess the fate of their host, with and without a vector, in soil, water, and activated sludge microcosms. In addition, they looked for the transfer of the BST-containing plasmid from their host to indigenous bacteria in these microcosms. While their host for the production of BST was a K-12 strain called 4300, they also examined strain HB101 with and without pBR322 in order to take advantage of the data base on the lack of survival and persistence of this strain in environmental microcosms. In each study samples were plated in triplicate and, whenever possible, bacterial population estimates were based on data from the dilution that yielded plate counts of 30 to 300 colony-forming units (CFU). In those cases in which none of the dilutions yielded counts within this range, estimates were based on data from the dilution with counts closest to the range. For each study, the data were evaluated by analysis of variance.

Three different soil samples were used in this study. All were classified as sandy loams but with distinct mineral differences. About 2 grams of each soil were placed into sterile tubes and capped with metal closures. The tubes were individually inoculated with one of the four selected strains of *E. coli* K-12 to a cell density of about 10^7 CFU per gram. One set of tubes remained uninoculated to serve as a control. The moisture content was adjusted to 60% of capacity and the tubes were incubated at 25 °C in a humidified chamber. Samples were removed at days 0, 7, 14, 28, 42, 56, and 70 diluted with 18 ml of sterile saline and thoroughly mixed. After the solids settled the liquid was serially diluted and plated on various media to quantitate the indigenous aerobic bacteria as well as the *E. coli* cells. Populations of all four strains decreased from an initial concentration of greater than 10^7 CFU per gram of soil to levels below the detection limit of 10^2 CFU per gram within 6 to 8 weeks. Under these same conditions the indigenous populations did not decline. Similar results were found with the host vector used by Monsanto (Bogosian, G., personal communication).

Smolin et al. also examined water from a river, stream, and lake, and activated sewage sludge samples from three separate sources. These microcosms were inoculated with aliquots of overnight cultures of each of the four strains to a level of about 10^8 CFU per ml. Uninoculated samples served as controls. These flasks were incubated with shaking at 25 °C in a humidified chamber and were sampled on days 0, 7, 14, 21, 28, and 42. The data from these studies established that the proposed BST production organism did not demonstrate increased or unusual survival relative to its plasmid-free parent strain or the other two *E. coli* K-12 strains monitored in the experiment. These results were observed in each of the various water or sewage samples taken from different natural environments. Populations of all four strains decreased from an initial concentration of about 10^8 CFU per ml to levels below the detection limit of 10 CFU in 3 to 4 weeks. Under these same conditions the indigenous bacteria displayed a much slower rate of decline.

The group from American Cyanamid also looked for gene transfer in each microcosm using a hybridization assay. A digoxygenin-labeled (non-radioactive) probe was prepared for the BST gene fragment contained in the BST expression plasmid and tested under model conditions which demonstrated its utility. In order to minimize the detection of a positive signal from the strain itself, the surviving microbial population from one each of the three soil, water, and sewage sludge microcosms was plated onto non-selective agar (Trypticase Soy) when the recombinant *E. coli* production strain could no longer be detected. The plating protocol was designed to get 200 to 300 CFU per plate. About 10000 CFU from each of the three microcosms were examined with the probe. The results of this study did not demonstrate any transfer of the BST gene from the recombinant to any other microorganism in any of the three microcosms. There was no true positive signal observed even under conditions where a strong signal was found with the positive control colony which had the BST gene on a prophage. Although these results do not rule out the possibility of a gene transfer event, such an event clearly did not affect a major portion of the microbial population.

In vivo studies

The inability of the commercial *E. coli* K-12 strains used by Eli Lilly and The Upjohn Company to survive in

Fischer-344 rats or mice, respectively, is the subject of the final two papers in this issue. Muth et al. demonstrate that their tetracycline-resistant host vector used for the production of bovine somatotropin was unable to survive in male or female Fischer-344 rats even when the antibiotic tetracycline was included in the feed.

Similarly, Yancey et al. found that their *E. coli* K-12 host vector was unable to colonize the intestinal tracts in conventional antibiotic-treated mice. These investigators took their studies further and looked for the transfer of genetic material from the recombinant *E. coli* to indigenous microorganisms. Two *E. coli* strains were isolated from the feces of the mice and marked by isolating a spontaneous mutant resistant to streptomycin. These indigenous marked strains were re-introduced to mice fed streptomycin in order to assist the colonization of the intestinal tract by these strains. The gene transfer experiment was to see whether the indigenous streptomycin resistant *E. coli* could pick up plasmid DNA (ampicillin resistance) or chromosomal DNA (tetracycline resistance) from the added recombinant *E. coli*. There was no evidence for gene transfer in any of these studies using hybridization assays.

CONCLUSIONS

All of these studies provide overwhelming support for the hypothesis that commercial *E. coli* strains are not intrinsically any different than the laboratory K-12 strains. Furthermore, growing these strains to high cell densities does not confer upon them any advantage to survive outside of the laboratory. The absence of gene transfer in all of these microcosms indicates that there is no environmental danger. While it is impossible to prove a negative, all of the studies reported here and elsewhere [2] indicate that no gene transfer from recombinant *E. coli* K-12 strains to indigenous microorganisms could be demonstrated even with state-of-the-art techniques.

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