ATTACHMENT I--FINAL RISK ASSESSMENT OF ESCHERICHIA COLI K-12 DERIVATIVES

(February 1997)

I. INTRODUCTION

Escherichia coli is one of a number of microorganisms which are normal inhabitants of the colons of virtually all warm-blooded mammals. *E. coli* belongs to the taxonomic family known as *Enterobacteriaceae*, which is one of the best-defined groups of bacteria. The strain *E. coli* K-12 is a debilitated strain which does not normally colonize the human intestine. It has also been shown to survive poorly in the environment, has a history of safe commercial use, and is not known to have adverse effects on microorganisms or plants. Because of its wide use as a model organism in research in microbial genetics and physiology, and its use in industrial applications, *E. coli* K-12 is one of the most extensively studied microorganisms.

History of Commercial Use and Products Subject to TSCA Jurisdiction

E. coli K-12 has a history of safe use. Its derivatives are currently used in a large number of industrial applications, including the production of specialty chemicals (e.g., L-aspartic, inosinic, and adenylic acids) and human drugs such as insulin and somatostatin (Dynamac, 1990). Further, *E. coli* can produce a number of specialty chemicals such as enzymes which would be regulated under TSCA. An insulin-like hormone for use as a component of cell culture media, resulting from a fermentation application in which *E. coli* was used as the recipient, has already been reviewed under TSCA (Premanufacture Notice P87-693). EPA recently reviewed a submission (94-1558) for use of *E. coli* K-12 to produce indigo for use as a dye. In general, *E. coli* K-12 is one of the most extensively studied bacteria, and has been used in genetic studies in laboratories worldwide.

Experience with the use of *E. coli* is reflected in its classification under the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines, U.S. Department of Health and Human Services, 1986). With the exception of strains which are known to be pathogenic, *E. coli* is considered a Class 1 Agent under the NIH Guidelines. Class 1 consists of all organisms which are not either human or animal pathogens. Most experiments involving *E. coli* K-12 have been exempted from the NIH Guidelines based on an analysis of safety,

except in certain circumstances (see Appendix C-II of the NIH Guidelines). Moreover NIH, under section III-D-4 of the NIH Guidelines, exempts transfers of genetic material between species that exchange DNA by known physiological processes with the genus *Escherichia*. Included in this exemption are exchanges between *Escherichia* and the closely related genera of *Shigella*, *Salmonella*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Erwinia*, *Pseudomonas aeruginosa*; also included are the species *Pseudomonas putida*, *Pseudomonas fluorescens*, *Serratia marcescens*, and *Yersinia enterocolitica*.

II. IDENTIFICATION AND TAXONOMY

A. Overview

E. coli belongs to the family *Enterobacteriaceae*. All *Enterobacteriaceae* are defined as Gram-negative, non-sporeforming rods that are facultative anaerobes. During the 1960's and 1970's, large amounts of information were generated regarding the phenotypic characteristics of the *Enterobacteriaceae*. The reasons for this increase in knowledge were two-fold. First, beginning in the early 1970's, a number of methods became available for the identification of enteric bacteria. These methods were based on biochemical or phenotypic reactions and could be performed with minimum labor and cost. Second, a major shift in nosocomial infections from Gram-positive to Gram-negative bacteria occurred in hospital patients during the 1960's and the early 1970's. Therefore, clinical microbiology laboratories, faced with the pressing need for accurate identification systems for enteric bacteria, carried out an extensive characterization of the members of this group of bacteria, including *E. coli*.

B. Taxonomy and Characterization

Escherichia coli is a member of the family *Enterobacteriaceae* and has been described by Brenner (1984). *Escherichia* is a Gram-negative rod which can be motile by peritrichous flagella or nonmotile. *Escherichia* is also a facultative anaerobe which has both a respiratory and a fermentative type of metabolism, and commonly occurs in the intestinal tract of humans and other animals.

E. coli K-12 was originally isolated from a convalescent diphtheria patient in 1922 (Bachmann, 1972). Because it lacks virulence characteristics, grows readily on common laboratory media, and has proven to be a valuable tool for microbial physiology and genetics research, it has become the standard bacteriological strain used in microbiological research and teaching. *E. coli* K-12 is now considered an enfeebled organism

as a result of being maintained in the laboratory environment for over 70 years (Williams-Smith, 1978).

E. coli can be readily differentiated from closely related bacteria by a number of standard tests. Classically, this has been accomplished by testing for production of indole from tryptophan, production of acid from glucose media using the dye methyl red as an indicator, lack of production of acetoin as a metabolic endproduct (also known as the Voges-Proskauer reaction), and the inability to utilize citrate as a sole source of carbon. Collectively, these reactions are known as the IMViC battery. The IMViC battery was developed for the analysis of water samples where it was important to differentiate *E. coli*, which was found to be always associated with fecal contamination of water, from other closely related bacteria which could be found naturally in water sources. Further refinements of the IMViC tests are used today and are available as commercial test kits.

C. Related Species of Concern

Taxonomically, the four species of the genus *Shigella* are closely related to *E. coli*. *Shigella* species cause diarrhea in humans and are classified as Class 2 agents under the NIH Guidelines. The *Shigella* species and *E. coli* share a high level of DNA sequence homology and many protein and polysaccharide capsular antigens. [These capsular antigens can be used to distinguish between *E. coli* strains and the pattern of capsular antigens determine the organism's "serotype" (Smith 1977).] The two genera can be distinguished based on the fact that *E. coli* has a unique colony morphology when grown on certain differential laboratory media (Jawetz et al., 1987). Commercially prepared kits for distinguishing between these organisms are available.

Most *E. coli* serotypes are benign and may even contribute to normal function and nutrition in the gastrointestinal tract. A few *E. coli* serotypes are pathogens.

E. coli K-12 strains in use today are from standard culture collections (Bachmann, 1972), such as the American Type Culture Collection and are not recent environmental isolates. As a result, these K-12 strains are well-characterized and should be expected to remain as pure cultures under standard microbiological practices. K-12 strains are distinguishable from both *Shigella* and other Escherichia (Cooke, 1974, Orskov 1978, Schmidt 1973).

III. HAZARD ASSESSMENT

The Proceedings of the Falmouth Workshop held in June 1977 served as a primary source for this assessment (Gorbach, 1978).

A. Human Health Hazards

The potential of K-12 strains to present risks to human health are examined in this assessment by analyzing K-12 in terms of (1) the phenotypic traits relevant to colonization of the colon, and (2) toxin production.

1. Colonization and Pathogenicity

E. coli is an inhabitant of the human colon, and it is thought that the primary means through which humans acquire their intestinal flora is through ingestion. Workers in fermentation facilities would not be expected to ingest *E. coli* under standard good practice, which prohibits the ingestion of food in work areas; however, some inhaled bacteria could be swallowed.

In order to evaluate K-12's potential to colonize the human intestine the following should be addressed: (1) the characteristics relevant to *E. coli* colonization of the human colon, and how K-12 compares to other *E. coli* in terms of these traits, and (2) data relevant to colonization potential of K-12 strains.

The binding of an *E. coli* to the mucosal surface of the colon requires two factors. The first factor is the production of a specific glycocalyx or fimbriae from the surface of the bacterium. This specific glycocalyx recognizes a specific lectin on the surface of the enterocyte lining of the human colon. The glycocalyxes appear to bind to structures such as the mucus glycoproteins elaborated from the goblet cells of the intestine. In Gram-negative bacteria, the polysaccharide chains arising from the core of the lipopolysaccharides in the outer membrane appear to be the major ones which affect binding to the colon.

E. coli K-12 is defective in at least three cell wall characteristics. The outer membrane has a defective lipopolysaccharide core which affects the attachment of the O-antigen polysaccharide side chains (Curtiss, 1978). Second, it does not have the type of glycocalyx required for attachment to the mucosal surface of the human colon (Edberg, 1991) as a result of the altered O-antigen properties noted above. Finally, K-12 strains do not appear to express capsular (K) antigens, which are heat-labile polysaccharides important for colonization and virulence (Curtiss, 1978).

K-12, thus, is not able to colonize the human intestinal tract under normal conditions, even after ingestion of billions of organisms (Anderson, 1975, Cohen et al., 1979., Levy and Marshall, 1981; Levy et. al, 1980, Smith, 1975). As noted above, K-12 is defective in cell wall components relevant to the ability

to recognize and adhere to the mucosal surface of colonic cells (Curtiss, 1978). The normal flora in residence in the colon thus can easily exclude K-12, and prevent it from colonizing the human colon.

A number of experiments have been conducted to measure the ability of K-12 to colonize in comparison to other *E. coli*. These experiments basically fall into two categories. First, those in which the normal human and/or animal flora was substantially reduced in order to provide the best opportunity for K-12 to colonize. The second category, known as co-colonization experiments, were to determine which strains could out-compete which other strains (Anderson, Gillespie and Richmond 1973; Anderson 1974; Burton et al. 1974; Anderson 1975; Smith 1975; Freter 1978; Laux, Cabelli and Cohen 1982; Myhal, Laux and Cohen 1982; Levine et al. 1983).

For the first category of experiments, normal flora were reduced through treatment with antibiotics or the experiments were performed using germ-free mice. In the second category, normal flora were reduced and two strains of *E. coli* were introduced to determine whether one strain could establish itself at the expense of the other.

These sets of experiments indicate that K-12 is a poor colonizer. Most strains of *E. coli*, including K-12, can colonize the intestine when the normal flora is substantially reduced. K-12 thus can colonize individuals whose normal intestinal flora is reduced through antibiotic therapy or is affected by other variables that can affect colonization such as anti-motility drugs. Similarly, *E. coli* K-12 can colonize germ-free mice, but is quickly displaced when the mice are fed other *E. coli* (Curtiss, 1978). In co-colonization experiments, *E. coli* K-12 has consistently been outcompeted by other *E. coli*. These studies indicate that K-12 is a poor colonizer and that indigenous intestinal microorganisms have a large competitive advantage over K-12 strains.

Aside from K-12's inherent decreased ability to colonize the gut, the culture conditions under which a K-12 strain is grown decrease the ability of the strain to colonize the gastrointestinal tract. Indeed, organisms grown under laboratory or fermentation conditions in general are not particularly competitive in comparison to microorganisms isolated from the environmental niche of the organism. Similarly, *E. coli*, which had been grown in continuous-flow cultures, were poor colonizers in mice with a normal cecal flora (Freter et al. 1983).

The medium in which *E. coli* are introduced to the GI tract is also important. Even in germ-free mice, the survival of K-12 strains is minimal unless the cultures of bacteria are introduced

in a basic buffer such as bicarbonate, since K-12 strains are killed by stomach acidity (Freter et al., 1983).

These two considerations further reduce the probability that K-12 strains grown under commercial fermentation conditions will be able to colonize the GI tract of workers. Such cultures would not be metabolically adapted to the human gut, and the microorganisms would not usually be grown in basically buffered fermentation media.

In order to cause disease, a microorganism must colonize a site of the human body and express virulence characteristics. On the basis of colonization alone, *E. coli* K-12 is innately defective as a pathogen, and a very low likelihood of acting as a pathogen of humans or animals.

The ability of certain *E. coli* serotypes to cause disease appears to be associated with certain specific capsular antigens. These serotypes of *E. coli* cause diarrhea, urinary tract infection, bacteremia and neonatal meningitis (Silverblatt and Weinstein 1979). It appears that colonization of the colon by the pathogenic serotype is a precondition for these illnesses (Moxon, Glode, Sutton 1977; Silverblatt and Weinstein 1979). The likelihood of these types of infections occurring with K-12 is thus very low for two reasons. First, since *E. coli* K-12 is a very poor colonizer it is unlikely to establish itself in the colon. Second, K-12 lacks adhesion and other virulence factors necessary for pathogenesis (Curtiss, 1978, Gorbach 1978, Edberg, 1991).

2. Toxin Production

A number of toxins affecting humans have been identified in *E. coli*: shiga-like toxins (SLT-I and II) and heat labile and heat stable toxins. The related genus *Shigella* produces shiga toxin (ST). These toxins are factors through which pathogenic serotypes cause diarrhea.

E. coli K-12 appears to lack the ability to produce significant quantities of toxins that affect humans (Edberg, 1991). There are two studies on production of toxins in K-12 strains. O'Brien and Holmes (1987) state that all strains of *E. coli* K-12 examined produced low levels of SLT as detected by monoclonal and polyclonal antibodies; however, DNA probes for SLT-I did not hybridize with DNA from these strains. These conflicting results are still unexplained. Edberg (1992) noted that strain K-12 was found to be in the low level toxin producing category ($2X10^2$ - $6X10^2$ CD50 per ml of sonic lysate). However, it is unclear whether this cell line-based assay is measuring an actual toxin or a protein that may exert activity against the cell.

No records of K-12 enterotoxin-induced disease for fermentation workers were located in the literature. This is not surprising since the K-12 would have to colonize and invade the tissue of the GI tract of workers in order to deliver the toxin to its site of action.

3. Conclusions

E. coli K-12 is not considered a human or animal pathogen nor is it toxicogenic. Any concerns for *E. coli* K-12 in terms of health considerations are mitigated by its poor ability to colonize the colon and establish infections.

B. Environmental Hazards

1. Hazards to Animals

A number of experiments with *E. coli* K-12 have been conducted in animal models (Burton et al. 1974; Freter *et al.*, 1983; Cohen and Laux, 1985; Laux *et al.* 1982; Myhal *et al.*).

These experiments demonstrate that *E. coli* K-12 will not under normal conditions colonize the GI tract of animals. A number of researchers have tried in vain to implant K-12 in the GI tract of laboratory animals under normal conditions (Freter 1978, Curtiss 1978). Negative results have been noted in mice, pigs, chickens, pigs and calves. K-12 is also unlikely to behave as a pathogen
(Gorbach 1978).

2. Hazards to Plants

There are no data that suggest that *E. coli* K-12 strains have adverse effects on plants. The genus *Escherichia* is not considered a plant pest species by the U.S. Department of Agriculture (7 CFR 330, et seq.).

3. Hazards Posed to Other Microorganisms

No evidence exists relative to hazardous effects of *E. coli* K-12 strains on other microorganisms in the environment (Sayre, 1991).

4. Conclusions

E. coli K-12 strains are very unlikely to pose a hazard to either animals, plants, or other microorganisms.

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

E. coli K-12 is considered an exempt host system under the NIH Guidelines. This microorganism also falls under the Class 1 Containment under the European Federation of Biotechnology guidelines (Frommer et al., 1989).

No data were available for assessing the release and survival specifically for fermentation facilities using *E. coli*. Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Area samples were taken in locations where the potential for worker exposure was considered to be potentially greatest, i.e., near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use area monitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area

sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Fate of the Organism

The natural habitat of *E. coli* is the large bowel of mammals. However, *E. coli* K-12 has lost the ability to colonize the gut and cannot survive in the bowel for very long. The ability of *E. coli* to survive under environmental conditions is also limited. In one study, *E. coli* HB101 introduced into non-sterile soil in saline declined to levels below detection (1-20 cells/g soil) after 21 days. *E. coli* also declines rapidly in seawater. There are of course many factors that determine the survival of these organisms in the environment and it is unlikely that all the introduced *E. coli* would die. However, it is reasonable to assume that over the long term, populations of *E. coli* K-12 in soil would be very low. *E. coli* released to air would not be expected to survive well because of the low nutrient levels and drying conditions (LaVeck, 1991).

2. Releases

Estimates of the number of *E. coli* organisms released during production are tabulated in Table 1 (Reilly, 1991). The uncontrolled/untreated scenario assumes no control features for the fermentor off-gases, and no inactivation of the fermentation broth for the liquid and solid waste releases. The containment criteria required for the full exemption scenario assume the use of features or equipment that minimize the number of viable cells in the fermentor off-gases. They also assume inactivation procedures resulting in a validated 6-log reduction of the number of viable microorganisms in the liquid and solid wastes relative to the maximum cell density of the fermentation broth.

TABLE 1. Estimated Number of Viable *E. coli* Organisms Released During Production

Release Media	Uncontrolled/ Untreated (cfu/day)	Full Exemption (cfu/day)	Release (days/yr)
Air Vents	$2x10^{8}$ - $1x10^{11}$	$<2x10^{8}$ - $1x10^{11}$	350
Rotary Drum Filter	250	250	350
Surface Water	$7x10^{16}$	$7x10^{10}$	90
Soil/Landfill	$7x10^{18}$	$7x10^{12}$	90

Source: Reilly, 1991

These are "worst-case" estimates which assume that the maximum cell density in the fermentation broth for bacteria is 10^{11} cfu/ml, with a fermentor size of 70,000 liters, and the separation efficiency for the rotary drum filter is 99 percent.

3. Air

Specific data which indicate the survivability of *E. coli* in the atmosphere after release are currently unavailable. Survival of vegetative cells during aerosolization is typically limited due to stresses such as shear forces, desiccation, temperature, and UV light exposure. Survival of *E. coli* K-12 after release is expected to be poor because its normal habitat is the human intestinal tract. However, as with naturally-occurring strains, human exposure may occur via inhalation as the organisms are dispersed in the atmosphere attached to dust particles, or lofted through mechanical or air disturbance.

Air releases from fermentor off-gas could potentially result in nonoccupational inhalation exposures due to point source releases. To estimate exposures from this source, the sector averaging form of the Gaussian algorithm described in Turner (1970) was used. For purposes of this assessment, a release height of 3 meters and downward contact at a distance of 100 meters were assumed. Assuming that there is no removal of organisms by controls/equipment for off-gases, potential human inhalation dose rates are estimated to range from 3.0×10^{3} to 1.5×10^{6} cfu/year for the uncontrolled/untreated scenario and less than that for systems with full exemptions. It should be noted that these estimates represent hypothetical exposures under reasonable worst case conditions (Versar, 1992).

4. Water

The concentrations of *E. coli* in surface water were estimated using stream flow values for water bodies receiving process wastewater discharges from facilities within SIC Code 283 (drugs, medicinal chemicals, and pharmaceuticals). The surface water release data (cfu/day) tabulated in Table 1 were divided by the stream flow values to yield a surface water concentration of the organism (cfu/l). The stream flow values for SIC Code 283 were based on discharger location data retrieved from the Industrial Facilities Dischargers (IFD) database on December 5, 1991, and surface water flow data retrieved from the RXGAGE database. Flow values were obtained for water bodies receiving wastewater discharges from 154 indirect (facilities that send their waste to a POTW) and direct dischargers facilities that have a NPDES permit to discharge to surface water). Tenth

percentile values indicate flows for smaller rivers within this distribution of 154 receiving water flows and 50th percentile values indicate flows for more average rivers. The flow value expressed as 7Q10 is the lowest flow observed over seven consecutive days during a 10-year period. The use of this methodology to estimate concentrations of *E. coli* in surface water assumes that all of the discharged organisms survive wastewater treatment and that growth is not enhanced by any component of the treatment process. Estimated concentrations of *E. coli* in surface water for the uncontrolled/untreated and the full exemption scenarios are tabulated in Table 2 (Versar, 1992).

TABLE 2. *E. coli* Concentrations in Surface Water

Flow	Receiving Stream Flow (MLD*)		Organisms (cfu/l)	
	Mean	7Q10	Mean	7Q10
Uncontrolled/Untreated				
10th Percentile	156	5.60	4.5×10^{8}	1.25×10^{10}
50th Percentile	768	68.13	9.11×10^{7}	1.03×10^{9}
Full Exemption				
10th Percentile	156	5.60	4.5×10^{2}	1.25×10^{4}
50th Percentile	768	68.13	9.11×10^{1}	1.03×10^{3}

*MLD = million liters per day
Source: Versar, 1992

5. Soil

Since *E. coli* is not a normal inhabitant of the soil, its survival would not be expected under these conditions. According to LaVeck (1991), *E. coli* HB101 introduced into non-sterile soil in saline declined to levels below detection (1-20 cells/g soil) after 21 days. Based on this study, and other studies cited in the section on environmental hazards and LaVeck (1991), survival of various strains of *E. coli* could range from 0 - 10^{4} cells/gram of soil.

6. Summary

Although direct monitoring data are unavailable, worst case estimates do not suggest high levels of exposure of *E. coli* K-12 to either workers or the public resulting from normal fermentation operations.

V. INTEGRATION OF RISK

A. Discussion

E. coli K-12 is a well-studied bacterium which has been the subject of extensive research in microbial physiology and genetics and commercially exploited for a variety of industrial uses. The natural habitat of the parent species, *E. coli,* is the large bowel of mammals. *E. coli* K-12 has a history of safe use. Its derivatives are currently used in a large number of industrial applications, including the production of specialty chemicals (e.g., L-aspartic, inosinic, and adenylic acids) and human drugs such as insulin and somatostatin. An insulin-like hormone for use as a component of cell culture media, resulting from a fermentation application in which *E. coli* was used as the recipient, has already been reviewed under TSCA (Premanufacture Notice P87-693).

E. coli K-12 was originally isolated from a convalescent diphtheria patient in 1922 (Bachmann, 1972). Because it lacks virulence characteristics, grows readily on common laboratory media, and has proven to be a valuable tool for microbial physiology and genetics research, it has become the standard bacteriological strain used in microbiological research and teaching. *E. coli* K-12 is now considered an enfeebled organism as a result of being maintained in the laboratory environment for over 70 years (Williams-Smith, 1978). As a result, K-12 strains are unable to colonize the intestines of humans and other animals under normal conditions.

E. coli K-12 strains are not likely to pose a risk to human or animal health, to plants, or to other microorganisms. *E. coli* K-12 has been utilized for 70 years, often in industrial settings with high volumes and cell densities. Moreover, *E. coli* K-12 has been employed extensively in research laboratories. In the industrial setting with the use of appropriate industrial practices for handling microorganisms, and with good laboratory practices in the research setting, the potential for K-12 strains to colonize the human colon is quite low.

Likewise, the ecological risks associated with the use of *E. coli* K-12 are low. Industrial fermentation uses of this organism are expected to result in a low number of microorganisms released from the fermentation facility. Given its natural habitat of the large bowel of mammals, *E. coli* will not likely survive for long periods in soil, water, or air. *E. coli* K-12 has lost the ability to colonize the gut and has been shown to have poorer survival characteristics in soil and water than other *E. coli*. The ability of *E. coli* to survive under environmental conditions

is thus very limited. *E. coli* K-12 has no known survival mechanisms in the environment, such as the ability to produce spores.

In conclusion, the use of *E. coli* K-12 under contained conditions in fermentation facilities present low risk.

B. Recommendations

E. coli K-12 is recommended for the tiered exemption.

VI. REFERENCES

7 CFR 330, et seq., as amended.

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