ORIGINAL

BEFORE THE ILLINOIS POLLUTION CONTROL BOARD

BIOMEDICAL TECHNOLOGY SOLUTIONS, INC., a Colorado Corporation, Petitioner,	)	PCB 07-149 (Variance Petition)
v.	)	
ILLINOIS ENVIRONMENTAL PROTECTION AGENCY, Respondent.	)	

NOTICE OF FILING

To: Division of Legal Counsel
Illinois Environmental Protection Agency
1021 North Grand Avenue East
P.O. Box 19276
Springfield, Illinois 62794-9276

PLEASE TAKE NOTICE that I have today filed with the Office of the Clerk of the Pollution Control Board the Petition for Variance of BioMedical Technology Solutions, Inc., a copy of which is herewith served upon you.

Dated: June 28, 2007

/s/ Jason B. Elster
Jason B. Elster

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CERTIFICATE OF SERVICE

I, Jason B. Elster, an attorney, certify that I have caused a true and correct copy of the foregoing PETITION FOR VARIANCE and NOTICE OF FILING to be served before 5:00 p.m. via First Class Mail, postage pre-paid, on the following:

Division of Legal Counsel
Illinois Environmental Protection Agency
1021 North Grand Avenue East
P.O. Box 19276
Springfield, Illinois 62794-9276

Dated: June 28, 2007

/s/ Jason B. Elster
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BIOMEDICAL TECHNOLOGY SOLUTIONS, INC., a Colorado Corporation,	)	
Petitioner,	)	
v.	)	PCB 07-149 (Variance Petition)
ILLINOIS ENVIRONMENTAL PROTECTION AGENCY,	)	
Respondent.	)	

PETITION FOR VARIANCE

Petitioner BioMedical Technology Solutions, Inc. ("BMTS"), by and through its undersigned attorneys, hereby petitions the Illinois Pollution Control Board (the "Board") for a variance from a provision of 35 IAC 1422. BMTS, which manufactures a countertop medical waste incinerator, the Demolizer®, seeks a variance from 35 IAC 1422, which requires the use of a particular microorganism, *Bacillus subtilis* (ATCC 19659), to determine the initial efficacy of the technology. In conducting the initial efficacy test required under the Board's regulations, BMTS seeks permission to use a different colored indicator microorganism that is otherwise *identical* to the indicator microorganism currently required under the Board's regulations. This petition for a variance (the "Petition") is brought pursuant to Section 35 of the Illinois Environmental Protection Act (the "Act"), 415 ILL. COMP. STAT. 5/35, and Part 104 of Chapter 35 of the Illinois Administrative Code, 35 IAC 104. In support of its Petition, BMTS states as follows:

I. Introduction

BMTS manufactures medical waste treatment devices that, employing Demolizer® technology, destroy potentially infectious microorganisms through the use of dry-heat. Prior to conducting a treatment cycle, medical wastes, including "sharps," are placed into the device, which is approximately the size of the common microwave. Through the course of a treatment cycle, the waste is sterilized and rendered into a non-recognizable solid waste that can then be disposed of as any other refuse. Businesses that generate relatively low volumes of medical waste such as nursing homes, medical, dental and veterinary offices, and pharmacies can use BMTS devices on-site as a safe and efficient method of treating and disposing these materials. It also avoids having to ship medical waste off-site for treatment and disposal. In fact, BMTS devices can be found throughout the United States and BMTS has begun marketing the technology world-wide. The technology is formally approved or meets statutory requirements in 46 states.

In order to sell its devices in Illinois, the Board's regulations require that BMTS demonstrate that its Demolizer® technology is effective in eliminating potentially harmful microorganisms by performing an Initial Efficacy Test ("IET"). The purpose of an IET is to validate the sterilization efficacy of a treatment device. Currently, the Board's regulations specify that a particular microorganism, ATCC 19659 *Bacillus subtilis* ("Custom Indicator" or "Yellow Indicator"), must be used in the IET. However, ATCC 19659[1] is not commercially available in a certified form, and the procedure for growing and certifying ATCC 19659 to the same standards achieved using an otherwise

[1] The American Type Culture Collection, commonly known as the ATCC, is an international nonprofit organization that provides biological products and technical services to the scientific community. The biological samples deposited with the ATCC are used internationally as the reference standard for biological materials. *See* ATCC, http://www.atcc.org/About/AboutATCC.cfm (last visited June 20, 2007).

identical *Bacillus subtilis* certified microorganism could take close to two and a half years and cost upwards of $320,000 - which would require that BMTS sell numerous additional Demolizer® units just to cover these costs.

The alternative to ATCC 19659 is a variant of the same species, ATCC 9372 *Bacillus subtilis* var. *niger*, also known as *Bacillus atrophaeus* ("Certified Indicator" or "Brown Indicator"), which is commercially available in a certified form and is the industry standard in 46 states as well as the international community. The Brown and Yellow Indicators are *identical* - the only difference between the subspecies is their color response under certain culture conditions. BMTS is requesting relief from the Board's requirement of using the Yellow Indicator in the IET and seeks permission to demonstrate the effectiveness of its devices by conducting the IET using the Brown Indicator. Currently, out of the 46 states that have approved the Demolizer® or for which the Demolizer® meets statutory requirements, Illinois is the *only* state that has required use of the Yellow Indicator in the IET for the Demolizer®.

II. Regulatory Requirements For Conducting An Initial Efficacy Test

35 IAC 104.204(a) requires that the Petition contain a statement describing the regulation from which a variance is sought. Pursuant to 35 IAC 1422.124, "[t]he manufacturer, owner or operator of a treatment unit shall conduct an Initial Efficacy Test, pursuant to Appendix A of this Part, for each model prior to its operation." 35 IAC 1422.124(a). The IET is a scientifically-controlled demonstration that the treatment unit does in fact eliminate the infectious potential from potentially infectious medical waste. Section 1422.Appendix A ("Appendix A"), titled Initial Efficacy Test Procedures, sets

forth the procedures for conducting an IET for three classes of treatment units. *See* 35 IAC 1422.Appendix A.

The IET procedure that applies to BMTS involves placing carriers of indicator microorganisms inside the device, conducting a treatment cycle, and then measuring the number of indicator microorganisms that remain viable. *See id.* Appendix A identifies three indicator microorganisms to be used in an IET for treatment units that use thermal treatment and maintain the integrity of the container of indicator microorganisms (*e.g.*, incinerators, autoclaves, and radiation-based processes): 1) *Bacillus subtilis* (ATCC 19659); 2) *Bacillus stearothermophilus* (ATCC 7953); and 3) *Bacillus pumilus* (ATCC 27142). *See* 35 IAC 1422.Table B ("Table B"). However, two of these options are not appropriate for validating BMTS' dry-heat based technology, which in effect turns Table B into a single-option requirement.[2] The variance sought by this Petition pertains to Table B's requirement that dry-heat based treatment systems use the Yellow Indicator in the IET.

III. Background

35 IAC 104.204(b) requires a complete and concise description of the nature of BMTS' activity that is the subject of the proposed variance. BMTS was incorporated in 2005 as a Colorado corporation. BMTS produces medical waste treatment devices that employ Demolizer® technology, which is based on a dry-heat treatment process that was developed and broadly approved throughout the United States in the mid-1990s. The Demolizer® technology has demonstrated broad-scale efficacy through studies at

[2] It is beyond dispute, and the Agency agrees, that the second and third indicator microorganisms, *Bacillus stearothermophilus* and *Bacillus pumilus*, are not scientifically appropriate options for verifying the efficacy of the Demolizer® devices because they are not recognized for the validation of a dry-heat treatment systems - they are used to test the efficacy of steam (autoclave) and radiation-based sterilization processes, respectively. *See* authorities contained in Group Exhibit G, *infra*.

Stanford University, Kansas State University, and various private laboratories. BMTS has customers in almost every state and has begun marketing the technology world-wide.

A. BMTS' Initial Efficacy Test Using the Certified Indicator

In 2006, BMTS commissioned Dr. James Marsden, Regent's Distinguished Professor at Kansas State University, to conduct an initial efficacy test for its updated Demolizer® technology that could be used to secure regulatory approval both in the United States and internationally (the "KSU Efficacy Test"). In selecting an appropriate indicator microorganism, Dr. Marsden conducted a comprehensive review of the scientific literature prior to initiating the efficacy trial.

In his preparations for the KSU Efficacy Test, Dr. Marsden discovered that the Yellow Indicator was not commercially available in a certified spore carrier form. However, the Brown Indicator, which is the industry standard for validating dry-heat sterilization technologies, was readily available from multiple certified manufacturers including STERIS Corporation, NAMSA, Raven Laboratories, STS, and Charles River Laboratories, to name a few. Through his literature review, Dr. Marsden concluded that the Brown and Yellow Indicators are equivalent and are only differentiated because of a pigmentation response to certain media. In fact, over 99.8% of their genetic material is *identical* - meaning that, but for their color, the Brown and Yellow Indicators are indistinguishable.[3] Therefore, it was the recommendation of Dr. Marsden, consistent with the overwhelming body of scientific literature, to use the commercially available Brown Indicator in the KSU Efficacy Test.

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[3] *See* Group Exhibit G, *infra*, K.S. Blackwood, C.Y. Tureene, D. Harmsen, and A.M. Kabini,, *Reassessment of Sequence-Based Targets for the Identification Bacillus Species*, J. CLINICAL MICROBIOLOGY, 42, No. 2 (2004).

The results from the KSU Efficacy Test conclusively established that the Demolizer® technology is an effective sterilization treatment for potential infectious medical waste. Since complete elimination or destruction of all forms of microbial life is difficult to prove, sterilization is usually expressed as a probability function in terms of the number of microorganisms surviving a particular treatment process. Under the Board's regulations, a valid sterilization process must demonstrate a one-millionth survival probability in the indicator microorganism population.[4] The Demolizer® devices used in the KSU Efficacy Test unequivocally demonstrated their ability to meet Illinois' requirements for sterilization devices.

B. Historical Classification and Subsequent Sub-Classification of the *Bacillus Subtilis* Species

Until 1989, the scientific community did not differentiate between the Brown Indicator and the Yellow Indicator. Migula first described the species now know as *Bacillus subtilis* in 1900. *See* Migula, W., *System der Bakterien*, vol. 2. JENA: GUSTAV FISCHER (1990). In 1952, Smith *et al.* noted that certain strains of *Bacillus subtilis* produced different colored pigments when exposed to varying culture conditions, but otherwise found no other discriminatory property between the strains other than pigmentation. *See* Smith, N.R., Gordon, R. E. & Clark, F.E., *Aerobic Spore-forming Bacteria*, AGRICULTURE MONOGRAPH NO. 16, Washington, DC: United States Department of Agriculture (1952). In that same work, Smith *et al.* allocated certain strains, including what would eventually become known as the Brown and Yellow Indicator strains, into a subspecies variety called *Bacillus subtilis* var. *niger*. *See id.*

[4] The Board's regulations express this probability function is a 6 Log_{10} reduction, *i.e.*, a 99.9999% reduction in microbial life.

However, in 1973, these different varieties were once again subsumed into the broader species designation *Bacillus subtilis* through the work of Gordon *et al.* due to the lack of differentiation between varieties. *See* Gordon, R.E., Haynes, W.C. & Pang, C. H.-N., *The Genus Bacillus*, AGRICULTURE HANDBOOK NO. 427, Washington, DC: United States Department of Agriculture (1973). In 1989, Nakamura re-examined the pigment-producing strains of *Bacillus subtilis* and, just like Smith *et al.*, once again differentiated certain subspecies based on pigmentation. *See* Group Exhibit G, *infra*, Nakamura, L.K., *Taxonomic Relationship of Black-Pigmented* Bacillus Subtilis *Strains and a Proposal for* Bacillus Atrophaeus *sp. nov.*, INT. J. SYST. BACTERIOLOGY 39, 295-300 (1989).

This time, Nakamura created a new subspecies designation, *Bacillus atrophaeus*, which included 21 of the 25 strains that had previously been designated as *Bacillus subtilis* var. *niger*. *See id.* Henceforth, the Brown Indicator belonged to the subspecies *atrophaeus* while the Yellow Indicator remained part of the subspecies *subtilis*. In making this distinction between strains, Nakamura noted that the species descriptions of *Bacillus subtilis* and *Bacillus atrophaeus* are not affected by the re-classification because, "except for the colour of the soluble pigment, all of the strains were indistinguishable by the standard characterization method; *i.e.* they exhibited the traits typical of *B. subtilis*." *Id.*; *see also* Fritze, D. and Pukall, R., *Reclassification of Bioindicator Strains* Bacillus Subtilis *DSM 675 and* Bacillus Subtilis *DSM 2277 as* Bacillus Atrophaeus, INT'L. J. SYSTEMATIC EVOLUTIONARY MICROBIOLOGY, 51, 35-37 (2001).

Since Nakamura's 1989 re-classification of *Bacillus subtilis* strains, the scientific community has consistently and unanimously found that the Brown and Yellow Indicators are phenotypically identical except for color. *See generally*, Group Exhibit G,

infra. Therefore, the Board's regulations state what is in effect a mandatory color preference of otherwise equal indicator microorganisms.

C. BMTS' Regulatory Approval Efforts

As part of the KSU Efficacy Test, extensive trials were conducted on the updated Demolizer® technology utilizing an array of organisms under varying conditions as required by the Illinois statutes and other state agencies across the United States. These results have been exhaustively reviewed by many of the states that formally approve such technologies and resulted in the issuance of technology approval letters. Only three states specifically identify the Yellow Indicator for use in validation procedures: Arizona, Illinois, and Delaware. In fact, both Arizona and Delaware have reviewed the KSU Efficacy Test that used the Brown Indicator and issued approval for the technology based on its findings. To date, BMTS' Demolizer® technology is either approved or meets statutory requirements in 46 states. Historically, the technology has been reviewed favorably by over 75 federal, state, and local agencies, and it meets statutory requirements for treatment across the United States and throughout the international community.

In mid-October 2006, BMTS contacted the Illinois Environmental Protection Agency (the "Agency") to request that the Agency consider a continuous monitoring system as an alternative to monthly testing.[5] After speaking with an Agency representative, BMTS submitted a formal request that included the KSU Efficacy Test results on October 19, 2006. Over the next few months, BMTS periodically contacted the Agency to check on the status of its request and was told that a response would be

[5] Formal approval from the Agency is required in order for a manufacture like BMTS to use a continuous monitoring approach to periodic verification initiatives.

issuing shortly. In January 2007, BMTS received a formal response from the Agency stating that, in the Agency's opinion, the KSU Efficacy Test did not conform with the IET requirements. A true and correct copy of the Agency's January 5, 2007 Letter is attached hereto as Exhibit A.

After receiving the Agency's January 5, 2007 letter, BMTS agreed to provide the Agency with additional information to resolve the issue regarding the IET, which was transmitted on January 10, 2007. A true and correct copy of BMTS' January 10, 2007 Correspondence is attached hereto as Exhibit B. Over the next four months, BMTS periodically contacted the Agency to inquire as to its review of the additional information BMTS provided. It was not until May 7, 2007 that BMTS received a response from the Agency that reiterated its prior position.[6] A true and correct copy of the Agency's April 4, 2007 Letter is attached hereto as Exhibit C. In response to the Agency's letter, on May 9, 2007, BMTS contacted the Agency to discuss why the Agency was unwilling to reconsider its position. During this conversation, the Agency's representative referred BMTS to Agency attorney Bill Ingersoll.

From May 8, 2007 through early June 2007, BMTS exchanged correspondence with Mr. Ingersoll regarding the IET. A true and correct copy of the e-mail correspondence between BMTS and Mr. Ingersoll is attached hereto as Exhibit D. In that correspondence, Mr. Ingersoll recognized that the Yellow Indicator was not commercially available.[7] Even so, Mr. Ingersoll stated that "it seems that we are unable

[6] Although the Agency's letter was dated April 4, 2007, which appears in a different type-font than the rest of the letter, BMTS received the letter on May 7, 2007.

[7] The Yellow Indicator cannot be purchased in a certified form. However, it is available in freeze-dried form, which would require the purchaser to grow a viable population. However, this method necessitates that the purchaser conduct rigorous testing to certify that the custom-grown population has the proper

to help you . . ." *See* Exhibit D. Pursuant to the suggestion of Mr. Ingersoll, BMTS initiated the present Petition.

IV. Difficulties Meeting 35 IAC § 1422.Table B

35 IAC 104.204(c) requires that the Petition contain data describing the nature and extent of the difficulties in meeting the regulation at issue. In developing the specific protocol used for demonstrating treatment efficacy, BMTS attempted to acquire the Yellow Indicator in a certified carrier form since this subspecies is specifically cited in Illinois state regulations. Unfortunately, this subspecies is not available commercially in a certified carrier form.

With the help of researchers at Kansas State University, BMTS completed a comprehensive scientific literature review and identified an equivalent subspecies, the Brown Indicator, as *the industry standard for the validation of dry-heat sterilization processes*. The overwhelming use of the Brown Indicator as *the* indicator organism for dry-heat processes stems from its pigmentation response that allows for a clear differentiation between a positive and negative result. The Yellow Indicator's response is actually less visible. The Brown Indicator is cited in numerous national and international standards including the U.S. Pharmacopoeia, the International Standards Organization, and over three dozen scientific papers related to the validation of sterilization processes. *See* Group Exhibit G, *infra*.

BMTS made the decision to use the Brown Indicator because: 1) the indicators are effectively identical except for color; 2) the Brown Indicator is nearly universally recognized as *the* appropriate indicator microorganism to demonstrate the effectiveness

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resistance properties to validate a treatment process. In most cases, the purchaser will have to grow and test several populations in order to certify a custom-grown population.

of dry-heat treatment processes, the underlying treatment technology of the Demolizer®; and 3) use of a Certified Indicator comports with the best practices of the scientific community since Custom Indicator populations must be grown in more non-controlled laboratory environments where it is possible to inadvertently compromise the resistance and growth properties. Since the Certified Indicator is indisputably equivalent to the Custom Indicator and, unlike the Custom Indicator, is available in a certified form that comports with the industry's best practices, BMTS used the Certified Indicator in the KSU Efficacy Test.

V. Description of Efforts Necessary for BMTS to Achieve Immediate Compliance

35 IAC 104.204(d) requires that the Petition contain a description of the efforts required to come into immediate compliance. Under the Agency's current interpretation of the Board's regulations, it is impossible for BMTS to achieve immediate compliance, which could take as long as two and a half years due to the time and resources required to grow and certify a Custom Indicator to the same standards already demonstrated in the KSU Efficacy Test. However, BMTS has already conducted a successful IET using the different colored strain of the same indicator microorganism species identified in the regulations. Therefore, if the Board were to interpret Table B as allowing the use of a Certified Indicator, BMTS would be in immediate compliance with the Board's regulations.

VI. Immediate Compliance Would Impose an Arbitrary and Unreasonable Hardship

35 IAC 104.204(e) requires that BMTS set forth reasons why immediate compliance with the regulation would impose arbitrary and unreasonable hardship. Table

B's requirement of using a Yellow Indicator over a Brown Indicator is arbitrary because it is based solely on a color preference.

Most states modeled their statutes and regulations off of a report titled *Technical Assistance Manual: State Regulatory Oversight of Medical Waste Treatment Technologies* that was prepared by the State and Territorial Association on Alternate Treatment Technologies (the "STAATT Report").[8] True and correct portions of the STAATT Report are attached hereto as Exhibit E. The STAATT Report identified the Yellow Indicator strain as a representative example of *Bacillus subtilis*. However, the STAATT Report stressed that the Yellow Indicator spore was only a representative strain of the species and was not selected based on any special resistance properties. Further, the STAATT Report stated that "the guidelines developed through this series of meetings should serve only to provide guidance to states in the development of a review and approval process for medical waste treatment technologies." Exhibit E, STAATT Report at p. 3.

As explained by Dr. Nelson S. Slavik, the primary author of the STAATT Report, BMTS' "selection of *B. subtilis* ATCC 9372 spores is consistent with the criteria provided by STAATT in their publication. This strain [the Brown Indicator] provides the dry-heat resistance which is appropriate for your treatment process." Letter from Nelson S. Slavik to Diane Gorder, June 11, 2007, a true and correct copy of which is attached hereto as Exhibit F. Table B's requirement that dry-heat based sterilization processes use the Yellow Indicator as opposed to the Brown Indicator in the IET is clearly arbitrary.

[8] The STAATT Report was a culmination of conferences and debates beginning in 1992, the conclusions of which were widely disseminated prior the publication of the final STAATT Report in April 1994.

Moreover, BMTS will incur significant and unreasonable costs if it is required to repeat the KSU Efficacy Test using a different colored, but otherwise equivalent, indicator microorganism. After learning of the Agency's position, BMTS requested that KSU prepare an estimate to repeat the KSU Efficacy Test using the Custom Indicator. In preparation of this estimate, BMTS again contacted Dr. Marsden, who would be responsible for repeating the study. Dr. Marsden informed BMTS that, in order to grow a Custom Indicator and ensure comparable quality standards, the study would require two major phases.

The first phase would involve growing a culture population of the Custom Indicator and certifying its resistance properties through exhaustive D-value studies.[9] Dr. Marsden would use standard protocols for validating the resistance of the culture similar to those used throughout the industry. This study will likely need to be repeated several times until a population is grown to the standards comparable to a Certified Indicator like those obtained from certified manufactures.

Dr. Marsden provided an estimate of a minimum of $60,000 for a single D-value evaluation of a population. It is very possible that repeated trials could result in a **total cost approaching $250,000** to properly certify the population with a **total time frame of up to two years**. These estimates are phase-one costs only.

Once a Custom Indicator population has been grown and certified, Dr. Marsden would begin the second phase, which involves repeating the Demolizer® efficacy study using appropriate replicates, load conditions, etc. This requires a **minimum of 2-4 months** to coordinate and report the study. Upon completion of both phases, validation

[9] An organism's D-value is the treatment time required for 90% deactivation (sterilization), *i.e.*, a measure of an organism's resistance to a particular treatment method - here, dry-heat.

results comparable to those already reported could be obtained. The estimate provided by Dr. Marsden for phase two of the validation study using ATCC 19659 is **$40,000**. In addition to these costs, BMTS would incur **direct costs totaling more than $30,000**, which includes the cost of three dedicated systems and the cost of BMTS staff time to be on-site at Kansas State University to facilitate the trial.

Therefore, the total cost for repeating the efficacy study using a Custom Indicator is estimated to be between $130,000 and $320,000 dollars and could take up to two and a half years to complete. BMTS would have to sell numerous additional Demolizer® units to make up for the cost of repeating the IET with the Yellow Indicator. Given that the Custom and Certified Indicators are equivalent, requiring BMTS to repeat the same efficacy test using a Custom Indicator is an arbitrary and unreasonable hardship.

VII. Compliance Plan

35 IAC 104.204(f) requires that the Petition provide a description of a compliance plan. The requirements in the Board's regulations for a description of a compliance plan are not applicable to this Petition. It is undisputed that the results from the KSU Efficacy Test completely satisfy Illinois' IET requirements in every way if the Board were to accept the use of a Certified Indicator. Instead, BMTS would rather seek to change the current regulatory requirements.

VIII. No Environmental Impact

35 IAC 104.204(g) requires that the Petition describe the environmental impact of the activity. BMTS' activities and the variance requested will have no impact on human, plant, or animal life. This is established by the studies described herein.

IX. Supporting Documents

35 IAC 104.204(h) requires that the Petition cite supporting documents and legal authority. With respect to documents, Exhibits A through H are attached to this Petition and are specifically referenced herein. In addition, for the convenience of the Board, true and correct copies of relevant portions of the scientific authorities cited in this Petition are attached collectively hereto as Group Exhibit G. The scientific literature discussed in this Petition establishes that the Brown and Yellow Indicators are equivalent.

In the scientific community, both the Brown and Yellow Indicators have been used to demonstrate efficacy of a particular sterilization technology. In all cases, there was no reported difference in the performance of the two substrains. *See generally*, Group Exhibit G; *see* U.S. Food and Drug Administration, *Guidance on Premarket Notification [510(k)] Submissions for Sterilizers Intended for Use in Health Care Facilities* (March 1993) (listing both the ATCC 9372 and the ATCC 19659 *B. subtilis* samples as equivalent indicator organisms to validate dry-heat sterilizers); *see also* U.S. Food and Drug Administration, *Guidance on Premarket Notification [510(k)] Submissions for Sterilizers Intended for Use in Health Care Facilities; Draft Guidance for Industry and FDA Reviewers* (May 2001) (updated publication listing only the Brown Indicator to validate dry-heat sterilization treatments).

Nakamura and others state that, "[e]xcept for colour of the soluble pigment, all of the strains were indistinguishable by the standard characterization method; i.e., they exhibited the traits typical of *B. subtilis*." Group Exhibit G, Nakamura, *supra*. Blackwood reported that the RNA sequences of various substrains of *B. subtilis* are indistinguishable with a reported sequence mapping of over 99%. *See* Group Exhibit G,

Blackwood, *supra*. Moreover, Blackwood also reported that the only way to differentiate between the substrains would be to observe oxidative activity since they are identical with the exception of pigmentation differences. *See id.*

In 2000, the European Commission Health and Consumer Protection Directorate-General stated that "*B. atrophaeus* is distinguishable from *B. subtilis* only by pigmentation." Group Exhibit G, European Commission, Health and Consumer Protection Directorate-General, *Opinion of the Scientific Committee on Animal Nutrition on the Safety of Use of Bacillus Species in Animal Nutrition* (Feb. 17, 2000). In a 2004 Environmental Technology Verification Report conducted by Battelle, both the Yellow and Brown Indicators were used to validate the effectiveness of a formaldehyde-based decontamination technology, and there were no reported qualitative differences in the resistance of the two samples. *See* Group Exhibit G, Battelle, *Environmental Technology Verification Report prepared for CERTEK, Inc.* (Aug. 2004).

In a 2001 comparative study by Khadre and Yousef, the resistance of both the Brown and Yellow Indicators were shown to be equivalent during an evaluation of ozone and hydrogen peroxide sterilization technologies. *See* Group Exhibit G, M.A. Khadre, A.E. Yousef, *Sporicidal Action of Ozone and Hydrogen Peroxide: A Comparative Study*, INT'L. J. OF FOOD MICROBIOLOGY, 71, 131-138 (2001). In fact, Khadre and Yousef concluded that "differences among these strains were not significant ($p<0.05$)." *Id.* Similarly, in a study by Sagripanti, *et al.*, the Brown and Yellow Indicators were evaluated along with other various strains for sporicidal activity against a broad range of treatment technologies and found to have resistances "within 1 Log_{10} of each other." Group Exhibit G, J-L. Sagripanti, *et al.*, *Virulent Spores of Bacillus Anthracis and other*

Bacillus Species Deposited on Solid Surfaces Have Similar Sensitivity to Chemical Decontaminants, J. APPLIED MICROBIOLOGY, 102, 11-21 (2007).

The following international standards list the Brown Indicator for dry-heat processes:

1. **US Pharmacopoeia**. USP28-NF23 USP. Monographs: Biological Indicator for Dry-Heat Sterilization, Paper Carrier; Rockville, MD; 2005.
2. **FDA**. Guidance on Premarket Notification [510(k)] Submissions for Sterilizers Intended for Use in Health Care Facilities. Infection Control Devices Branch, Division of General and Restorative Devices (March 1993).
3. **FDA**. Premarket Notifications [510(k)] for Biological Indicators Intended to Monitor Sterilizers Used in Health Care Facilities; Draft Guidance for Industry and FDA Reviewers; U.S. Department of Health and Human Services, Food and Drug Administration, Center for Devices and Radiological Health, Infection Control Devices Branch (March 2001).
4. **British Pharmacopoeia Commission**. Methods of sterilization. London, UK: British Pharmacopoeia Commission; British Pharmacopoeia Appendix XVIII (2003).
5. **European Pharmacopoeia Commission**. Biological indicators of sterilization. Strasbourg, France: European Pharmacopoeia Commission; European Pharmacopoeia EP 5.1.2 (1997).
6. **Japanese Pharmacopoeia**. JP14e.partII.15 JP. Terminal Sterilization and Sterilization Indicators.
7. **ISO.** Sterilization of health care products – Biological indicators; Geneva (Switzerland): International Organization for Standardization/ANSI; ISO 11138-2:1994.
8. **ISO.** Sterilization of single-use medical devices incorporating materials of animal origin – Validation and routine control of sterilization by liquid chemical sterilants. Geneva (Switzerland): International Organization for Standardization/ANSI; ISO 14160:1998.

See Group Exhibit G.

X. No Pending Permit Application

35 IAC 104.204(i) requires that any relevant permit or permit application be attached to this Petition. There is no pending permit application associated with this Petition. The use of the Demolizer® by Illinois generators to treat their own waste is

exempted from permitting in 35 IAC 1422. BMTS has requested acceptance of its use of continuous parametric monitoring as a substitute to monthly spore testing consistent with the requirements in 35 IAC 1422.

XI. Suggested Conditions

35 IAC 104.204(j) requires that BMTS suggest any conditions for the variance. BMTS seeks no additional conditions. This variance is simply a request that the Board recognize BMTS' use of the Certified Indicator in conducting the IET.

XII. Proposed Beginning and End of the Variance

35 IAC 104.204(k) requires that BMTS propose a beginning and ending date for the variance. BMTS is requesting that the variance commence upon the Board's issuance of the Order granting BMTS the requested variance. BMTS further requests that the variance extend for the maximum allowable period under the Board's regulations.

XIII. Consistency with Other Law

35 IAC 104.204(l) requires that the Petition discuss consistency with federal law. State, federal, and international authorities recognize the use of the Brown Indicator as an appropriate indicator microorganism for sterilization validation procedures.

BMTS' Demolizer® devices have been approved or meet statutory requirements in 46 states based on the results of the KSU Efficacy Test. While some of the states that have approved Demolizer® technology do not specify a particular strain of indicator microorganism, *e.g.*, California, New York, Michigan, Connecticut, North Carolina, South Carolina, Georgia, and Louisiana, others such as Florida specify only that the species *B. subtilis* be used to validate sterilization treatments. Of the three states that particularly identify the Yellow Indicator, Arizona, Delaware, and Illinois, BMTS has

already received approval from both Arizona and Delaware based on the KSU Efficacy Test.

The federal government recognizes the appropriateness of using the Brown Indicator to validate sterilization procedures. The U.S. Food and Drug Administration identifies the Brown Indicator as the appropriate test organism for dry-heat based sterilization procedures. *See* Group Exhibit G, *Guidance on Premarket Notification [510(k)] Submissions for Sterilizers Intended for Use in Health Care Facilities* (March 1993); *Guidance on Premarket Notification [510(k)] Submissions for Sterilizers Intended for Use in Health Care Facilities; Draft Guidance for Industry and FDA Reviewers* (May 2001), *supra*. In addition, the U.S. Pharmacopeia states that an appropriate biological indicator for dry-heat sterilization should "compl[y] substantially with the morphological, cultural, and biochemical characteristics of the strain of *Bacillus subtilis*, ATCC No. 9372 [the Brown Indicator], designated subspecies *niger* . . ." Group Exhibit G, U.S. Pharmacopeia, Monographs: Biological Indicator for Dry-Heat Sterilization, Paper Carrier, USP28-NF23 USP (2005), *infra*.

Moreover, the international community has identified the Brown Indicator as the standard indicator microorganism for validating dry-heat processes. For example, the British Pharmacopoeia, the European Pharmacopoeia, the Japanese Pharmacopoeia, and the International Organization for Standardization all list the Brown Indicator as the biological indicator to validate dry-heat sterilization treatments. The world-wide acceptance of the Brown Indicator as the industry standard further supports BMTS' assertion that the Brown and Yellow Indicators are equivalent.

XIV. Affidavit Verifying Facts

35 IAC 104.204(m) requires that the Petition contain an affidavit verifying the facts submitted herein. The affidavit of BMTS' Director of Regulatory Compliance, Diane Gorder, verifying both that the facts stated in this Petition are true and that the attached exhibits are true and accurate copies, is attached hereto as Exhibit H.

XV. Hearing Requested

35 IAC 104.204(n) requires a statement requesting or denying that a hearing should be held. BMTS hereby requests a hearing on the Petition.

CONCLUSION

BMTS therefore asks that this Board, pursuant to its authority under Section 35 of the Act and the Board's regulations under 35 IAC 104, grant BMTS a variance from the provisions of 35 IAC 1422.Table B regarding the requirement that the Yellow Indicator be used in an IET for medical waste treatment units that utilize dry-heat technology.

Respectfully Submitted,

BIOMEDICAL TECHNOLOGY SOLUTIONS, INC.

By: /s/ Neal H. Weinfield
One of Its Attorneys

Dated: June 28, 2007

Neal H. Weinfield
Jason B. Elster
GREENBERG TRAURIG, LLP
Firm No. 36511
77 West Wacker Drive, Suite 2500
Chicago, Illinois 60601
312-456-8400 (Telephone)
312-456-8435 (Facsimile)
weinfieldn@gtlaw.com
elsterj@gtlaw.com

EXHIBIT A

ILLINOIS ENVIRONMENTAL PROTECTION AGENCY

1021 NORTH GRAND AVENUE EAST, P.O. BOX 19276, SPRINGFIELD, ILLINOIS 62794-9276 – (217) 782-3397
JAMES R. THOMPSON CENTER, 100 WEST RANDOLPH, SUITE 11-300, CHICAGO, IL 60601 – (312) 814-6026

ROD R. BLAGOJEVICH, GOVERNOR DOUGLAS P. SCOTT, DIRECTOR

217/524-3300

January 5, 2007

Diane Gorder
BioMedical Technology Solutions, Inc.
9800 Mt. Pyramid Court
Suite 350
Englewood, Colorado 80112

Re: 9080000000 – Colorado
BioMedical Technology Solutions, Inc.
Log No. PS06-165
Permit File

Dear Ms. Gorder:

Thank you for your letter of October 19, 2006, in which you request to use an alternative method to comply with the periodic verification test requirements in 35 Illinois Administrative Code 1422. You propose the use of continuous monitoring of critical control operating parameters as an alternative periodic test for your Demolizer unit designed to treat potentially infectious medical waste (PIMW).

The initial efficacy testing data included in your submittal indicated that testing was performed on the Demolizer using several microorganisms. However, none of the microorganisms used were the ATCC number required by 35 Illinois Administrative Code 1422.AppendixA.TableA. For example, Demolizer testing used *Staphylococcus aureus* ATCC 33591, while the Illinois regulations require *S. aureus* ATCC 6538. 35 Ill. Adm. Code 1422 requires the use of these specific microorganisms, including ATCC number.

The composition and placement of challenge loads used in the efficacy testing appear to comply with the PIMW regulations. The submitted data indicates that the Demolizer is capable of achieving a 6 $\log_{10}$ reduction for all of the microorganisms used.

An alternative periodic verification test (PVT) may be approved and used only when the initial efficacy test (IET) has been performed completely in accordance with 35 Ill. Adm. Code 1422, and the alternative PVT has been directly correlated to the results obtained in the IET.

Units designed to treat potentially infectious medical waste may be used in Illinois without a permit from the Illinois Environmental Protection Agency (Illinois EPA), provided the treatment

unit is accepting and treating only PIMW generated on-site. The efficacy testing results for the treatment unit must be kept on-site and made available to the Illinois EPA upon request.

In order for the Demolizer treatment unit to be used in Illinois, testing must be performed on the unit to demonstrate compliance with 35 Illinois Administrative Code: Subtitle M, or you could seek an Adjusted Standard from the Illinois Pollution Control Board. They may be reached at 312/814-3620.

I hope this satisfies your inquiry. If you have further questions, please feel free to contact Beverly Albarracin of my staff at 217/524-3289.

Sincerely,

Stephen F. Nightingale, P.E.
Manager, Permit Section
Bureau of Land

SFN:BA/mls/073626.doc

EXHIBIT B

Diane Gorder

From: Diane Gorder [dgorder@bmtscorp.com]
Sent: Wednesday, January 10, 2007 12:04 PM
To: beverly.albarracin@epa.state.il.us
Subject: Demolizer technology and the Initial Efficacy Testing
Follow Up Flag: Follow up
Flag Status: Red

Beverly,

Attached is a response to your concerns expressed in the January 5, 2007 letter. We are sending this by email and hope it provides the information you need to complete the evaluation of our alternative quality monitoring approach. While our customers will likely not require a permit for use of the system within the state, we are diligently working to make certain we have the appropriate approvals in place to provide the best guidance possible on state regulatory compliance issues.

If you need any more information or have any questions about the information, please call me on my mobile at (719) 661-2296. I currently work between two offices so my mobile is the easiest way to reach me.

Thank you again for your time and consideration. If you could please send me a quick reply that you have received the information, I would greatly appreciate it.

Diane Gorder

BioMedical Technology Solutions, Inc.
9800 Mt. Pyramid Ct., Suite 350
Englewood, CO 80112
Direct: (719) 260-2331
Main Office: (303) 653-0100
Fax: (303) 653-0120

SENT VIA EMAIL

January 10, 2007

Ms. Beverly Albarracin
Illinois Environmental Protection Agency
PO Box 19276, BOL Permit #33
Springfield, IL 62794

Su: 5 January 2007 Letter from the Illinois EPA, Log No. PS06-165

Dear Beverly:

Thank you for returning my call so promptly this morning. As we discussed, I am responding in writing to your letter dated 5 January 2007 related to your concerns about the Initial Efficacy Testing results provided in our October submittal.

As we discussed, we recently completed supplemental efficacy testing under the leadership and guidance of Dr. James Marsden, Regent's Distinguished Professor – Kansas State University. The purpose of this effort was to substantiate the claim that the changes in the electronics, process control capabilities, and the shape of the Collector do not affect the efficacy of the Demolizer™ technology that has been approved and in use throughout the U.S. since the mid-1990s.

In developing a protocol in collaboration with the research team at Kansas State University, we diligently reviewed the numerous protocols defined by various state agencies and developed a scientifically-sound approach for this supplemental efficacy data based on the follow major criteria:

1. The waste load should be reflective of representative sharps or red bag waste loads and should pose the most difficult challenge for a dry heat treatment process. The waste loads must also be at full capacity.
2. The inoculation approach should represent a worst-case loading scenario with a high bacterial recovery rate.
3. Bacterial species should broadly meet state efficacy testing requirements and, more importantly, be selected based on their appropriateness for a dry heat treatment process.
4. Microbiological techniques, experimental design, and analytical analysis should conform to generally accepted scientifically sound approaches.
5. The microbiological challenge must be conducted under normal operating conditions for the device. This approach was necessary since performing tests exactly conforming to general requirements for alternative technologies (whether chemical, microwave, etc.) and meeting the rigorous protocol guidelines discussed above, would have required months of testing and hundreds of thousands of dollars.

The recent efficacy testing of the Demolizer™ technology at Kansas State University was consistent with Options 3 of Appendix A and Section 1422.122 (a)(1)(A) of the Illinois regulations. The regulations, as we have interpret them, allow for the demonstration of a 6 $\log_{10}$ kill of indicator microorganism spores as an alternative test for thermal treatment systems that maintain the integrity of the spore carrier. For the efficacy trials, the tamper-resistant lid was altered to allow for retrieval of the carriers at the end of a treatment cycle. *Bacillus atrophaeus* (formerly known as *Bacillus subtilis* var. *niger,* ATCC 19659) was utilized as the USP recognized indicator spore organism for dry heat and ethylene oxide treatment processes. Note: Spore strips of ATCC 19659 are no longer commercially available and have been substituted with the species used in the trial.

Further the protocol largely conforms to the other requirements listed in the regulations. Specifically, five carriers were used for each replication with the carriers placed near the geometric center of the load away from the hot, radiating sides of the metal collector. The composition of the loads was selected to be both *representative* of the types of waste to be treated in the Demolizer™ system and to pose the most rigorous challenge to a dry heat process. Specifically, we used both a sharps and red bag waste load. The Sharps load was comprised of 370 g of syringes with a small amount of added residual liquid (~11% by weight). This low moisture environment demonstrated a 6 $\log_{10}$ reduction of resistant *B. subtilis* spores, the USP indicator organism for dry heat processes. Reducing the moisture by a tablespoon to hit the target of 5% moisture is not believed to be a meaningful difference and would therefore not impact the results.

The red bag waste load represented the greatest challenge and was thus evaluated using a broad array of microbial species. The load was comprised of over 80% by volume of highly insulating adsorbent material (3-ply gauze and cotton), about 8% by volume of non-adsorbent material (syringes and gloves), and 12% by volume of organic material. By weight, the breakdown was approximately 42% moisture and 30% organic (equine serum and TSB broth). Importantly, even at this moisture level content (w/w%), the waste load was very dry with only a small portion of the gauze moistened. The carriers were essentially embedded within the dry, insulating gauze material near the geometric center of the load, representing a worst-case loading challenge for the dry heat process. Note, the moisture content was very near the mid-point guideline specified in the Illinois regulations. Reducing the content to 5% moisture would have been an unrealistic loading condition for a typical bloody waste load (only 1 T of total liquid in the 1 gallon collector).

Finally, the requirement for a 70% organic load is not directly relevant for the dry heat process. For those processes relying on an oxidizing chemistry, a high organic load could neutralize the reactive sites thereby impacting the efficacy of the sterilization process. Dry heat does not rely on oxidative chemistry, instead the kill is based on heat and dehydration of organisms. As such, increasing the organic load from 30% to 70% would have no impact on the results.

While not required under Option 3, we used a broad range of other representative microorganisms to evaluate the effectiveness of the Demolizer™ treatment process. These included gram positive and negative bacteria (Methicillin resistant *Staphylococcus aureus* and *Escherchia coli), Candida albicans, Mycobacterium phlei* and *Bacillus subtilis.* While many of the specimens have ATCC numbers different from those indicated in Table A, they are scientifically considered alternatives and represent the commercially available equivalent. These organisms have been accepted by numerous state Departments of Health and the Environment including New York, Delaware, West Virginia, Florida, Michigan, Pennsylvania, South Carolina, North Carolina, Louisiana, etc.

The mycobacterium and bacillus species represent the toughest challenge for the Demolizer™ technology because these are the most heat resistant organisms. In fact, most states are modifying their regulations to be consistent with the STAATT II and III guidelines that call for using the appropriate indicator spore organism and one of three *Mycobacterium* species for the demonstration of efficacy of alternative treatment technologies. *Bacillus subtilis* was selected because it is the recognized indicator organism for dry heat processes and *Mycobacterium phlei* was selected due to its susceptibility to heat and its BioSafety Level II classification.

In previous trials, the Demolizer™ dry heat process has demonstrated a minimum 6 $\log_{10}$ reduction of the following additional organisms: *Pseudomonas aeruginosa*, *Giardia* spp. (oocysts), Duck Hepatitis B, *Mycobacterium bovis*, and *Mycobacterium fortuitum*. Please refer to the background information provided in the October submittal.

We did not specifically evaluate *Trichophyton metagrophytes* arthrospores because our research shows that Illinois and Delaware are the only states that include this in the list of indicator organisms for a dry heat process. This organism has been shown to be "extremely susceptible to moderate heat (above 50°C)" as reported by Hashimoto and Blumenthal (1978 Feb; 35(2):274-7; Appl Environ Microbiol). Due to their low heat resistance, they are not considered appropriate indicator organisms for a dry heat process.

We hope this provides additional information to substantiate our claim that the recent efficacy trials conform to the Illinois requirements. While our customers will not fall under the permit requirements of the state, we are seeking official acceptance of our quality control monitoring programs as a scientifically sound alternative to periodic verification testing using spore strips. We strongly believe that the continuous monitoring of critical control points provides a significantly higher level of assurance and is consistent with other recognized quality programs such as 6-Sigma and HACCP initiatives.

Please keep me apprised of your review of this material. If there is any other information you wish us to provide to support our submittal, please call me on my mobile at (719) 661-2296 or send me an email at dgorder@bmtscorp.com.

Sincerely,

Diane Gorder
Director of Regulatory Compliance

Cc: Don Cox, President/CEO
Dr. James Marsden, KSU

EXHIBIT C

ILLINOIS ENVIRONMENTAL PROTECTION AGENCY

1021 NORTH GRAND AVENUE EAST, P.O. BOX 19276, SPRINGFIELD, ILLINOIS 62794-9276 – (217) 782-3397
JAMES R. THOMPSON CENTER, 100 WEST RANDOLPH, SUITE 11-300, CHICAGO, IL 60601 – (312) 814-6026

ROD R. BLAGOJEVICH, GOVERNOR DOUGLAS P. SCOTT, DIRECTOR

217/524-3300

April 4, 2007

Diane Gorder
BioMedical Technology Solutions, Inc.
9800 Mt. Pyramid Court
Suite 350
Englewood, Colorado 80112

Re: 9080000000 – Colorado
BioMedical Technology Solutions, Inc.
Log No. PS07-022
Permit File

Dear Ms. Gorder:

Thank you for your e-mail with attached letter of January 10, 2007, in which you provide a response to our letter of January 5, 2007.

Your letter stated that the testing performed was consistent with Option 3 of Appendix A and Section 1422.122(a)(1)(A) of 35 Illinois Administrative Code: Subtitle M. Your letter also stated that *Bacillus atrophaeus* was used as a substitute for *Bacillus subtilis* var. *niger*, ATCC 19659 in the testing. You noted that spore strips of ATCC 19659 are no longer commercially available and have been substituted with *B. atrophaeus*. Your letter also indicated that many of the specimens used in your testing have ATCC numbers different from those indicated in 35 Ill. Adm. Code 1422.Appendix A(Table A), but they are scientifically considered alternatives and represent the commercially-available equivalent.

The regulations for efficacy testing found in 35 Ill. Adm. Code: Subtitle M require the use of at least one of the Indicator Microorganisms found in Section 1422.Appendix A(Table B). It appears as though *B. subtilis*, ATCC 19659, is still available through ATCC, although not in spore strip form. In addition, if *B. subtilis* is not available, there are two other microorganisms that can be used for the efficacy testing, *B. stearothermophilus* (ATCC 7953) or *B. pumilus* (ATCC 27142).

The initial efficacy testing data included in your submittal indicated that testing was performed on the Demolizer using several microorganisms. However, none of the microorganisms used were the ATCC number required by 35 Illinois Administrative Code 1422.AppendixA.TableA. For example, Demolizer testing used *Staphylococcus aureus* ATCC 33591, while the Illinois

regulations require *S. aureus* ATCC 6538. 35 Ill. Adm. Code 1422 requires the use of these specific microorganisms, including ATCC number.

The composition and placement of challenge loads used in the efficacy testing appear to comply with the PIMW regulations. The submitted data indicates that the Demolizer is capable of achieving a 6 $\log_{10}$ reduction for all of the microorganisms used.

An alternative periodic verification test (PVT) may be approved and used only when the initial efficacy test (IET) has been performed completely in accordance with 35 Ill. Adm. Code 1422, and the alternative PVT has been directly correlated to the results obtained in the IET. In order for an alternative PVT to be approved, the Initial Efficacy Test must be performed in accordance with the regulations found in 35 Ill. Adm. Code: Subtitle M. Another option is to seek an Adjusted Standard from the Illinois Pollution Control Board. They may be reached at 312/814-3620.

Units designed to treat potentially infectious medical waste may be used in Illinois without a permit from the Illinois Environmental Protection Agency (Illinois EPA), provided the treatment unit is accepting and treating only PIMW generated on-site. The efficacy testing results for the treatment unit must be kept on-site and made available to the Illinois EPA upon request.

I hope this satisfies your inquiry. If you have further questions, please feel free to contact Beverly Albarracin of my staff at 217/524-3289.

Sincerely,

Stephen F. Nightingale, P.E.
Manager, Permit Section
Bureau of Land

SFN:BGA:bjh\072792s.doc

EXHIBIT D

Diane Gorder

From: William Ingersoll [William.Ingersoll@illinois.gov]
Sent: Monday, June 04, 2007 1:16 PM
To: Diane Gorder
Subject: RE: Inquiry re PIMW and Bacillus Subtilus

Diane,
I sat down with Bureau of Land Permit Section staff and management earlier today to discuss this matter in more detail. To recap your issue, I believe that your company proposed to use bacillus subtilus (ATCC 9362) in its performance testing, while the Illinois regulations require the use of bacillus subtilsu (ATCC 19659).

One of the issues you raised was that the bacillus subtilsu (ATCC 19659) is no longer commercially available. I am told that while this strain may not be "off-the-shelf" at this time, it can still be purchased. In addition, I am told that all of the relevant facilities currently permitted in Illinois used the regulatorily required strain.

Therefore, it seems that we are unable to help you in seeking an interpretive resolution to your regulatory problem.

Bill Ingersoll
Manager of Enforcement Programs
Illinois EPA
217-782-9827
fax: 217-782-9807

Please note change of e-mail address to:
william.ingersoll@illinois.gov

>>> "Diane Gorder" <dgorder@bmtscorp.com> 6/1/2007 10:09 AM >>>

Bill,

I just left a voicemail for you inquiring about whether we have made any progress on this matter. I would very much appreciate if we could touch basis at the first of next week to see if we can work on a resolution to move this forward.

Thank you and have a great weekend.

My direct number is 303-653-0111. I will be in the office Monday and Tuesday of next week, but will be out of the office on Wednesday.

Sincerely,

Diane Gorder
BMTS, Inc.
-----Original Message-----
From: Diane Gorder [mailto:dgorder@bmtscorp.com]
Sent: Tuesday, May 08, 2007 10:46 AM
To: 'William Ingersoll'
Subject: RE: Inquiry re PIMW and Bacillus Subtilus

Thank you for sending your information and working to see if we can come to a quick resolution. My contact information is

Diane Gorder
Director of Regulatory Compliance
BioMedical Technology Solutions, Inc. (BMTS) 9800 Mt Pyramid Court, Suite 350 Englewood,

CO 80112
(303) 653-0111 (direct)
(719) 661-2296 (mobile)

As a summary of our conversation, we used Bacillus subtilis var niger in our efficacy studies. We used the commercially available isolate (ATCC 9372, NRRL #B4418) since the Bacillus subtilis var niger (ATCC 19659) is not commercially available in a certified form.

In 1993, the FDA listed either ATCC 9372 or 19659 as appropriate organisms for testing dry heat sterilization technologies. Multiple international organizations and state departments of health and environment recognize the isolate of Bacillus subtilis var niger used in our studies as the ideal species for demonstrating efficacy of dry heat treatment processes, even though many have outdated regulations that specify the ATCC 19659 isolate. The following organizations recognize ATCC 9372 as the appropriate biological indicator for dry heat processes: US Pharmacopia (USP), ISO 11138-4:2006, FDA pre-market clearance requirements, and EN 866 guidelines.

All of the major U.S. and international biological indicator manufacturers market the Bacillus subtilis (ATCC 9372) as THE indicator organism for dry heat. This includes STERIS Corporation, Raven Laboratories, Charles River Laboratories, NAMSA, etc. We obtained our spores strips from STERIS Corporation, the largest and most well-known company in this industry.

We believe an adjusted standard should not be necessary since we used the organism listed in Item 1 of Appendix A. It has been suggested that we repeat the extensive trials using one of the other two organisms; however, that is not a scientifically sound recommendation since B.
stearothermophilus is the USP indicator for steam sterilization processes and B. pumilus is recognized internationally for radiation processes.
Neither is appropriate for dry heat processes.

If you need additional information, please give me a call or send me an email. Again, thank you for your time and hopefully we can find a way to work through this over the near term.

Sincerely,

Diane Gorder

-----Original Message-----
From: William Ingersoll [mailto:William.Ingersoll@illinois.gov]
Sent: Tuesday, May 08, 2007 9:47 AM
To: dgorder@bmtscorp.com
Subject: Inquiry re PIMW and Bacillus Subtilus

Diane,
Here is my contact info:

Bill Ingersoll
Manager of Enforcement Programs
Illinois EPA
217-782-9827
fax: 217-782-9807

Please note change of e-mail address to:
william.ingersoll@illinois.gov

EXHIBIT E

TECHNICAL ASSISTANCE MANUAL: STATE REGULATORY OVERSIGHT OF MEDICAL WASTE TREATMENT TECHNOLOGIES

April 1994

A Report of the State and Territorial Association on Alternate Treatment Technologies

EXECUTIVE SUMMARY

I. Introduction

The purpose of this report is to establish a framework or guideline that defines medical waste treatment technology efficacy criteria and delineates the components required to establish an effective state medical waste treatment technology approval process. The recommendations made in this report are an attempt to find commonality on many of the issues and criteria required in the medical waste treatment technology review process. Recognizing that all states may not totally agree with these recommended criteria or protocols, the guidelines developed should serve only to provide guidance to the states in the development of an approval process for medical waste treatment technologies.

The establishment of qualitative and quantitative parameters that ensure effective and safe medical waste treatment are required in defining treatment technology efficacy criteria and delineating the components necessary to establish an effective state medical waste treatment technology approval process. Recommendations are provided in this report for the following:

- Medical Waste Treatment Technology Efficacy Assessment
- Medical Waste Treatment Technology Approval Process
- Permitting and Site Authorization Issues
- Research and Development

II. Medical Waste Treatment Technology Efficacy Assessment Criteria

This report recommends that all medical waste treatment technologies meet the following microbial inactivation criteria:

> Inactivation of vegetative bacteria, fungi, lipophilic/hydrophilic viruses, parasites, and mycobacteria at a 6 Log_{10} reduction or greater; and inactivation of B. stearothermophilus spores or B. subtilis spores at a 4 Log_{10} reduction or greater.

In meeting these criteria, selected pathogen surrogates which represent vegetative bacteria, fungi, parasites, lipophilic/hydrophilic viruses, mycobacteria, and bacterial spores are recommended. Formulas and methods of calculations are recommended and are based on microbial inactivation ("kill") efficacy as equated to "Log_{10} Kill", which is defined as the difference between the logarithms of the number of viable test microorganisms before and after treatment.

report was distributed for review and comment to all state and territorial regulatory agencies involved in medical waste regulatory activities.

To gain additional input into the development of a uniform guideline for the assessment of medical waste treatment technologies, a third meeting was conducted on June 14-16, 1993, in Washington, D.C. with invited participants from all state and territorial medical waste regulatory agencies. The report prepared from the Atlanta meeting served as a basis of discussion. With invited input from all state and territorial representatives, the primary objective of the meeting was to seek consensus on the key topic areas listed above.

This report details the discussions and recommendations of the participants from the three meetings. It should be emphasized that the recommendations made in this report are an attempt to find commonality on many of the issues and criteria required in the medical waste treatment technology review process. As such, consensus agreement was sought on key issues to demonstrate support for the recommendations made in this report. However, consensus support for a recommendation does not necessarily imply unanimity for the position taken. Recognizing that all states may not totally agree with these recommended criteria or protocols, the guidelines developed through this series of meetings should serve only to provide guidance to states in the development of a review and approval process for medical waste treatment technologies.

Logistical support for all three meetings was provided by the USEPA. Roger Greene, Rhode Island Department of Environmental Management, Diann J. Miele, Rhode Island Department of Health, and Dr. Nelson S. Slavik, President, Environmental Health Management Systems, Inc., cofacilitated each of the meetings. A listing of all participants attending the New Orleans, Atlanta, and Washington, D.C. meetings is found in Appendix D.

The committee realized that there might be circumstances under which a state may allow relaxation of the Level III requirement. These exceptions would by necessity need to be made on a case-by-case basis, would require the equipment manufacturer to provide a rationale for relaxation, and would require adequate supporting documentation to substantiate that rationale.

The committee also debated if laboratory wastes (i.e. discarded cultures and stocks of pathogenic agents) should require sterilization (i.e. meet Level IV criteria) on the basis that these wastes may contain high concentrations of known pathogens. The committee took the position that Level III criteria remained the standard for all medical waste categories. The committee emphasized, however, that laboratories should be aware that cultures and stocks of disease-causing agents may require sterilization before disposal. In addition to guidelines set by the Centers for Disease Control in Biosafety in Microbiological and Biomedical Laboratories, (1993) and standards of the College of American Pathologists (CAP), some states require laboratory cultures to be incinerated or autoclaved (i.e., steam sterilized) before leaving the laboratory or before being disposed of. Although no specific recommendations for medical waste disposal are made under the Clinical Laboratory Improvement Amendments (CLIA), medical waste disposal practices are receiving increased scrutiny during routine inspections.

2.3 Representative Biological Indicators

In the absence of an ultimate pathogen surrogate to represent all defined microbial groups, the selection of pathogen surrogates representing vegetative bacteria, fungi, parasites, viruses, mycobacteria, and bacterial spores was considered necessary to define and facilitate any state approval process. Criteria defining surrogate selection should include that any surrogate recommended:

- Not affect healthy individuals;
- Be easily obtainable;
- Be an ATCC registered strain, as available;
- Be easily cultured and maintained; and
- Meet quality control requirements.

Microorganism strains obtained from the American Type Culture Collection (ATCC) and methods prescribed by the Association of Official Analytical Chemists (AOAC) assist in fulfilling these recommendations by (1) providing traceable and pure cultures of known characteristics and concentration and (2) providing recognized culturing protocols and detailed sampling and testing protocols.

Provided in Table II are the biological indicators recommended by the committee for testing microbial inactivation efficacy in medical waste treatment processes. The selection of these representatives was based on each microorganism:

- Meeting, where possible, the criteria established above;
- Representing, where possible, those organisms associated with medical waste; and
- Providing a biological challenge equivalent to or greater than that associated with microorganisms found in medical waste.

Biological indicators selected to provide documentation of relative resistance to an inactivating agent should be chosen after evaluation of the treatment process as it relates to the conditions used during comparative resistance research studies described in the literature. Literature studies support the assertion that the degree of relative resistance of a microorganism to an inactivating agent can be dependent on various factors (i.e., pH, temperature). Conditions used in literature studies that demonstrate a relatively high degree of resistance of a particular microorganism may be significantly different to the conditions found within the treatment process. A comparison of the conditions used in the literature to those used in the treatment process should be made to determine if relative microbial resistance can be altered (i.e., lowered) as a result of treatment process conditions.

The committee emphasized that although the microorganisms selected represent pathogen surrogates, these selected surrogates may have the potential to be pathogenic under certain conditions. As such, the committee recommended that all testing be conducted using recognized microbial techniques. For those pathogen surrogates that still retain some higher degree of pathogenicity (e.g., Cryptosporidium, Giardia, and Mycobacteria), efficacy testing should be conducted only by qualified laboratory personnel.

TABLE II - RECOMMENDED BIOLOGICAL INDICATORS

Vegetative Bacteria	-	Staphylococcus aureus (ATCC 6538) Pseudomonas aeruginosa (ATCC 15442)
Fungi	-	Candida albicans (ATCC 18804) Penicillium chrysogenum (ATCC 24791) Aspergillus niger
Viruses	-	Polio 2, Polio 3 MS-2 Bacteriophage (ATCC 15597-B1)
Parasites	-	Cryptosporidium spp. oocysts Giardia spp. cysts
Mycobacteria	-	Mycobacterium terrae Mycobacterium phlei Mycobacterium bovis (BCG) (ATCC 35743)

Bacterial Spores - B. stearothermophilus (ATCC 7953)
B. subtilis (ATCC 19659)

The committee recommended that one or more of the representative microorganisms from each microbial group be used in efficacy evaluation. Specific criteria for the selection of these microorganisms are provided below in Table III:

TABLE III - BIOLOGICAL INDICATOR SELECTION CRITERIA

Vegetative Bacteria	-	Staphylococcus aureus and Pseudomonas aeruginosa were selected to represent both gram-positive and gram-negative bacteria, respectively. Both are currently required by the Association of Official Analytical Chemists (AOAC) use-dilution method and both have been shown to be resistant to chemical inactivation.
Fungi	-	The selection of Candida albicans and Penicillium chrysogenum was based on reported data indicating these organisms representing yeast and molds, respectively, are the most resistant to germicides. Although Trichophyton mentagrophytes is the AOAC test organism for molds, Penicillium chrysogenum is reported to be more resistant to germicides. The inclusion of Aspergillus niger as an indicator organism was based on its familiarity as a common mold.
Viruses	-	Lipophilic (enveloped) viruses are less resistant to both thermal and chemical inactivation than the hydrophilic (nonenveloped) viruses. As such, enveloped viruses such as HIV, Herpes simplex virus and Hepatitis B virus are less resistant than enveloped viruses such as Poliovirus, Adenovirus, and Coxsackievirus. Polio 2 (attenuated vaccine strain) and Polio 3 virus were selected based on their relative higher chemical and thermal resistance. Additionally, the use of an enterovirus (e.g., Polio 2 or Polio 3) can provide a stringent measure of efficacy for irradiation treatment processes. MS-2 bacteriophage was selected as a Hepatitis virus surrogate in that this bacteriophage offers a comparable degree of chemical and thermal resistance, is safe to handle and easy to culture.

Parasites	-	Both Cryptosporidium spp. oocysts and Giardia spp. cysts are used as test organisms to demonstrate germicidal effectiveness. Cryptosporidium has been demonstrated to have a higher chemical resistance and Cryptosporidium spp. oocysts are more readily available than Giardia spp. cysts. Both are significantly pathogenic (both have an infectious dose of 10 cysts) and care is advised when using these microorganisms as parasitic biological indicators.
Mycobacteria	-	Mycobacterium phlei has a demonstrated measure of disinfectant resistance, is a rapid grower and is pigmented for easy identification. M. bovis (BCG) is used in the AOAC Tuberculocidal Method and is analogous to M. tuberculosis in that it is in the same group or complex. Individuals exposed to M. bovis (BCG, ATCC strain) may skin test convert although no actual infectivity or disease occurs. Risk of exposure would come from those mechanisms that grind the waste. Mycobacterium terrae is equivalent to M. tuberculosis in resistance to chemical inactivation. In Europe it is recommended for disinfectant testing. M. terrae does not grow as rapidly as M. bovis or M. tuberculosis.
Bacterial Spores	-	Both B. stearothermophilus and B. subtilis spores are commonly used as biological indicators for both thermal and chemical resistance. B. stearothermophilus spores exhibit more thermal and chemical resistance than spores from B. subtilis.

After discussion on the rationale for selection of the representative biological indicators presented above, consensus by the committee was attained on recommending the use of these biological indicator strains for treatment technology efficacy testing.

2.4 Quantification of Microbial Inactivation

Establishing the mechanisms to quantify the level of microbial inactivation is essential in developing the format and requirements of the guidance protocols. As presented and discussed, microbial inactivation ("kill") is equated to "Log_{10}Kill" which is defined as the difference between the logarithms of number of viable test microorganisms before and after treatment. This definition is translated into the following formula:

Roger Greene, Rhode Island Department of Environmental Management, Diann J. Miele, M.S., Rhode Island Department of Health, and Nelson S. Slavik, Ph.D., President, Environmental Health Management Systems, Inc., were primarily responsible for facilitating consensus among participants during each of the three meetings that were held to discuss state review of medical waste treatment technologies.

Nelson S. Slavik, Ph.D., prepared this final document which reflects the discussions and consensus reached at these meetings.

The following state officials served as a steering committee for these meetings:

Charles H. Anderson
Louisiana Department of Health and Hospitals

Lawrence Chadzynski, M.P.H.
Michigan Department of Public Health

Robert M. Confer
New Jersey Department of Environmental Protection & Energy

Carolyn Dinger
Louisiana Department of Environmental Quality

Roger Greene
Rhode Island Department of Environmental Management

Diann J. Miele, M.S.
Rhode Island Department of Health

Phillip R. Morris
South Carolina Department of Health and Environmental Control

Ira F. Salkin, Ph.D.
New York Department of Health

Wayne Turnberg
Washington Department of Ecology

John Winn, R.E.H.S.
California Department of Health Services

A complete listing of all participants attending the New Orleans, Atlanta, and Washington, D.C. meetings may be found in Appendix D.

EXHIBIT F

EHMS

ENVIRONMENTAL HEALTH MANAGEMENT SYSTEMS, INC.
2617 Korn Street, Niles, Michigan 49120
269/683-8444(O), 269/683-8441(F)

June 11, 2007

Diane Gorder
BioMedical Technology Solutions, Inc.
9800 Mt. Pyramid Court, Suite 350
Englewood, CO 80112

Dear Ms. Gorder,

I am writing pursuant to your request for historical background concerning biological indicator strains used during treatment efficacy studies on medical waste treatment devices and equipment. I hold a doctorate in microbiology from the University of Illinois at Urbana-Champaign and I served as co-facilitator and medical waste consultant to the State and Territorial Association on Alternate Treatment Technologies (STAATT). This was a select group of state regulatory representatives gathered to prepare and adopt a cohesive approach to evaluate the microbiological inactivation effectiveness of medical waste treatment equipment. This group was first convened in late 1992 and was supported and funded by the U.S. Environmental Protection Agency with the primary mission to establish qualitative protocols and quantitative measures by which to evaluate the efficacy of microbial kill of these devices. This effort culminated in the document entitled *Technical Assistance Manual: State Regulatory Oversight of Medical Waste Treatment Technologies* published in April of 1994. I was the author of that document.

This document was the first attempt at creating a comprehensive protocol and evaluative mechanism to determine the treatment efficacy of medical waste treatment equipment. We relied on documents that provided a semblance of guidance as they related to clinic evaluation of microbial kill. We realized that in creating this document that revisions would be required as knowledge advanced or as necessary to enhance the use of the protocols. We also realized that states might view this document as a path to regulatory development and incorporate portions of the document into regulatory language. As such, we stated clearly in the document's "Introduction" that "the guidelines developed through this series of meetings should serve only to provide guidance to states in the development of a review and approval process for medical waste treatment technologies." The document was never intended to be the final word on treatment efficacy of medical waste treatment equipment, but rather a first start of a work-in-progress.

As part of our qualitative measure, it was required that we assign specific challenge (surrogate) organisms to each microbiological category requiring testing (i.e., vegetative bacteria, viruses, fungi, parasites, mycobacterium, and bacterial spores). The aforementioned categories (with the exception of bacterial spores) represented the types

of microorganisms that could be found in medical waste that potentially could transmit disease. Bacterial spores were to be tested to provide a "margin of safety from the variables inherent in the treatment of medical waste (i.e., waste packaging, waste composition, waste density, and factors influencing the homogeneity of the treatment process)" since "*B. subtilis* and *B. stearothermophilus* spores both display significantly more heat resistance than microorganisms in the aforementioned groups." There was no effort to single out a specific strain of *B. subtilis* and *B. stearothermophilus* as the most resistant for chemical or thermal resistance or as having a specific desirable characteristic. The recommended strains selected were those that met the following criteria:

- "Not affect healthy individuals;
- Be easily obtainable;
- Be an ATCC registered strain, as available;
- Be easily cultured and maintained; and
- Meet quality control requirements."

It was recognized by the committee that other strains not provided in the "Technical Assistance Manual" could also meet these criteria and be acceptable as microbial challenge surrogates.

I have reviewed your efforts to use the appropriate spore surrogate for your device and have found that your selection of *B. subtilis* ATCC 9372 spores is consistent with the criteria provided by STAATT in their publication. This strain provides the dry-heat resistance which is appropriate for your treatment process. It is readily available through a certified manufacturer and each manufactured lot has a traceable background and certification analysis that quantifies dry-heat resistance (e.g., D-value) to demonstrate the quality assurances required of the STAATT criteria.

I hope that this brief summary into the development of the "Technical Assistance Manual" and its recommendations will provide you with the information you need. I can be reached at the numbers listed above or by e-mail at enmed@aol.com.

Sincerely,

Nelson S. Slavik, Ph.D.
President

GROUP EXHIBIT G

Guidance on
Premarket Notification [510(k)] Submissions
for
Sterilizers
Intended for Use in Health Care Facilities

Infection Control Devices Branch

Division of General and Restorative Devices

March, 1993

H. Biological Performance Tests

1. General

The applicant must unequivocally demonstrate that the device can sterilize, to an acceptable SAL, all the medical products identified in the labeling, when used in accordance with the directions for use.

2. Test Organisms

Since a consistent type and concentration of bioburden cannot be assured or realistically evaluated in a health care facility, an overkill sterilization is necessary. The sterilization cycle is based upon an initial concentration of at least 10^6 CFU (or Plaque Forming Units - PFU)/unit of a highly resistant organism to the process. Typically, the most resistant organism to a sterilization process is used based upon determination of D-values. Table 1 lists the commonly recognized test organisms for the classified sterilizers.

TABLE 1

TEST ORGANISMS FOR CLASSIFIED STERILIZERS

Sterilizer	Organism
steam	Bacillus stearothermophilus (ATCC 7953)
dry heat	Bacillus subtilis var. niger (ATCC 9372 or 19659)
EtO	Bacillus subtilis var. niger (ATCC 9372 or 19659)

The biological lethality profile of a nontraditional sterilization technology must be exhaustively evaluated since the most resistant organism is initially unknown. Table 2 identifies recommended organisms to test for determination of the most resistant organism.

TABLE 2

TEST ORGANISMS FOR NONTRADITIONAL STERILIZERS

A. Bacterial Spores

Bacillus subtilis var. niger (ATCC 9372 or 19659)
Bacillus stearothermophilus (ATCC 7953)
Clostridium sporogenes (ATCC 3584)

B. Mycobacteria

Mycobacterium tuberculosis var. bovis
(or other representative mycobacterium)

C. Nonlipid Viruses

poliovirus Type II

D. Fungi

Trichophyton mentagrophytes (with conidia)

E. Vegetative Bacteria

Staphylococcus aureus
Salmonella choleraesius
Pseudomonas aeruginosa

F. Lipid Viruses

herpes simplex

G. THE LITERATURE OR OTHER INFORMATION MAY SUGGEST ADDITIONAL TEST ORGANISMS DEPENDING UPON THE TECHNOLOGY OR THE TYPICAL BIOBURDEN ENCOUNTERED BY THE ARTICLES INTENDED FOR REPROCESSING IN THE STERILIZER.

Premarket Notifications [510(k)] for Biological Indicators Intended to Monitor Sterilizers Used in Health Care Facilities; Draft Guidance for Industry and FDA Reviewers

See Related Information

Draft Guidance – Not for Implementation

This guidance document is being distributed for comment purposes only.

Draft released for comment on May 21, 2001

This document will supersede the document "FDA Guide for Validation of Biological Indicator Incubation Time" dated January 1, 1986 once this draft guidance is finalized.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Devices and Radiological Health

Infection Control Devices Branch
Division of Dental, Infection Control and General Hospital Devices
Office of Device Evaluation

Preface

Public Comment

16. Emergency and additional information: Provide a telephone number for emergencies or for additional information.

H. Efficacy Data

Health care facilities use biological indicators to monitor sterilization processes. As defined in 21 CFR §880.2800, a biological indicator accompanies devices through sterilization processes and monitors sterilization adequacy by its growth or failure to grow. Biological indicators do not indicate that any given sterilizer load or device is rendered sterile. Instead, biological indicators indicate that conditions to inactivate the biological indicator organisms were achieved at the biological indicator location in a particular cycle. When the user places biological indicators in the most difficult to sterilize location in a device load, the biological indicator result provides some assurance that organisms in devices were inactivated. Health care facilities use biological indicators as part of an infection control quality assurance program along with physical and chemical monitoring.

Biological indicators are of two types: paper strip, which require a separate culture medium; and self-contained, which include the culture medium. Some self-contained biological indicators include growth indicators such as pH sensitive dyes. Some biological indicators include two spore species to allow the same product to monitor either steam or ethylene oxide processes. Additionally, biological indicators are marketed in test packs (see Section III.J.2), with separate chemical indicators (see Section III.J.3), or with indicators that allow for rapid interpretation (prior to the visible growth of spores) on the basis of an enzyme or chemical reaction (see Section I.B).

1. Indicator (Test) Organisms

Bacterial spores are used as indicator organisms because they have high resistance to the various sterilization processes. Spore resistance is complex and many aspects are not well understood. Factors involved include: intrinsic (innate) resistance of the spore species and strain, environmental conditions during sporulation, biological indicator preparation, storage, exposure, incubation, and recovery, and biological indicator carrier and packaging materials. The following Bacillus species and strains are accepted for the uses listed in Table 2 (USP, 2000).

Table 2

Sterilizer type:	Indicator Organism/Spore:
Steam	Bacillus stearothermophilus (ATCC 7953 or 12980)
Dry Heat	Bacillus subtilis var. niger (ATCC 9372)
Ethylene Oxide	Bacillus subtilis var. niger (ATCC 9372)

For biological indicators intended to monitor sterilization processes other than those listed in Table 2, you should justify the indicator organism using valid science. To do so, you may conduct testing and submit data, or rely on published literature, if an adequate body of knowledge exists.

Because resistance involves many factors other than spore species and strain, you should characterize and validate biological indicators in the final finished form for your specific indications for use (see Section III.H.3 below).

2. Efficacy Study Reports

Efficacy study reports should provide complete details and include data to support product effectiveness claims. Study reports should meet standards for publication in peer-reviewed scientific journals. Reports should include the following information:

JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2004, p. 1626–1630 Vol. 42, No. 4
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.4.1626–1630.2004

Reassessment of Sequence-Based Targets for Identification of *Bacillus* Species

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The *Bacillus* genus is a large heterogeneous group in need of an efficient method for species differentiation. To determine the current validity of a sequence-based method for identification and provide contemporary data, PCR and sequencing of a 500-bp product encompassing the V1 to V3 regions of the 16S rRNA gene were undertaken using 65 of the 83 type strains of this genus. This region proved discriminatory between most species (70.0 to 100% similarity), the exceptions being clinically relevant *B. cereus* and *B. anthracis* as well as nonpathogenic *B. psychrotolerans* and *B. psychrodurans*. Consequently, 27 type and clinical strains from the *B. cereus* group were used to test alternate targets (*rpoB*, *vrrA*, and the 16S-23S spacer region) for identification. The *rpoB* gene proved the best alternate target, with a conserved 4-nucleotide difference between *B. cereus* and *B. anthracis*. The high 16S rRNA gene sequence similarities between some strains demonstrated the need for a polyphasic approach to the systematics of this genus. This approach is one focus of the Ribosomal Differentiation of Medical Microorganisms mandate. Accordingly, the 16S rRNA gene sequences generated in this study have been submitted for inclusion into its publicly accessible, quality-controlled database at http://www.ridom_rdna.de/.

The *Bacillus* genus is an extensive heterogeneous group encompassing 83 validly described species to date (http://www.bacterio.cict.fr/b/bacillus.html). Many species in this taxon are of major clinical importance, such as the *B. cereus* group (comprised of *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, and *B. weihenstephanensis*), but unfortunately, members of this group share a great deal of morphological and biochemical similarities (3, 8, 16). In contrast, the environmental and nonpathogenic species of this genus exhibit a wide range of physiology, DNA base content, and nutritional requirements (2, 4, 15). Since the biochemical approach for species identification can be tedious, expensive, and inaccurate, a rapid, definitive method is greatly needed. Molecular procedures are increasingly being used for rapid species identification. However, some methods used for this genus such as restriction digests of a target gene (i.e., 16S rRNA gene) (11) or randomly amplified polymorphic DNA analysis (22) are limiting in discriminating between a large group of species (6). Sequencing of the 16S rRNA gene and select housekeeping genes has shown to be particularly useful, generating large public sequence databases due to the tangible, exact nature of sequence data. With the increasing use of these methods and decreased expense of running sequencing reactions after the initial equipment investment, more laboratories are relying on sequence data for species identification (21).

A previous study using the 16S rRNA gene for rapid identification of the *Bacillus* genus was undertaken by Goto et al. (6). At this time, the validity of using a hypervariable region (nucleotides [nt] 70 to 344) of the gene was proven adequate to discriminate between all the species except between *B. cereus* and *B. anthracis* and between *B. mojavensis* and *B. atrophaeus*. However, new sequence data were only acquired for 19 of the species, with the rest obtained from preexisting sequences available from the National Center for Biotechnology Information GenBank. The GenBank nucleotide database is well known for the non-quality-controlled nature of its data, including base errors, ambiguous base designation, and incomplete, short sequences. Several recent studies have examined the problems surrounding the use of non-quality-controlled databases such as GenBank and the Ribosomal Database Project for identification purposes and have shown the benefits of standardized, maintained databanks that include subsidiary information, such as Ribosomal Differentiation of Medical Microorganisms (RIDOM) (7, 21).

With the available data on this genus incomplete and the many problems associated with public database use for similarity searches, a fragment of the 16S rRNA gene (*Escherichia coli* nt 54 to 510) for species of the *Bacillus* genus was sequenced for submission to RIDOM. Current sequence technologies allow the acquisition of unambiguous, error-free data for definitive identification. This is only one of many collaborative ongoing efforts to collect quality-controlled sequence data for RIDOM for free access to others. Second, alternate sequence targets for identification of the closely related *B. cereus* group were reviewed and tested for inclusion into RIDOM.

MATERIALS AND METHODS

A total of 65 of 83 *Bacillus* type strains were currently available for this study (Table 1). The partial 16S rRNA gene sequence (corresponding to primers for *E. coli* 16S rRNA positions 8 to 27 and 536 to 518) (21) was determined using standard 16S rRNA gene primers for PCR and sequencing. For the members of

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TABLE 1. *Bacillus* species type strains used in this study

Species	Identifier[a] (accession no.)	Species	Identifier[a] (accession no.)
B. agaradhaerens	DSM 8721^T	*B. jeotgali*	YKJ-10^T (AF221061)
B. alcalophilus	DSM 485^T (X76436)	*B. laevolacticus*	DSM 442^T
B. amyloliquefaciens	ATCC 23350^T	*B. lentus*	ATCC 10840^T
B. anthracis	ATCC 14578^T	*B. lichenformis*	ATCC 14580^T
B. arseniciselenatis	ATCC 700614^T	*B. luciferensis*	LMG 19422^T (AJ419629)
B. atrophaeus	DSM 7264^T	*B. megaterium*	ATCC 14581^T
B. azotoformans	DSM 1046^T	*B. methanolicus*	C1 (X64465)
B. badius	ATCC 14574^T	*B. mojavensis*	DSM 9205^T
B. benzoevorans	DSM 5391^T	*B. mucilaginosus*	AF006077
B. carboniphilus	LMG 19001^T	*B. mycoides*	ATCC 6462^T
B. cereus	ATCC 14579^T	*B. naganoensis*	DSM 10191^T
B. chitinolyticus	DSM 11030^T	*B. nealsonii*	FO-092 (AF234863)
B. circulans	ATCC 4513^T	*B. neidei*	BD-87 (AF169520)
B. clarkii	DSM 8720^T	*B. niacini*	DSM 2923^T
B. clausii	DSM 8716^T	*B. okuhidensis*	GTC854/AB047684
B. coagulans	ATCC 7050^T	*B. oleronius*	DSM 9356^T
B. cohnii	DSM 6307^T	*B. pallidus*	DSM 3670^T
B. decolorationis	LMG 19507^T (AJ315075)	*B. pseudalcaliphilus*	DSM 8725^T
B. edaphicus	T7 / AF006076	*B. pseudofirmus*	DSM 8715^T
B. ehimensis	DSM 11029^T	*B. pseudomycoides*	DSM 12442^T
B. endophyticus	2DT^T (AF295302)	*B. psychrodurans*	DSM 11713^T (AJ277984)
B. fastidiosus	DSM 91^T	*B. psychrosaccharolyticus*	DSM 6^T
B. firmus	ATCC 14575^T	*B. psychrotolerans*	DSM 11706^T (AJ277983)
B. flexus	DSM 1320^T	*B. pumilus*	ATCC 7061^T
B. fumarioli	LMG 19448^T	*B. pycnus*	NRS-1691 (AF169531)
B. funiculus	NAF001/AB049195	*B. schlegelii*	ATCC 43741^T (AB042060)
B. fusiformis	DSM 2898^T	*B. selenitireducens*	ATCC 700615^T
B. gibsonii	DSM 8722^T	*B. silvestris*	DSM 12223^T
B. halmapalus	DSM 8723^T	*B. simplex*	DSM 1321^T
B. haloalkaliphilus	DSM 5271^T	*B. siralis*	DSM 13140^T
B. halodenitrificans	DSM 10037^T	*B. smithii*	DSM 4216^T
B. halodurans	DSM 497^T	*B. sonorensis*	DSM 13779^T
B. halophilus	DSM 4771^T	*B. sphaericus*	ATCC 14577^T
B. horikoshii	DSM 8719^T	*B. sporothermodurans*	DSM 10599^T
B. horti	DSM 12751^T	*B. subterraneus*	—[b]
B. infernus	DSM 10277^T	*B. subtilis* subsp. *subtilis*	ATCC 6051^T
B. insolitus	ATCC 23299^T	*B. subtilis* subsp. *spizizenii*	NRRL B-23049^T
B. vallismortis	DSM 11031^T	*B. thermantarcticus*	M1 (AJ493665)
B. vedderi	DSM 9768^T	*B. thermoamylovorans*	LMG 19084^T
B. vulcani	DSM 13174^T	*B. thermocloaceae*	DSM 5250^T
B. weihenstephanensis	DSM 11921^T	*B. thuringiensis*	ATCC 10792^T
B. tusciae	DSM 2912^T		

[a] Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany; ATCC, American Type Culture Collection, Manassas, Va.; LMG, Belgian Coordinated Collections of Microorganisms, Laboratorium voor Microbiologie, Universiteit Gent (RUG), Ghent, Belgium; NRRL, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.

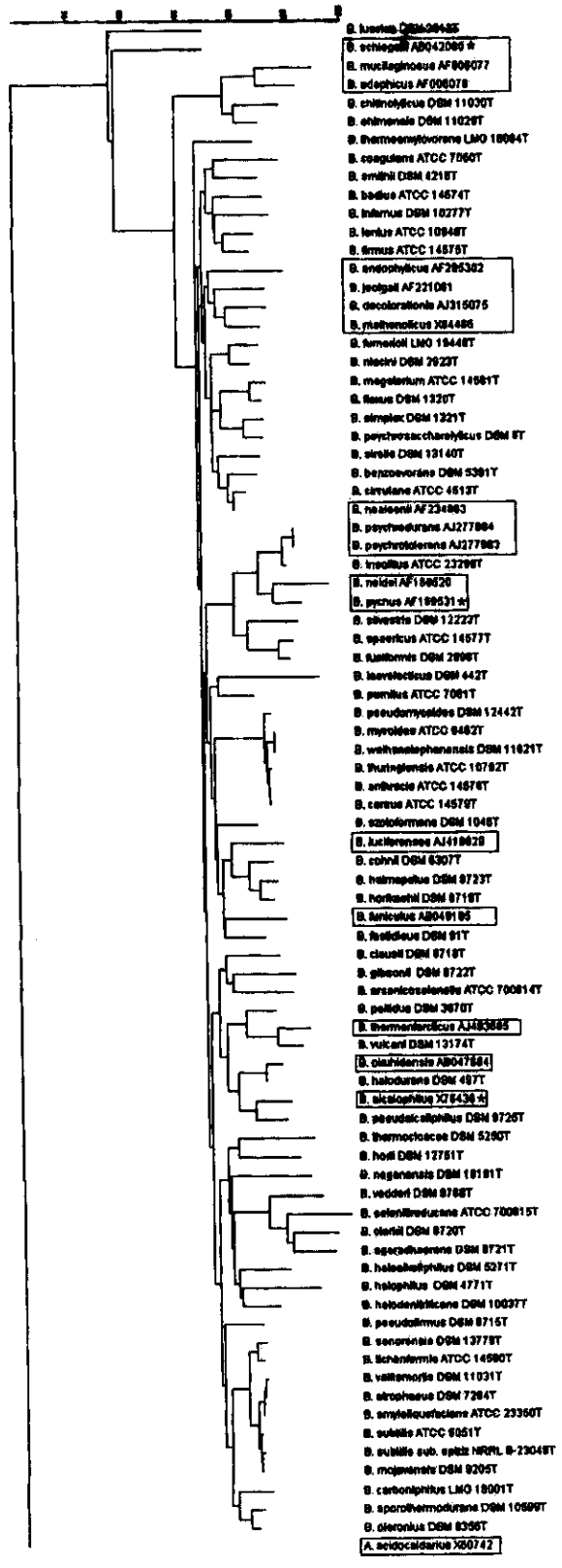
[b] —, identifier not applicable; GenBank sequence not available.

the *B. cereus* clade, *rpoB* gene amplification and sequencing were undertaken with previously published primers (positions 1482 to 1500 and positions 2281 to 2300 of the *B. subtilis rpoB* gene) (17). Both forward and reverse strands were sequenced using standard procedures of cycle sequencing with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems).

Alignments and phylogenetic analysis of the 16S rRNA gene sequences completed in-house were performed using nucleotide sequences from position 54 to 510. For complete analysis of the genus, sequences of 17 *Bacillus* species that we were unable to obtain in this study were chosen from GenBank. Except for three species noted in Fig. 1, these sequences were deemed free of any questionable deletions, insertions, or ambiguous bases (accession numbers are noted in Table 1). In addition, one newly described species, *B. subterraneus* ATCC BAA 136^T, did not have a 16S sequence available in GenBank. *rpoB* gene sequences were analyzed using a fragment from position 1821 to 1995 of the *B. subtilis rpoB* gene. Multiple alignments and the construction of a neighbor-joining phylogenetic tree subjected to a bootstrapping analysis of 1,000 simulations to assess topology were performed with Bionumerics (version 2.50; Applied Maths) default parameters. The sequences obtained from GenBank were highlighted in the tree to distinguish them from the strains sequenced in-house. *Alicyclobacillus acidocaldarius* (X60742) was used as the outgroup to compare our results with those of Goto et al. (6). The sequences determined in the study have been submitted to RIDOM to be available in the near future for similarity searches.

RESULTS

Interspecies sequence identity results of the 16S rRNA gene sequences from bp 54 to 510, which includes hypervariable regions V1 to V3, demonstrated a range of 70.0 to 100% similarity (data not shown), with the closest related species (excluding the *B. cereus* clade) being two recently published environmental species, *B. psychrotolerans* and *B. psychrodurans* (1), which showed 100% identity. Within the *B. subtilis* group, between *B. atrophaeus* and *B. vallismortis*, as well as *B. subtilis* subsp. *spizizenii* and *B. mojavensis*, a 1-bp difference was observed (99.8% identity). *B. atrophaeus* and *B. mojavensis* have 100% sequence identity in the region used in previously published studies (nt 70 to 344) but can be differentiated due to a



3-bp difference in the V3 region. The most distantly related *Bacillus* species were *B. tusciae* and *B. neidei*, presumably due to several regions of deletions detected in *B. tusciae*. Use of this fragment of the gene for phylogenetic analysis shows similar clade assignments compared to phylogenetic trees constructed using the near complete 16S rRNA gene sequences as illustrated in previous publication (6) (Fig. 1).

A review of current chromosomal targets for identification of the medically relevant *B. cereus* group prompted us to examine the use of the *vrrA* region (10), 16S-23S spacer region (4, 8), and the *rpoB* (17) gene for sequence-based identification. The *vrrA* region does not include a known, conserved housekeeping gene, and the variability observed is much more suitable for subtyping instead of identification (12). The 16S-23S spacer region shows a single base insertion difference between *B. cereus* and *B. anthracis*. The *rpoB* was the best alternate target, allowing discrimination between *B. cereus* and *B. anthracis* by a conserved 4-bp difference over a region of 175 bp in all isolates tested in this study as well as previous research (17). As illustrated in Fig. 2, the similarity index indicates 100% identity in the *rpoB* sequences of *B. anthracis*, making it an ideal target for identification purposes.

DISCUSSION

A generally accepted concept in bacterial taxonomy is that the DNA base (GC) composition of species within a genus should not differ by more than 10 to 12 %mol G+C (15). Nonetheless, values within the *Bacillus* genus ranged from 33 to 65 %mol G+C in 1993, although many of the species did cluster at 40 to 50 %mol G+C (15). Subsequently, recent phylogenetic analyses have reclassified some of the *Bacillus* species into new genera, including *Paenibacillus*, *Geobacillus*, and *Brevibacillus* (4). Due to these recent advances, it has become increasingly difficult to classify species within the *Bacillus* genus, as many share similar physiology, metabolism, and morphology as well as highly conserved 16S rRNA genes. Fox et al. (5) indicate that a new species should be created when the organism has a sequence difference of 1.5% (over 1,000 bp) in conjunction with phenotypic differences. However, these studies on *Bacillus globisporus* and *Bacillus psychrophilus* demonstrated a 16S rRNA gene sequence similarity of 99.5%. These data revealed that although 16S rRNA gene sequences can be routinely used to identify and establish relationships between genera and well-resolved species, very recently diverged species may not be identified (5, 14).

It is important to note that ideally a polyphasic approach to the systematics of this genus (and all genera) should be practiced to fully understand and classify organisms, as a reliance on a singular molecular method such as 16S rRNA gene sequencing cannot account for slight evolutionary events and

FIG. 1. Neighbor-joining phylogenetic tree based on the V1-V3 region of the 16S rRNA gene (*E. coli* nt 54 to 510) of *Bacillus* species used in this study. Sequences we were unable to obtain in this study were taken from GenBank (boxed). Three strains (*) had one ambiguous base pair (n). The branching pattern is rooted using *A. acidocaldarius* as the outlier. Created with Bionumerics (version 2.50).

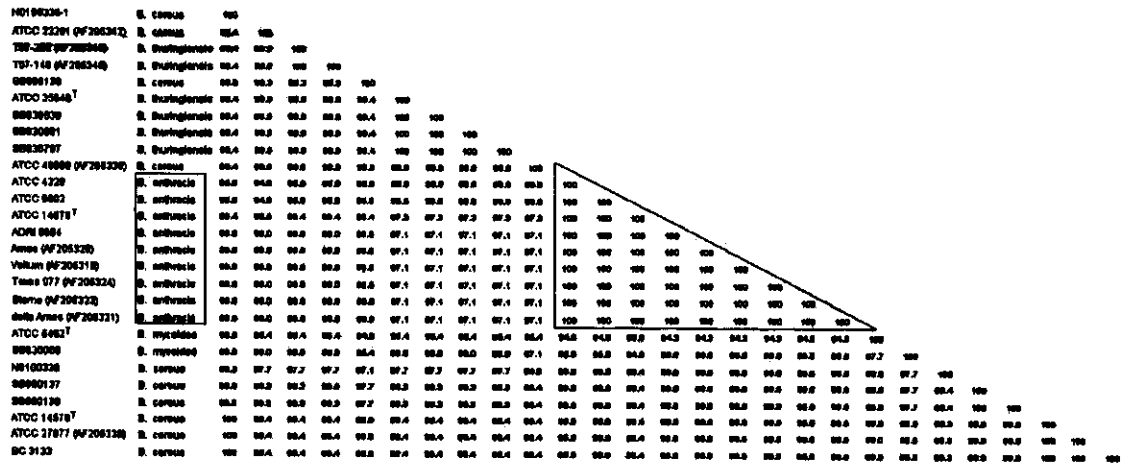


FIG. 2. *B. cereus* group members (clinical as well as type strains) used for *rpoB* gene analysis. The similarity matrix (pairwise comparison) and corresponding phylogenetic tree (neighbor joining) were created with Bionumerics (version 2.5).

may "overspeciate" the genus of study (i.e., may subdivide the genus into too many species). In contrast, two species may exist with identical 16S rRNA sequences yet have phenotypic differences or may differ in clinical relevance. Therefore, in practice, a number of phenotypic and phylogenetic properties should be examined to establish taxonomic positions of groups of related strains as a strain or a species (20).

Several examples of applying a polyphasic approach to delineate a new species from a group of similar strains were observed within this genus, specifically among the recently or newly described species. *B. psychrotolerans* and *B. psychrodurans* are newly described psychrotolerant species that have 100% sequence identity with the region of the 16S rRNA gene chosen in this study, but they can be differentiated further downstream of the 16S rRNA gene, as well as by biochemical characteristics (1). This is also evident for members recently established within the *B. subtilis* group, i.e., *B. atrophaeus* and *B. mojavensis*, which can be differentiated by both a 3-nt difference in the region tested and phenotypic differences such as oxidase activity. Thus, in the case of a nontype strain of these two species with a possible 16S rRNA sequence polymorphism(s), testing for oxidase activity could support identification to the species level (18).

In contrast, other closely related organisms within this genus can share phenotypic properties as well but have been classified as different species based on DNA reassociation values. This is observed between *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*, which share phenotypic profiles but are segregated based on DNA reassociation values of 58 to 69%, in addition to minor polymorphisms in the 16S rRNA gene between the type strains (13). Furthermore, *B. mojavensis* and *B. subtilis* subsp. *spizizenii* have only a 1-bp difference in the 16S rRNA gene and can only be distinguished from each other by sexual isolation, divergence in DNA sequences of the *rpoB* and *gyrA* genes, and fatty acid composition (13). These are a examples where reliance on only biochemical-based identification could lead to inaccurate identification of an organism.

The above discussion focuses on harmless saprophytes which are currently not of clinical importance, for which a rapid turnaround time to identification is less critical. However, *B. cereus* and *B. anthracis*, which can be extremely pathogenic, have 100% sequence identity across the entire 16S rRNA gene. The *B. cereus* group is highly homologous, as shown by genomic DNA-DNA hybridization, and the validity of classifying each as a species on the basis of pathogenicity has been questioned (9, 17). Although the species belonging to the *B. cereus* group can generally be differentiated from each other with conventional biochemical tests, such as capsular staining, motility, hemolysis, and observing the presence of intracellular *para*-crystalline formation (8, 9, 17), these tests are time-consuming and, in the case of genetically modified strains, may not even be useful for identification to the species level.

Although a recent publication by Sacchi et al. cites differences in the complete 16S rRNA gene (19), the single difference present over the entire 1,554-bp gene between *B. anthracis* and three *B. cereus* strains is a W (representing A or T) versus an A. This difference at bp 1146 of the gene (beyond the region examined in this study) may only be a reflection of base pair variation between multiple ribosomal operons in *Bacillus* species and not a true interspecies difference. The disadvantage of using this target for identification is twofold. First, the sequencing technology has to be PCR and not clone based in order to detect the "mixed" nucleotide caused by multiple ribosomal operons, and second, multiple primers would be necessary to obtain the complete sequence, which is not as rapid and unmistakable as using an alternate, smaller target with greater sequence variability. Several alternate chromosomal targets have been studied, although most suffer from inadequacy in some aspect, such as the Ba813 marker which has been detected in both *B. cereus* and *B. thuringiensis* (17). The *vrrA* region tested in this study has been noted as a possible credible method of distinguishing *B. anthracis* from *B. cereus* due to specific allele patterns defined for *B. anthracis*; however, only a limited amount of *B. cereus* and *B. thuringiensis* isolates were tested (12). Furthermore, as mentioned earlier, this target is useful primarily for subtyping and not for routine

identification in a clinical laboratory. The use of a conserved, housekeeping gene necessary for the survival of the organism such as *rpoB* is a desirable alternative.

In conclusion, the *Bacillus* genus requires a polyphasic approach to definitive species identification, including alternate gene targets as well as chemotaxonomic and clinical information (20). RIDOM is attempting to fill this niche by means of a quality-controlled, error-free 16S rRNA gene sequence-based identification database that also includes both secondary targets (such as the 16S-23S spacer region, and possibly the *rpoB* gene in the near future) and ancillary information regarding phenotypical characteristics. Consequently, when newly described pathogenic *Bacillus* species that have 16S ribosomal DNA sequences almost identical or identical to those of preexisting species are validated, the accumulation of a variety of strain characteristics in such a database is critical in the establishment of taxonomic positions. From a clinical standpoint, rapid, presumptive identification to the level of a certain group is useful to confirm medical diagnosis and aid in further differentiation.

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NOTE

Reclassification of bioindicator strains *Bacillus subtilis* DSM 675 and *Bacillus subtilis* DSM 2277 as *Bacillus atrophaeus*

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On the basis of high DNA–DNA reassociation values and confirmatory automated RiboPrint analysis, two aerobic spore-forming strains hitherto allocated to *Bacillus subtilis* and used as bioindicators (DSM 675, hot-air sterilization control; DSM 2277, ethylene oxide sterilization control) are reclassified as *Bacillus atrophaeus*.

Keywords: sterilization control, '*Bacillus globigii*', red strain, '*Bacillus subtilis* var. *niger*', *Bacillus atrophaeus*

Strains of the species *Bacillus subtilis* are used in a variety of applications, an important one being sterilization control. Strains of this species produce spores of specific resistance to, for example, dry heat or ethylene oxide and are thus proposed for testing the effectiveness of such methods for sterilization (Kelsey, 1967; Russell *et al.*, 1992; US Pharmacopeia, 1995; CEN–European Committee for Standardization, 1997a, b). *B. subtilis* DSM 675, originally designated as the 'red strain', was especially suited for routine use because of its distinctly coloured colonies.

Modern taxonomic methods have led to numerous reclassifications and rearrangements of strains, species and genera. This has been particularly true for the genus *Bacillus*, which has undergone a wide range of taxonomic developments in recent years. Most of these investigations are usually based on type strains; only rarely are additional strains of the species in question included. Thus, strains of practical importance, e.g. test and control strains, are often not taken into account.

The long history of strain DSM 675, the 'red strain'

In 1900, Migula described the species '*Bacillus globigii*'. When Smith *et al.* (1952) re-examined a number of strains received under this name, they had to allocate all of them to other more established species. Strains with traits corresponding to the original description were transferred to *Bacillus licheniformis*, because the original description of '*B. globigii*' by Migula was judged to be synonymous with that for *B. licheniformis*. Those strains not corresponding to the original description were allocated to *Bacillus circulans*, *Bacillus pumilus* and '*B. subtilis* var. *niger*'. Two strains from the Bacon Laboratories (the 'red strain' and the 'brown strain') were allocated to the latter species and were designated as NRS-1221A and NRS-1221B, respectively. In the same work, the authors concurrently reduced '*Bacillus niger*' from species to variety because they had found no discriminatory property, other than pigmentation, between *B. subtilis*, '*Bacillus aterrimus*' and '*B. niger*'. This property was shown to be susceptible to culture conditions (e.g. cultivation on media containing glucose or cultivation at a high incubation temperature). Clarifying the situation, Smith *et al.* (1952) stated (p. 83) that "the characterization of *B. subtilis* serves for '*B. subtilis* var. *niger*' by adding the words substrate blackened to the description of the growth on mediums containing tyrosine".

Later, Gordon *et al.* (1973) found 'varieties' unsatisfactory and subsumed them under *B. subtilis* knowing that this was a 'lumped' group; this group, with the arrival of better tests and methods, could then be taken apart again and 'good' species described. Indeed, since then, a number of new species have been separated from the species *B. subtilis sensu stricto* and validly published (Priest *et al.*, 1987; Nakamura, 1989; Roberts *et al.*, 1994, 1996; Nakamura *et al.*, 1999).

Nakamura (1989) re-examined the black-pigment-producing strains of *B. subtilis* and, on the basis of pigment production (on two different media) and DNA hybridization studies, he was able to discriminate between three groups of strains. Group 3 did not produce any pigment on either medium and included the type strain of *B. subtilis*. Group 2 was a pigment-forming variant but still belonged to *B. subtilis sensu*

Table 1. *Bacillus* strains investigated in this study

ATCC, American Type Culture Collection; BMTU, Boehringer Mannheim Tutzing; CCM, Czech Collection of Microorganisms; CIP, Collection de l'Institut Pasteur; DSM, DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen; IFO, Institute for Fermentation, Osaka; NCDO, National Collection of Dairy Organisms; NCIB, National Collection of Industrial Bacteria; NCTC, National Collection of Type Cultures; NRRL, Northern Regional Research Laboratory; NRS, Nathan R. Smith.

DSM no.	History	Other collection nos
B. subtilis DSM 675	← BMTU ← ATCC ← N. R. Smith (1221A, '*B. subtilis* var. *niger*') ← Frederick S. Bacon Laboratories, Watertown, Massachusetts, 1947 ('*Bacillus globigii*', 'red strain') ← C. R. Phillips, Fort Detrick, USA ← Elisabeth McCoy	ATCC 9372, NCIB 8058, CIP 77.18 NRS 1221A, IFO 13721, NCDO 738
B. subtilis DSM 2277	← NCTC ← J. C. Kelsey, London ← C. R. Phillips, Fort Detrick, USA ('*B. globigii*')	NCTC 10073, NCIB 8649, CIP 103406
B. atrophaeus DSM 7264^T	← NRRL ← NRS-213 ('*B. subtilis* var. *niger*')	NRRL–NRS 213^T, ATCC 49337^T
B. subtilis DSM 10^T	← ATCC ← H. J. Conn, strain Marburg	NRS 744^T, ATCC 6051^T, CCM 2216^T, NCIB 3610^T, NCTC 3610^T, IFO 12210^T

Table 2. Percentage DNA–DNA similarity

The DNA–DNA similarity values are the means of at least two determinations.

Strain	DSM 2277	DSM 675	DSM 7264^T	DSM 10^T
DSM 2277	–	87	98	30
DSM 675		–	88	32
B. atrophaeus DSM 7264^T			–	ND
B. subtilis DSM 10^T				–

ND, Not determined.

stricto according to the high DNA–DNA similarity values between groups 2 and 3. Both groups (2 and 3) represent the species *B. subtilis*. Group 1, which produced a brownish-black pigment on one medium and a brown pigment on the other, showed low levels of DNA hybridization with groups 2 and 3. Thus, group 1 was described as the new species *Bacillus atrophaeus*. Twenty-one of the 25 strains in this group had previously been designated as '*B. subtilis* var. *niger*'.

Unfortunately, neither '*B. subtilis* var. *niger*' DSM 675 (or any of its equivalents in other collections) nor '*B. subtilis* var. *niger*' DSM 2277 was included in this study. To reveal the taxonomic position of these important sterilization control strains, spectroscopic DNA–DNA hybridizations (Huß *et al.*, 1983) and automated RiboPrint (Qualicon) analyses (Bruce, 1996) were performed on all relevant strains (Table 1, Table 2, Fig. 1).

The present study reveals high DNA–DNA homology values between the two strains and the type strain of *B. atrophaeus* (DSM 7264^T) and low hybridization values with *B. subtilis* DSM 10^T. In addition, RiboPrint patterns for all of the strains involved were generated and compared with each other and with other *Bacillus* type strains. Strains DSM 675 and DSM 2277 showed a close association with *B. atrophaeus*, and a separation from *B. subtilis* was confirmed (the similarity coefficients of the RiboPrint patterns were approximately 0·92 and 0·94, respectively; see Fig. 1).

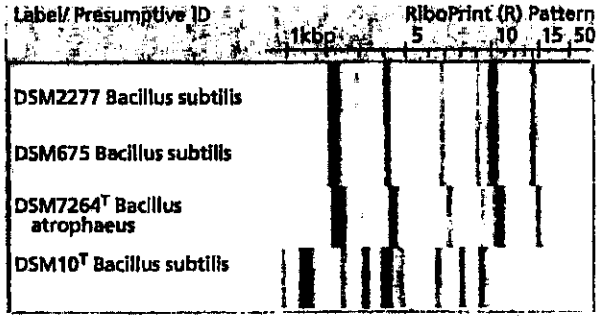

Fig. 1. Normalized RiboPrint pattern found within '*Bacillus subtilis*' strains DSM 675 and DSM 2277, related to the type strain of *Bacillus atrophaeus*, compared with the ribotype pattern of the type strain of *B. subtilis*.

Thus, both sterilization control strains DSM 675 and DSM 2277, previously named '*B. globigii*', '*B. niger*', '*B. subtilis* var. *niger*' and, finally, *B. subtilis*, have to be reclassified as members of the species *B. atrophaeus*. Species descriptions of *B. subtilis* and *B. atrophaeus* are not affected by this reclassification, as Smith *et al.* (1952) had classified the 'red strain' as '*B. subtilis* var. *niger*' after its substrate blackening of media containing tyrosine. Nakamura (1989) described the soluble pigment as 'brownish black' or 'dark brown' and stated that 'except for the colour of the soluble pigment, all of the strains were indistinguishable by the standard characterization method; i.e. they exhibited the traits typical of *B. subtilis*'.

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Sporicidal action of ozone and hydrogen peroxide: a comparative study

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Abstract

Elimination of contaminating spores on packaging materials and food-contact surfaces remains a challenge to the food industry. Hydrogen peroxide and chlorine are the most commonly used sanitizers to eliminate these contaminants, and ozone was recommended recently as an alternative. Hence, we compared the sporicidal action of ozone and hydrogen peroxide against selected foodborne spores of *Bacillus* spp. Under identical treatment conditions, 11 μg/ml aqueous ozone decreased spore counts by 1.3 to 6.1 $\log_{10}$ cfu/ml depending upon the bacterial species tested. Hydrogen peroxide (10%, w/w), produced only 0.32 to 1.6 $\log_{10}$ cfu/ml reductions in spore counts. Thus, hydrogen peroxide, at ~10,000-fold higher concentration, was less effective than ozone against *Bacillus* spores. Resistance of spores to ozone was highest for *Bacillus stearothermophilus* and lowest for *B. cereus*. Therefore, spores of *B. stearothermophilus* are suitable for testing the efficacy of sanitization by ozone. Electron microscopic study of ozone-treated *B. subtilis* spores suggests the outer spore coat layers as a probable site of action of ozone.

Keywords: Ozone; Hydrogen peroxide; Sporicidal; *Bacillus*

1. Introduction

The bacterial endospore is resistant to a variety of harsh treatments including heat, irradiation, chemicals and desiccation. Spores can survive for long periods in the absence of moisture and exogenous nutrients. Bacterial spores survive treatments with commercial sterilants and disinfectants (Sagripanti and Bonifacino, 1999). Spores also possess a swift and highly efficient mechanism for reverting to the vegetative state when nutrients, in aqueous solutions, become available (Gould et al., 1994). Therefore, presence of *Bacillus* and *Clostridium* spores in food constitutes a challenge to the industry.

Clostriadum botulinum spores are widely distributed in the environment (Smith and Sugiyama, 1988); these spores are occasionally isolated from food (Franciosa et al., 1999). Bacterial spores, present as contaminants in food, may survive processing, grow during storage, and cause spoilage of food or diseases to consumers. Meer et al. (1991) noted that *Bacillus cereus* survives adverse environmental conditions, adapts and eventually multiplies in foods. Some strains of *B. cereus* grew to ~10^6 cfu/g and produced toxin in refrigerated foods (Dufrenne et al., 1995). Sporeforming bacilli were reported to cause spoilage of pasteurized, aseptically packed apple juice (Cerny et al., 1985; Splittsoesser et al., 1994). Con-

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centrated orange juice from different suppliers has been recently found to contain spores of *Alicyclobacillus* spp. (Eiroa et al., 1999). Additionally, Komitopoulou et al. (1999) reported the ability of *Alicyclobacillus acidoterrestris* to grow in orange juice, grapefruit juice and apple juice, and the resistance of its spores under normal juice pasteurization conditions. Elimination of such spores from equipment surfaces, packaging materials and the food itself is a prerequisite for successful production of aseptically packaged products.

To inactivate contaminating spores in the processing environment, hydrogen peroxide (Yokoyama, 1990) and chlorine (Marriott, 1999) are commonly used. Ozone was recommended recently as an alternative to chlorine (Kim, 1998) and hydrogen peroxide (Khadre and Yousef, 2001). Ozone use in the processing environment may become feasible if the sporicidal action of this sanitizer is demonstrated. Therefore, this study was initiated to compare the effectiveness of ozone and H_2O_2 against a variety of foodborne bacterial spores.

2. Materials and methods

2.1. Ozone

Ozone demand-free glassware was prepared as described previously (Kim et al., 1999). Aqueous ozone was produced by bubbling ozone gas into sterile deionized water at controlled flow rates. Ozone gas was produced from purified extra dry oxygen by an ozone generator (U.S. Filter/Polymetrics T-816, San Jose, CA). The desired ozone concentration in water was attained by adjusting the flow rate of gaseous ozone. Approximate concentration of ozone solubilizing in water was monitored by measuring absorbance at 258 nm (A_{258}), using a spectrophotometer (Spectronic 1201, Milton Roy, Rochester, NY), as indicated in a previous study (Kim and Yousef, 2000). Ozonation of water continued until the targeted ozone concentration (~ 10 μg/ml) was attained. Final ozone concentration in water was measured using the indigo method (Bader and Hoigne, 1981). The resulting aqueous ozone solution (11 μg/ml) was tested against spores of eight *Bacillus* spp. This concentration was chosen based on preliminary experiments on the sensitivity of spores of *B. subtilis* OSU494 to varying concentrations of ozone (0.2 to 14 μg/ml). All experimental work with ozone was done under a chemical hood. Excess ozone was neutralized by diverting the gas stream into a reservoir containing 2% potassium iodide solution or to an ozone-decomposing catalytic column. Protective masks and ozone-resistant gloves were worn during the experiments.

2.2. Hydrogen peroxide

Hydrogen peroxide solution (30% w/w) (Sigma, St. Louis, MO) was stored at 4 °C, as recommended by the manufacturer. Lower concentrations of hydrogen peroxide were prepared by dilution in sterile deionized water, and kept at 4 °C until used.

2.3. Catalase enzyme

Lyophilized catalase enzyme (Sigma) contained 3260 units/mg, and it was stored at −18 °C. Catalase enzyme solutions were prepared according to the manufacturer's specifications and used within 30 min, during which it was kept at 4 °C.

2.4. Bacterial cultures

Eight *Bacillus* spp. were obtained from the culture collection of the Department of Microbiology at the Ohio State University and tested in this study. These strains were *B. subtilis* OSU494, *B. subtilis* OSU848, *B. subtilis* var niger ATCC 9372, *B. subtilis* ATCC 19659, *B. cereus* OSU11, *B. polymyxa* OSU443, *B. megaterium* OSU125 and *B. stearothermophilus* OSU24. Stock cultures of these bacteria were grown in nutrient broth (Difco Laboratories, Detroit, MI) at 37 °C for 24 h, and their spores were prepared as indicated later.

2.5. Spore suspensions

Spore suspensions were prepared as described by Sala et al. (1995). Briefly, cultures of *Bacillus* spp. were spread onto sporulation agar medium and inoculated plates were incubated for 6–8 days at 37 °C. The sporulation medium consisted of nutrient agar supplemented with 500 ppm Bacto-dextrose (Difco

Laboratories) and 3 ppm manganese sulfate (Mallinckrodt, Paris, KY). Sporulation was verified by microscopic inspection of the growth under phase contrast. Spores were harvested and treated in a sonicator (FS-28, Fisher, Pittsburgh, PA) to disperse clumps. The sonicated suspensions were washed six times by centrifugation (8000 × *g* for 20 min at 4 °C) and resuspension in sterile deionized water. After an additional centrifugation, the spore pellet was resuspended in 0.1% sodium chloride solution to obtain ~ 10^9 spores/ml. The spore suspension was stored at 4 °C until used.

2.6. Ozone treatment

A portion of the spore suspension (0.2 ml) was dispensed in a 4-oz stomacher bag and 20 ml, 11 μg/ml aqueous ozone (22 °C) was added. The mixture was stomached immediately for 1 min, and 1.0-ml aliquot was transferred to a test tube containing 9-ml sterile peptone water to neutralize excess ozone. In some experiments, 2 ml sodium thiosulfate solution (0.206 g/l) (Fisher Scientific, Fair Lawn, NJ) was added to the contents of the stomacher bag to neutralize excess ozone before counting the survivors. These two methods were equally effective in neutralizing excess ozone. Additionally, sodium thiosulfate, at the amount used, had no effect on the viability of the treated spores (data not shown).

2.7. Hydrogen peroxide treatment

Spores of the eight *Bacillus* spp. were treated with 10% hydrogen peroxide solution (i.e., 100,000 μg/ml) as follows. A spore suspension aliquot (0.2 ml) was dispensed in a sterile 500-ml Erlenmeyer flask and 20 ml hydrogen peroxide solution (22 °C) was added. The mixture was stirred for 1 min using a magnetic stirrer. A solution (2 ml) containing enough catalase enzyme to neutralize excess hydrogen peroxide was added to the flask with continuous stirring until frothing stopped and most of the bubbles dissipated. Catalase enzyme at the concentrations used did not have any sporicidal effect. A 1.0-ml aliquot was transferred to a test tube containing 9-ml sterile peptone for dilution and plating. A similar procedure was used to test the activity of 1% to 30% hydrogen peroxide against spores of *B. subtilis* OSU494.

2.8. Microbiological analysis

For enumerating surviving bacterial spores, sanitizer-treated and untreated spore suspensions were heat-shocked at 80 °C for 30 min, and counts were determined in plate count agar using the pour-plating technique. Plates were incubated for 48 h at 35 °C and colonies were counted.

2.9. Electron microscopy

A spore suspension (0.2 ml) was mixed with 20 ml ozone–water (22 °C) in a 4-oz stomacher bag and the mixture was stomached immediately for 1 min. Sodium thiosulfate (2 ml, 0.206 g/l) was added to the bag contents to neutralize excess ozone. The control treatment was exposed to 20 ml deionized water instead of ozone–water. The following procedure was recommended by the Department of Imaging and Microscopy, the Ohio State University. Spores were centrifuged at 8000 × *g* for 20 min, the pellet was suspended in 1.5 ml, 4% gluteraldehyde in 0.1 M cacodylate buffer, pH 7.2, and kept at 4 °C overnight for fixation. Spores were centrifuged and rinsed three times in 0.1 M cacodylate buffer, pH 7.2 (referred to as buffer hereafter), at 25 °C. Spores were fixed in 1% osmium tetroxide in buffer for 1.5 h, and rinsed twice in buffer with centrifugation and resuspension. After centrifugation and removal of most of the buffer, spores were suspended in a small quantity of 2% agarose, which was allowed to gel. After the agarose–spores mixture was cooled in an ice-bath, it was cut into pieces not larger than 1 mm^3 and left in buffer overnight at 4 °C. Samples were rinsed twice in distilled water and en bloc stained in 1% uranyl acetate for 90 min. Samples were rinsed twice in distilled water and gradually dehydrated in solutions containing 50% to 100% ethanol. Samples were put into propylene oxide for 20 min and infiltrated in 1:1 propylene oxide/Spurr resin for 24 h. Samples were embedded in Spurr resin in flat embedding molds and polymerized overnight at 60 °C. Sections were cut at 70 nm on a Reichert Ultracut E ultramicrotome and picked up on formvar-coated 200 mesh copper grids. Grids were stained in 2% aqueous uranyl acetate for 15 min, followed by Reynolds lead citrate for 5 min. Grids were examined in a Philips CM 12 transmission electron microscope at 60 kV.

2.10. Data analysis

Population of spores, which was inactivated during the ozone treatment ($\log_{10}$ cfu/ml untreated–$\log_{10}$ cfu/ml treated sample), was analyzed using MINITAB statistical program (Minitab, State College, PA). One-way analysis of variance was performed for the effect of spore strain on the degree of inactivation by ozone. Multiple comparison of means was done using Fisher's range test at an error rate of 0.05.

3. Results

3.1. Relative resistance of spores to ozone

Treatment of spore suspensions with 11 μg/ml aqueous ozone for 1 min followed by neutralization of excess ozone, reduced spore counts by 1.3 to 6.1 $\log_{10}$ cfu/ml depending upon the bacterial strain (Table 1). Resistance of spores to ozone was highest for *B. stearothermophilus* OSU24, *B. polymyxa* OSU443, *B. megaterium* OSU125 and *B. subtilis* OSU494; differences among these species were insignificant ($p < 0.05$). Spores of *B. subtilis* OSU848 had an intermediate resistance to ozone. Compared to other tested strains, spores of *B. subtilis* ATCC 19659, *B. cereus* OSU11 and *B. subtilis* var Niger ATCC 9372 were the most sensitive to ozone; differences among these three strains were not significant ($p < 0.05$).

Table 1
Decrease in spore populations ($\log_{10}$ cfu/ml untreated control–$\log_{10}$ cfu/ml treated sample)[a] after exposure of different *Bacillus* spp. to ozone (11 μg/ml) or hydrogen peroxide (100,000 μg/ml) for 1 min at 22 °C, followed by neutralization with sodium thiosulfate or catalase, respectively

Bacillus spp.	Ozone		Hydrogen peroxide	
	Average[b,c]	SD[d]	Average[c,e]	SD[d]
B. cereus OSU11	6.1^{A}	1.0	1.6^{A}	0.22
B. megaterium OSU125	2.1^{C}	0.49	0.93^{AD}	0.29
B. polymyxa OSU443	1.9^{C}	0.50	0.58^{D}	0.11
B. stearothermophilus OSU24	1.3^{C}	0.07	0.64^{CD}	0.19
B. subtilis OSU494	2.7^{C}	0.83	0.32^{D}	0.14
B. subtilis OSU848	4.8^{B}	0.57	1.2^{ABC}	0.68
B. subtilis ATCC 19659	6.1^{AB}	0.85	0.64^{BD}	0.03
B. subtilis var Niger ATCC 9372	5.7^{AB}	0.43	1.3^{A}	0.44

[a] Average initial count is 1.3×10^7 spore/ml.
[b] Data represent averages of two to seven repeats.
[c] Averages, within the same column, with the same capital letter are not significantly different (Fisher's LSD at $p = 0.05$).
[d] Sample Standard Deviation.
[e] Data represent averages of three repeats.

3.2. Relative resistance of spores to hydrogen peroxide

When spores of eight *Bacillus* strains were treated with 10% H_2O_2 for 1 min at 22 °C, the counts decreased 0.32 to 1.6 $\log_{10}$ cfu/ml, depending on the bacterial species tested (Table 1). Spores of *B. subtilis* OSU494, *B. polymyxa* OSU443, *B. stearothermophilus* OSU24, *B. subtilis* ATCC 19659 and *B. megaterium* OSU125 were the most resistant to the hydrogen peroxide treatment, and differences among these strains were not statistically significant ($p < 0.05$). Spores of *B. subtilis* OSU848 had intermediate resistance, whereas spores of *B. cereus* OSU11 and *B. subtilis* var Niger ATCC 9373 were the most sensitive to the hydrogen peroxide treatment.

Results in Table 1 illustrate the superiority of ozone to hydrogen peroxide as a sporicidal agent; H_2O_2, at ~10,000-fold higher concentration, was less effective than ozone against *Bacillus* spores. Since *B. subtilis* OSU494 showed the highest resistance to 10% H_2O_2 solution, this strain was tested at a range of H_2O_2 concentrations. The count of *B. subtilis* OSU494 spores decreased modestly when the concentration of H_2O_2 increased from 1% to 15%, and appreciably at 20% to 30% (Fig. 1).

3.3. Mechanism of action of ozone on spores

Correlation between susceptibility of spores to ozone and hydrogen peroxide may reflect similarity in the mechanism of spore inactivation by these two oxidizing agents. Spores, treated or untreated with ozone, were examined by transmission electron microscope (TEM). Inspecting these micrographs re-

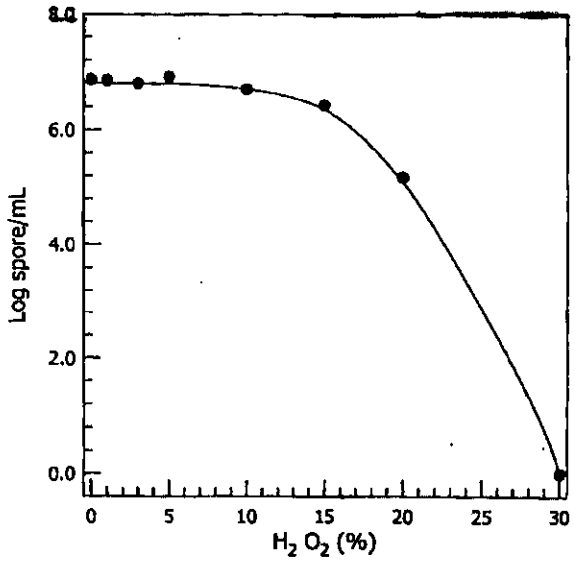

Fig. 1. Inactivation of spores of *B. subtilis* OSU494, 7.3×10^6 initially, when treated with a varying concentration of hydrogen peroxide (1% to 30%) at 22 °C for 1 min.

vealed damage to the surface layer, the outer spore coat, and to some extent to the inner spore coat layer in ozone-treated spores, which may have lead to exposing the cortex to the action of ozone (Fig. 2). Spore structure designations followed that of Henrique and Moran (2000).

4. Discussion

4.1. Spores and ozone

Our study demonstrates the ability of ozone in water at low concentrations to produce significant reduction in spore counts, compared to hydrogen peroxide. Sensitivity of bacterial spores to ozone, compared to other sanitization factors, is of interest to food processors who are also interested in identifying an indicator microorganism for this sanitizer. *B. stearothermophilus* may serve as a suitable indicator for ozone sanitization. In addition to its resistance to ozone (Table 1), spores of *B. stearothermophilus* also are extremely resistant to heat (Russell, 1982). Spores of *B. subtilis* var niger ATCC 9372 are used as indicators in dry heat and ethylene oxide sterilization (Anonymous, 1995, 1999). Spores of *B. subtilis* ATCC 19659 and *B. subtilis* var niger ATCC 9372 are used commercially in sterility testing of aseptic fillers (e.g., the spore-strip kit of North American Science Associates, Northwood, OH). These two strains, however, are sensitive to ozone (Table 1).

4.2. Spores and hydrogen peroxide

Compared to ozone in water, hydrogen peroxide was substantially inferior in sporicidal activity. Set-

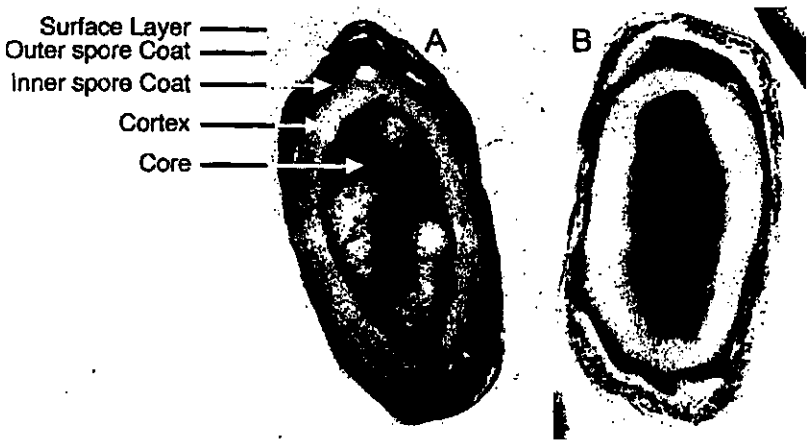

Fig. 2. Transmission electron microscopic micrograph of *B. subtilis* OSU494 spores, untreated (A), or treated (B) with ozone. Ozone-treated spores were exposed to aqueous ozone (10 μg/ml) at 22 °C for 1 min followed by neutralization with sodium thiosulfate. Note that the surface layer and the outer spore coat are the structures most apparently damaged by ozone treatment.

low and Setlow (1993) found *B. subtilis* spores resistant to treatment with 4 M hydrogen peroxide solution for 20 min. It is of interest to note also that the antimicrobial power of hydrogen peroxide increases as the temperature rises (Toledo, 1975), while that of ozone increases as the temperature decreases below ambient (Herbold et al., 1989). In this study, hydrogen peroxide at a concentration of 15% (22 °C) for 1 min decreased *B. subtilis* spores 0.44 $\log_{10}$ cfu/ml, whereas Shin et al. (1994) observed 4.7 $\log_{10}$ reduction of similar spores using 15% hydrogen peroxide at 60 °C for 30 min. Therefore, for effective sporicidal action in the food processing environment, treatment with H_2O_2 (at 30%) is followed by application of hot air (Yokoyama, 1990). Detectable changes in the physical structure of spores required 10 μg/ml ozone at 22 °C for 1 min (Fig. 2) or 15% hydrogen peroxide at 60 °C for 120 min (Shin et al. 1994). Cerf and Metro (1977) suggested that hydrogen peroxide in the immediate vicinity of spores is destroyed by an associated spore catalase enzyme. Lawrence (1957) indicated that intact spores have demonstrated catalase activity independent of the vegetative residue or the presence of germinated spores.

When spores were compared, *B. cereus* OSU11 and *B. subtilis* var niger ATCC 9372 were the most sensitive, whereas *B. subtilis* OSU494 and *B. polymyxa* OSU443 were the most resistant to hydrogen peroxide, under the conditions tested in this study. Spores of *B. subtilis* ATCC 19659 and *B. subtilis* var niger ATCC 9372, which are commonly used in sterility testing of aseptic fillers, varied in sensitivity to H_2O_2; ATCC 19659 was moderately resistant but ATCC 9372 was sensitive to the sanitizer. *B. stearothermophilus* produces one of the most heat-resistant spores known (Russell, 1982); this bacterium was also fairly resistant to hydrogen peroxide and ozone (Table 1). Resistance of spores to inactivation by hydrogen peroxide and tertiary butyl hydroperoxide has been reported for *B. stearothermophilus*, *B. subtilis* and *B. megaterium* (Marquis et al., 1994). It appears that there is a threshold concentration for the sporicidal action of H_2O_2. According to our data (Fig. 1), 15% was the threshold of action of hydrogen peroxide against *B. subtilis* OSU494. Therefore, in aseptic processing, high concentration of H_2O_2 should be maintained for effective sanitization of equipment surfaces and packaging materials.

4.3. Mechanism of action of ozone on spores

The precise killing mechanism of spores by ozone and similar oxidizing agents are not fully understood. Setlow and Setlow (1993) found no increase in mutation frequency and no DNA damage among survivors of H_2O_2-treated spores of *B. subtilis*. In contrast, *B. subtilis* spores treated with H_2O_2 showed clear degradation of outer spore layers including spore coats and cortex (Shin et al. 1994). Our present study on ozone supports the notion that oxidizing agents including ozone and H_2O_2 probably kill spores by degrading outer spore components, and exposing the spore core to the action of the sanitizer (Fig. 2).

Coats comprise ~50% of the spore volume. These coats contain ~80% of the spore proteins and they constitute barriers to damaging enzymes such as lysozyme (Murrell, 1967; Aronson and Horn, 1972; Marquis et al., 1994). Spore coats are probably disrupted by oxidizing sporicidal agents such as hydrogen peroxide and hypochlorite, which may cause extraction of spore coat material, facilitating the penetration of these sanitizers into the cortex and protoplast (Bayliss and Waites, 1976). It is important to note that extracted spores, i.e., spores in which the spore coats have been removed, retain their dipicolinic acid, and refractility. These extracted spores are resistant to heat and radiation, and are fully viable but they become sensitive to lysozyme (Russell, 1982; Marquis et al., 1994). Hydrogen peroxide was shown to remove protein from the spore coats in *B. cereus* and *C. bifermentans* (Russell, 1982).

In spite of the evidence that oxidizing agents target spore coats, damage to DNA may partially explain spore inactivation by these agents. Setlow and Setlow (1993) believe that hydrogen peroxide, or possibly the free hydroxyl radicals resulting from its degradation, gained access to the core of spores of certain *B. subtilis* mutants and killed these spores at least in part by DNA damage. Similarly, Shin et al. (1994) found that H_2O_2-treated (15%, at 60 °C for 30 min) spores of *B. megaterium* greatly lost viability (> 5 $\log_{10}$ reduction in viability) with almost no loss in optical density, change in the phase micro-

scopic appearance of the spores, or observable changes in the fine structure of the spores. Ozone, in our study, damaged the outer spore coat but slightly affected the inner coat and spared the cortex (Fig. 2); the vast majority of these spores lost viability. Gerhardt et al. (1972) suggested that molecules greater than 200 Da penetrate ~40% of the spore volume.

5. Conclusion

It is evident that ozone is superior to hydrogen peroxide in killing bacterial spores. The comparatively low concentration needed to eliminate large population of spores at ambient temperature in short-time periods makes ozone best suited for industrial settings. Effectiveness of ozone in disinfecting food-contact surfaces may be tested using spores of *B. stearothermophilus* as indicators.

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Taxonomic Relationship of Black-Pigmented *Bacillus subtilis* Strains and a Proposal for *Bacillus atrophaeus* sp. nov.

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The taxonomic position of *Bacillus subtilis* strains that produce soluble black pigment is unclear. To assess the genetic relatedness between the pigmented and nonpigmented strains, deoxyribonucleic acid (DNA) reassociation was measured spectrophotometrically. Among the 40 pigmented strains examined, two distinct DNA relatedness groups were found. A total of 25 strains (group 1) showed 24 to 34% DNA relatedness and 15 strains (group 2) showed 70 to 100% relatedness to *Bacillus subtilis* type strain NRRL NRS-744. The intragroup DNA relatedness values for each group ranged from 85 to 100%; the intergroup relatedness values ranged from 20 to 35%. A multilocus enzyme electrophoresis analysis revealed a low level of similarity between group 1 and group 2 or the nonpigmented group. The group 2 strains and the nonpigmented strains clustered in a common group, indicating the close genetic relationship of these organisms. My results strongly suggest that group 2 is a pigmented variant of *B. subtilis*, but group 1 is a new species, for which the name *Bacillus atrophaeus* is proposed. The type strain of the new species is strain NRRL NRS-213.

Smith et al. (13) observed and studied two black-pigmented varieties of *Bacillus subtilis*. One variety, designated "*Bacillus subtilis* var. *aterrimus*," developed a soluble black pigment in media containing glucose or other utilizable carbohydrates; the other, called "*Bacillus subtilis* var. *niger*," formed a soluble dark pigment in tyrosine-containing media. Early workers presumed that pigmentation in "*B. subtilis* var. *niger*" resulted from tyrosinase activity. Because it was repeatedly observable in the crude agar media available to Smith et al. (13), black-pigment development was considered to be a stable characteristic and, therefore, a dependable and distinctive basis for varietal designation.

Some black-pigmented *B. subtilis* strains have important uses or characteristics. For example, "*B. subtilis* var. *niger*" strains produce 1-deoxynojirimycin, a substance with antibiotic as well as glucosidase-inhibiting activities (10). Selected "*B. subtilis* var. *niger*" strains are also used as standards for autoclave sterility testing (*Catalogue of Bacteria, Phages, and rRNA Vectors*, 16th ed., American Type Culture Collection, Rockville, Md.).

Except for pigment production, the colored strains are generally phenotypically indistinguishable from nonpigmented *B. subtilis* strains. However, in an extensive numerical phenetic survey carried out by Priest et al. (8), *B. subtilis* and "*B. subtilis* var. *niger*" did segregate into distinct but adjacent clusters. Furthermore, studies based on a small number of strains have indicated that strains classified as "*B. subtilis* var. *aterriums*" are genetically unrelated to strains classified as "*B. subtilis* var. *niger*" (2). In this study I augmented the sparse previously existing taxonomic data with guanine-plus-cytosine (G+C) and deoxyribonucleic acid (DNA) relatedness measurements and with enzyme electrophoresis pattern analyses of 40 black-pigmented and 12 nonpigmented strains identified as *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the pigmented and nonpigmented *B. subtilis* strains used in this study. Also used in this study were *Bacillus alvei* Cheshire and Cheyne 1885 NRRL B-383^T (T = type strain), *Bacillus badius* Batchelor 1919 NRRL NRS-663^T, *Bacillus brevis* Migula 1900 NRRL NRS-604^T, *Bacillus coagulans* Hammer 1915 NRRL NRS-609^T, *Bacillus firmus* Bredemann and Werner 1933 NRRL B-14307^T, *Bacillus licheniformis* (Weigmann) Chester 1901 NRRL NRS-1264^T, *Bacillus polymyxa* (Prazmowski) Mace 1889 NRRL NRS-1105^T, and *Bacillus pumilus* Meyer and Gottheil 1901 NRRL NRS-272^T. The Northern Regional Research Laboratory (NRRL) strain designations include the prefixes B- and NRS-; the prefix B- indicates strains that were obtained directly from a source or strains that were isolated at the Northern Regional Research Center, and the prefix NRS- indicates strains that were obtained from N. R. Smith. Working cultures were grown at 30°C in soil extract agar (5) until sporulation occurred, and they were stored at 4°C.

DNA investigations. The cells were grown in TGY broth (6) with agitation and were harvested by centrifugation at 5°C in the mid- or late logarithmic growth phase. All cultures were checked microscopically for the absence of sporulation before harvesting. Previous publications have described the procedure used for preparing highly purified DNA samples by hydroxyapatite chromatography and the method used for measuring the extent of DNA reassociation by determining DNA renaturation rates spectrophotometrically with a model 250 ultraviolet spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a model 2527 thermoprogramer (7). The equation of De Ley et al. (3) was used to calculate DNA relatedness values.

The G+C contents of DNA samples were determined by measuring buoyant densities by CsCl density centrifugation in a Beckman model E ultracentrifuge (9). *Micrococcus luteus* (synonym, "*Micrococcus lysodeikticus*") DNA with a buoyant density of 1.724 g/cm^3, which was purchased from Sigma Chemical Co., St. Louis, Mo., was used as an internal standard.

Characterization. The physiological, morphological, and biochemical characteristics were determined as described previously (5, 7).

Enzyme electrophoresis. Cells were grown at 30°C for 24 h in 3 liters of TGY broth with agitation, harvested by centrifugation at 30,000 × *g* for 10 min, and suspended in 10 ml of pH 6.8 buffer containing 10 mM tris (hydroxymethyl) aminomethane (Tris), 1 mM ethylenediaminetetraacetate, and 0.5 mM $NaH(PO_4)_2$. The cells were disrupted by passage through a chilled French pressure cell at 10,000 lb/in^2. After centrifugation at 30,000 × *g* for 15 min at 4°C, portions of the

TABLE 1. List of *B. subtilis* strains used in this study

NRRL no.	Received as strain(s):	Source[a]	Strain history[b]
B-357	NRS-242	1	From soil
B-360	NRS-230	1	C. Thom, from corn
B-361	NRS-259	1	I. C. Hall
B-362	NRS-624	1	J. R. Porter from F. W. Fabian
B-363	NRS-212	1	From Colorado soil
B-364	NRS-220	1	AMNH from Kral Collection
B-365	NRS-356	1	"*B. subtilis* var. *niger*"[c]
B-447	20	2	"*Bacillus mesentericus*"
B-554	12-H	3	
B-627		4	"*B. subtilis* var. *niger*"
B-765	ATCC 6633	5	N. R. Smith 231 from K. F. Kellerman
B-971	398	6	
B-4418	GL 100	7	"*B. subtilis* subsp. *niger*"
NRS-162	NRS-162	1	D. M. Webley FFT, from grass compost
NRS-163	NRS-163	1	D. M. Webley HFT, from grass compost
NRS-193	NRS-193	1	W. Bohrer, C-1889, from okra
NRS-211, NRS-213^T, NRS-214 to NRS-216, NRS-218, NRS-219, NRS-221 to NRS-224, NRS-226 to NRS-228	NRS-211, NRS-213^T, NRS-214 to NRS-216, NRS-218, NRS-219, NRS-221 to NRS-224, NRS-226 to NRS-228	1	N. R. Smith, "*B. subtilis* var. *niger*," from Colorado soil
NRS-229	NRS-229		N. R. Smith, "*B. subtilis* var. *niger*," from Utah soil
NRS-253	NRS-253		N. R. Smith, "*B. subtilis* var. *niger*," from air
NRS-261, NRS-262	NRS-261, NRS-262	1	I. C. Hall 620, I. C. Hall 621A, "*B. subtilis* var. *aterrimus*"
NRS-263	NRS-263	1	I. C. Hall 798B from W. W. Ford, "*Bacillus aterrimus*"
NRS-264	NRS-264	1	I. C. Hall 799, "*Bacillus niger*," from W. W. Ford from Kral collection
NRS-265	NRS-265	1	I. C. Hall 1509, (ATCC 6455)[d]
NRS-274	NRS-274	1	USDA, from air
NRS-275	NRS-275	1	USDA, from Maryland soil, (ATCC 6461)
NRS-276	NRS-276	1	USDA, from Maryland soil
NRS-330	NRS-330	1	NCTC 2590, "*Bacillus aterrimus*," from W. W. Ford 5A
NRS-561	NRS-561	1	NIH 4
NRS-651	NRS-651	1	NCA, "*Bacillus niger*," from R. S. Breed from NCTC 2592, from W. W. Ford 6
NRS-652	NRS-652	1	NCA, "*Bacillus aterrimus*," from R. S. Breed from NCTC 2590 from W. W. Ford 5A
NRS-653	NRS-653	1	NCA, "*Bacillus aterrimus*," from R. S. Breed from NCTC 2591 from W. W. Ford 5B
NRS-655	NRS-655	1	NCA, "*Bacillus lactis niger*," from R. S. Breed from C. Gorini 2
NRS-704	NRS-704	1	M. L. Rakieten C3, "*B. subtilis* (*niger*)"
NRS-730	NRS-730	1	ATCC 7003, "*Bacillus graveolens*," from F. S. Orcutt
NRS-740	NRS-740	1	ATCC 4295, "*Bacillus nigrificans*," from F. W. Fabian from pickle brine
NRS-744^T	NRS-744^T	1	ATCC 6051^T from H. J. Conn from NCTC 3610^T
NRS-748	NRS-748	1	USDA, from decomposed wheat

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[b] AMNH, American Museum of Natural History, Washington, D.C.; ATCC, American Type Culture Collection, Rockville, Md.; USDA United States Department of Agriculture, Washington, D.C.; NCTC, National Collection of Type Cultures, London, England; NIH, National Institutes of Health, Washington, D.C.; NCA, National Canners Association, San Francisco, Calif.

[c] Names in quotation marks are not on the Approved Lists of Bacterial Names (12) and have not been validly published since January 1980.

[d] Designations in parentheses are equivalent strain designations.

supernatant were transferred to capped, 1-ml plastic centrifuge tubes and stored at −20°C. Fresh cell lysates were prepared at weekly intervals.

Enzymes were separated by vertical electrophoresis through polyacrylamide slab gels (0.75 mm by 15.5 cm by 16 cm). A stacking gel (0.75 mm by 4.5 cm by 16 cm) was also used. The separating gel (12%) contained 11.68% acrylamide, 0.32% *N,N'*-methylene bisacrylamide, 0.05% ammonium persulfate, and 0.05% *N,N,N',N'*-tetramethylethylenediamine. The stacking gel (4%) contained 3.9% acrylamide, 0.1% *N,N'*-methylene bisacrylamide, 0.05% ammonium persulfate, and 0.1% *N,N,N',N'*-tetramethylethylenediamine. The separating gel buffer was 0.375 M Tris hydrochloride (pH 8.8), and the stacking gel buffer was 0.125 M Tris hydrochloride (pH 6.8). The running buffer (pH 8.3) was a mixture of 0.123 M Tris and 0.959 M glycine. Electro-

TABLE 2. DNA relatedness of pigmented *B. subtilis* strains

Strain (NRRL no.)	% Reassociation with DNA from strain[a]:			Soluble pigment color on[b]:	
	NRRL NRS-213[T]	NRRL NRS-261	NRRL NRS-744[T]	TGY agar	Glycerol-glutamate agar
Group 1					
B-363	97	29	35	Brownish black	Brown
B-364	95	28	27	Brownish black	Brown
B-365	93	27	25	Brownish black	Brown
B-627	90	33	35	Brownish black	Brown
B-4418	98	34	27	Brownish black	Brown
NRS-211	88	27	25	Brownish black	Brown
NRS-213[T]	(100)[c]	24	30	Brownish black	Brown
NRS-214	100	26	25	Brownish black	Brown
NRS-215	100	29	23	Brownish black	Brown
NRS-216	94	26	30	Brownish black	Brown
NRS-218	94	33	29	Brownish black	Brown
NRS-219	91	27	30	Brownish black	Brown
NRS-221	99	24	25	Brownish black	Brown
NRS-222	97	26	22	Brownish black	Brown
NRS-223	91	29	30	Brownish black	Brown
NRS-224	98	26	36	Brownish black	Brown
NRS-226	100	34	25	Brownish black	Brown
NRS-227	96	32	32	Brownish black	Brown
NRS-228	96	28	32	Brownish black	Brown
NRS-229	91	30	25	Brownish black	Brown
NRS-253	96	26	33	Brownish black	Brown
NRS-265	94	31	27	Brownish black	Brown
NRS-651	88	30	30	Brownish black	Brown
NRS-704	96	24	30	Brownish black	Brown
NRS-748	92	29	25	Brownish black	Brown
Group 2					
B-360	20	100	96	Brown	Bluish black
B-361	29	100	93	Brown	Bluish black
B-362	25	100	96	Brown	Bluish black
NRS-261	25	(100)	96	Brown	Bluish black
NRS-262	30	84	92	Brown	Bluish black
NRS-263	24	93	99	Brown	Bluish black
NRS-264	21	95	100	Brown	Bluish black
NRS-274	23	98	100	Brown	Bluish black
NRS-275	25	90	85	Brown	Bluish black
NRS-276	26	93	92	Brown	Bluish black
NRS-330	35	99	98	Brown	Bluish black
NRS-652	27	100	98	Brown	Bluish black
NRS-653	27	90	97	Brown	Bluish black
NRS-655	29	99	70	Brown	Bluish black
NRS-740	30	90	95	Brown	Bluish black
Group 3					
B-357	25	100	100	None	None
B-447	23	100	98	None	None
B-554	30	100	93	None	None
B-765	30	75	89	None	None
B-971	28	80	70	None	None
NRS-161	25	91	92	None	None
NRS-162	30	96	95	None	None
NRS-163	28	100	91	None	None
NRS-193	36	94	88	None	None
NRS-561	33	93	100	None	None
NRS-730	22	95	98	None	None
NRS-744[T]	30	96	(100)	None	None

[a] Reassociation values are averages of two determinations; the maximum difference found between determinations was 7%.

[b] Brownish black pigmentation of group 1 strains was observed after 2 to 6 days in TGY agar, and brown pigmentation of group 2 strains was observed after 14 days. Brown pigmentation of group 1 strains in glycerol-glutamate agar was observed after 6 to 13 days, and bluish black pigmentation of group 2 strains was observed after 1 to 2 days.

[c] Values in parentheses indicate that, by definition, the reassociation value was 100%.

phoresis was carried out at 5°C at a constant amperage of 13 mA per slab to stack the samples and 18 mA per slab to effect enzyme separation. Sample proteins were diluted to a concentration of 600 μg/ml in 0.125 M Tris hydrochloride (pH 6.8) containing 10% glycerol and 0.00125% bromophenol blue; 50-μl portions of the diluted samples were analyzed electrophoretically.

The 12 enzymes studied were alanine dehydrogenase (EC 1.4.1.1), alcohol dehydrogenase (EC 1.1.1.1), aspartate dehydrogenase (EC 1.4.3.×), fumarase (EC 4.2.1.2), glucose-

TABLE 3. Levels of DNA relatedness of group reference strains and selected *Bacillus* spp. type strains

Strain	G+C content (mol%)[a]	% Reassociation with DNA from group reference strain[b]: NRRL NRS-213^T	NRRL NRS-261
B. firmus NRRL B-14307^T	41.5	27	23
B. pumilus NRRL NRS-272^T	42.0	17	24
B. badius NRRL NRS-663^T	43.8	26	30
B. polymyxa NRRL NRS-1105^T	44.5	37	23
B. alvei NRRL B-383^T	44.6	29	32
B. coagulans NRRL NRS-609^T	45.0	29	25
B. licheniformis NRRL NRS-1264^T	46.5	21	22
B. brevis NRRL NRS-604^T	47.5	28	30

[a] Data from reference 4.
[b] Reassociation values are averages of two determinations; the maximum difference found between determinations was 7%.

6-phosphate dehydrogenase (EC 1.1.1.49), glutamate dehydrogenase (EC 1.4.1.2), hexokinase (EC 2.7.1.1), indophenol oxidase (EC 1.15.1.1), leucine dehydrogenase (EC 1.4.3.2), lysine dehydrogenase (EC 1.4.3.×), malic dehydrogenase (EC 1.1.1.40), and phosphoglucose isomerase (EC 5.3.1.9). The enzymes were stained by using the method of Selander et al. (11).

The relative mobilities of alternative forms of each enzyme in the strains were compared directly on the electrophoresis gels. These allozymes (electromorphs) were assumed to be encoded by chromosomal genes and thus were equated with alleles at each locus. The electromorphs were numbered in order of increasing anodal mobility, and the combination of electromorphs at the 12 enzyme loci was determined for each strain. The absence of enzyme activity was scored as a null allele. Each distinctive combination of alleles was designated an electrophoretic type (ET).

Levels of similarity among strains were determined by using the simple matching coefficient, and clustering was based on the unweighted pair group arithmetic average algorithm (14). Computations were carried with an DTK AT computer by using the TAXAN program of David Swartz, University of Maryland, College Park.

RESULTS

B. subtilis strains that produced a soluble black pigment segregated into two groups on the basis of DNA relatedness (Table 2). Group 1 strains (which produced a brownish black pigment) showed 88 to 100% DNA relatedness to reference strain NRRL NRS-213^T and a range of relatedness to reference strains NRRL NRS-261 (which produced a bluish black pigment) and NRRL NRS-744^T (nonpigmented) of 22 to 35%. Strains in group 2 (which produced a bluish black pigment) had levels of DNA complementarity of 84 to 100 and 70 to 100% with reference strains NRRL NRS-261 and NRRL NRS-744^T, respectively. The levels of DNA relatedness of group 2 strains to strain NRRL NRS-213^T ranged from 20 to 35%. The nonpigmented group 3 strains showed 70 to 100% DNA relatedness to reference strain NRRL NRS-744^T and 75 to 100% DNA relatedness to strain NRRL NRS-261. The levels of DNA relatedness between group 3 strains and strain NRRL NRS-213^T ranged from 22 to 36%. The intragroup DNA relatedness values (data not shown) for all three groups ranged from 85 to 100%.

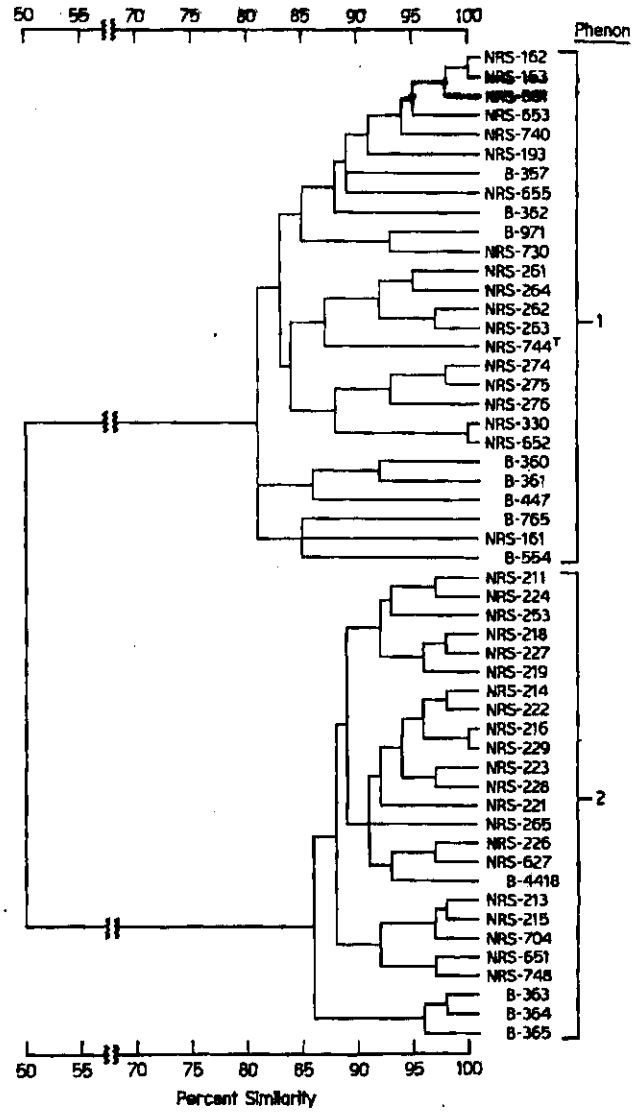

FIG. 1. Relationships of pigmented *B. subtilis* strains. The dendrogram was generated by unweighted average linkage clustering from a matrix of simple matching coefficients based on 12 enzyme loci.

The data in Table 3 show that reference strains NRRL NRS-213^T and NRRL NRS-261 yielded low DNA complementarity values (17 to 37%) with the following type strains: *B. alvei* NRRL B-383, *B. badius* NRRL NRS-663, *B. brevis* NRRL NRS-604, *B. coagulans* NRRL NRS-609, *B. firmus* NRRL B-14307, *B. licheniformis* NRRL NRS-1264, *B. polymyxa* NRRL NRS-1105, and *B. pumilus* NRRL NRS-272. These species had G+C contents ranging from 40.5 to 47.5 mol%, a range that includes the values (41 to 43 mol%) exhibited by the pigmented strains.

Analyses of the multilocus enzyme electrophoresis data revealed 49 ETs. The overall genetic diversity (11) of the 49 ETs was 0.39. The dendrogram in Fig. 1 shows the relationships of ETs based on the enzyme electrophoresis data. At a level of about 50% similarity, two distinct phena were identified. Phenon 1, with a genetic diversity of 0.3, contained 27 strains that represented 25 ETs (2 ETs contained

two strains each; the other ETs were one-member entities). Within this phenon, two enzymes were monomorphic, and 10 were polymorphic. The strains included in this phenon correspond exactly to the strains in DNA relatedness groups 2 and 3. Phenon 2, with a genetic diversity of 0.27, contained 25 strains that were equivalent to 24 ETs (1 ET contained two strains, and the other ETs contained one strain each). The strains in this phenon were identical to the strains found in DNA relatedness group 1. Of 12 enzymes, 4 were monomorphic and 8 were polymorphic.

Except for the color of the soluble pigment, all of the strains were indistinguishable by the standard characterization method (data not shown); i.e., they exhibited the traits typical of *B. subtilis* (5). The G+C contents of all of the strains ranged from 41 to 43 mol%. All group 1 strains produced a soluble brownish black pigment in 2 to 6 days in TGY agar (5); group 2 strains produced a brown pigment slowly in TGY agar. On the glycerol-glutamate medium of Arai and Mikami (1), group 2 strains synthesized a distinctly blue pigment in 1 to 2 days, the color of which intensified to a bluish black hue after 6 days. Group 1 strains produced only a brown pigment in glycerol-glutamate agar in 6 to 13 days.

DISCUSSION

The results of DNA relatedness studies indicate that the soluble pigment-forming strains of *B. subtilis* consist of two distinct genetically unrelated groups. Low DNA relatedness values show that the producers of the brownish black pigment are genetically unrelated to the bluish black strains and the nonpigmented strains. Moreover, the brownish black-pigmented organisms are also not closely related genetically to previously described species with G+C contents ranging from about 40 to 48 mol% (Table 3). Thus, the brownish black pigment producers, once classified as "*B. subtilis* var. *niger*," are members of a separate species. High DNA relatedness levels indicate that the bluish black pigment producers and nonpigmented *B. subtilis* strains are closely related genetically and thus are cospecific. Thus, the bluish black-pigmented strains are truly variants of *B. subtilis*.

The results of multilocus enzyme electrophoresis analyses supported the conclusions drawn from the DNA relatedness studies. Basically, the reduction of the genetic diversity value from 0.39 to about 0.30 upon segregation into the brownish black- and bluish black-pigmented groups suggested genetic heterogeneity of the whole group. If the group were genetically homogeneous, subgrouping should not have affected the genetic diversity value. Furthermore, organisms that form tight DNA relatedness groups are closely related on the basis of enzyme electrophoresis comparisons. While subgroups occur in phenon 1 at the 84 to 85% similarity level, the blue-pigmented strains are dispersed in a roughly even pattern among these subgroups. This suggests that mutations causing blue pigmentation occurred independently of mutations causing allelic enzyme variation.

Although conventional classification procedures barely differentiate one pigmented group from the other, DNA relatedness and multilocus enzyme electrophoresis analyses have established clearly that the *B. subtilis*-like organisms which produce a soluble brownish black pigment are members of a distinct, previously unnamed species. Since it is phenotypically virtually identical to *B. subtilis*, the pigmented taxon can be differentiated from *B. alvei*, *B. badius*, *B. brevis*, *B. coagulans*, *B. firmus*, *B. licheniformis*, *B. polymyxa*, and *B. pumilus* on the same bases as *B. subtilis* is. These brownish black pigment producers represent between 10 and 15% of the 300 organisms identified as *B. subtilis* in the Agricultural Research Service Culture Collection. Based on their demonstrated distinctiveness, rather common occurrence in nature, and usefulness, these organisms merit designation as members of a new species, for which I propose the name *Bacillus atrophaeus*. A description of the species is given below.

Bacillus atrophaeus sp. nov. *Bacillus atrophaeus* (a.tro.phae'.us L. adj. *ater*, black; Gr. adj. *phaeus*, brown; M.L. adj. *atrophaeus*, dark brown) vegetative cells are rods that are 0.5 to 1.0 μm wide by 2.0 to 4.0 μm long (as determined by phase microscopy) and occur singly and in short chains. Motile. Gram positive. Produces ellipsoidal spores centrally or paracentrally in unswollen sporangia.

Agar colonies are opaque, smooth, circular, entire, and 1.0 to 2.0 mm in diameter after 2 days at 28°C. A dark brown pigment is formed in 2 to 6 days in media containing an organic nitrogen source.

Catalase is produced. Oxidase is not produced. Aerobic. Acetylmethylcarbinol (Voges-Proskauer test) is produced. Hydrogen sulfide, indole, and dihydroxyacetone are not produced. The pH in Voges-Proskauer broth ranges from 5.3 to 5.7. Nitrate is reduced to nitrite. Starch and casein are hydrolyzed. Citrate but not propionate is utilized. Egg yolk lecithin, Tween 80, and urea are not decomposed. The pH in litmus milk is alkaline; casein is digested.

Arginine, lysine, ornithine, phenylalanine, and tyrosine are not decomposed.

The optimum growth temperature ranges from 28 to 30°C, the maximum growth temperature ranges from 50 to 55°C, and the minimum growth temperature ranges from 5 to 10°C. Grows at pH 5.6 or 5.7 and in the presence of 7% NaCl. Growth is usually inhibited by 0.001% lysozyme.

Acid but no gas is produced from L-arabinose, D-fructose, D-glucose, mannitol, salicin, sucrose, trehalose, and D-xylose. Acid production from cellobiose, D-galactose, maltose, D-mannose, D-ribose, L-rhamnose, and sorbitol is variable. Lactose and melibiose are not fermented.

The DNA buoyant density ranges from 1.6946 to 1.6966 g/cm^3, and the G+C contents determined from these values are 41 to 43 mol%.

The description above is virtually identical to that of *B. subtilis*. The new species is differentiated from *B. subtilis* on the basis of DNA relatedness and multilocus enzyme electrophoresis analyses, as well as pigment production.

Isolated mainly from soil.

The type strain is strain NRS-213, which has been deposited as NRRL NRS-213 in the Agricultural Research Service Culture Collection, Peoria, Ill.

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Environmental Technology Verification Report

ETV Building Decontamination Technology Center

CERTEK, Inc.
1414RH Formaldehyde Generator/Neutralizer

by

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3.2 Test Design

Coupons were cut from larger pieces of the representative materials for each of the seven indoor surfaces (Section 3.1). These coupons measured 3/4 x 3 in (1.9 x 7.5 cm) and varied in thickness from about 1/32 in (0.079 cm) to 3/8 in (0.95 cm), depending upon the material. In triplicate, the coupons were placed into a biological agent safety hood, and aliquots of an aqueous suspension of the biological agent were added to the surface of each coupon. Based upon the concentration of the spores in the aqueous suspension, the number of spores added to each coupon was calculated. The coupons were allowed to dry overnight. After drying, the inoculated coupons intended for decontamination were transferred into a custom-modified glove box and placed horizontally on a wire rack. Both blank (uncontaminated; N=2) and control (inoculated with spores, but not decontaminated; N=3) coupons were prepared, together with the inoculated coupons that were to be decontaminated (N=3).

Efficacy of the 1414RH unit was determined by comparing the number of viable spores on the control coupons (not decontaminated) to the number present on the decontaminated coupons, expressed as a log reduction. Following extraction of spores from the test, control, and blank coupons, efficacy was further evaluated for each biological agent or surrogate by transferring each coupon into liquid growth medium and assessing bacterial growth after 1 and 7 days.

Physical degradation of the indoor materials used as test surfaces was evaluated informally in conjunction with the efficacy testing procedure. After decontaminating the test coupons, the appearance of the decontaminated coupons was observed; and any obvious changes in the color, reflectivity, and apparent roughness of the coupon surfaces were noted.

3.3 Agents and Surrogates

The following biological agent was used for verification testing:

- *Bacillus anthracis* spores (Ames strain).

To provide correlations with the biological agent results, two biological surrogates also were used:

- *Bacillus subtilis* spores (ATCC 19659)
- *Geobacillus stearothermophilus* spores (ATCC 12980).

Biological indicators and spore strips that were used to evaluate decontamination efficacy included:

- Biological indicators (Apex Laboratories, Apex, North Carolina), approximately 1 x 10^6 spores each: *Bacillus subtilis* (ATCC 19659) and *Geobacillus stearothermophilus* (ATCC 12980) spores on steel disks and sealed Tyvek pouches

- Spore strips (Raven Biological Laboratories, Omaha, Nebraska): with *Bacillus atrophaeus* (ATCC 9372) spores, approximately 1 x 10^6 spores per strip on a filter paper matrix in sealed glassine envelopes.

3.4 Test Sequence

In Table 3-1, a summary of the verification testing of the 1414RH unit is presented. Verification testing was performed during a 7-week period that commenced in November 2003 and concluded in January 2004.

Table 3-1. Test Sequence and Parameters

Test Procedure	Parameters Evaluated	Data Produced
Biological Efficacy Test	Enumerations	Log reduction (Efficacy)
	B. anthracis	
	B. subtilis	
	G. stearothermophilus	
	Liquid culture assessment of coupons	Positive/negative bacterial growth (1 and 7 days)
	B. anthracis	
	B. subtilis	
	G. stearothermophilus	
	Biological indicators/spore strips	Positive/negative bacterial growth (1 and 7 days)
	B. subtilis	
	G. stearothermophilus	
	B. atrophaeus	
Coupon Damage	Damage to test coupons	Visual observation of every test coupon in all biological efficacy tests before and after decontamination

3.5 Coupon-Scale Testing

Coupon-scale testing was used to evaluate the decontamination efficacy of the 1414RH unit by extracting and measuring the viable biological spores on test coupons.

3.5.1 Preparation of Test Materials

Coupons used for biological agent decontamination were cut to about 3/4 x 3 in (1.9 x 7.5 cm) and prepared as shown in Table 3-2 by Battelle staff. Test coupons were visually

A liquid culture growth assessment at 1 and 7 days post-decontamination was performed to determine whether viable *B. subtilis* spores remained on the test materials following the extraction step (Table 6-6). As stated above, each test material (or non-inoculated blank) was wiped with 70% isopropanol prior to inoculation with *B. subtilis* spores; however, this isopropanol wash does not guarantee sterility, especially with the porous materials. Therefore, growth observed in some of the test materials not inoculated with *B. subtilis* spores may have resulted from growth of other microorganisms not affected by the 70% isopropanol wash. This type of assessment may not discriminate between the growth of *B. anthracis* and/or other microorganisms.

Table 6-6. Liquid Culture Assessment of *Bacillus subtilis* Spores

Test Material		Day 1				Day 7			
		S1	S2	S3	Bl	S1	S2	S3	Bl
Industrial-Grade Carpet (IC)	Control	-	-	-	-	-	+	-	-
	Decontaminated	-	-	-	-	-	-	-	-
Bare Wood (BWD)	Control	+	+	+	+	+	+	+	+
	Decontaminated	-	-	-	-	-	-	+	-
Glass (GS)	Control	+	+	+	-	+	+	+	-
	Decontaminated	-	-	-	-	-	-	-	-
Decorative Laminate (DL)	Control	+	+	+	-	+	+	+	+
	Decontaminated	-	-	-	-	-	-	+	-
Galvanized Metal Ductwork (GM)	Control	+	+	+	-	+	+	+	-
	Decontaminated	-	-	-	-	-	-	-	-
Painted Wallboard Paper (PW)	Control	+	+	+	-	+	+	+	+
	Decontaminated	-	-	-	-	+	-	-	-
Painted Concrete (PC)	Control	-	+	+	-	+	+	+	-
	Decontaminated	-	-	-	-	-	-	-	-

S1 = Sample 1
S2 = Sample 2
S3 = Sample 3
Bl = Blank (not inoculated with *B. subtilis* spores)
"+" = growth; "-" = no growth

Qualitative assessment of biological indicators and spore strips are shown in Tables 6-7, 6-8, and 6-9. For all tests using *B. subtilis*, the biological indicators and spore strips not exposed to formaldehyde using the 1414RH unit exhibited growth in the liquid cultures at both 1 and 7 days. No growth in the liquid cultures was observed at 1 and 7 days for the biological indicators and spore strips subject to formaldehyde exposure using the 1414RH unit, with the exception of a single spore strip exhibiting growth at Day 7 for week one of *testing.*

Table 6-7. Liquid Culture Assessment of Biological Indicators/Spore Strips (Week 1 *B. subtilis* Decontamination)

Indicator (Organism)		Day 1		Day 7	
		S1	S2	S1	S2
Biological Indicator (*B. subtilis* ATCC 19659)	Control	+	+	+	+
Spore Strip (*B. atrophaeus* ATCC 9372)	Control	+	+	+	+
Biological Indicator (*B. subtilis* ATCC 19659)	Decontaminated	-	-	-	-
Spore Strip (*B. atrophaeus* ATCC 9372)	Decontaminated	-	-	-	+

S1 = Sample 1
S2 = Sample 2
"+" = growth; "-" = no growth

Table 6-8. Liquid Culture Assessment of Biological Indicators/Spore Strips (Week 2 *B. subtilis* Decontamination)

Indicator (Organism)		Day 1		Day 7	
		S1	S2	S1	S2
Biological Indicator (*B. subtilis* ATCC 19659)	Control	+	+	+	+
Spore Strip (*B. atrophaeus* ATCC 9372)	Control	+	+	+	+
Biological Indicator (*B. subtilis* ATCC 19659)	Decontaminated	-	-	-	-
Spore Strip (*B. atrophaeus* ATCC 9372)	Decontaminated	-	-	-	-

S1 = Sample 1
S2 = Sample 2
"+" = growth; "-" = no growth

Table 6-9. Liquid Culture Assessment of Biological Indicators/Spore Strips (Week 3 *B. subtilis Decontamination*)

Indicator (Organism)		Day 1			Day 7		
		S1	S2	S3	S1	S2	S3
Biological Indicator (*B. subtilis* ATCC 19659)	Control	+	+	+	+	+	+
Spore Strip (*B. atrophaeus* ATCC 9372)	Control	+	+	+	+	+	+
Biological Indicator (*B. subtilis* ATCC 19659)	Decontaminated	-	-	-	-	-	-
Spore Strip (*B. atrophaeus* ATCC 9372)	Decontaminated	-	-	-	-	-	-

S1 = Sample 1
S2 = Sample 2
S3 = Sample 3
"+" = growth; "-" = no growth

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ORIGINAL ARTICLE

Virulent spores of *Bacillus anthracis* and other *Bacillus species* deposited on solid surfaces have similar sensitivity to chemical decontaminants

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anthrax, *Bacillus anthracis*, decontamination, disinfection, simulants, spores, sporicidal test, sterilization.

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Abstract

Aims: To compare the relative sensitivity of *Bacillus anthracis* and spores of other *Bacillus* spp. deposited on different solid surfaces to inactivation by liquid chemical disinfecting agents.

Methods and Results: We prepared under similar conditions spores from five different virulent and three attenuated strains of *B. anthracis*, as well as spores of *Bacillus subtilis*, *Bacillus atrophaeus* (previously known as *Bacillus globigii*), *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus megaterium*. As spore-surface interactions may bias inactivation experiments, we evaluated the relative binding of different spores to carrier materials. The survival of spores deposited on glass, metallic or polymeric surfaces were quantitatively measured by ASTM standard method E-2414-05 which recovers spores from surfaces by increasing stringency. The number of spores inactivated by each decontaminant was similar and generally within 1 log among the 12 different Bacillus strains tested. This similarity among Bacillus strains and species was observed through a range of sporicidal efficacy on spores deposited on painted metal, polymeric rubber or glass.

Conclusions: The data obtained indicate that the sensitivity of common simulants (*B. atrophaeus* and *B. subtilis*), as well as spores of *B. cereus*, *B. thuringiensis*, and *B. megaterium*, to inactivation by products that contain either: peroxide, chlorine or oxidants is similar to that shown by spores from all eight *B. anthracis* strains studied.

Significance and Impact of the Study: The comparative results of the present study suggest that decontamination and sterilization data obtained with simulants can be safely extrapolated to virulent spores of *B. anthracis*. Thus, valid conclusions on sporicidal efficacy could be drawn from safer and less costly experiments employing non-pathogenic spore simulants.

Introduction

Bacillus spores are among the life forms most resistant to inactivation, with examples of spores revived from amber 25–40 million old (Cano and Borucki 1995) or from brine inclusions dated 250-million years old (Vreeland *et al.* 2000). Spores of *Bacillus anthracis* have been considered to be potentially effective biological weapons, and at different times this pathogen has been included in the biological arsenals of several nations (Sherman 1995). The resilience of spores of *B. anthracis* can make the decontamination of surfaces very difficult, making imperative the availability of chemical disinfectants whose efficacy is well known. Remediation of contaminated buildings after the delivery of

anthrax spores via the US mail (Dewan *et al.* 2002) involved multimillion-dollar budgets (with the Trenton and Brentwood postal facilities decontaminated at a cost estimated in $200 million; reviewed in Canter 2005). Scientific issues and commercial considerations promoted a recent increase on the number of products that reportedly inactivated *B. anthracis* spores. However, the vast majority of these products have been tested against Bacillus spores others than *B. anthracis* (Spotts-Whitney *et al.* 2003).

Many genes encoding structural and regulatory proteins are similar in all Bacilli (Driks 2002). In particular, *Bacillus subtilis* and *Bacillus atrophaeus* (formerly named *Bacillus globigii*) spores are extremely similar because of their close phylogenic relationship (Priest 1993). However, there are structural and molecular differences between spores of *B. anthracis* and *B. atrophaeus* or *B. subtilis* spores. These differences could be important as *B. atrophaeus* or *B. subtilis* are generally used as simulants of *B. anthracis* in decontamination studies. Spores of *B. anthracis* differ from spores of *B. subtilis* and *B. atrophaeus* in the composition of proteins in the outer coat (Driks 2002; Kim *et al.* 2004). In addition, spores of *B. anthracis* are surrounded by an exosporium which is absent in spores of *B. subtilis* or *B. atrophaeus*. These differences in outer coat composition and in the presence or absence of exosporium could potentially result in differences in sensitivity to chemical inactivation between *B. anthracis* and *B. subtilis* or *B. atrophaeus*.

Given less stringent biosafety requirements, abundant data are available on decontamination of spores derived from non-pathogenic *Bacillus* species (reviewed in Block 2001). Bacillus spores exposed to biocides in commonly used sporicidal formulations, including glutaraldehyde, formaldehyde, peracetic acid, hydrogen peroxide, chlorine, phenol and heavy metals showed various degrees of inactivation, from a relatively high level (reducing spore contamination by one-million fold which is considered a 6 log reduction) or more, to practically negligible (with survival similar to spores exposed to water as a control) (Sagripanti 1992; Sagripanti and Bonifacino 1996a,b; 1997). Data on the relative efficacy of various sporicidal commercial products on Bacillus spores suggested that commercial liquid sterilants and disinfectants were less effective on contaminated surfaces tan generally acknowledged (Sagripanti and Bonifacino 1999).

Information on the inactivation of *B. anthracis* spores is largely derived from the effect of chlorination treatment on spores in suspension. An earlier report suggested that *B. atrophaeus* spores in suspension could be more resistant to chlorine than *B. anthracis* (Brazis *et al.* 1958). Additional studies have suggested slight differences in sensitivity to chlorine between spores suspensions of *B. anthracis* Ames strain (virulent) and the attenuated Sterne strain (Rose *et al.* 2005). Differential sensitivity has also been reported between *B. anthracis* Sterne spores and spores of *Bacillus thuringiensis* or *B. anthracis* Ames strain (Rice *et al.* 2005). It is difficult to correlate previous data obtained with spores in liquid suspensions to the sensitivity of dry spores on contaminated surfaces as it has been shown that some bacteria are on average 300-fold more resistant to germicides when deposited on contaminated surfaces than in suspension (Sagripanti and Bonifacino 2000).

A review by the Centers for Disease Control and Prevention on available data from 1930 to 2002 made evident the lack of quantitative data comparing the sensitivity of *B. anthracis* spores to that of other Bacillus spores grown and analysed under similar conditions (Spotts-Whitney *et al.* 2003). In addition, (i) the use of spore preparations containing vegetative bacteria or germinated spores, (ii) the potentially different binding to and recovery from carrier materials, and (iii) the use of methods that do not account for all challenged spores or that have unknown recovery may further compromise the limited information available.

It remains unclear whether decontamination protocols used in building and environmental remediation or in medical sterilization/disinfection procedures to be used after a biological attack will be effective in inactivating spores of *B. anthracis*. Great savings in effort and speed in the development of knowledge and countermeasures could be accomplished if all members of the Bacillus family were shown to have similar sensitivity to sporicidal agents. In contrast, grave risk would be taken if assumptions drawn from experiments with simulants proved not to be valid for pathogenic anthrax. The goal of this study was to compare the sensitivity of virulent and attenuated spores of *B. anthracis*, as well as to establish the relative sensitivity of other Bacillus spores grown under similar conditions to inactivation by chemical agents that may be used to decontaminate civilian and military assets after a biological attack.

Materials and methods

Disinfectants

Decon-Green consisting in a mixture of 0·090 g of K_2CO_3, 0·024 g of K_2MoO_4, 1 ml of 50% H_2O_2, 2·8 ml of propylene carbonate and 1 ml Triton X-100 was prepared and used undiluted as previously described (Wagner and Yang 2002). Sodium hypochlorite 6% (commercial Clorox, The Clorox Company, Oakland, CA, USA) was diluted with distilled water and used at a concentration of 5% (v/v chlorine, without adjusting pH) as recommended in the Handbook Medical Management of Biological Casualties (Eitzen *et al.* 1998). DF100 and DF200 are formulations developed by Sandia National Laboratory, US patent number 6566·574 B1 and commercialized by

EnviroFoam Technologies, Inc. (Huntsville, AL, USA). These products were used as recommended by the manufacturer on the product label (http://www.sandia.gov/SandiaDecon/factsheets/factsheets.htm).

Carriers

Rubber

Black rubber material was obtained from the exterior and interior of the face piece of M-40 series military gas protective masks (meeting ECBC/US Army Specification EA-F-1379). The rubber material is made of a proprietary silicone and butyl rubber blend, formulation '2J02' produced by ILC Dover Corporation (Frederica, DE 19946-2080) or formulation '2G06' manufactured by Mine Safety Appliances (Pittsburgh, PA, USA). A number of protective masks were randomly selected, marked with a ruler and cut into 5 × 5 mm using a pair of scissors. The coupons were washed with ethanol (70%) and rinsed with distilled water before storing them. The carriers (together with biosterility markers) were sterilized in an autoclave at 121°C for a minimum of 15 min.

Metal

Light armour used to protect high mobility multipurpose-wheeled vehicles (HMMWV) was obtained by the Engineering Directorate (Edgewood Chemical Biological Center, ECBC, Aberdeen Proving Ground, MD, USA) from the manufacturer AM General Corporation (South Bend, IN, USA, http://www.amgeneral.com). The exterior of this material consisted in an aluminium alloy 5052-H34 camouflage coated with polyurea/polyurethane paint (Chemical Agent Resisting Coating, CARC military specification DIL 64159). A piece of light armour plate was randomly chosen from a large supply and custom-cut at the machine shop of the Aberdeen Proving Ground into 5 × 5 × 1 mm pieces. The metal coupons were cleaned with ethanol, rinsed with distilled water and sterilized in the same way as described for the rubber carriers.

Glass

Clear microscopy glass slides were custom-cut into 5 × 5 × 1 mm pieces by Erie Scientific Company (Portsmouth, New Hampshire, USA). Before use, the carriers were washed with ethanol, rinsed with distilled water, and then autoclaved in the same way described for the other carriers.

Bacillus species and strains

Several virulent strains were generously provided by Melissa Longnecker (US Army Research Institute of Infectious Diseases [USAMRIID], Ft. Detrick, MD, USA) including: (i) *B. anthracis* USAMRIID ba 1087; (ii) *B. anthracis* USAMRIID ba 1029; and (iii) *B. anthracis* LA1 (know also as USAMRIID ba 1088). Some of these strains have been used previously in research at USAMRIID (Little and Knudson 1986). *Bacillus anthracis* Ames was generously provided by Robert Buell [Biological Defense Research Division, US Navy, Washington, DC,]. *Bacillus anthracis* Vollum 1B (V1B) was provided under contract by Amanda Schilling (Naval Surface Warfare Centre, Dahlgren, VA, USA). Attenuated *B. anthracis* strains included Sterne and delta-Sterne provided by Dr Lisa Collins (Edgewood Chemical Biological Center) and Pasteur USAMRIID ba 3132 provided by USAMRIID (Fort Detrick). Other strains used in this study included *B. subtilis 1031*, *B. atrophaeus* ATCC B-385 (formerly known as *B. globigii*), *Bacillus cereus* ATCC 10702, *B. thuringiensis* 4055 (Microbial Genomic and Bioprocessing Research Unit, NCAUR, Peoria, IL, USA), and *Bacillus megaterium* CDC 684 (Carolina Biological Supply Company, Burlington, NC, USA). The identity of stocks of microbial strains was confirmed by analysis with The Crystal Identification System (Becton-Dickenson, Sparks, MD, USA) and by gas chromatographic analysis of fatty acids using instrumentation and software purchased from MIDI Inc (Newark, DE, USA). The plasmid composition of *B. anthracis* strains was confirmed by PCR analysis and it is indicated in Table 1.

Preparation of spores

Pathogenic *B. anthracis* spores were prepared in the BSL3 facility of the Edgewood Chemical Biological Center. All strains of *B. anthracis* and all *Bacillus species* studied here were grown under comparable conditions as previously described (Carrera *et al.* 2006). Fresh overnight cultures of each *Bacillus species* were incubated by rotation at 37°C in 5–10 ml tryptic soy agar (TSA, Difco, Kansas City, MO, USA). Aliquots (400 μl) were spread over the surface of each 150 mm plates (six per strain) containing a modified medium derived from the Schaeffer Sporulation medium (described as sporulation medium S in Schaeffer *et al.* 1965). The agar plates were incubated at 25–37°C until 90–99% phase-bright spores were observed by phase-contrast light microscopy (see below). Spores were harvested and washed with cold sterile distilled ionized (DI) water as previously described (Carrera *et al.* 2006) and stored in DI water at 4°C until use for up to 2 weeks, changing the water at least once a week, or in the freezer at −20°C for up to a month.

Quality control of spores

The quality of spores was determined by two complementary criteria previously established to validate the

Table 1 Characteristics of *Bacillus anthracis* strains used in this study

Strain denomination				
Name	Alternate	Pathogenesis	Plasmids*	Origin†
Ames		Virulent	+/+	Originally isolated in Texas, USA
Vollum 1B	V1B	Virulent	+/+	Derived from Vollum which was isolated in the UK from a cow with anthrax in 1944
Albia	USAMRIID ba 1029	Virulent	+/+	Albia, Iowa, 1963. Originally distributed by Iowa State University. With relatively lower virulence and forming rough colonies
ba 1087	USAMRIID ba 1087	Virulent	+/+	Dundee, Scotland. Isolated from a child treated for cutaneus anthrax
LA1	USAMRIID ba 1088	Virulent	+/+	Isolated in 1983 from an elephant (Loxodonta africana = LA) with anthrax in Etosha, Namibia
Pasteur	USAMRIID ba 3132	Attenuated	−/+	Derived from the original strain attenuated by Pasteur and used as vaccine in 1881
Sterne		Attenuated	+/−	South Africa, Isolated by Sterne in 1937 and used as vaccine in livestock
Delta-Sterne		Attenuated	−/−	As Sterne after the removal of the remaining plasmid

*The presence (+) or absence (−) of capacity to synthesize capsule and toxin are indicated, respectively.
†Origins as reported by Little, S.F., Knudson, G.B. (1986), and by Keim *et al.* (1997) and Price *et al.* (1999).

presence of dormant spores (Sagripanti and Bonifacino 1996a; ASTM 2414-05, 2005). The criteria consisted in the evaluation of (i) the absence of vegetative cells (rods) determined by microscopic examination as described below, and (ii) the survival of spores in hydrochloric acid (2·5 N).

Microscopic analysis

Phase-contrast microscopy was performed using a Leica DMR optical microscope (Leica Microsystems Inc. Bannockburn, IL, USA) to distinguish spores at early stages of germination, which appeared phase dark, from dormant spores, which appeared phase bright. Imaging analysis was achieved with a Leica DC-480 camera (Leica Microsystems Inc. Bannockburn, IL, USA) and Image Pro Express software (Media Cybernetics L.P Silver Spring, MD, USA) as previously described (Carrera *et al.* 2006). Digital pictures were taken of every spore preparation and 200 microbial particles in each preparation were classified as vegetative cells or spores either in phase bright or in phase dark. All preparations used in this study contained less than 11% germinated spores, vegetative- or sporulating-cells, and consisted in 89% or more spores in phase bright as examined by phase-contrast light microscopy.

Acid resistance

Ten microlitres of each spore suspension was mixed with 90 μl of HCl 2·5 N and incubated for 5 min (vortexing every minute) and immediately neutralized with 900 μl of Luria Bertani's (LB) broth + 90 μl NaOH 2·5 N. The titre of spores treated with acid was compared with the *titre of spores without acid* treatment and incubated in distilled sterile water as a control. Spore preparations were acceptable if 90% of spores challenged survived acid treatment.

Sporicidal testing

The efficacy of decontaminant agents was evaluated by employing the ASTM standard E 2414-05 (ASTM 2005) which is a quantitative three-step method (TSM) to determine the sporicidal efficacy of liquids, liquid sprays and vapour and gases on contaminated carrier surfaces (Fig. 1). This method fully recovers treated spores by differential elution (in fractions A, B and C) with increasing stringency (nearly 100% spore recovery calculated as previously reported by the ratio of [the sum spores in fractions A + B + C after treatment with water as a control, divided by the number of spores loaded on each device] × 100, Sagripanti and Bonifacino 1996a,b, 1999). The forces to dislodge spores in each step are different and not interchangeable. Spores loosely attached to carriers are released by washing in A. Those spores bound with higher affinity are released by sonication in B, and those spores still remaining on the coupons are recovered after incipient germination in C (Fig. 1). Briefly, each clean and sterile carrier received 10 μl of a spore suspension containing between 1×10^9 and 5×10^9 organisms ml^{-1} (resulting in a microbial load between 1 and 5×10^7 spores per carrier) and was then dried during 2–4 h at 20–25°C. The carrier loaded with spores was placed inside of a 1·5-ml microcentrifuge tube (labelled A). The disinfectant was added to this tube assuring that the inoculum in the carrier was completely submerged in the fluid. Control carriers did not receive disinfectant but instead received an equal volume of sterile DI water. After 30-min incubation with the disinfectant at room tempera-

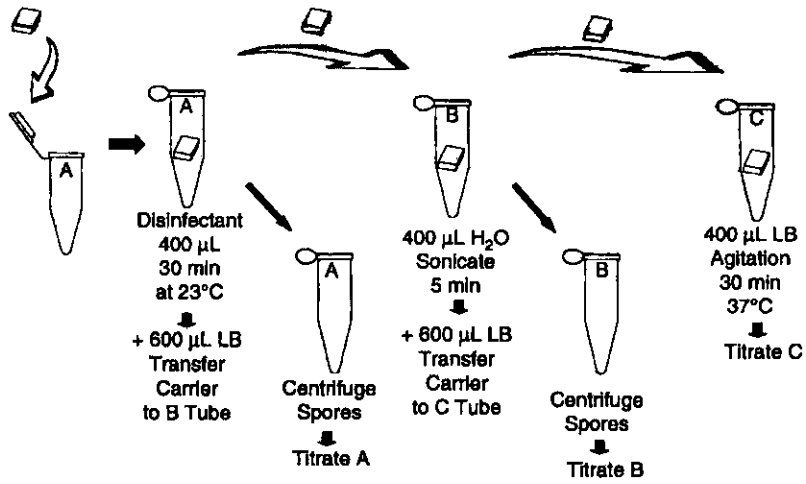

Figure 1 Schematics of the three-step sporicidal method used in this work. Reprinted, with permission, from E2414-05 Standard Test Method for Quantitative Sporicidal Three-Step Method to Determine Sporicidal Efficacy of Liquids, Liquid Sprays, and Vapour or Gases on Contaminated Carrier Surfaces, copyright ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA 19428.

ture (21 ± 3°C), ice-cold LB medium was added. Each carrier was immediately transferred to a new 1·5-ml microcentrifuge tube (labelled B) containing sterile DI water at room temperature and sonicated for 5 min in a low power water-bath sonicator (rated at 400–500 watts, and generally used for cleaning jewellery and other small objects). Ice-cold LB medium was added after which, the carrier was transferred to a new 1·5-ml microcentrifuge tube (labelled C) with LB medium. The tubes were incubated in a rotator inside of an incubator at 37°C for 30 min. Ice-cold LB was added to the tube (C) and the carrier, free from remaining spores, was discarded. The surviving spores in each fraction (A, B and C) were titrated by serial dilution and spread on petri dishes containing nutrient agar medium. Culture plates were incubated overnight at 37 ± 1°C and colonies were counted. Total spores surviving treatment with disinfectant were calculated by adding the spores counted in fraction A, plus spores in fraction B, plus spores in fraction C. The $\log_{10}$ reduction (that is 90% spore inactivation corresponds to 1 $\log_{10}$ reduction, 99% spore inactivation to 2 $\log_{10}$, etc.) of the total spores exposed to the disinfectant was calculated by subtracting the total number of surviving spores from the total number of spores in the controls incubated with sterile water. The assay allowed measuring a 10^7-fold reduction (7 $\log_{10}$) in spore survival relative to those in the untreated controls (Sagripanti and Bonifacino 1996a).

Results

Quality of spores

To properly compare spores from diverse *Bacillus species and different strains of B. anthracis* (described in Table 1), we prepared spores in various media until we identified one (Medium S which is a modification of Schaeffer *et al.* 1965 as described in 'Materials and methods') able to sustain efficient growth and sporulation of all *Bacillus species* studied. A series of techniques involving a variety of reagents, including lysozyme (Prentice *et al.* 1972) and renographin (Tamir and Gilvarg 1966), have been used in other studies to purify spores from their plate or liquid cultures, separating the cells and the germinated spores from the dormant ones. To prevent any reagent from altering the true sensitivity of spores to decontaminating agents, we eliminated cells and accompanying culture debris from our preparations by repeated centrifugation and washing of spore pellets with sterile DI water. A high concentration of cells in logarithmic phase at the time of inoculation in sporulating media was critically necessary in order to obtain spore preparations that passed our quality criteria (as described in 'Materials and methods') with the relatively high proportion of spores shown in Table 2. Acid resistance and microscopic analyses demonstrated that the spores to be challenged with decontaminating agents consisted largely of (phase bright) dormant spores (Fig. 2). Preparing spores of good quality and nearly free of vegetative cells was essential in obtaining reproducible data on the sensitivity of spores to disinfecting agents.

All *B. anthracis* sporulated after 5–6 days of plating. In contrast, *B. cereus* and *B. megaterium* sporulated quite rapidly, achieving 90–95% sporulation between 48 and 72 h after plating. By growing bacteria in TSB media and sporulating in medium S, yields ranged from $6{\cdot}0 \times 10^9$ spores plate^{-1} (*B. anthracis* LA1) to $2{\cdot}2 \times 10^{10}$ spores plate^{-1} (*B. megaterium*).

Two or more batches of each Bacillus spores were prepared and tested below to account for any difference in sporulation between batches.

Table 2 Quality control of spore preparation*

Species and strains	Spores phase bright (%)	Spores phase dark (%)	Cells (%)
Bacillus anthracis ba 1029	90	1·0	9·0
B. anthracis LA-1	89	1·0	10·0
B. anthracis Vollum V1B	90	0·5	9·5
B. anthracis ba 1087	97	1·0	2·0
B. anthracis Ames	90	1·0	9·0
B. anthracis Sterne	97	1·0	2·0
B. anthracis Delta-Sterne	96	1·5	2·5
B. anthracis Pasteur	96	1·0	3·0
Bacillus cereus	99	0·5	0·5
Bacillus thuringiensis	95	1·0	4·0
Bacillus megaterium	98	0·5	1·5
Bacillus subtilis	95	3·0	2·0
Bacillus atrophaeus	96	2·5	1·5

*Spores in early stages of germination (which appear phase dark), dormant spores (which appear as phase bright) and vegetative bacteria (rod shaped) were distinguished by microscopic observation and photographic analysis as described in 'Materials and methods'.

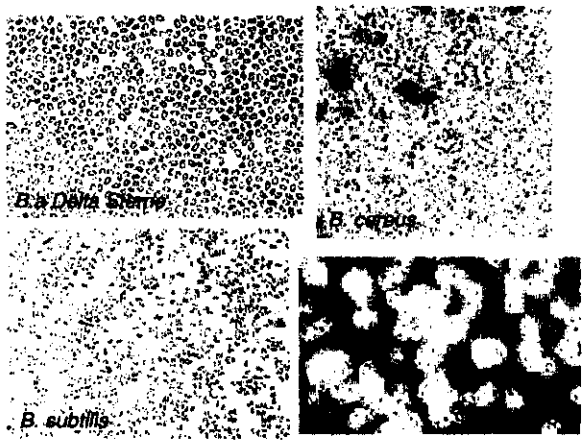

Figure 2 Quality of spores. Phase contrast microscopy at 1000× (total magnification) of *Bacillus anthracis* delta Sterne, *B. anthracis* ba 1087, *Bacillus cereus*, and *Bacillus subtilis* showing more than 95% of phase bright dormant spores.

Effect of surface material

To quantitatively evaluate the interaction of various Bacillus spores with surface materials, we exposed the contaminated carriers to water as a non-sporicidal control and released the spores from the carriers by three steps of increasing stringency (fractions A, B and C). The fractioned elution of *B. subtilis* spores dried onto glass carriers after exposure to water was A > B > C, as expected from a relatively smooth and low-binding material, with

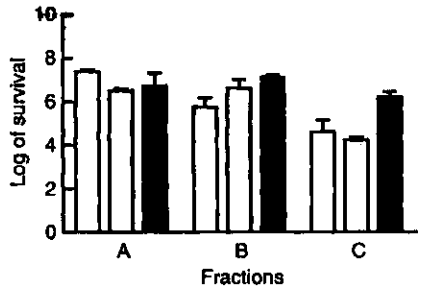

Figure 3 Effect of surface materials. *Bacillus subtilis* spores dried onto glass (empty bars), metal (grey bars), or rubber (black bars) carriers were treated with water (in absence of disinfectants) and eluted in three steps of increasing stringency. Each fraction A, B and C was titrated separately as described in 'Materials and methods'. Bar height represents the mean log of spore survival and the bracket over the bars indicate the standard error obtained in triplicate determinations.

1·6 $\log_{10}$ and 1·1 $\log_{10}$ difference between steps, respectively (empty bars in Fig. 3).

A survey (Engineering Directorate, ECBC, US Army Material Command) revealed that gas masks (for their expected protective role) and light armour (for its wide distribution on military vehicles) were materials whose decontamination was of critical importance. Therefore, we dried spores onto silicone rubber employed in military protective gas mask production and onto a painted metal aluminium alloy used as light armour in military vehicles. The elution profile of *B. subtilis* from glass, metal and rubber is shown in Fig. 3. In both military materials, the mean number of spores in fractions A to B remained relatively constant in contrast to the progressive decrease observed in glass.

Sequential elution of virulent *B. anthracis* spores after drying in military materials and exposure to water is shown in comparison to *B. athropheus* in Fig. 4. In addition, spores from attenuated strains of *B. anthracis* and the other *Bacillus species* studied were also eluted with increasing stringency from metal and rubber carriers (data not shown). To compare any effect of the carrier material, we first counted the number of spores recovered in each fraction (A, B or C) after water treatment of each *Bacillus species* or strain (listed in Tables 1 and 3). Then, we calculated the average $\log_{10}$ number for each fraction (A, B or C) eluted from either metal or rubber among all spore strains and species tested. The $\log_{10}$ averages (± standard deviation, SD, in a number of experiments $n = 12$) from metal and from rubber carriers were 7·25 ± 0·52 and 7·30 ± 0·44 for fraction A; 6·58 ± 0·48 and 6·59 ± 0·53 for fraction B; and 4·82 ± 0·59 and 4·98 ± 0·91, respectively. These similar results obtained for each fraction ruled out a systematic effect of the carrier material in the recovery of spores from metal or rub-

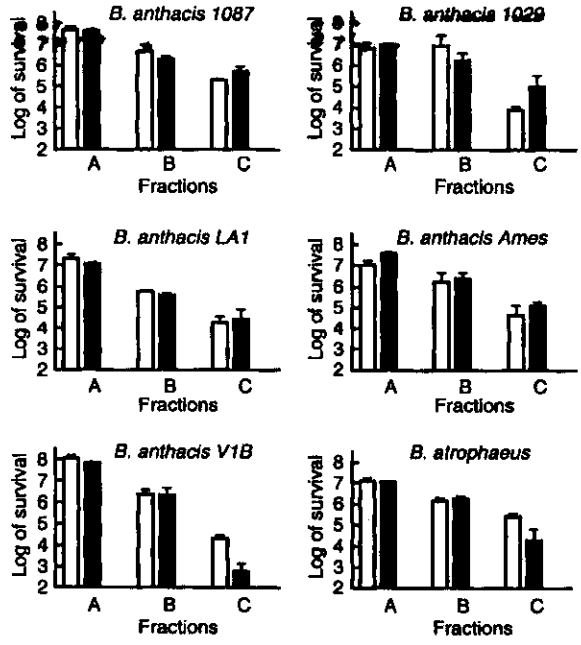

Figure 4 Elution of *Bacillus anthracis* spores from metal or rubber. The different strains of virulent *B. anthracis* indicated in the graphs were tested on metal carriers (grey bars) or rubber carriers (black bars). The elution profile of *Bacillus atrophaeus* is included for comparison. Bar height represents the mean log of spore survival and the bracket over the bars indicate the SE ($n \geq 3$).

ber. The SD of the mean number of spores (obtained for all 13 different Bacillus spores) eluted in each fraction (A, B and C) by water from metal or rubber was 0·3 $\log_{10}$ ($n = 75$). Spores of *B. anthracis* Vollum V1B released relatively easily from rubber (most spores in fractions A and B and fewer in C, Fig. 3). In contrast, the number of spores recovered in fraction C from rubber remained relatively high for *B. subtilis* (Fig. 2) and for *B. cereus* (data not shown) suggesting a relatively stronger interaction between these spores and this particular carrier material.

Sensitivity of *Bacillus* strains

The sensitivity of various strains of *B. anthracis* deposited in military surfaces to a common decontaminating agent (Chlorox) was compared with the sensitivity of *B. subtilis* and *B. atrophaeus* spores. The inactivation by chlorine was similar among all these spores as shown by the results presented in Fig. 5. We investigated whether these similarities would extend to spores of other *Bacillus species* and to treatment with chemically different decontaminating agents. Therefore, we determined the inactivation produced by three additional decontaminating agents that have been proposed for use in biodefense and with chemical compositions that included peroxides and other oxidants. We compared the effect on spores of the same strains of *B. anthracis* tested with chlorine and five addi-

Table 3 Comparative inactivation sensitivity of *Bacillus* spores

	Log reduction							
	Decon green		Clorox		Sandia DF100		Sandia DF200	
	Rubber	Metal	Rubber	Metal	Rubber	Metal	Rubber	Metal
Bacillus anthracis 1029	6·61 ± 0·48	5·84 ± 0·10	6·99 ± 0·17	6·30 ± 0·23	0·05 ± 0·16	0·75 ± 0·36	7·09 ± 0·01	6·50 ± 0·25
B. anthracis V1B	6·10 ± 0·20	8·06 ± 0·01	7·41 ± 0·78	8·06 ± 0·01	3·33 ± 0·03	3·90 ± 0·35	7·86 ± 0·02	8·06 ± 0·01
B. anthracis Ames	4·97 ± 0·10	5·33 ± 0·49	6·32 ± 0·60	5·99 ± 0·77	0·49 ± 0·22	2·54 ± 0·07	6·36 ± 0·44	6·77 ± 0·17
B. anthracis 1087	>7·67*	7·13 ± 0·20	>7·67	7·53 ± 0·40	0·16 ± 0·10	0·22 ± 0·11	7·51 ± 0·27	5·55 ± 0·13
B. anthracis LA-1	6·13 ± 0·40	6·19 ± 0·36	6·10 ± 0·93	6·16 ± 0·88	0·79 ± 0·16	0·85 ± 0·16	6·85 ± 0·30	6·91 ± 0·24
B. anthracis Sterne	5·96 ± 1·02	>7·06	5·94 ± 1·04	6·30 ± 0·97	2·04 ± 0·35	1·75 ± 0·03	>6·97	>7·06
B. anthracis D Sterne	6·74 ± 0·24	5·73 ± 0·27	6·93 ± 0·32	6·70 ± 0·49	1·34 ± 0·04	0·87 ± 0·03	6·75 ± 0·21	5·92 ± 0·03
B. anthracis Pasteur	6·69 ± 0·37	5·93 ± 0·48	7·05 ± 0·94	7·12 ± 0·08	1·70 ± 0·12	0·78 ± 0·10	>7·60	>8·05
Bacillus cereus	6·32 ± 0·28	5·62 ± 0·09	6·33 ± 0·38	5·52 ± 0·09	1·45 ± 0·06	1·08 ± 0·04	6·40 ± 0·28	5·80 ± 1·06
Bacillus thuringiensis	6·77 ± 0·17	6·36 ± 0·04	>6·87	6·91 ± 0·07	1·40 ± 0·56	1·16 ± 0·01	6·7 ± 0·28	7·17 ± 0·54
Bacillus megaterium	7·18 ± 0·64	6·44 ± 0·24	7·09 ± 0·18	7·02 ± 0·72	0·09 ± 0·12	0·02 ± 0·08	7·51 ± 0·24	6·71 ± 0·28
Bacillus subtilis	5·51 ± 0·19	4·68 ± 0·57	6·30 ± 0·33	6·29 ± 0·23	1·90 ± 0·23	1·57 ± 0·11	6·18 ± 0·06	5·73 ± 0·74
Bacillus atrophaeus	5·95 ± 0·11	6·34 ± 0·13	6·28 ± 0·37	6·71 ± 0·23	1·97 ± 0·74	1·76 ± 0·22	6·47 ± 0·74	6·52 ± 0·22

The log of spore reduction relative to the amount of spores in the controls (identically processed after exposure to water). In each independent experiment, the three-step method protocol was performed with spores of one *Bacillus* strain deposited on triplicate carriers of each material and exposed to each decontaminant. The values are the mean log reduction ± SD (standard deviation, $n \geq 3$).

*> in the Table indicates the detection limit when no surviving colonies were obtained.

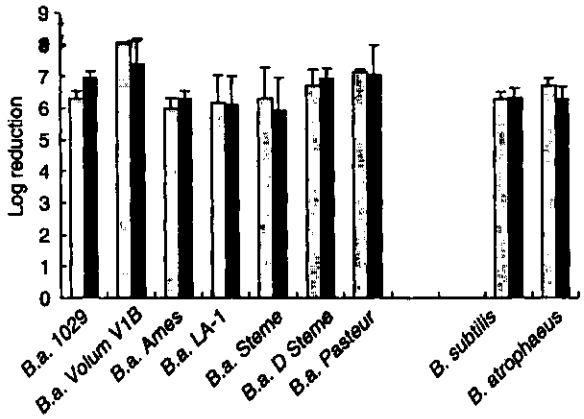

Figure 5 Comparison of *Bacillus anthracis* and its simulants. Bar height represents the log reduction of the number of spores deposited either on metal carriers (grey bars) or rubber carriers (black bars). Log reduction was calculated by subtracting the total number of spores surviving treatment with sodium hypochlorite 5% (v/v) from the total number of spores recovered from carriers exposed to water as a control. Total spores surviving treatment with either hypochlorite or water were calculated by adding the spores counted in fraction A, plus spores in fraction B, plus spores in fraction C, respectively, as described in 'Materials and methods'. The bracket over the bars represents the Standard Error obtained in each of the experiments from triplicate samples. 'B. a'. represents *B. anthracis* spores of the strain specified in the *x*-axis. Gap separates *B. anthracis* strains from other *Bacillus species* (simulants).

tional *Bacillus species* deposited on silicone rubber (protective mask material) or on aluminium alloy (light armour) with the results shown in Table 3. The different spores showed similar resistance to inactivation by the different decontaminating agents. The total number of spores inactivated by each agent was also similar on spores dried on both materials. Three decontaminating agents currently considered for use in military decontamination had a high and similar efficacy (generally above 6 log killing in Table 2). A decontaminating formulation previously considered for use (DF100) showed relatively low sporicidal activity.

The average inactivation from the eight treatments for *B. anthracis* spores (rows in Table 3) ranged between 4·8 log reduction for the Ames strain to 6·6 log reduction for the V1B strain. The average SD for the mean inactivation of all spores and all treatments in Table 3 was 0·31 $\log_{10}$ ($n = 95$), nearly identical to the SD obtained on the binding experiments discussed in the previous section. The average sensitivity (rows) of spores from the five (non-anthracis) *Bacillus species* ranged between 4·8 $\log_{10}$ for *B. subtilis* to 5·4 $\log_{10}$ for *B. thuringiensis*, within the range obtained for spores of *B. anthracis*. Therefore, the relative sensitivities to the tested disinfecting agents appeared similar for the various spores species and strains studied.

Discussion

Some often overlooked parameters that can potentially bias the results from spore inactivation experiments include the use of preparations containing vegetative bacteria or germinated spores, and the use of tests that do not account for all challenged spores.

We subjected each spore preparation to quality-acceptance criteria before testing in order to avoid inactivation results from being confounded by the presence of germinated spores, by more sensitive vegetative cells, or by the chemical reactivity of disinfectants being scavenged by cell debris. As the goal of this study was to compare different spores and not to evaluate the effect of growth conditions, we employed the same growth and sporulation media to sustain the growth and sporulation of all Bacilli used in this study.

Full recovery of all the spores in the inoculums from contaminated negative controls required the fractionated elution of spores in three fractions: (i) consisting in spores loosely attached the surface, (ii) spores dislodged by sonication and (iii) spores released by a short incubation with agitation at 37°C. ASTM Standard E-2414-05 generally known as the TSM (Sagripanti and Bonifacino 1996a,b) was rapid, inexpensive, generated very little waste and quantitatively accounted for all spores challenged.

The lack of significant differences in the data pooled for all spores in rubber or in metal carriers precluded a difference in the relative binding of spores to surfaces that could bias subsequent decontamination studies in military gas mask or light armour. However, spores from different strains of *B. anthracis* and other *Bacillus species* seem to interact slightly different with each carrier, as shown by their elution profiles (Figs 3 and 4). The relative strength of spore binding to metal or rubber was independent of growth conditions (as *B. anthracis* and all other spores were prepared similarly) and was not correlated to virulence or presence of exosporium. Screening for spore binding by the sequential elution method described in this study could assist in identifying surfaces and materials better suited for microbial decontamination and in avoiding other materials where bacterial spores persist more readily.

We observed a similar sensitivity to chlorine among different strains of *B. anthracis* on contaminated surfaces (Fig. 5). This finding is consistent with the fact that strains of *B. anthracis* form a very monophyletic group as shown by genomic sequencing (Price *et al.* 1999). Our findings appear in disagreement with previous observa-

tions where the Ames strain (virulent) appeared slightly less susceptible to chlorination conditions used in water treatment than the attenuated Sterne strain (Rose *et al.* 2005), but lack of standard deviation and slight differences in initial inoculum and chlorine concentration make difficult to assess the statistical significance of the differences previously reported. In a subsequent study, spores of *B. anthracis* Sterne and *B. cereus* in suspension were more sensitive (between 1 and 2 $\log_{10}$) to chlorine than spores of *B. thuringiensis* ssp. Israelensis or *B. anthracis* Ames strain (Rice *et al.* 2005). There is apparent discrepancy between the similar sensitivity among strains of *B. anthracis* that we observed and the slight differences reported by others. Apparently contradicting results could be due either to (i) a differential sensitivity between spores in suspensions as reported previously and similar sensitivity on surfaces, as we observed; (ii) differences in inoculums or preparation conditions among strains or species in studies where these variables were not identical; or (iii) the differences previously reported could be below statistical significance.

In previous studies, *B. atrophaeus* spores in suspension appeared to be more resistant (approximately 2 $\log_{10}$) to free active chlorine than *B. anthracis* spores up to pH 8·6, above which resistance of both species appeared to be equal (Brazis *et al.* 1958). However, when chlorine was expressed in terms of hypochlorous acid, the same concentration was required to produce similar inactivation. We also observed a similar sensitivity of *B. anthracis* and *B. atrophaeus* on contaminated surfaces to unadjusted chlorine (whose pH is near 10).

The mean log reduction of different spores from five different virulent and three attenuated strains of *B. anthracis*, as well as *B. subtilis*, *B. atrophaeus* and the near neighbours *B. cereus*, *B. thuringiensis* and *B. megaterium* inactivated by each decontaminant that we tested was similar and generally within 1 $\log_{10}$ of each other (Table 3). This similarity among Bacillus strains and species was observed after treatment with any of the three agents with high activity as well as after exposure to the product showing low sporicidal activity. Although sporadic and relatively small differences in mean spore reduction were obtained for a given species or strain under a single combination (e.g. the relatively lower value for *B. subtilis* on metal exposed to Decon Green), these differences were not apparent under other conditions, and hence, can be attributed to the statistical variation expected on a relatively large body of data.

Virulent *B. anthracis* Ames strain and *B. subtilis* spores on contaminated surfaces exhibited no significant differences to inactivation by gaseous hydrogen peroxide in five of seven surfaces used as interior building materials (Rogers *et al.* 2005). Thus, the similar sensitivity to liquid agents that we observed for spores on surfaces generally agrees with the similar sensitivity of *B. anthracis* and *B. subtilis* spores reported after gas inactivation. The difference in sensitivity to gaseous inactivation (approximately 1·5 $\log_{10}$) between *B. anthracis* Ames and *B. subtilis* previously reported for the other two substrates (industrial carpet and pine wood) paralleled 1 log reduction difference (10%) in the experimental recovery of both organisms obtained in the untreated controls (Rogers *et al.* 2005). Thus, the apparent difference in sensitivity to gaseous peroxide previously reported could relate to differential recovery, the impact effect of which on sporicidal testing has been discussed previously (Sagripanti and Bonifacino 1996a,b, 1999). Moreover, the previous report of differences to gaseous inactivation could be traced to different conditions reported to prepare spores (*B. anthracis* Ames using a BioFlo fermentor in the laboratory *vs B. subtilis* purchased from a commercial source, Rogers *et al.* 2005).

Overall, the data reported here indicate that the sensitivity of common simulants (*B. atrophaeus* and *B. subtilis*) to inactivation by products that contain peroxide, chlorine or oxidants is similar to that of all the *B. anthracis* strains studied. Our findings of similar spore sensitivity to chemical agents is consistent with the similar sensitivity to UV inactivation (same UV inactivation kinetics) exhibited by *B. anthracis* Sterne and *B. subtilis* spores, as long as both spores were prepared and assayed under identical conditions. (Nicholson and Galeano 2003).

The similar sensitivity that we observed with spores from different species and strains suggests that members of the *Bacillus* genera share an energetically comparable biochemical pathway that ultimately leads to spore inactivation. The comparative results of the present study suggest that decontamination and sterilization data obtained with simulants can be safely extrapolated to spores of *B. anthracis* and indicate that valid conclusions on sporicidal efficacy can be drawn from safer and less costly experiments employing non-pathogenic spore simulants. These findings should assist government agencies and commercial companies involved in biodefense to develop and evaluate more effective sporicidal products.

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EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate B - Scientific Health Opinions
Unit B3 - Management of scientific committees II

OPINION OF THE SCIENTIFIC COMMITTEE ON ANIMAL NUTRITION ON THE SAFETY OF USE OF *BACILLUS* SPECIES IN ANIMAL NUTRITION

(EXPRESSED ON 17 FEBRUARY 2000)

1. BACKGROUND

In its report on the use of certain micro-organisms as feed additives expressed on 26 September 1997, the Scientific Committee on Animal Nutrition (hereafter SCAN) stated that the use of *Bacillus* species may be ill-advised and should be accepted only for clearly defined strains which have been tested negative for toxicity and pathogenicity *in vitro* and *in vivo*.

In June 1999, Denmark drew the attention of the Commission to a number of scientific publications describing the detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. Strains of these species are used in animal nutrition either as microbial feed additives or as a source of enzymes used as feed additives.

2. TERMS OF REFERENCE

In the light of its previous report and of newly available scientific data, SCAN is requested to reassess the safety of the use of bacteria of the genus *Bacillus* (*Bacillus cereus* and other species) in animal nutrition. SCAN is also requested to identify the scientific data which should be provided for the safety evaluation of products using strains of *Bacillus* species submitted for authorisation as feed additives.

3. INTRODUCTION

The genus *Bacillus* contains a number of industrially important species. The large range of physiological types found amongst the bacilli (attributed to the genetic diversity of the genus) and the fact that most species are non-pathogenic and are relatively easy to manipulate and to grow, makes *Bacillus* spp. preferred hosts in the fermentation industry (Arbige *et al.*, 1993). Approximately half of the present commercial production of bulk enzymes derives from strains of *Bacillus* spp. These include proteases (from *B. alcalophilus, B. amyloliquefaciens, B. lentus, B. licheniformis*), α-amylases (from *B. amyloliquefaciens, B. licheniformis, B. stearothermophilus)* glucose isomerase (from *B. coagulans*) and pullulanase (from *B. acidopullulyticus*). Strains of *B. subtilis* are used for the preparation of nucleic acid bases such as inosine which are precursors of flavour enhancing nucleotides for use in the food industry (Priest and Harwood, 1994). These bacteria also produce

lipopeptide surfactants and a diversity of polypeptide "antibiotics" with activity against bacteria and fungi. Some *Bacillus* species (*B. cereus, B. subtilis, B. licheniformis*) have also found use in the animal feed industry. Their addition to diets of pigs, poultry and calves is said to improve performance and the health of livestock. Several products of this nature have temporary approval and are now seeking permanent authorisation for use as feed additives.

Publications appearing in the scientific literature in 1998/9 have suggested that toxin production amongst *Bacillus* species may be far more widespread than previously thought (Beattie and Williams, 1999). One reason for this is that the introduction of more sensitive test methods has allowed the detection of toxigenic effects at much lower concentrations (Andersson *et al.*, 1999; Finlay *et al.*, 1999; Salkinoja-Salonen *et al.*, 1999). The detection of toxin production by current industrial strains would bring into question their continuing use despite a history of *apparent* safe use. Application of a precautionary approach would argue that if genes encoding toxins are present, the level of expression could not be predicted or guaranteed under all circumstances. Where the organism itself may enter the human food chain it would appear prudent to avoid the use of those strains which are potentially toxigenic. However, where bacilli are used as a source of fermentation products the same stringency may not be required. Fermentation conditions are standardised and it is reasonable to assume that toxins, in the unlikely event of their presence, would be produced at a constant low concentration. In these cases, the hazard arises from the possible inclusion and concentration of the toxin(s) in the final product (e.g. enzyme). Since the producer organism itself does not enter the food chain, monitoring of the final product for the absence of toxigenic material may provide sufficient safeguard.

This Opinion examines the extent to which toxin production may be an unrecognised problem amongst some species of *Bacillus* and the implications this may have for their continuing commercial use. Knowledge of the genetic and biochemical basis for toxin production and methods for the detection of *Bacillus* toxins are reviewed and recommendations made for how best to ensure the absence of toxins (or a capacity for toxin production) given the present state of knowledge.

4. TAXONOMY OF *BACILLUS CEREUS* AND RELATED SPECIES

Bacteria that differentiate into endospores under aerobic conditions have traditionally been placed in the genus *Bacillus*. Over the past three decades, this genus has expanded to accommodate more than 100 species (see www.dsmz.de/bactnom/nam0379.htm). A pioneering analysis of 16S ribosomal RNA sequences from numerous *Bacillus* species indicated that the genus *Bacillus* should be divided into at least five genera or rRNA groups (Ash *et al.*, 1991). With the subsequent isolation of many new species this number of "genera" has increased to about 16. Within this framework, *Bacillus subtilis*, the type species, is accommodated in rRNA group 1 or *Bacillus sensu stricto*. Two species groups of interest to this report are included in rRNA group 1, the *B. cereus* group and the *B. subtilis* group. These present very different taxonomic structures.

4.1. The Bacillus cereus group

Bacillus anthracis, B. cereus, B. mycoides, B. thuringiensis and more recently *B. pseudomycoides* (Nakamura 1998) and *B. weihenstephanensis* (Lechner *et al.*, 1998) comprise the *B. cereus* group. These bacteria have highly similar 16S and 23S rRNA sequences indicating that have diverged from a common evolutionary line relatively recently. The guidelines for the delineation of a bacterial species require strains within a species to share more than 70% chromosomal DNA hybridisation and between species less than 70% hybridisation. Strains of *B. anthracis* conform to these guidelines; it is the most distinctive member of this group, both in its highly virulent pathogenicity and taxonomically. On the other hand, DNA from strains of *B. cereus* and *B. thuringiensis* hybridises beyond the 70% limit and extensive genomic studies have shown that there is no taxonomic basis for separate species status (Carlson *et al.*, 1996). Nevertheless, the name *B. thuringiensis* is retained for those strains that synthesise a crystalline inclusion (Cry protein) or delta-endotoxin that may be highly toxic to insects. The *cry* genes are usually located on plasmids and loss of the relevant plasmid(s) makes the bacterium indistinguishable from *B. cereus*. It is now clear that most strains in the *B. cereus* group, including *B. thuringiensis*, carry enterotoxin genes (see section 7).

4.2. The *Bacillus subtilis* group

The *B. subtilis* group traditionally comprises four species: *B. amyloliquefaciens, B. licheniformis, B. pumilus* and *B. subtilis* itself (Claus and Berkeley, 1986; Priest *et al.*, 1988). Recent ecological studies, however, have identified some very close relatives of *B. subtilis*: *B. atrophaeus* (Nakamura, 1989) *B. mojavensis* (Roberts *et al.*, 1994) and *B. vallismortis* (Roberts *et al.*, 1996) and have subdivided *B. subtilis* into subsp. *subtilis* and subsp. *spizizenii* (Nakamura *et al.*, 1999). These taxa all conform to the DNA hybridisation guidelines for bacterial species noted above (section 4.1). The 16S rRNA gene sequences differ between representative species of the *B. subtilis* group, but such data are not available for the recently-described "ecological" group. Species of the traditional group can be distinguished phenotypically, but *B. mojavensis, B. subtilis* and *B. vallismortis* are indistinguishable and can only be identified by molecular means while *B. atrophaeus* is distinguished from *B. subtilis* only by pigmentation. One of the main implications of the inability to distinguish the members of the ecological group is that strains of "*B. subtilis*" being used by industry may actually belong to *B. mojavensis, B. vallismortis* or to other species.

5. *BACILLUS* SPP. AS A HUMAN HEALTH PROBLEM

5.1. Gastrointestinal diseases caused by *Bacillus cereus* and related species

B. cereus is well recognised as a food poisoning organism. Outbreaks can be divided into two types according to their symptomatology. The diarrhoeal type is far more frequent in Europe and USA while the emetic type appears more prevalent in Japan. While the poisonings are usually mild, both types of intoxications have caused deaths. Typical foods implicated are stews,

Biological Indicator for Dry-Heat Sterilization, Paper Carrier

» Biological Indicator for Dry-Heat Sterilization, Paper Carrier, is a defined preparation of viable spores made from a culture derived from a specified strain of *Bacillus subtilis* subspecies *niger*, on a suitable grade of paper carrier, individually packaged in a container readily penetrable by dry heat, and characterized for predictable resistance to dry-heat sterilization. The packaged Biological Indicator for Dry-Heat Sterilization, Paper Carrier, has a particular labeled spore count per carrier of not less than 10^4 and not more than 10^9 spores. When labeled for and subjected to dry-heat sterilization conditions at a particular temperature, it has a survival time and kill time appropriate to the labeled spore count and to the decimal reduction value (*D value*, in minutes) of the preparation, specified by:

Survival time (in minutes) = not less than (labeled *D value*) × (log labeled spore count per carrier − 2); and

Kill time (in minutes) = not more than (labeled *D value*) × (log labeled spore count per carrier + 4).

Packaging and storage—Preserve in the original package under the conditions recommended on the label, and protect from light, toxic substances, excessive heat, and moisture. The packaging and container materials do not adversely affect the performance of the article used as directed in the labeling.

Expiration date—The expiration date is determined on the basis of stability studies and is not less than 18 months from the date of manufacture, the date of manufacture being the date on which the first determination of the total viable spore count was made.

Labeling—Label it to state that it is a Biological Indicator for Dry-Heat Sterilization, Paper Carrier; to indicate its *D value* and the method used to determine such *D value*, i.e., by spore count or fraction negative procedure after graded exposures to the sterilization conditions; the survival time and kill time under the specified sterilization conditions stated on the label; its particular total viable spore count, with a statement that such count has been determined after preliminary heat treatment; and its recommended storage conditions. State in the labeling the size of the paper carrier, the strain and ATCC number from which the spores were derived, and instructions for spore recovery and for safe disposal of the indicator. Indicate in the labeling that the stated *D value* is reproducible only under the exact conditions under which it was determined, that the user would not necessarily obtain the same result, and that the user should determine the suitability of the biological indicator for the particular use.

Identification—The biological indicator organism complies substantially with the morphological, cultural, and biochemical characteristics of the strain of *Bacillus subtilis*, ATCC No. 9372, designated subspecies *niger*, detailed for that biological indicator organism under *Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier*.

Resistance performance tests—

D value—Proceed as directed for the relevant procedure for *D Value* under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. The requirements of the test are met if the determined *D value* is within 20% of the labeled *D value* for the selected sterilizing temperature, and if the confidence limits of the estimate are within 10% of the determined *D value*.

Survival time and *kill time*—Proceed as directed for *Survival Time and Kill Time* in the section *Dry-Heat Sterilization, Paper Carrier*, under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. The requirements of the test are met if all of the specimens subjected to dry-heat sterilization for the survival time show evidence of growth, while none of the specimens subjected to dry-heat sterilization for the kill time shows growth. If for either the survival time test or the kill time test not more than 1 specimen out of both groups fails the survival requirement or the kill requirement (whichever is applicable), continue the corresponding test with 4 additional groups, each consisting of 20 specimens, according to the procedure described. If all of the additional specimens subjected to dry-heat sterilization either meet the survival requirement for the survival time test or meet the kill requirement for the kill time test, whichever is applicable, the requirements of the test are met.

Total viable spore count—Proceed as directed for *Total Viable Spore Count* under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. The requirements of the test are met if the log average number of viable spores per carrier is not less than 0.3 log of the labeled spore count per carrier and does not exceed the log labeled spore count per carrier by 0.48.

Purity—

Presence of contamination by other microorganisms—By examination of the spores on a suitable plate culture medium, there is no evidence of contamination with other microorganisms.

Disposal—Prior to destruction or discard, sterilize it by steam at 121° for not less than 30 minutes, or by not less than an equivalent method recommended by the manufacturer. This includes a test strip employed in any test procedures for the strips themselves.

Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier

» Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier, is a defined preparation of viable spores made from a culture derived from a specified strain of *Bacillus subtilis* subspecies *niger* on a suitable grade of paper carrier, individually packaged in a suitable container readily penetrable by ethylene oxide sterilizing gas mixture, and characterized for predictable resistance to sterilization with such gas mixture. The packaged Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier, has a particular labeled spore count per carrier of not less than 10^4 and not more than 10^9 spores. Where labeled for and subjected to particular ethylene oxide sterilization conditions of a stated gaseous mixture, temperature, and relative humidity, it has a survival time and kill time appropriate to the labeled spore count and to the decimal reduction value (*D value*, in minutes) of the preparation, specified by:

Survival time (in minutes) = not less than (labeled *D value*) × (log labeled spore count per carrier − 2), and

Kill time (in minutes) = not more than (labeled *D value*) × (log labeled spore count per carrier + 4).

Packaging and storage—Preserve in the original package under the conditions recommended on the label, and protect it from light, toxic substances, excessive heat, and moisture. The packaging and container material shall be such that it does not adversely affect the performance of the article used as directed in the labeling.

Expiration date—The expiration date is determined on the basis of stability studies and is not less than 18 months from the date of manufacture, the date of manufacture being the date on which the first determination of the total viable spore count was made.

Labeling—Label it to state that it is a Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier; to indicate its *D value*, the method used to determine such *D value*, i.e., by spore count or fraction negative procedure after graded exposures to the sterilization conditions; the survival time and kill time under specified

sterilization conditions stated on the label; its particular total viable spore count, with a statement that such count has been determined after preliminary heat treatment; and its recommended storage conditions. State in the labeling the size of the paper carrier, the strain and ATCC number from which the spores were derived, and instructions for spore recovery and for safe disposal of the indicator. Indicate in the labeling that the stated *D value* is reproducible only under the exact conditions under which it was determined, that the user would not necessarily obtain the same result, and that the user should determine the suitability of the biological indicator for the particular use.

Identification—The biological indicator organism complies substantially with the morphological, cultural, and biochemical characteristics of the strain of *Bacillus subtilis*, ATCC No. 9372, designated subspecies *niger:* under microscopic examination it consists of Gram-positive rods of width 0.7 to 0.8 µm, and length 2 to 3 µm; the endospores are oval and central and the cells are not swollen; when incubated aerobically in appropriate media at 30° to 35°, growth occurs within 24 hours, and similar inoculated media incubated concomitantly at 55° to 60° show no evidence of growth in the same period; agar colonies have a dull appearance and may be cream or brown-colored; when incubated in nutrient broth it develops a pellicle, and shows little or no turbidity; when examined under conventional biochemical tests for microbial characterization, it develops a black pigment with tyrosine, it liquefies gelatin, utilizes citrate but not propionate or hippurate, reduces nitrate, and hydrolyzes both starch and glucose with no gas production; it shows a positive catalase reaction and gives a positive result with the Voges-Proskauer test.

Resistance performance tests—

D value—Proceed as directed for the relevant procedure for *D value* under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. The requirements of the test are met if the determined *D value* is within 20% of the labeled *D value* for the selected sterilizing temperature and if the confidence limits of the estimate are within 10% of the determined *D value*.

Survival time and *kill time*—Proceed as directed for *Survival Time and Kill Time* in the section *Ethylene Oxide Sterilization, Paper Carrier*, under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. The requirements of the test are met if all of the specimens subjected to the ethylene oxide sterilization conditions for the survival time show evidence of growth, while none of the specimens subjected to the ethylene oxide sterilization conditions for the kill time shows evidence of growth. If for either the survival time test or the kill time test, not more than 1 specimen out of both groups fails the survival requirement or the kill requirement (whichever is applicable), continue the corresponding test with 4 additional groups, each consisting of 20 specimens, according to the procedure described. If all of the additional specimens subjected to ethylene oxide sterilization meet either the survival requirement for the survival time test or the kill requirement for the kill time test, whichever is applicable, the requirements of the test are met.

Total viable spore count—Follow the procedure for *Total Viable Spore Count* in the section *Ethylene Oxide Sterilization, Paper Carrier*, under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. The requirements of the test are met if the log average number of viable spores per carrier is not less than 0.3 log of the labeled spore count per carrier and does not exceed the log labeled spore count per carrier by 0.48.

Purity—

Presence of contamination by other microorganisms—By examination of the spores on a suitable plate culture medium, there is no evidence of contamination with other microorganisms.

Disposal—Prior to destruction or discard, sterilize it by steam at 121° for not less than 30 minutes, or by not less than an equivalent method recommended by the manufacturer. This includes a strip used in test procedures for strips themselves.

Biological Indicator for Steam Sterilization, Paper Carrier

» Biological Indicator for Steam Sterilization, Paper Carrier, is a defined preparation of viable spores made from a culture derived from a specified strain of *Bacillus stearothermophilus*, on a suitable grade of paper carrier, individually packaged in a suitable container readily penetrable by steam, and characterized for predictable resistance to steam sterilization. The packaged Biological Indicator for Steam Sterilization, Paper Carrier, has a particular labeled spore count per carrier of not less than 10^4 and not more than 10^9 spores. When labeled for and subjected to steam sterilization conditions at a particular temperature, it has a survival time and kill time appropriate to the labeled spore count and to the decimal reduction value (*D value*, in minutes) of the preparation, specified by:

Survival time (in minutes) = not less than (labeled *D value*) × (log labeled spore count per carrier − 2); and

Kill time (in minutes) = not more than (labeled *D value*) × (log labeled spore count per carrier + 4).

Packaging and storage—Preserve in the original package under the conditions recommended on the label, and protect it from light, toxic substances, excessive heat, and moisture. The packaging and container materials do not adversely affect the performance of the article used as directed in the labeling.

Expiration date—The expiration date is determined on the basis of stability studies and is not less than 18 months from the date of manufacture, the date of manufacture being the date on which the first determination of the total viable spore count was made.

Labeling—Label it to state that it is a Biological Indicator for Steam Sterilization, Paper Carrier; to indicate its *D value*, the method used to determine such *D value*, i.e., by spore count or fraction negative procedure after graded exposures to the sterilization conditions; the survival time and kill time under specified sterilization conditions stated on the label; its particular total viable spore count with a statement that such count has been determined after preliminary heat treatment; and its recommended storage conditions. State in the labeling the size of the paper carrier, the strain and ATCC number from which the spores were derived, and instructions for spore recovery and for safe disposal of the indicator. Indicate in the labeling that the stated *D value* is reproducible only under the exact conditions under which it was determined, that the user would not necessarily obtain the same result and that the user should determine the suitability of the biological indicator for the particular use.

Identification—The biological indicator organism complies substantially with the morphological, cultural, and biochemical characteristics of the strain of *Bacillus stearothermophilus*, ATCC No. 7953 or 12980, whichever is stated in the labeling: under microscopic examination it consists of Gram-positive rods with oval endospores in subterminally swollen cells; when incubated in nutrient broth for 17 hours and used to inoculate appropriate solid media, growth occurs when the inoculated media are incubated aerobically for 24 hours at 55° to 60°, and similar inoculated media incubated concomitantly at 30° to 35° show no evidence of growth in the same period. When examined under conventional biochemical tests for microbial characterization, it shows a delayed weak positive catalase reaction, it does not utilize citrate, propionate or hippurate, it reduces nitrate, but it does not liquefy gelatin, and it gives a negative result with the Voges-Proskauer test. Organisms derived from ATCC strain No. 7953 show negative egg yolk and starch hydrolysis reactions, while those derived from ATCC strain No. 12980 show positive reactions in both tests.

...istance performance tests—

...D value—Proceed as directed for the relevant procedure for *D ... value* under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. The requirements of the test are met if the determined *D value* ...within 20% of the labeled *D value* for the selected sterilizing ...perature, and if the confidence limits of the estimate are within ...% of the determined *D value*.

...rvival time and *kill time*—Follow the procedure for *Survival ...e and Kill Time* in the section *Steam Sterilization, Paper Carrier*, ...der *Biological Indicators—Resistance Performance Tests* ⟨55⟩. ...e requirements of the test are met if all of the specimens subjected ...the steam sterilization for the survival time show evidence of ...owth, while none of the specimens subjected to the steam ...ilization for the kill time shows growth. If for either the survival ...e test or the kill time test, not more than 1 specimen out of both ...ups fails the survival requirement or the kill requirement ...ichever is applicable), continue the corresponding test with 4 ...itional groups, each consisting of 20 specimens, according to the ...cedure described. If all of the additional specimens subjected to ...am sterilization either meet the survival requirement for the ...rvival time test or meet the kill requirement for the kill time test, ...ichever is applicable, the requirements of the test are met.

...tal viable spore count—Proceed as directed for *Total Viable ...re Count* in the section *Steam Sterilization, Paper Carrier*, under ...logical Indicators—Resistance Performance Tests ⟨55⟩. The ...irements of the tests are met if the log average number of viable ...es per carrier is not less than 0.3 log of the labeled spore count ...carrier and does not exceed the log labeled spore count per ...ier by 0.48.

...ity—

...resence of contamination by other microorganisms—By exam...ion of the spores on a suitable plate culture medium, there is no ...ence of contamination with other microorganisms.

...osal—Prior to destruction or discard, sterilize it by steam at ...° for not less than 30 minutes, or by not less than an equivalent ...hod recommended by the manufacturer. This includes a test strip ...loyed in any test procedures for the strips themselves.

...ological Indicator for Steam ...erilization, Self-Contained

...Biological Indicator for Steam Sterilization, Self-...tained, is a Biological Indicator for Steam Sterili...ion, Paper Carrier individually packaged in a suitable ...tainer readily penetrable by steam and designed to ...d an appropriate bacteriological culture medium, so ...o enable the packaged carrier, after subjection to ...ated steam sterilization conditions, to be incubated ...e supplied medium in a self-contained system. The ...plied medium may contain a suitable indicator as a ...venience for determining by a color change whether ...not spores have survived. The design of the self-...tained system is such that, after exposure to the ...cified sterilization conditions and inoculation of the ...dium under closed conditions as stated in the ...eling, there is no loss of medium and inoculum ...ng subsequent transport and handling, if done ...ording to the provided instructions. The materials ...which the self-contained system are made are such ...there is no retention or release of any substance that ...y cause inhibition of growth of surviving spores ...er the incubation conditions stated in the labeling.

Packaging and storage—Preserve in the original package under the conditions recommended on the label, and protect from light, from substances that may adversely affect the contained microorganisms, from excessive heat, and from moisture.

Expiration date—The expiration date is determined on the basis of stability studies and is not less than 18 months from the date of manufacture, the date of manufacture being the date on which the first determination of the total viable spore count was made.

Labeling—Label it to state that it is a Biological Indicator for Steam Sterilization, Self-Contained; to indicate the *D value* of the self-contained system, the method used to determine such *D value* (i.e., by spore count or fraction negative procedure after graded exposures to the sterilization conditions); the survival time and kill time under the specified conditions stated on the label; its particular total viable spore count, with a statement that such count has been determined after preliminary heat treatment; and its recommended storage conditions. State on the labeling that the supplied bacteriological medium will meet requirements for growth-promoting ability, the strain and ATCC number from which the spores were derived, and the instructions for spore recovery and for safe disposal of the indicator unit. Also indicate in the labeling that the stated resistance characteristics are reproducible only under steam sterilization conditions at the stated temperature and only under the exact conditions under which it was determined, that the user would not necessarily obtain the same result, and that the user should determine the suitability of the biological indicator for the particular use.

Identification—It meets the requirements of the *Identification* test under *Biological Indicator for Steam Sterilization, Paper Carrier*.

Resistance performance tests—

D value—Proceed as directed for the relevant procedure for *D Value* under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. The requirements of the test are met if the determined *D value* is within 20% of the labeled *D value* for the selected sterilizing temperature and if the confidence limits of the estimate are within 10% of the determined *D value*.

Survival time and *kill time*—Follow the procedure for *Survival Time and Kill Time* in the section *Steam Sterilization, Self-Contained*, under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. The requirements of the test are met if all of the specimens subjected to the steam sterilization for the survival time show evidence of growth, while none of the specimens subjected to the steam sterilization for the kill time shows growth. If for either the survival time or the kill time requirement, not more than 1 specimen out of both groups fails the test, whichever is applicable, continue the corresponding test with 4 additional groups, each consisting of 10 specimens, according to the procedure described above. If all of the additional specimens subjected to steam sterilization either meet the survival requirement for the survival time test or meet the kill requirement for the kill time test, whichever is applicable, the requirements of the test are met.

Total viable spore count—Proceed as directed for *Total Viable Spore Count* under *Biological Indicators—Resistance Performance Tests* ⟨55⟩ using the procedure applicable to *Biological Indicator for Steam Sterilization, Paper Carrier*. The requirements of the test are met if the average number of viable spores per carrier is not less than 0.3 log of the labeled spore count per carrier and does not exceed the log labeled spore count per carrier by 0.48.

Medium suitability—

Sterility—Incubate 10 self-contained biological indicator systems at 55° to 60°, or at the optimal recovery temperature specified by the manufacturer, for 48 hours, making sure that there is no contact between the individual spore strips and the supplied medium. Examine the incubated medium visually (for change in color indicator or turbidity) and microscopically (for absence of microbial growth).

Growth promotion of medium prior to sterilization treatment—Submerge 10 self-contained units in a water bath maintained at 95° to 100° for 15 minutes. Start timing when the temperature of the container contents reach 95°. Cool rapidly in an ice-water bath (0° to 4°). Remove the units from the ice-water bath, submerge each spore strip with the self-contained medium, incubate at 55° to 60°, or at the optimal recovery temperature specified by the manufacturer, and examine visually after 48 hours for growth (for turbidity or change in color), and microscopically (for microbial growth). All the speci-

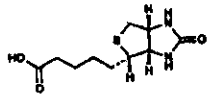
mens under test show growth. If one or more of the specimens do not show growth, repeat the test with 20 additional units. The additional units all show growth.

Growth promotion of medium after exposure to sterilization conditions—Expose the specified number of units for both the *Survival Time* and *Kill time* stated in the labeling, as described in the section *Biological Indicator for Steam Sterilization, Self-Contained* under *Biological Indicators—Resistance Performance Tests* ⟨55⟩. Incubate the spore strips submerged in the self-contained medium according to the instructions of the manufacturer. At the end of the incubation period confirm the existence of growth in each of the specimens that were exposed for each *Survival time* and the absence of growth in each of the specimens that were exposed for each *Kill time* by visual inspection (turbidity or color indicator change) and by separate microscopic examination of each specimen and confirm, where applicable, correspondence of the labeled color to the appearance of growth in the supplied medium.

Ability of medium to support growth after exposure to the sterilization conditions—Take a stated number of units (e.g., 10) after they have been exposed for each *Kill time* stated in the labeling as directed in the preceding section. Aseptically remove and pool the medium from each unit. Prepare a suspension of the indicator microorganism as directed for *Total Viable Spore Counts* under *Biological Indicator for Steam Sterilization, Paper Carrier*. Prepare a dilution of that suspension so as to contain 100 to 1000 viable microorganisms in one mL. Inoculate the pooled medium with enough suspension to contain a total of 100 to 1000 microorganisms in a 10 mL aliquot of not more than the volume from 10 units of the pooled medium. Incubate the inoculated pooled medium as directed for *Total Viable Spore Count*. Clear evidence of growth is obtained within 7 days.

Disposal—Prior to destruction or discard, sterilize it by steam at 121° for not less than 30 minutes, or by not less than an equivalent method recommended by the manufacturer. This includes test strips employed in any test procedures for the strips themselves.

Biotin



$C_{10}H_{16}N_2O_3S$ 244.31

1*H*-Thieno3,4-*d*imidazole-4-pentanoic acid, hexahydro-2-oxo-, 3a*S*-(3aα,4β,6aα)-.

(3a*S*,4*S*,6a*R*)-Hexahydro-2-oxo-1*H*-thieno3,4-*d*imidazole-4-valeric acid [*58-85-5*].

» Biotin contains not less than 97.5 percent and not more than 100.5 percent of $C_{10}H_{16}N_2O_3S$.

Packaging and storage—Store in tight containers.

USP Reference standards ⟨11⟩—*USP Biotin RS*.

Identification, *Infrared Absorption* ⟨197K⟩.

Specific rotation ⟨781S⟩: between +89° and +93°.

Test solution: 20 mg per mL, in 0.1 N sodium hydroxide.

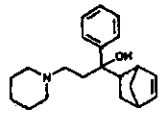
Organic volatile impurities, *Method V* ⟨467⟩: meets the requirements.

Solvent—Use dimethyl sulfoxide.

(Official until July 1, 2007)

Assay—Mix about 500 mg, accurately weighed, of Biotin with 100 mL of water, add phenolphthalein TS, and titrate the suspension slowly with 0.1 N sodium hydroxide VS, while heating and stirring continuously. Each mL of 0.1 N sodium hydroxide is equivalent to 24.43 mg of $C_{10}H_{16}N_2O_3S$.

Biperiden



$C_{21}H_{29}NO$ 311.46

1-Piperidinepropanol, α-bicyclo[2.2.1]hept-5-en-2-yl-α-phenyl-.

α-5-Norbornen-2-yl-α-phenyl-1-piperidinepropanol [*514-65-8*].

» Biperiden contains not less than 98.0 percent and not more than 101.0 percent of $C_{21}H_{29}NO$, calculated on the dried basis.

Packaging and storage—Preserve in well-closed, light-resistant containers.

USP Reference standards ⟨11⟩—*USP Biperiden RS*.

Identification—

A: *Infrared Absorption* ⟨197K⟩.

B: *Ultraviolet Absorption* ⟨197U⟩—

Solution: 900 mg per mL. Transfer about 180 mg of it, accurately weighed, to a 200-mL volumetric flask, add 1 mL of lactic acid, dilute with water to volume, and mix. Absorptivities, at about nm, calculated on the dried basis, do not differ by more than

C: Dissolve about 20 mg in 5 mL of phosphoric acid: a color is produced.

D: Dissolve 200 mg in 80 mL of water with the aid of 0.5 3 N hydrochloric acid, warming, if necessary, to effect solution, then cool. To 5 mL of the solution add 1 drop of hydrochloric and several drops of mercuric chloride TS: a white precipitate formed. To a second 5-mL portion of the solution add bromine dropwise: a yellow precipitate forms which redissolves on shaking, and finally, upon the addition of more bromine TS, a permanent precipitate is formed.

Melting range, *Class I* ⟨741⟩: between 112° and 116°.

Loss on drying ⟨731⟩—Dry it at 105° for 3 hours: it loses not more than 1.0% of its weight.

Residue on ignition ⟨281⟩: not more than 0.1%.

Ordinary impurities ⟨466⟩—

Test solution: methanol.

Standard solution: methanol.

Eluant: a mixture of methanol and ammonium hydroxide (100 : 1.5).

Visualization: 17.

Organic volatile impurities, *Method IV* ⟨467⟩: meets requirements.

(Official until July 1,

Assay—Dissolve about 500 mg of Biperiden, accurately weighed, in 20 mL of benzene, add 2 drops of crystal violet TS, and titrate with 0.1 N perchloric acid VS to a blue endpoint. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 31.15 mg of $C_{21}H_{29}NO$.

Biperiden Hydrochloride

$C_{21}H_{29}NO \cdot HCl$ 347.92

1-Piperidinepropanol, α-bicyclo[2.2.1]hept-5-en-2-yl-α-phenyl-, hydrochloride.

α-5-Norbornen-2-yl-α-phenyl-1-piperidinepropanol hydrochloride [*1235-82-1*].

ORIGINAL

BEFORE THE ILLINOIS POLLUTION CONTROL BOARD

BIOMEDICAL TECHNOLOGY SOLUTIONS, INC., a Colorado Corporation,	)	
Petitioner,	)	
v.	)	PCB 07-149 (Variance Petition)
ILLINOIS ENVIRONMENTAL PROTECTION AGENCY,	)	
Respondent.	)	

AFFIDAVIT OF DIANE R. GORDER

I, Diane R. Gorder, pursuant to 35 IAC 104.204(m) and in support of BioMedical Technology Solutions, Inc.'s ("BMTS") Petition for Variance ("Petition"), state as follows:

1. I am the Director of Regulatory Compliance at BMTS.

2. My responsibilities as Director of Regulatory Compliance include securing the relevant state certifications from each state in which BMTS does business.

3. As the Director of Regulatory Compliance, I have been involved with, and have personal knowledge of, the facts contained in the Petition.

4. Having read the Petition and associated exhibits, I hereby certify that, to the best of my knowledge, information, and belief, the information set forth in the Petition is true and correct and the associated exhibits are true and accurate copies.

Further Affiant Sayeth Naught,

Diane R. Gorder

SUBSCRIBED AND SWORN to before me this 27th day of June, 2007.

NOTARY PUBLIC

DENISE COX NOTARY PUBLIC STATE OF COLORADO My Commission Expires 04-02-2011

GREENBERG TRAURIG, LLP
OPERATING ACCOUNT

012845

Seventy-five and 00/100**********

07/02/07 12845 $ ***75.00***

Illinois Pollution Control Board

BANK ONE

"012845" :071000013: 5330089549"

DATE DESCRIPTION REFERENCE

Check #: 12845 Vendor ID: 55944 Check Date: Jul 02/07

Disb Date	Disbursement Description	Amount
07/02/07	Filing Fees PAYEE: Illinois Pollution Control Board; REQUEST#: 865396; DATE: 7/2/2007. 075745.010100 Fee for Petition for Variance	75.00

Transaction Date: 6/28/07

ILLINOIS POLLUTION CONTROL BOARD
100 W. RANDOLPH ST.
SUITE 11 - 500
CHICAGO, ILLINOIS 60601

INVOICE
28373

PURCHASER/FILER:

Name: JASON B. ELSTER
Firm: GREENBERG TRAURIG, LLP
Address: 77 W. WACKER DRIVE
STE. 2500
City/State/Zip: CHICAGO IL 60601
Phone: 312/456-8400

TRANSACTION TYPE		PAYMENT TYPE		
Opinion/Transcript Document Sale	Filing Fee	Cash	Check	Billable
	X		#012845	

Description	Unit Price	Unit	Amount
RB 07-149	75 00	1	75 00
BOMEDICAL TECHNOLOGY SOLUTIONS, INC.			
V.			
IEPA TOTAL			$75 00

PAID JUN 28 2007