ATTACHMENT I--FINAL RISK ASSESSMENT OF BACILLUS SUBTILIS

(February 1997)

I. INTRODUCTION

Bacillus subtilis is a ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. The bacterium produces an endospore that allows it to endure extreme conditions of heat and desiccation in the environment. *B. subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. However, under most conditions the organism is not biologically active but exists in the spore form (Alexander, 1977). *B. subtilis* is considered a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low.

History of Commercial Use and Products Subject to TSCA Jurisdiction

B. subtilis is one of the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include production of amylase, protease, inosine, ribosides, and amino acids. TSCA uses of proteases include cleaning aids in detergents and dehairing and batting in the leather industry. TSCA uses of amylases include desizing of textiles and starch modification for sizing of paper (Erikson, 1976).

The Agency has reviewed, under TSCA, three PMNs of genetically modified *B. subtilis* for production of a protease (P87-1030), alpha-amylase (P89-227), and lipase (P91-1154). EPA found that there were no unreasonable risks associated with the use of these recombinant strains for enzyme production in fermentation facilities.

II. IDENTIFICATION AND TAXONOMY

A. Overview

B. subtilis is a ubiquitous soil microorganism that contributes to nutrient cycling when biologically active due to the various enzymes produced by members of the species. Although

the actual numbers in existence in the environment for this species has not been determined, bacilli occur at population levels of 10^6 to 10^7 per gram of soil (Alexander, 1977). However, unless a soil has been recently amended with organic matter providing readily utilizable nutrients, the bacilli exist in the endospore stage. It is thought that 60 to 100% of soil bacilli populations exist in the inactive spore state (Alexander, 1977). Like most members of the genus, *B. subtilis* is aerobic, except in the presence of glucose and nitrate, some anaerobic growth can occur (Claus and Berkeley, 1986).

B. Taxonomy and Characterization

The genus *Bacillus* consists of a large number of diverse, rod-shaped Gram positive (or positive only in early stages of growth) bacteria that are motile by peritrichous flagella and are aerobic. Members of the genus are capable of producing endospores that are highly resistant to unfavorable environment conditions (Claus and Berkeley, 1986). The genus consists of a diverse group of organisms as evidenced by the wide range of DNA base ratios of approximately 32 to 69 mol% G + C (Claus and Berkeley, 1986), which is far wider than that usually considered reasonable for a genus (Norris et al., 1981).

B. subtilis is the type species of the genus. Historically, prior to the monographs of Smith in 1946 and 1952, *B. subtilis* was a term given to all aerobic endospore-forming bacilli (Logan, 1988). Numerous species that appeared in the early literature are no longer recognized as official species. Former species designations that are now considered to be members of the species *B. subtilis* include *B. aterrimus, B. mesentericus, B. niger, B. panis, B. vulgarus, B. nigrificans,* and *B. natto* (Gibson, 1944 and Smith et al., 1946 as cited by Gordon, 1973). Although in the past it has been designated as a separate species, the latest edition of Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986) listed *B. amyloliquefaciens* as a member of the species *B. subtilis*. However, recently it has again achieved the status of a separate species (Priest et al., 1987).

The *Bacillus* species *subtilis, licheniformis,* and *pumilus* are closely related and there has been difficulty distinguishing among the three species that historically were grouped together as the subtilis-group or subtilis-spectrum (Gordon, 1973). These three species clustered together (78%) in the "*subtilis*" group in a numerical classification based on 118 unit characteristics of 368 strains of *Bacillus* (Priest et al., 1988). However, this major cluster contained four subclusters that could be identified as *B. subtilis, B. licheniformis, B. pumulis,* and *B. amyloliquefaciens*. Recent data in the literature have suggested that it is possible to differentiate *B. subtilis* from *B. licheniformis* and *B. pumulis* by the use of pyrolysis-gas chromatography (O'Donnell et al., 1980) or by the use of API

tests (Logan and Berkeley, 1981). In addition, *B. subtilis* and *B. amyloliquefaciens* show little DNA sequence homology to each other (Seki et al., 1975; Priest, 1981) and can also be distinguished from each other by pyrolysis-gas chromatography (O'Donnell et al., 1980) and by a few phenotypic properties including the production of acid from lactose (Priest et al., 1987).

In conclusion, it appears that *B. subtilis* can be distinguished from other closely related species. However, because of changes in the classification of the genus, and the recent development of new methods for taxonomic purposes, older strains may not actually be *B. subtilis* under present-day definitions.

C. Related Species of Concern

There are several species of the genus that are known pathogens. These include *B. anthracis* which is pathogenic to humans and other animals, and *B. cereus* which is a common cause of food poisoning (Claus and Berkeley, 1986; Norris et al., 1981). *B. thuringiensis, B. larvae, B. lentimorbus, B. popilliae*, and some strains of *B. sphaericus* are pathogenic to certain insects. Other species in the genus are considered "opportunistic pathogens".

In a numerical classification using 118 characteristics of 368 species of *Bacillus*, the species *B. thuringiensis, B. cereus*, and *B. mycoides* clustered together at 89 - 92% similarity (Priest et al., 1988). The *B. subtilis* group joined the *B. cereus* group at 72% relatedness. There is no difficulty in distinguishing between the toxin-producing strains of *Bacillus* and *B. subtilis*.

III. HAZARD ASSESSMENT

A. Human Health Hazards

1. Colonization

B. subtilis is widely distributed throughout the environment, particularly in soil, air, and decomposing plant residue. It has shown a capacity to grow over a wide range of temperatures including that of the human body (Claus and Berkeley, 1986). However, *B. subtilis* does not appear to have any specialized attachment mechanisms typically found in organisms capable of colonizing humans (Edberg, 1991). Given its ubiquity in nature and the environmental conditions under which it is capable of surviving, *B. subtilis* could be expected to temporarily inhabit the skin and gastrointestinal tract of humans, but it is doubtful that this organism would colonize other sites in the human body (Edberg, 1991).

2. Gene Transfer

The transfer of gene sequences between strains of *B. subtilis* has been demonstrated when the strains were grown together in soil (Graham and Istock, 1979). In addition, Klier et al. (1983) demonstrated the ability of *B. subtilis* and *B. thuringiensis* to exchange high frequency transfer plasmids. Other studies have shown that *B. subtilis* has the ability to express and secrete toxins or components of the toxins that were acquired from other microorganisms through such transfers of genetic material. *B. subtilis* expressed subunits of toxins from *Bordatella pertussis* (Saris et al., 1990a, 1990b), as well as subunits of diphtheria toxin (Hemila et al., 1989) and pneumolysin A pneumococcal toxin (Taira et al., 1989). Although *B. subtilis* does not appear to possess indigenous virulence factor genes, it is theoretically possible that it may acquire such genes from other bacteria, particularly from closely related bacteria within the genus.

3. Toxin Production

A review of the literature by Edberg (1991) failed to reveal the production of toxins by *B. subtilis*. Although it has been associated with outbreaks of food poisoning (Gilbert et al., 1981 and Kramer et al., 1982 as cited by Logan, 1988), the exact nature of its involvement has not been established. *B. subtilis*, like other closely related species in the genus, *B. licheniformis, B. pumulis,* and *B. megaterium*, have been shown to be capable of producing lecithinase, an enzyme which disrupts membranes of mammalian cells. However, there has not been any correlation between lecithinase production and human disease in *B. subtilis*.

B. subtilis does produce an extracellular toxin known as subtilisin. Although subtilisin has very low toxigenic properties (Gill, 1982), this proteinaceous compound is capable of causing allergic reactions in individuals who are repeatedly exposed to it (Edberg, 1991). Sensitization of workers to subtilisin may be a problem in fermentation facilities where exposure to high concentration of this compound may occur. Exposure limits to subtilisin are regulated by Occupational Safety and Health Administration (OSHA) (29 CFR 1900, et seq.)

4. Measure of the Degree of Virulence

B. subtilis appears to have a low degree of virulence to humans. It does not produce significant quantities of extracellular enzymes or possess other virulence factors that would predispose it to cause infection (Edberg, 1991). There are a number of reports where *B. subtilis* has been isolated from human infections. Earlier literature contains references to infections caused by *B. subtilis*. However, as previously stated,

the term *B. subtilis* was synonymous for any aerobic sporeforming bacilli, and quite possibly, many of these infections were associated with *B. cereus*. In a recent British review article, Logan (1988) cites more recent cases of *B. subtilis* infections in which identification of the bacterium appeared reliable. Infections include a case of endocarditis in a drug abuse patient; fatal pneumonia and bacteremia in three leukemic patients; septicemia in a patient with breast cancer; and infection of a necrotic axillary tumor in another breast cancer patient. Isolation of *B. subtilis* was also made from surgical wound-drainage sites, from a subphrenic abscess from a breast prosthesis, and from two ventriculo-atrial shunt infections (as cited by Logan, 1988).

Reviews of *Bacillus* infections from several major hospitals suggest that *B. subtilis* is an organism with low virulence. Idhe and Armstrong (1973) reported that *Bacillus* infections were encountered only twelve times over a 6-1/2 year period. Species identification of these *Bacillus* infections was not made. In another hospital study over a 6-yr. period, only two of the 24 cases of bacteremia caused by *Bacillus* (of a total of 1,038 cases) were due to *B. subtilis* (as cited by Edberg, 1991). Many of these patients were immunocompromised or had long term indwelling foreign bodies such as a Hickman catheter.

B. subtilis has also been implicated in several cases of food poisoning (Gilbert et al., 1981 and Kramer et al., 1982 as cited by Logan, 1988).

As previously mentioned, *B. subtilis* produces a number of enzymes, including subtilisin, for use in laundry detergent products. There have been a number of cases of allergic or hypersensitivity reactions, including dermatitis and respiratory distress after the use of these laundry products (Norris et al., 1981).

5. Conclusions

B. subtilis is not a human pathogen, nor is it toxigenic like some other members of the genus. The virulence characteristics of the microorganism are low. According to Edberg (1991) either the number of microorganisms challenging the individual must be very high or the immune status of the individual very low in order for infection with *B. subtilis* to occur.

B. Environmental Hazards

1. Hazards to Animals

B. subtilis has been isolated from a number of cases of bovine and ovine abortions, however, the microorganism has never

been identified as the causal agent (Logan, 1988). Reports on association of *B. subtilis* with livestock abortions are fairly rare, and of much lower frequency than with other *Bacillus* species, which are rare compared to all other microorganisms, especially viruses and fungi. *B. subtilis* has also been reported in 17 cases of bovine mastitis in which it was thought to be the causal agent (Fossum et al., 1986). However, the limited number of cases of mastitis associated with *B. subtilis* also is rare compared to mastitis caused by other microorganisms.

B. subtilis has also been shown to be capable of infecting and causing mortality of the 2nd instar larvae of the mosquito, *Anophelis culicifacies*, which is the primary insect vector of malaria in central India (Gupta and Vyas, 1989). *B. subtilis* was being investigated for use as a biocontrol agent in this study.

2. Hazards to Plants

B. subtilis is not considered to be a plant pathogen (7 CFR 330, et seq.; Claus and Berkeley, 1986). However, there are several reports in the literature that associate *B. subtilis* with certain plant diseases. Kararah et al. (1985) produced soft rot of garlic cloves by injecting *B. subtilis* into them. Bergey's Manual of Systematic Bacteriology notes that pectin and polysaccharides of plant tissues can be decomposed by *B. subtilis* and that this microorganism can cause soft rot of potato tubers (Claus and Berkeley, 1986). There are several abstracts obtained in a literature review that suggests that *B. subtilis* may cause other plant diseases, however, no more information was obtainable. One abstract reported that *B. subtilis* was the cause of a broad open cancer ulcera on Norway maples in forests in the Urals (Yakovleva et al., 1990). Another reported that an organism tentatively identified as *B. subtilis* was consistently isolated from glasswort (*Salicornia*) plants suffering from a soft-rot disease (Stanghellini and Rasmussen, 1989).

3. Hazards to Other Microorganisms

B. subtilis has been shown to produce a wide variety of antibacterial and antifungal compounds (Katz and Demain, 1977; Korzybski et al., 1978). It produces novel antibiotics such as difficidin and oxydifficidin that have activity against a wide spectrum of aerobic and anaerobic bacteria (Zimmerman et al., 1987) as well as more common antibiotics such as bacitracin, bacillin, and bacillomycin B (Parry et al., 1983). The use of *B. subtilis* as a biocontrol agent of fungal plant pathogens is being investigated because of the effects of antifungal compounds on *Monilinia fructicola* (McKeen et al., 1986), *Aspergillus flavus* and *A. parasiticus* (Kimura and Hirano, 1988), and *Rhizoctonia* (Loeffler et al., 1986).

Although *B. subtilis* produces a variety of antibiotic compounds in culture media, the importance of antibiotic production in the environment is unknown (Alexander, 1977).

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

B. subtilis is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986). 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 *B. subtilis*. 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

B. subtilis is a common saprophytic inhabitant of soils and is thought to contribute to nutrient cycling due to the variety of proteases and other enzymes members of the species are capable of producing. Growth normally occurs under aerobic conditions, but in complex media in the presence of nitrate, anaerobic growth can occur (Claus and Berkeley, 1986). Under adverse environmental conditions, *B. subtilis* produces endospores that are resistant to heat and desiccation (Claus and Berkeley, 1986). Specific data comparing the survivability of industrial and wild-type strains of *B. subtilis* were not available in the existing literature. However, the ability of *B. subtilis* to produce highly resistant spores and grow under a wide range of conditions indicates that released strains are likely to survive outside of containment.

2. Releases

Estimates of the number of *B. subtilis* 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 *Bacillus subtilis* 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 *B. subtilis* 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. However, its ability to survive in a broad habitat range and produce endospores suggests that this organism may survive after release. 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 *B. subtilis* 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 *B. subtilis* 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 *B. subtilis* in surface water for the uncontrolled/untreated and the full exemption scenarios are tabulated in Table 2 (Versar, 1992).

TABLE 2. *Bacillus subtilis* 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

The natural habitat for *B. subtilis* is soil. Therefore, long-term survival in soil may be expected to occur. Human exposures via dermal and ingestion routes, and environmental exposures [i.e., to terrestrial, avian, and aquatic organisms (via runoff)] may occur at the discharge site because of the establishment of *B. subtilis* within the soil.

6. Summary

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

V. INTEGRATION OF RISK

A. Discussion

Bacillus subtilis is a ubiquitous, saprophytic, soil bacterium which is thought to contribute to nutrient cycling due to its ability to produce a wide variety of enzymes. This latter feature of the microorganism has been commercially exploited for over a decade. *B. subtilis* has been used for industrial production of proteases, amylases, antibiotics, and specialty chemicals. The Agency has reviewed three submissions for production of enzymes using genetically modified *B. subtilis* and found no unreasonable risks to human health or the environment from the use of this microorganism in fermentation facilities.

Historically, *B. subtilis* was a term given to all aerobic endospore-forming bacilli. Later, *B. subtilis* and two closely related species, *B. licheniformis*, and *B. pumilus*, were grouped taxonomically into what was known as the subtilis-group. However, recently methods have been developed that allow *B. subtilis* to be distinguished from these other species.

B. subtilis is not a frank human pathogen, but has on several occasions been isolated from human infections. Infections attributed to *B. subtilis* include bacteremia, endocarditis, pneumonia, and septicemia. However, these infections were found in patients in compromised immune states. There must be immunosuppression of the host followed by inoculation in high numbers before infection with *B. subtilis* can

occur. There also have been several reported cases of food poisoning attributed to large numbers of *B. subtilis* contaminated food. *B. subtilis* does not produce significant quantities of extracellular enzymes or other factors that would predispose it to cause infection. Unlike several other species in the genus, *B. subtilis* is not consider toxigenic. *B. subtilis* does produce the extracellular enzyme subtilisin that has been reported to cause allergic or hypersensitivity reactions in individuals repeatedly exposed to it.

Overall, *B. subtilis* has a low degree of virulence. Although the possibility of human infection is not non-existent, it is low in the industrial setting where exposure to the bacterium is expected to be low and where highly immunocompromised individuals would not be present. In an industrial setting with the use of proper safety precautions, good laboratory practices, and proper protective clothing and eyewear, the potential for infection of workers should be quite low. The only human health concern for workers in the fermentation facility is the potential for allergic reactions with chronic exposure to subtilisin. As previously stated, OSHA has established an exposure limit to subtilisin which must be met in the industrial setting.

Likewise, the ecological hazards associated with the use of *B. subtilis* are low. There are several reports in the literature on the association of *B. subtilis* with abortions in livestock. However, these few reports indicate that this association must be fairly rare, and typically, the animals were immunocompromised. In addition, *B. subtilis* has not been shown to be a causal agent and is not considered an animal pathogen. Likewise, *B. subtilis* is not considered a plant pathogen. Although it produces enzymes such as polygalacturonase and cellulase that are sometimes associated with the ability to produce soft rot in plant tissue, there are many organisms that are capable of producing a soft rot when injected beneath the outer protective epidermal layers.

The use of *B. subtilis* in an industrial setting should not pose an unreasonable risk to human health or the environment. First, human health and environmental hazards of *B. subtilis* are low. Second, the number of microorganisms released from the fermentation facility is low. In addition, *B. subtilis* is ubiquitous in the environment, and the releases expected from the fermentation facilities will not significantly increase populations of this bacterium in the environment.

In conclusion, the use of *B. subtilis* in fermentation facilities for the production of enzymes or specialty chemicals has low risk. Although not completely innocuous, the industrial use of *B. subtilis* presents low risk of adverse effects to human health or the environment.

B. Recommendations

Bacillus subtilis is recommended for the tiered exemption.

VI. REFERENCES

7 CFR 330, et seq., as amended.

29 CFR 1900, et seq., as amended.

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