BEFORE THE ILLINOIS POLLUTION CONTROL BOARD

BIOMEDICAL TECHNOLOGY SOLUTIONS, INC., a Colorado Corporation,	)	
Petitioner,	)	AS 08-6
v.	)	~~PCB 07-~~
	)	(Adjusted Standard Petition)
ILLINOIS ENVIRONMENTAL PROTECTION AGENCY,	)	HEARING WAIVED
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 filed with the Office of the Clerk of the Pollution Control Board the Petition for Adjusted Standard of BioMedical Technology Solutions, Inc., a copy of which is herewith served upon you.

Dated: November 28, 2007

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PETITION FOR ADJUSTED STANDARD

Petitioner BioMedical Technology Solutions, Inc. ("BMTS"), by and through its undersigned attorneys, hereby petitions the Illinois Pollution Control Board (the "Board") for an Adjusted Standard from a provision of 35 IAC 1422.[1] BMTS, which manufactures a countertop medical waste treatment device, the Demolizer® technology, seeks a technology-specific Adjusted Standard 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 subspecies of *Bacillus subtilis* commonly referred to as *Bacillus subtilis* var. *niger* (recently reclassified as *Bacillus atrophaeus*) that is the preferred and most appropriate biological indicator organism for the validation of dry heat sterilization processes.

The proposed Adjusted Standard exhibits superior dry heat resistance and can be distinguished from the generic *Bacillus subtilis* primarily through differences in color or pigmentation response to certain media. Importantly, the proposed Adjusted Standard is nationally and internationally recognized by microbiologists and governing standards

[1] BMTS and the Illinois Environmental Protection Agency have agreed to waive a hearing for this petition.

organizations as the preferred and most appropriate biological indicator organism for the validation of dry heat sterilization technologies, the underlying technology of the Demolizer® system. Further, it is the only *Bacillus subtilis* organism available in a tested, certified carrier form. This petition for an Adjusted Standard (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* ("Chemical Indicator"), must be used in the IET. However, ATCC 19659[2] is not commercially available in a certified form, and the procedure for growing and certifying ATCC 19659 to the same standards achieved using the most appropriate *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 "Dry Heat Indicator"), which is commercially available in a certified form and is the scientifically-recognized standard in 46 states as well as the international community for the validation of dry heat sterilization processes due to its superior growth and heat resistance properties.

The Certified and Chemical Indicator organisms are very similar organisms. The Chemical Indicator, *Bacillus subtilis,* is commonly used for the validation of chemical disinfectants and is, therefore, most appropriate for the validation of alternative technologies employing a chemical sterilization agent. The Chemical Indicator is not recognized by international standards organizations or in the scientific literature for the validation of dry heat sterilization technologies.

[2] 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).

The Certified Indicator, *Bacillus subtilis* var. *niger* (reclassified as *Bacillus atrophaeus* in 2004), exhibits enhanced resistance in dry heat applications compared to a generic *Bacillus subtilis* organism, typical of the Chemical Indicator. In a definitive study conducted by Gurney and Quesnel, the dry heat resistance performance of a generic *Bacillus subtilis* and *Bacillus subtilis* var. *niger* were compared at dry heat treatment temperatures ranging form 140 to 170°C. At all temperatures, *Bacillus subtilis* var *niger* demonstrated superior dry heat resistance. The study definitively found that "the var. *niger* strain is clearly the organism of choice as an indicator of dry heat sterilization..." *See* Group Exhibit J,[3] Gurney, T.R. & Quesnel, L.B., *Thermal Activation and Dry-heat Inactivation of Spores of Bacilus subtilis MD2 and Bacillus subtilis var. niger,* J. APPLIED BACTERIOLOGY, 48, 231-247 (1980).

Based on these findings and the preponderance of evidence in the scientific community, the Certified Indicator has been universally adopted as the preferred and most appropriate biological indicator organism for the validation of dry heat sterilization. The following international standards organizations specify the proposed Adjusted Standard, *Bacillus subtilis* var. *niger* (ATCC 9372) as the preferred biological indicator organism for dry-heat processes. Each standards organization convenes an expert panel of microbiologists and specialists in sterilization assurance that review the body of scientific evidence to substantiate their recommendations and published standards. Manufacturers of certified biological indicators must then test each production lot against

[3] True and correct copies of relevant portions of the scientific authorities cited in this Petition are attached collectively hereto as Group Exhibit J.

these standards meeting stringent performance requirements for resistance as measured in D-values and z-values.[4]

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 and ANSI.** Sterilization of health care products – Biological indicators; Part 4: Biological indicators for dry heat processes. Geneva (Switzerland): International Organization for Standardization/ANSI; ISO 11138-4:2006.

BMTS is requesting relief from the Board's requirement of using the Chemical Indicator in the IET and seeks permission to demonstrate the effectiveness of its devices by conducting the IET using the Certified 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 Chemical Indicator in the IET for the Demolizer® technology rather than the Certified or Dry Heat Indicator for the validation of the dry heat sterilization technology.

[4] The D-value is the time required to destroy 90% (1 log10 reduction) of cells under specified conditions while the z-value is the increase in temperature required to reduce the thermal death time by a factor of 10.

II. Regulatory Requirements For Conducting An Initial Efficacy Test

35 IAC 104.406(a) requires that the Petition contain a statement describing the regulation from which an Adjusted Standard 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"). The Agency has agreed that the second and third indicator microorganisms are not scientifically appropriate for verifying the efficacy of the Demolizer® system because they are not recognized for the validation of dry heat systems. The effective date of the regulation is March 1993.

III. Statement of Applicability

As required by 35 IAC 104.406(b), the regulation of general applicability was not promulgated to implement, in whole or in part, the requirements of the CWA (33 USC 1251 et seq.), Safe Drinking Water Act (42 USC 7401 et seq.), or the State programs concerning RCRA, UIC, or NPDES [415 ILCS 5/28.1].

IV. Level of Justification

35 IAC 104.406(c) requires the Petitioner to state whether a specific level of justification is provided in the regulation of general applicability. 35 IAC 1422 does not specify a level of justification or other requirements.

V. Description of the Nature of the Petitioner's Activity

35 IAC 104.406(d) requires a complete and concise description of the nature of BMTS' activity that is the subject of the proposed Adjusted Standard. 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 technology heats one gallon of medical waste to a minimum treatment temperature of 350°F for a minimum of 90 minutes. The Demolizer® technology has demonstrated broad-scale efficacy under these treatment conditions through studies at Stanford University, Kansas State University, and various private laboratories. BMTS has customers in almost every state and has begun marketing the technology world-wide. Further, the temperature profile completely destroys sharps waste through a slow-melting of the plastic components of used syringes. The resulting

melted mass is further contained in the bottom of the metal collector for final disposal as ordinary solid waste.

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 Chemical Indicator was not commercially available in a certified spore carrier form. However, the scientifically similar Certified Indicator, which is the industry standard for validating dry-heat sterilization technologies due to superior heat resistance, 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 Chemical and Certified Indicators are essentially equivalent with primary differentiation based on pigmentation response to certain media. In fact, over 99.8% of their genetic material is *identical* - meaning that, but for their color, the Chemical and Certified Indicators are indistinguishable.[5]

Most importantly, Dr. Marsden determined that the international scientific community, including many of the world's most prestigious standards organizations, recognizes the Certified Indicator as the preferred and most appropriate biological

[5] *See* Group Exhibit J *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).

indicator for the validation of dry heat processes. As cited in the previously, *Bacillus subtilis* var. *niger,* the Certified Indicator, exhibits enhanced resistance in dry heat applications compared to a generic *Bacillus subtilis* organism, typical of the Chemical Indicator.

Therefore, it was the recommendation of Dr. Marsden, consistent with the overwhelming body of scientific literature, to use the commercially available Certified Indicator in the KSU Efficacy Test. This approach poses the most rigorous challenge for the Demolizer® technology and relies on the use of tested and standardized indicator spore carriers.

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.[6] 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

The following provides a discussion of the subspecies reclassification of the *Bacillus* genus that affects *Bacillus subtilis* organisms.

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

Until 1989, the scientific community recognized the Chemical and Certified Indicators as members of the *Bacillus* family commonly referred to as *Bacillus subtilis*. 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 into a subspecies variety called *Bacillus subtilis* var. *niger*. *See id.*

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 J, *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 Certified Indicator belonged to the subspecies

atrophaeus while the Chemical 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 members of the *Bacillus subtilis* and *Bacillus atrophaeus* are phenotypically identical except for color. *See generally*, Group Exhibit J, *infra*.

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 Chemical Indicator in their regulations for use in validation procedures: Arizona, Illinois, and Delaware. In fact, both Arizona and Delaware have reviewed the KSU Efficacy Test that used the Certified 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. Exhibit A, attached hereto, contains regulatory approval documentation from select states, including the States of Arizona and Delaware, which have accepted the Certified Indicator as equivalent to the Chemical Indicator. This information has been previously provided to the Illinois Bureau of Land in September 2007 in support of the Agency's review of this petition.

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 biological testing consistent with the provisions of 35 IAC 1422.125(a)(3).[7] 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 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 B.

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 C. Over the next four months, BMTS periodically contacted the Agency to inquire as to its review of the additional information

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

BMTS provided. On May 7, 2007, BMTS received a response from the Agency that reiterated its prior position.[8] A true and correct copy of the Agency's April 4, 2007 Letter is attached hereto as Exhibit D. The Agency's representative referred BMTS to Agency attorney Bill Ingersoll, who in turn referred BMTS to the Agency Attorney, Kyle Davis.

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 E. Mr. Ingersoll recognized that the Chemical Indicator was not commercially available.[9] Even so, Mr. Ingersoll stated that "it seems that we are unable to help you . . ." *See* Exhibit E. Pursuant to the suggestion of Mr. Ingersoll, BMTS filed a Variance Petition on or about June 24, 2007. (The Variance Petition was subsequently dismissed on July 26, 2007).

On August 24, 2007, BMTS, IEPA, and Agency attorney Kyle Davis, discussed concerns related to the Variance Petition. Dr. Marsden participated in this teleconference to try to answer specific technical questions on the appropriateness of the use of the Certified Indicator in the KSU Efficacy Study. As an outcome of this conference, BMTS agreed to provide additional information supporting the assertion that the Certified Indicator is the preferred and most appropriate biological indicator organism for the validation of dry heat sterilization processes. As part of this effort, BMTS provided the

[8] 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.

[9] The Chemical 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 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.

Agency with additional information regarding the acceptance of the Certified Indicator by other states. *See* Exhibit A.

Dr. Daniel Y.C. Fung, an internationally known food, environmental and public health microbiologist, and authority in the field of sterility control, reviewed the body of scientific literature and provided an assessment on the appropriateness on the use of the proposed Adjusted Standard for the validation of the Demolizer® technology. Specifically, Dr. Fung concludes:

> Based on the overwhelming evidence, it is my expert opinion that *Bacillus subtilis var. niger* (ATCC 9372, also known as *Bacillus atrophaeus)* is the *most appropriate* biological indicator organism for the validation of dry heat sterilization technologies. This specific subspecies of *Bacillus subtilis* demonstrates excellent growth and dry heat resistance characteristics. Standards for performance have been established by USP, ISO, and others to ensure that certified biological indicators for dry heat sterilization deliver predictable and standardized resistance.
>
> The Demolizer® technology is an alternative infectious waste treatment system that employs dry heat as the sterilization agent. As such, the most appropriate biological indicator organism for the validation of the efficacy of the Demolizer® technology is the ISO and USP recognized standard, *Bacillus subtilis* var. *niger* (also known as *Bacillus atrophaeus*). Further, certified carriers manufactured under rigorous quality standards should be used, wherever possible, since such carriers are tested for purity and performance meeting defined D-value and z-value performance criteria.

Letter from Dr. Daniel Y. C. Fung to Diane Gorder, August 27, 2007, a true and correct copy of which is attached hereto as Exhibit F.

Dr. Fung has published extensively in Food Microbiology, Applied Microbiology and Rapid Methods with more than 700 Journal articles, meeting abstracts, proceeding papers, book chapters and books in his career. He has served as the major professor for more than 90 M.S. and Ph.D. graduate students. The Kansas State University Rapid Methods and Automation in Microbiology Workshop, directed by Dr. Fung, has attracted

more than 3,500 participants from 60 countries and 46 states to the program in the past 27 years. Dr. Fung is a Fellow in the American Academy of Microbiology, Institute of Food Technologists (IFT), International Academy of Food Science and Technology and Institute for Food Science and Technology (UK). He has won more than 30 professional awards which included the International Award from IFT (1997), Waksman Outstanding Educator Award from The Society of Industrial Microbiology (2001), KSU College of Agriculture Excellence in Graduate Teaching Award (2005), and the Exceptional Achievement and Founder of the KSU International Workshop on Rapid Methods and Automation in Microbiology Award given by the Director of the Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, 2005. Dr. Fung received the B.A. degree from International Christian University, Tokyo, Japan in 1965, M.S.P.H. at University of North Carolina-Chapel Hill in 1967, and the Ph.D. in Food Technology from Iowa State University in 1969. He is currently a Professor of Food Science, Professor of Animal Sciences and Industry and Ancillary Professor of Biology at Kansas State University and Distinguished Professor Universitat Autonama de Barcelona, Spain.

Based on all of this information, the Agency has agreed to recommend to the Board that it grant this Petition for an Adjusted Standard.

VI. Difficulties Meeting 35 IAC § 1422.Table B

In developing the specific protocol used for demonstrating treatment efficacy, BMTS attempted to acquire the Chemical Indicator in a certified carrier form. Unfortunately, this subspecies is not available commercially in a certified carrier form.

With the help of researchers at Kansas State University, BMTS reviewed a comprehensive scientific literature survey and identified an equivalent subspecies, the

Certified Indicator, as the industry standard for the validation of dry-heat sterilization processes. The overwhelming use of the Certified Indicator as the preferred and most appropriate indicator organism for dry-heat processes stems from its demonstrated excellent dry heat resistance compared to dry heat sterilization compared to other *B. subtilis* organisms. *See* Exhibit F, Letter from Dr. Daniel Fung; Group Exhibit J Gurney, *et al*, for expanded discussion on the appropriateness of the Certified Indicator for the validation of dry heat sterilization processes. The Certified 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 J, *infra*.

BMTS made the decision to use the Certified Indicator because: 1) the indicators are phenotypically identical with the exception of pigmentation response; 2) the Certified Indicator is nearly universally recognized as the appropriate indicator microorganism to demonstrate the effectiveness of dry-heat treatment processes, the underlying treatment technology of the Demolizer® system; 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. Each manufacture must test all production lots against stringent dry heat resistance performance standards as expressed in D-values and z-values. Since the Certified Indicator is indisputably recognized as the most appropriate *Bacillus* indicator organism for dry heat sterilization processes and considered superior, from a heat resistance perspective, to the Chemical Indicator and, unlike the Chemical 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.

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

35 IAC 104.406(e) 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 Chemical Indicator to the same standards already demonstrated in the KSU Efficacy Test. However, BMTS has already conducted a successful IET using the preferred and most appropriate *Bacillus subtilis* indicator microorganism with a dry heat resistance, understood in the scientific community, to be superior to that of the specific species identified in the regulations. Therefore, if the Board were to accept the proposed Adjusted Standard recognizing the overwhelming evidence in the scientific community, BMTS would be in immediate compliance with the Board's regulations.

VIII. Immediate Compliance Would Impose an Arbitrary and Unreasonable Hardship

35 IAC 104.406(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 Chemical Indicator over a Certified or Dry Heat Indicator is inappropriate and would impose an arbitrary and unreasonable hardship because it does not take into consideration the body of scientific evidence that unequivocally supports the claim that the Certified Indicator is most appropriate due to enhanced heat resistance under dry heat conditions.

Further, 35 IAC 1422 has not been updated to include the Certified Indicator as an equivalent alternative *B. subtilis* organism for the validation of dry heat and gas sterilization technologies consistent with the market availability of such sterilization technologies and the consensus within the standards and scientific community. At the time of the adoption of 35 IAC 1422, prevalent sterilization technologies included incineration, steam sterilization, chemical disinfection and radiation. The selection of the specific subspecies in Table B are appropriate and consistent with scientifically recognized indicator organisms for these traditional sterilization processes but are inconsistent with domestic and international standards for the qualification of dry heat treatment processes. These international standards promulgated by the US Pharmacopoeia, International Standards Organization, the U.S. Food and Drug Administration, the European Pharmacopoeia Commission, and others are the primary reason why *B. subtilis* is only available commercially both domestically and internationally as the Certified Indicator used in the KSU Efficacy Study.

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").[10] True and correct portions of the STAATT Report are attached hereto as Exhibit G. The STAATT Report identified the Chemical Indicator strain as a representative example of *Bacillus subtilis*. However, the STAATT Report stressed that the Chemical Indicator spore was only a representative strain of the species and was not selected based on any special resistance properties.

[10] 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.

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 G, 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 Certified 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 H.

This opinion is supported by Dr. Daniel Y. C. Fung, internationally renowned microbiologist. *See* Section V-C of this Petition and Exhibit F for a review of Dr. Fung's analysis on the appropriateness of the Certified Indicator. The 35 IAC 1422 requirements that dry-heat based sterilization processes use the Chemical Indicator as opposed to the Certified or Dry Heat Indicator in the IET is clearly arbitrary.

Moreover, BMTS will incur significant and unreasonable costs if it is required to repeat the KSU Efficacy Test using a different colored indicator microorganism that is likely to exhibit inferior heat resistance in a dry heat sterilization process. After learning of the Agency's position, BMTS requested that KSU prepare an estimate to repeat the KSU Efficacy Test using the Chemical Indicator to the same quality standards as attained in the original KSU Efficacy Study. 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 to the previously conducted study using a certified carrier, 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.[11] 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 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.

[11] 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.

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. A true and current copy of the estimate is found in Exhibit I. This information was also provided to the Illinois Bureau of Land in September 2007 in support of the agency's review of this petition. BMTS would have to sell numerous additional Demolizer® units to make up for the cost of repeating the IET with the Chemical Indicator. Given that the Certified Indicator is reported to demonstrate greater heat resistance than other *Bacilus subtilis* isolates, requiring BMTS to repeat the same efficacy test using a Chemical Indicator is an arbitrary and unreasonable hardship.

Further, BMTS envisions continuous improvements of the technology which may necessitate future IET trials to validate such improvements have not adversely impacted treatment efficacy. The Certified Indicator is the scientifically recognized and widely accepted indicator organism for the validation of the Demolizer® technology. If the Adjusted Standard is not granted, BMTS will continue to incur substantial ongoing costs to conduct efficacy studies using two similar and likely equivalent organisms, the Certified Indicator and the Chemical Indicator organisms. Such duplicate effort is not scientifically justified and is an arbitrary and unreasonable hardship.

IX. Narrative Description of the Proposed Adjusted Standard

35 IAC 104.406(f) requires that the Petition provide a narrative description of the proposed adjusted standard as well as proposed language for a Board order that would impose the standard. The Adjusted Standard would simply involve formally recognizing the appropriateness of both the Certified and Chemical Indicators in Table B of 35 IAC 1422 for the validation of dry heat and chemical sterilization processes, respectively. The

proposed language for a Board order would involve amending Item 1 of Table B **from** "1. Bacillus subtilis (ATCC 19659)," **to** "1. Bacillus subtilis (ATCC 19659) or Bacillus subtilis var. niger (ATCC 9372)." If the Board wishes to recognize the recent change in species classification, the proposed language could read, "1. Bacillus subtilis (ATCC 19659) or Bacillus atrophaeus (ATCC 9372)."

35 IAC 104.406(f) further requires the Petition to describe efforts necessary to achieve this proposed standard and the corresponding costs must also be presented. BMTS has already completed an Initial Efficacy Test demonstrating a 6 $\log_{10}$ reduction of *B. subtilis* var. *niger* (ATCC 9372) under varying load conditions the Agency has been acknowledged meets the requirements of 35 IAC 1422 with the exception of the use of the Certified Indicator instead of the Chemical Indicator. Thus, no additional efforts are required by the Petitioner if the proposed standard is adopted.

X. No Environmental Impact

35 IAC 104.406(g) requires that the Petition describe the quantitative and qualitative description of the impact of the petitioner's activity on the environment if the Petitioner were to comply with the regulation of general applicability as compared to the quantitative and qualitative impact on the environment if the Petitioner were to comply only with the proposed Adjusted Standard. BMTS' activities, operating under either the regulation of general applicability or the proposed Adjusted Standard, have no adverse impact on human, plant, or animal life. This is established by the studies described herein. There are no emissions, discharges or releases from the use of the Demolize® technology. All infectious waste treated in a Demolizer® system meets the requirements for sterilization and final disposal outlined in the regulations.

XI. Justification for the Adjusted Standard

35 IAC 104.406(h) requires that the Petition explain how the Petitioner seeks to justify, pursuant to the applicable level of justification, the proposed adjusted standard. As presented in Section IV of this Petition, the regulation of general applicability does not describe a specific level of justification therefore the level of justification outlined in 35 IAC 104.426 applies. The following outlines a statement of justification for each of the four conditions outlined in 35 IAC 104.426.

A. Change in Factors Relied Upon by the are Substantially Different

35 104.426(a)(1) requires that the Petitioner demonstrate that factors relating to that petitioner are substantially and significantly different from the factors relied upon by the Board in adopting the general regulation applicable to that petitioner. At the time the Illinois regulations were drafted (1992-1993), infectious waste treatment technologies available both domestically and internationally primarily consisted of autoclave or steam sterilization, chemical disinfection, and radiation. The Agency identified scientifically recognized indicator organisms for these classes of sterilization technologies. *Bacillus stearothermophilus* is the internationally recognized indicator organism for the validation of steam sterilization technologies in the same manner that the Certified Indicator is the USP and ISO recognized indicator organism for dry heat. *Bacillus subtilis* (ATCC 19659), the Chemical Indicator, is commonly used for the validation of chemical disinfection processes, disinfectants and hand washing procedures. *Bacillus pumilis* is generally recognized as the appropriate indicator organism for radiation sterilization technologies. During the time period of the adoption of the Illinois regulation, the STAATT committee, a group of state regulatory personnel and infection control

scientists, strongly recommended that the specific subspecies (*Bacillus subtilis, Bacillus stearothermophilus, and Bacillus pumilis)* are for example purposes only and should not be integrated directly into regulations since future technologies may warrant the use of better suited indicator organisms. Section VIII and Exhibit H hereto provide additional supporting evidence to this effect.

In the late 1990s, the Demolizer® technology and other dry heat based systems were formally introduced in the United States for the treatment of infectious wastes. The regulations were, in fact, promulgated in 1993 before the Demolizer technology was formally introduced. Further, published standards for the validation of dry heat sterilization technologies both domestically and internationally converged on the selection of the Certified Indicator in the mid to late 1990s as the most appropriate indicator organism for the validation of such technologies. The specific selection of biological indicators in Table B is consistent with chemical disinfection, steam sterilization, and radiation-based technologies. Table B does not, however, include the Certified Indicator which is specifically optimal for the validation of dry heat sterilization processes.

The Agency acknowledged that an Adjusted Standard may be necessary to address emerging technologies in the Second Notice for Rulemaking (R91-20) published on March 25, 1993. On Pages 19 and 20 of this Notice, the Agency specifically cites the following:

> The record shows that the Study Group and the Agency proposed these provisions to allow easy consideration for new technologies that do not fit the definition of chemical, thermal or irradiation treatment. The Board supports this concept. (Note, dry heat is not specifically listed in the definition of thermal treatment provided in the regulation.)

> The Board emphasizes that it is sympathetic with the concerns of the Agency regarding the administrative burden of adjusted standards. An adjusted standard proceeding is resource consuming not only for the Board, but for the Agency and the petitioning party as well. Accordingly, reliance on the adjusted standard process must be contemplated with care that an unnecessary and onerous administrative burden is not created.
>
> By requiring the Board to grant adjusted standards consistent with Section 27(a), the statute requires the Board to consider the implications of certain site-specific conditions when granting an adjusted standard. As long as information requirements are met to the extent applicable, a technology-specific adjusted standard may be granted.

Therefore, at the time of adoption of the general regulation, dry heat sterilization had not been adapted for the treatment of infectious waste and was not included in the definition of thermal treatment. In the late 1990s, the Demolizer® and other dry heat treatment technologies became available in the U.S. and the international community. The specific organisms listed in Table B of 35 IAC 1422 are consistent with technologies available in the U.S. in the early 1990s. Table B, however, is not consistent with the application of dry heat to treat infectious waste. The Agency and the Board envisioned that adjusted standards may be warranted to include alternative biological indicators on a technology-specific basis. Therefore, the absence of dry heat alternatives at the time of drafting of the regulations is a factor that is substantially and significantly different than factors existing today and warrant adoption of a technology-specific, adjusted standard.

B. Existence of Those Factors Justifies an Adjusted Standard

35 104.406(a)(2) requires that the Petitioner demonstrate that existence of such factors justifies an adjusted standard. As stated above, 35 IAC 1422 is not consistent with the large body of scientific evidence for the selection of appropriate indicator microorganisms for the validation of dry heat medical waste treatment technologies. The scientific consensus in the domestic and international scientific community and the

overwhelming body of evidence justify the use of the Certified Indicator for the validation of dry heat sterilization technologies. Use of a different indicator organism, such as *B. stearothermophilus* or *B. pumilis,* are not recognized in the scientific community for the validation of dry heat technologies. The Chemical Indicator, *Bacillus subtilis* (ATCC 19659) is recognized in the scientific literature for the verification of chemical disinfectants, chemical disinfectant processes and hand washing procedures. The Chemical Indicator is not recognized in the scientific community for the validation of dry heat treatment processes. Similarly, *Bacillus subtilis* var. *niger* (ATCC 9372), the Certified or Dry Heat Indicator, is the biological indicator of choice for dry heat sterilization technologies due to its enhanced heat resistance under such conditions.

Further, the use of certified carriers for the validation of sterilization technologies represents best practices in the scientific community since such certified carriers are manufactured under strict international standards for quality and certification. The Chemical Indicator is not commercially available in a certified form, thus insistence on the use of a carrier that is not recognized for the validation of dry heat technologies and must be grown under non-controlled conditions actually results in a lower quality result. For these reasons, the factors presented hereto justify the proposed Adjusted Standard.

C. No Environmental or Health Effects

35 104.406(a)(3) requires that the Petitioner demonstrate that the requested standard will not result in environmental or health effects substantially and significantly more adverse than the effects considered by the Board in adopting the rule of general applicability. The extensive body of scientific evidence presented herein provides proof that the proposed Adjusted Standard has no adverse environmental or health effect

compared to the standard stipulated in the regulation of general applicability. In fact, the proposed Adjusted Standard is more beneficial. The proposed Adjusted Standard, the Certified Indicator, poses a more difficult challenge for the Demolizer® technology than the Chemical Indicator. BMTS has demonstrated that the Demolizer® technology delivers a minimum 6 $\log_{10}$ reduction of the Certified Indicator consistent with the regulatory disinfection standard.

D. Consistency with Applicable Federal Law

35 IAC 104.406(a)(4) requires that the Petitioner demonstrate that the adjusted standard is consistent with any applicable federal law. The treatment of infectious waste and the approval of alternative treatment technologies are not regulated at the federal level. However, state, federal, and international authorities recognize the use of the Certified Indicator as an appropriate indicator microorganism for dry heat 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 Chemical Indicator in their regulations, Arizona, Delaware, and Illinois, BMTS has already received approval from both Arizona and Delaware based on the KSU Efficacy Test. *See* Exhibit A. The State of New Mexico regulations have recently been updated effective August 2, 2007. The previous draft of the New Mexico

Administrative Code, Solid Waste Regulations cited the *B. subtilis* ATCC 19659 (the Chemical Indicator) as an indicator organism to demonstrate initial efficacy of alternative treatment technologies. In the recently amended N.M.A.C. 20.9.8.13, the State of New Mexico specifically recognizes *Geobacillus stearothermophilus* or *Bacillus atrophaeus* (the Certified Indicator) as appropriate and scientifically recognized indicator organisms for the validation of alternative technologies consistent with the facts and the evidence of scientific consensus described hereto.

The federal government recognizes the appropriateness of using the Certified Indicator to validate sterilization procedures. The U.S. Food and Drug Administration identifies the Certified Indicator as the appropriate test organism for dry-heat based sterilization procedures. *See* Group Exhibit J, *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 Certified Indicator], designated subspecies *niger* . . ." Group Exhibit J, U.S. Pharmacopeia, Monographs: Biological Indicator for Dry-Heat Sterilization, Paper Carrier, USP28-NF23 USP (2005), *infra*.

Moreover, the international community has identified the Certified 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 Certified Indicator as the biological indicator to validate dry-heat sterilization treatments. The world-wide acceptance of the Certified Indicator as the industry standard further supports BMTS' assertion that the Certified Indicator is the most appropriate organism for the validation of dry heat sterilization technologies.

XII. Consistency with Federal Law

35 IAC 104.406(i) requires that the Petition provide supporting reasons that the Board may grant the proposed standard consistent with federal Law. Section XI-D of this Petition provides such a statement. Infectious waste treatment standards are not governed at the federal level. There are no procedural requirements applicable to the Board's decision on the petition that are imposed by federal law.

XIII. Supporting Documents

35 IAC 104.406(k) requires that the Petition cite supporting documents and legal authority. With respect to documents, Exhibits A through Exhibit I 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 J. The scientific literature discussed in this Petition establishes that the Chemical and Certified Indicators are very similar[12], if not equivalent, with the Certified Indicator recognized internationally as the *most appropriate* biological indicator for the validation of dry heat sterilization processes.

[12] In the scientific community, both the Certified and Chemical Indicators have been used to demonstrate efficacy of a particular sterilization technology. The two substrains are very similar with the exception of pigmentation response to certain culture conditions and, prior to 2004, were classified in the same *Bacillus* species. 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 J, 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

Most importantly, Gurney and Quesnel established that the Certified Indicator is the preferred biological microorganism for the validation of dry heat treatment processes in a definitive comparative study. This work surveyed the compendium of published literature on dry heat resistance of *Bacillus subtilis* spores. Further, the authors completed extensive comparative resistance studies on the Certified Indicator and a generic *Bacillus subtilis* organism, typical of the Chemical Indicator, over a temperature

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Exhibit J, 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 J, 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).

Both strains have been used in the validation studies for various oxidative sterilization technologies. In all cases, there was no reported difference in the performance of the two substrains. The Chemical Indicator is broadly used for the validation of disinfectants and chemical disinfection processes. The Certified Indicator is broadly used and recognized as the preferred indicator organism for dry heat sterilization processes due to its demonstrated superior dry heat resistance. The Certified Indicator is also recognized for the validation of certain gas sterilization technologies, including ethylene oxide disinfection. *See generally*, Group Exhibit J; *see Gurney and Quesnel, 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 Certified Indicator (ATCC 9372) to validate dry-heat sterilization treatments).

In a 2004 Environmental Technology Verification Report conducted by Battelle, both the Chemical and Certified 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 J, 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 Certified and Chemical Indicators were shown to be equivalent during an evaluation of ozone and hydrogen peroxide sterilization technologies. *See* Group Exhibit J, 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 Chemical and Certified Indicators were evaluated along with other various strains for sporicidal activity against a broad range of oxidative treatment technologies and found to have resistances "within 1 Log_{10} of each other." Group Exhibit J, 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).

range of 140 to 170°C. At all temperatures, the Certified Indicator demonstrated superior heat resistance properties. Group Exhibit J, Gurney, T.R. & Quesnel, L.B., *Thermal Activation and Dry-heat Inactivation of Spores of Bacilus subtilis MD2 and Bacillus subtilis var. niger,* J. APPLIED BACTERIOLOGY, 48, 231-247 (1980).

The following domestic and international standards list the Certified Indicator for the validation of dry-heat processes. Each standard is developed by an expert panel of microbiologists and sterility assurance specialists who review the body of scientific and published literature to make recommendations based on overall resistance of organisms to a specific sterilization technology. Manufacturers of certified carriers, such as those used in the KSU Efficacy Study, must test each production lot of carriers to meet specific heat resistance targets, as measured in D-value and z-values under specific conditions, to ensure the proper standardization.

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 and ANSI.** Sterilization of health care products – Biological indicators; Part 4: Biological indicators for dry heat processes. Geneva (Switzerland): International Organization for Standardization/ANSI; ISO 11138-4:2006.

See Group Exhibit J.

XIV. Waiver of Hearing

Pursuant to 35 IAC 104.406(j), BMTS hereby waives 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 an Adjusted Standard from the provisions of 35 IAC 1422.Table B recognizing the Certified Indicator as the most appropriate biological indicator organism for the validation of dry heat sterilization technologies.

Specifically, BMTS requests that an Adjusted Standard be granted for the use of *Bacillus atrophaeus* (formerly *Bacillus subtilis var. niger,* also scientifically recognized as ATCC 9372 or NRRL B4418) for the IET of dry heat treatment technologies.

Respectfully Submitted,

BIOMEDICAL TECHNOLOGY SOLUTIONS, INC.

Dated: November 28, 2007

By: _____
One of Its Attorneys

Neal H. Weinfield
Jason B. Elster
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BEFORE THE ILLINOIS POLLUTION CONTROL BOARD

BIOMEDICAL TECHNOLOGY SOLUTIONS, INC., a Colorado Corporation,	)	
Petitioner,	)	
v.	)	PCB 07-_____ (Adjusted Standard Petition)
ILLINOIS ENVIRONMENTAL PROTECTION AGENCY,	)	HEARING WAIVED
Respondent.	)	

CERTIFICATE OF SERVICE

I, Neil H. Weinfield, an attorney, certify that I have caused a true and correct copy of the foregoing ADJUSTED STANDARD PETITION 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

and

Kyle Davis
Illinois Environmental Protection Agency
1021 North Grand Avenue East
P.O. Box 19276
Springfield, Illinois 62794-9276

Dated: November 28, 2007

Neal H. Weinfield

Exhibit A

BMTS
BioMedical Technology Solutions, Inc.

September 28, 2007

Mr. Neal H. Weinfield, J.D.
Greenberg Traurig
77 West Wacker Drive, Suite 2500
Chicago, IL 60601

Re: Regulatory Approvals

Dear Neal:

Based on your recent conversations with Kyle Davis of the Illinois Pollution Control Board, we are enclosing copies of the regulatory approvals in the State of Delaware and the State of Arizona. These are the two states directly referred to in the draft Adjusted Standard Petition that reference the ATCC 19659 strain in their infectious waste regulations. Both states reviewed the Kansas State University efficacy data in 2006 and reauthorized the approval of the upgraded technology.

In addition to these two states, the Demolizer® technology has been formally approved in 20 states and 16 counties. Further, the technology meets or exceeds regulatory requirements for treatment in 23 states that do not formally review technologies for the onsite treatment of low volumes of medical waste. Instead most of these states either formally recognize dry heat treatment consistent with the Demolizer® operating conditions or require generators to have appropriate documentation onsite that demonstrates compliance with state requirements.

We have also included listings from the few states that post approval status on their agency websites. These states include California, Oregon, West Virginia, North Carolina, and Michigan.

Sincerely,

Diane R. Gorder
Director of Regulatory Compliance

Cc: Mr. Don Cox, BMTS

9800 Mt. Pyramid Court
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STATE OF DELAWARE
DEPARTMENT OF NATURAL RESOURCES
& ENVIRONMENTAL CONTROL
DIVISION OF AIR & WASTE MANAGEMENT
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SOLID & HAZARDOUS WASTE
MANAGEMENT BRANCH

TELEPHONE: (302) 739-9403
FAX NO.: (302) 739-5060

September 12, 2006

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

Subject: Approval of Request for Re-Issue and Transfer of Approval Letter for the Demolizer Technology
Reference: File Code: 09.A

Dear Ms. Gorder:

The Delaware Department of Natural Resources and Environmental Control (DNREC), Solid and Hazardous Waste Management Branch (SHWMB) is in receipt of your letter dated September 5, 2006, providing additional information regarding the efficacy data for your company's alternative method to treat infectious waste. Based on the information provided in the September 5, 2006 letter, your company's alternative method to treat infectious waste does meet the regulations set forth in the *Delaware Regulations Governing Solid Waste* (DRGSW).

Please note, any facility installing the proposed system in the State of Delaware must fulfill all the requirements of the DRGSW and will be evaluated on an individual basis. Also, there are additional regulations that will apply if the treated waste is to be disposed of in a solid waste landfill or compacted. Chapter 11, Sections G (1) and (2) state:

> *"1. Infectious waste may not be disposed at a sanitary landfill unless the waste has been rendered noninfectious and non-recognizable.*
>
> *2. Compactors, grinders or similar devices may not be used by a generator to reduce the volume of infectious waste until after the waste has been rendered noninfectious, or unless the device is part of an approved treatment process which renders the waste noninfectious."*

While the DRGSW do afford disposal of infectious waste that is rendered noninfectious and non-recognizable into a solid waste landfill, the decision to accept such waste lies with the operator of the solid waste landfill. In Delaware, this entity is the Delaware Solid Waste Authority (DSWA). Therefore, it is necessary to contact the DSWA at (302) 739-5361 to obtain written disposal approval.

Should you have additional questions please feel free to contact me at (302) 739-9403.

Sincerely,

Nicole E. Hill

Nicole E. Hill
Environmental Scientist
Solid and Hazardous Waste Management Branch

NEH: jmr
NEH0669.doc

cc: Karen G. J'Anthony, Environmental Program Manager I, SHWMB

Janet Napolitano
Governor

ARIZONA DEPARTMENT OF ENVIRONMENTAL QUALITY

1110 West Washington Street • Phoenix, Arizona 85007
(602) 771-2300 • www.azdeq.gov

Stephen A. Owens
Director

December 22, 2006
PRU 06-362

CERTIFIED MAIL
Return Receipt Requested

Ms. Diane Gorder
Sr. VP of Operations
BioMedical Technology Solutions, Inc.
9800 Mt. Pyramid Court
Suite 350
Englewood, CO 80112

Re: Biohazardous Medical Waste Alternative Treatment
Method Registration No. MWAT221206.00
BioMedical Technology Solutions, Inc.

Dear Ms. Gorder:

The Arizona Department of Environmental Quality (ADEQ), Solid Waste Plan Review Unit (PRU), received your application dated September 5, 2006, which included product information, efficacy test results, and other supporting documents for the State of Arizona Biohazardous Medical Waste Alternative Treatment Method Registration for the Demolizer II System manufactured by your company. PRU reviewed your submittal and based on the information submitted, it appears that the biohazardous medical waste treatment technology for Demolizer II System is capable of achieving high level disinfection of biohazardous medical wastes without adversely impacting public health or the environment.

In accordance with Arizona Administrative Code (A.A.C.) R18-13-1414.A, ADEQ approves the Demolizer II System unit outlined in the operator's manual as a biohazardous medical waste alternative treatment technology system. This Biohazardous Medical Waste Alternative Treatment Method Registration shall be deemed effective as of the date of this letter and the accompanying certificate.

Administrative, Operational, and Other Conditions

1. This approval is granted only for the Demolizer II System technology used in the efficacy studies and should not be construed as a general endorsement of any other model(s) or system(s). Any modification of the approved system will require separate approval by ADEQ and may involve further efficacy testing.

2. This approval does not relieve BioMedical Technology Solutions, Inc. or any person, organization, or facility using or intending to use the Demolizer II System, from obtaining any other approvals which may be required by federal, state, county or local agencies, or Indian Nations which may have additional regulations within their respective jurisdiction.

3. This approval does not relieve BioMedical Technology Solutions, Inc. or any person, organization, or facility using or intending to use the Demolizer II System of its responsibility to comply with federal, state, county, or local requirements and shall not be construed as permission to create a public health hazard, environmental nuisance, or cause contamination to the environment as prohibited by Arizona Revised Statutes (A.R.S.) 49-141.A.8.

4. This system is not to be used to treat chemotherapy waste, radioactive waste, and anatomical wastes, or any identifiable human body parts.

5. Notification of liquid discharge management practices shall be submitted to the local water/waste water authority serving the facility where the Demolizer II System is installed prior to discharge. All discharge must comply with pretreatment requirements.

6. Arizona municipal solid waste landfills are prohibited from accepting liquids or waste that failed the EPA paint filter test, in accordance with 40 § CFR 258.28. Therefore, processed waste must be dewatered to the extent that it will pass the EPA paint filter liquid test prior to placement into the landfill.

7. Prior to operating a biohazardous medical waste treatment facility, the owner/operator shall obtain a facility plan approval in accordance with A.A.C. R18-13-1410, and shall meet the treatment standards as described in A.A.C. R13-1415, if off-site waste is to be received.

8. This Certificate is not transferable and is valid for 5 years from the issuance date. Please notify ADEQ within 14 days after any changes to the information in your application occur.

9. This Arizona Biohazardous Medical Waste Alternative Treatment Method Registration constitutes an appealable agency action pursuant to A.R.S. § 41-1092 et seq. To obtain an administrative hearing on ADEQ's decision, a Notice of Appeal ("Notice") must be filed with ADEQ within thirty (30) days of receipt of this letter. The notice must contain the following:

   i. The name of the person or party filing the appeal.

   ii. The address of the person or party filing the appeal. (The person or party must notify ADEQ of any change of address within five (5) days of the change).

   iii. The name of the Agency whose decision is being appealed. (In this matter, the agency is the Arizona Department of Environmental Quality).

   iv. The action being appealed.

   v. A concise statement of the reasons for the appeal.

   The notice must be filed with ADEQ. Send the notice to:

   Hearing Administrator
   Office of Administrative Counsel
   Arizona Department of Environmental Quality
   1110 West Washington Street
   Phoenix, Arizona 85007.

Notice should be filed by either mailing the notice by certified mail, return receipt requested, or by hand delivery to ADEQ. The hearing will be conducted by an administrative law judge from the Office of Administrative Hearings. ADEQ will notify the parties of the hearing date at least thirty (30) days prior to that date. If the appealing party wishes to try to settle this matter before the administrative hearing occurs, that party must file a request for an informal settlement conference with the Hearing Administrator. This request must be filed no later than twenty (20) days before the hearing date. ADEQ will hold the settlement conference within fifteen (15) days of receiving the request. Filing an informal settlement request does not change the date of the hearing.

If you have any questions about this letter, please call Maria Sachs, Solid Waste Permits Plan Review Unit, at (602) 771-4670 or toll free at (800) 234-5677, Ext. 771- 4670.

Sincerely,

Amanda E. Stone, Director
Waste Programs Division

cc: Mindi Cross, Manager, Inspection and Compliance Section

Enclosure

ARIZONA DEPARTMENT OF ENVIRONMENTAL QUALITY

BIOHAZARDOUS MEDICAL WASTE ALTERNATIVE TREATMENT METHOD Registration No. MWAT221206.00

In accordance with Arizona Administrative Code Title 18, Chapter 13, Article 14

Registration issued to: ***BioMedical Technology Solutions, Inc.***

This Registration for Arizona Biohazardous Medical Waste Alternative Treatment is issued to the above named company or entity and is to be used for treatment of biohazardous medical waste only in accordance with instructions supplied by the company. This registration is deemed effective on the Issue Date below and expires on the Expiration Date below (5 years after the Issue Date).

This registration is granted based upon the information provided in the Application for Arizona Biohazardous Medical Waste Alternative Treatment Registration. This registration is not transferable from one company or entity to another.

Amanda E. Stone, Director
Waste Programs Division

ISSUE DATE: ***December 22, 2006***
EXPIRATION DATE: ***December 22, 2011***

Alternative Medical Waste Treatment Technologies Approved by the California Department of Public Health

Effective Date: August 22, 2007

The technologies listed below have been approved by the Department of Public Health to treat medical waste in California. The approval may be limited to certain types of wastes. Please review the information provided to verify the approved uses of each technology. Individuals interested in the products described in this document are encouraged to contact the company directly.
Alternative Technology Approval is based solely on a product's demonstration of pathogen destruction. Putting the technologies to use may require permitting as on-site or off-site medical waste treatment. Sections 118130 and 118135 of the Medical Waste Management Act require that any offsite medical waste treatment facility obtain a permit from the Department before treating medical waste. Permitting of onsite facilities is addressed in 117925 (a) and 117950 (a) of the Medical Waste Management Act.

Company	Device	Address	Telephone	Web Site	Type of Treatment	Approved for treatment of:
BioMedical Technology Solutions, Inc.	Demolizer System	9800 Mt. Pyramid Court, Suite 350 Englewood, CO 80112	303-653-0100 866-525-BMTS	www.bmtscorp.com	Heat	red bag /sharps
Earth-Shield Company	Sharp-Shield	304 Yampa Street Bakersfield, CA 93307	661-322-0300	www.earth-shield.com	encase in cement	sharps
GMS Marketing Services	Sterimed	191Hempstead Turnpike West Hempstead, NY	516 (800)483-1403	www.globalmarketingservices.org	chemical	red bag/sharps
InEnTec Medical Services, LLC	Plasma Enhanced Melter	1935 Butler Loop Richland, WA 99352	509-946-5700 949-472-3713	www.inentec.com	heat	red bag /sharps /path /trace chemo /pharms
International Marketing	Needlyzer	2119 North Kenmore Ave. Chicago, IL 60614	773-528-2652	www.needlyzer.com	heat	sharps
Isolyser	Sharps Management System (SMS)	6054 Corte Del Cedro Carlsbad, CA 92009	866-436-9264	www.wcminc.net	encapsulate within container	sharps
Isolyser	ORex Processor	512 Lehmberg Road Columbus, MS 39702	662-327-1863 800-824-3207	www.orex.com	chemical	red bag
Kvaerner U.S. Inc	Encore 2000 RWP	116 Roddy Avenue South Attleboro, MA 02703-7974	508-399-6400		chemical	red bag/sharps
Medical Innovations, Inc	TAPS	P.O. Box 148 Wayland, MA 01778	508-358-8099 508-358-2131		heat	sharps
Medical Safe TEC	LFB 12-5, SF 15C	330 West Center Street North Salt Lake, Utah	801-209-6582 801-936-0112 Fax	www.medwastetec.com	chemical	red bag/sharps
Metrex Research Corp	PermiCide-CA	1717 W. Collins Ave. Orange, CA92867	800-841-1428	www.metrex.com	chemical	Suction canister only

Document can be found on the California Department of Health Services website at http://www.dhs.ca.gov/medicalwaste

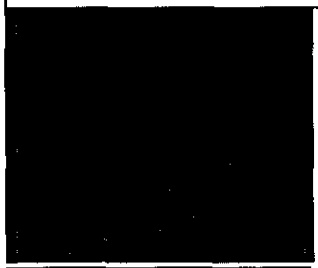
Oregon
Theodore R. Kulongoski, Governor

Department of Human Services
Public Health Division
800 NE Oregon Street
Portland, OR 97232-2162
(971) 673-1111 Phone
(971) 673-1100 Fax
(971) 673-0372 TTY-Nonvoice

August 01, 2007

ALTERNATIVE INFECTIOUS WASTE TREATMENT PROCESSES APPROVED BY THE PUBLIC HEALTH DIVISION FOR USE IN OREGON

PROCESS	***DATE APPROVED***
MEDWASTE TEC	11/18/1991
MODEL Z-5000 HC	
MODEL Z-12,500	
LFB 12-5/MST Z-12,500	11/19/2004
ECOMED I	12/24/1992
WINFIELD CONDOR	02/02/1993
MEDICLEAN IWP-1000	02/08/1993
STERICYCLE ETD	03/15/1993
DEMOLIZER	
TWT	03/06/1995
BMTS	10/17/2006
ABB SANITEC MICROWAVE	03/21/1995
STERIS/ECOMED ECOCYCLE 10	07/24/1995
MEDICLAVE	11/13/1995
STI CHEM-CLAV/MODEL STI-2000CV	07/07/1997
STERIMED	02/17/1999
RED BAG SOLUTIONS SSM-150	03/27/1999
PREMICIDE	06/17/1999
STERIMED JUNIOR	07/02/2002
IET PLASMA ENHANCED MELTER	04/26/2004



Information

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Alternative Treatment Products

Autoclave Information

Definition of Infectious Medical Waste

Documents

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Alternative Treatment Technologies

Click here for information on how to submit a new alternative treatment technology for approval.

Approved Solidifiers

Product	Notes	Company	Address	City	State	Zip
Isolyser LTS **Plus**	24 hour hold time	Microtek Medical, Inc.	512 Lehmberg Road	Columbus	MS	39702
Premicide	12 hour hold time	OBF Industries, Inc.	2719 Curtiss St.	Downers Grove	IL	60515

*All solidification products are subject to a hold time prior to disposal. Hold times allow the product to effectively disinfect the contents of a suction canister, as well as to set up. Healthcare facilities are required to ensure the proper disposal of solidified liquids.

At this time, only these two products can be used to solidify and disinfect liquid infectious medical waste intended to be land filled.

Any other solidification products may be used as long as suction canisters are disposed in the infectious medical waste.

It is a violation of the West Virginia Infectious Medical Waste Rule if you do not follow the manufacturer's instructions for use for any of these products.

Alternative Treatment Technologies

Product	Notes	Company	Address	City	State	Zip
HGA-100S, M, & -250M	Microwave treatment	ABB Sanitec, Inc.	155 Route 46, West Plaza II	Wayne	NJ	07470
Model HGA-250-S	Microwave treatment	ABB Sanitec, Inc.	155 Route 46, West Plaza II	Wayne	NJ	07470
Demolizer II System	Heat treatment	BioMedical Technology Solutions, Inc.	9800 Mt. Pyramid Court, Suite 350	Englewood	CO	80112
DSI System 2000	Heat treatment w/ reusable sharps box	Disposal Sciences, Inc.	6352 320 Street Way P.O. Box 487	Cannon Falls	MN	55009
ZMD-M3	Stream treatment with shredding	GTH Roland North America, Inc.	7887 Katy Freeway, Suite 200	Houston	TX	77024
Isolyser LTS Plus	Liquid solidification and	Isolyser Company	4320 International Blvd.	Norcross	GA	30093

North Carolina Department of Environment and Natural Resources

DIVISION OF WASTE MANAGEMENT

Educating today for a cleaner tomorrow

Technical Assistance, Education & Guidance >> Innovative Technology >> Medical Waste

Alternative Medical Waste Treatment Technologies

Many hospitals and medical waste treatment facilities have discontinued use of incinerators because of increased c costs of operating medical waste incinerators have risen mainly because of the recently enacted EPA regulations fo Hospital/Medical/Infectious Waste Incinerators (HMIWI).

A number of new technologies have been developed which minimize or nearly eliminate environmental discharges. summary of some representative technologies is presented in USEPA Alternate Treatment Technologies Fact Shee

Applying for State Approval in North Carolina

It is necessary to obtain state approval to operate a new treatment technology in North Carolina. Vendors who woul seek approval of a medical waste technology may submit information to obtain approval. Vendors should submit:

- General description of the treatment system
- Efficacy data on representative microorganisms (a full report is required which includes complete information experimental design, unsummarized and summarized data, and qualifications of the testing laboratory)
- Description of any environmental discharges
- Information on worker safety aspects
- Additional information may be required.

For further information please contact the Solid Waste Section at (919-508-8512).

Guidance and Assistance in Evaluating New Treatment Technologies

Guidance for Evaluating Medical Waste Treatment Technologies - EPA publication which provides guidance on how evaluate new technologies

Some types of technology require registration under the Federal Insecticide, Fungicide, and Rodenticide Act

Technologies Approved for Use in North Carolina

The following is a list of alternative medical waste treatment technologies approved for use in North Carolina. Comp products previously listed are either out of business or no longer market formerly approved products in North Caroli

Company	Product Name	Technology Type	Contact Information
WPS Company Waste Processing Solutions 3431 Benson Ave. Suite 100 Baltimore, MD 21227	SSM 150/75 Approved: Feb. 5, 2003	Superheated Water	Sanford A. Glazer Directo Technology ph: 443-524-4245 ext.#14 Fax: 443-524-4250 Cell:301-254-2234 Email:sglazer@redbag.co redbagwps@aol.com Website: www.redbag.cor

M.C.M Environmental Technologies, Inc. One Parker Plaza Fort Lee, NJ 07024	SteriMed Approved: July 28,1999	Chemical Disinfection	Karen Albretsen Project Manager Ph: 201- 242-1222 1-800-783-7463 Fax: 201-592-0393 www.mcm-sterimed.com
Med WasteTec, Inc. 2200 South 400 West Salt Lake City, Utah 84115	LFB-12-5 (Medical SafeTEC "MST" series) Approved: November 15, 1991	Chemical Disinfection	Dennis Cox Ph:801-845-6550 877-485-6550 Cell:801-718-4904 Fax: 801-484-6417 Dennis.c@medwastetec.c www.medwastetec.com
Sterile Technology Industries, Inc. 5725 West Minnesota St. Indianapolis, IN 46241	STI ChemClave Approved: May 24,1996	Shred/Heat/ Chemical (sodium hydroxide)	Randall McKee, Pres., CE Ph:317-484-4200 Fax: 317-484-4201 http://www.wr2.net/
Steris Corporation 5960 Heisley Rd. Mentor, Ohio 44060	Ecocycle 10 – no longer Marketed, but company will continue to support models still in use. Approved: Oct. 20, 2001	Shred/chemical sterilant (peracetic acid)	Richard Snead Ph:1-800-989-7575 Ext.2 Fax:440-639-4450 www.steris.com
Biomedical Technology Solutions, Inc. 9800 Mt. Pyramid Court, Suite 350 Englewood, CO 80112	Demolizer Approved: Feb. 2, 1994	Thermal Treatment	Don Cox, Pres. Diane Gorden, Sr. VP of Operations Ph: 1 866-525-2687 Fax: 303-653-0120 BMTSCORP.com
Waste & Compliance Management, Inc. 6054 Corte Del Cedro Carlsbad, CA 92009	Isolyser Approved: Oct.13,1989	Sharps Disposal System	Keri Tucker Ph: 866-436-9264 Fax: 760-930-9225 www.wcminc.net email: service@wcminc.n
Waste Reduction By Waste Reduction, Inc. 2910-D Fortune Circle West Minnesota St. Indianapolis, IN 46241	WR^2 Animal Tissue Digester Approved: Sept.18, 2001	Alkaline Hydrolysis, Superheated water	Gordon I Kaye, Ph.D. Chairman Ph:317- 484- 4200 Fax: 317- 484- 4201 Website: http://www.wr2.r
Ozonator Industries 1850 Industrial Drive PO Box 26030 Regina, Saskatchawan Canada S4R 897	Ozonator Approved: July 17, 2006	Shred / Ozone	Randy Johnson Ph: 306-791-0900 Fax: 306-791-0905 www.ozonatorindustries.c

Revised November 2006, send questions to: William.Patrakis@ncmail.net

Regulated Medical Waste Treatment Providers

MICHIGAN DEPARTMENT OF ENVIRONMENTAL QUALITY
CURRENTLY APPROVED ALTERNATE TREATMENT TECHNOLOGIES

VENDOR	TECHNOLOGY	MEDICAL WASTE TREATED*
Baker, R.E., Inc.	Autoclave/Shredder	1,2,4
Biomedical Technology Solutions, Inc.	Dry Heat Oven	2,3,4
BioSteril Technology, Inc. (Biosiris)	Radiation Sterilization (Electron Beam)	1-5
Disposal Services, Inc.	Dry Heat	2,4
ECODAS	Autoclave	1,2,4
Healthcare Products Plus, Inc. (Needlyzer)	Electric Arc/ Disintegration	4
IET Plasma Enhanced Melter™ (PEM™)	Plasma Arc	2,3,4
Med Mark International, Inc (Medaway 1™)	Dry Heat (Infrared) Chamber	2,3,4
Medical Safe Tec	Shredder/Chemical	2,3,4
NIC Americas, Inc. (Nic Safe 1800)	Electric Oxidation	4
OBF Industries Inc. (Premisorb, Premicide, Vitalcide)	Solidifier/Sanitation	2
PEAT International, Inc. (Plasma Thermal Destruction and Recovery--PTDR™)	Plasma Arc	2,3,4
Peerless Waste Reduction, Inc. (formerly WR^2)	Chemical	1,2,3,5
Red Bag Solutions (Formerly Antaeus Group, Inc.) (SSM)	Autoclave/Maceration	1,2,4,5
San I Pack	Autoclave/Shredder	1-5
Sanitec	Microwave/Shredder	2,3,4
Spintech, Inc.	Dry Heat Oven	4
Stericycle, Inc.	Heat/Shredder	2,3,4
Sterimed	Chemical/Grinder/ Shredder	2,3,4
Steris Corp (Eco-cycle 10)	Chemical/Shredder	2,3,4
STI Chem Clave	Chemical/Autoclave	1,2,3,4
Tempico (Rotoclave ®)	Autoclave/Shredder	1-5
Thermokill, Inc.	Dry Heat Oven	2,3,4

*Medical Waste Categories
1. Cultures & Stocks
2. Liquid Human and Animal Waste
3. Pathological Waste
4. Sharps
5. Contaminated Animal Waste

09/26/2007

Exhibit B

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 C

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 D

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 E

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 F

K-STATE
Kansas State University
Food Science Institute
148 Waters Hall
Manhattan, KS 66506-4010
785-532-2202
Fax: 785-532-5861
E-mail: foodsci@k-state.edu
http://foodsci.k-state.edu

August 27, 2007

Diane Gorder

Director of Regulatory Compliance

Biomedical Technology Solutions, Inc.

9800 Mt. Pyramid Court – Suite 350

Englewood, CO 80132

Dear Ms Gorder:

I have evaluated the materials presented to the Illinois Environmental Protection Agency to substantiate the claim that *Bacillus subtilis* var. *niger* (ATCC 9372), alos known as *Bacillus atrophaeus)* is the *most appropriate* biological indicator organism for the validation of dry heat sterilization technologies.

Upon review of the large body of scientific citations and international standards, the following major findings are provided for your consideration.

The use of dry heat for the sterilization of surfaces, medical devices, medical waste, etc., has been studied extensively since as early as the late 1960s. The original research was performed primarily in the space industry to address sterilization requirements in a space environment. Subsequently, dry heat sterilization was broadly adopted for the sterilization of equipment and surfaces in the medical and dental applications. In the mid-1990s, dry heat technology was adapted and demonstrated to effectively sterilize infectious waste including infectious sharps waste consistent with state and local standards for disinfection.

Various scientific studies have focused on the identification of the most appropriate biological indicator organism for the validation of dry heat technologies.[1-12] These studies evaluated the inactivation of *Bacillus subtlis* var. *niger* under various treatment conditions using various substrates including paper strips, stainless steel, glass, epoxy resin, etc. By the late 1970s, the scientific community converged on the selection of *Bacillus subtilis var. niger*, and more specifically ATCC 9372, as a spore-forming organism exhibiting superior dry heat resistance. A wide variety of D-values and z-values had been reported for this species based on the specific variables of the experimental design.

In 1980, Gurney and Quesnel [6] initiated a comprehensive comparison of a generic *Bacillus subtilis* organism and *Bacillus subtilis* var. *niger*. During this timeframe, both generic *B. subtilis* and the subspecies *niger* had demonstrated excellence heat resistance in dry heat sterilization applications. Gurney and Quesnel's work extensively studied the growth properties and the thermal inactivation performance of both indicator organisms in a dry heat sterilization process and definitively concluded that *Bacillus subtilis var. niger* delivers superior growth and heat resistance properties. The purpose of this extensive study was to determine the optimal biological indicator organism for a dry heat treatment process. The authors devised a very simple and efficient method for dry heat treatment to support the extensive D-value and z-value determinations of both organisms. Statistical methods were employed

fsi

using appropriate experimental replicates (3-6 replications per condition) to derive regression lines of best fit from these D-values at different temperatures.

Gurney and Quesnel definitely concluded that "*Bacillus subtilis* var. *niger* germinated more readily and delivered higher percentage germination than *B. subtilis* MD2 on all media evaluated." This is an important finding for the selection of an optimal biological indicator since the objective of a validation study is to assure that the most resistant organisms are inactivated by the anti-microbial technology under evaluation.

The authors conducted extensive studies on decimal reduction times (D-values), comparing results with those published by other investigators. Specifically, the decimal reduction times for each of the spores were evaluated at various temperatures (between 140 and 160°C) using different media (TGE, CAA, and MGR). **At all temperatures var. *niger* showed greater dry-heat resistance than *B. subtilis*.** The difference in the shapes of the thermal death curves for *B. subtilis* var. *niger* and *B. subtilis* were found to be significant, with a sharp drop-off in the death curve noted for the *B. subtilis* spore. The authors reported that "The difference is due to the facts that MD2 spore germination is considerably enhanced by heat activation, while *niger* spores germinate readily and rapidly with little or no heat activation."

In summary, this work definitely found that *Bacillus subtilis* var. *niger* is the preferred and most appropriate biological indicator organism for the validation of dry heat sterilization due to its enhanced dry heat resistance at all temperatures evaluated and, importantly exhibited enhanced growth properties in each of the growth media evaluated. Specifically.

> **"From this study the var. *niger* strain is clearly the organism of choice as an indicator of dry-heat sterilization in the temperature range of 140 to 170°C."**

Based on this research and the compendium of research by others in the field of sterilization and microbiological control, multiple standards organizations have formally designated *Bacillus subtilis* var. *niger* (ATCC 9372) as the definitive preferred biological indicator organism for the validation of dry heat sterilization processes.[13-19] These standards are developed through review of the scientific literature and a consensus of international experts in the field of sterility control. The committees assess the morphological, growth, stability, and resistance characteristics of biological indicator organisms to select the most appropriate organism for a given application. While numerous standards identify the ATCC 9372 organism for the validation of dry heat processes, the U.S. Pharmacopeia's Official Monograph and the International Standard Organization (ISO) determinations are among the most notable.

U.S. Pharmacopeia (USP) is the official public standards-setting authority for all prescription and over-the counter medicines, dietary supplements, and other healthcare products manufactured and sold in the United States. These standards are recognized in over 130 countries. The USP Convention membership has approximately 450 members who represent U.S. colleges and state associations of medicine and pharmacy; governments of the U.S. and other countries; national and international health professional, scientific, and trade organizations; the pharmaceutical industry; and consumer organizations. The Council of Experts and Expert Committees are the bodies that make the USP's scientific and standards-setting decisions. The Microbiology and Sterility Assurance Expert Committee is currently chaired by Dr. James E. Akers, an internationally recognized expert in sterility assurance. Other distinguished members of the committee include Mr. James P. Agalloco, Mr. Ivan W. Chin, Dr. Anthony M. Cundell, Dr. Joseph K. Farrington, Dr. Dennis E. Guilfoyle, Dr. David Hussong, Dr. Leonard W. Mestrandrea, Dr. David A. Porter, Dr. Donald C. Singer, and Dr. Scott V.W. Sutton.

The official USP Monograph – Biological Indicator for Dry-heat Sterilization, Paper Carrier, USP28-NF23, 2005, provides species identification, packaging and storage requirements, and resistance performance testing standards for the production of biological indicators to be used to validate dry heat sterilization processes. Specifically, "Biological Indicator for Dry-Heat Sterilization, Paper Carrier, is a defined preparation of viable spores from a culture derived from a specified strain of *Bacillus subtilis*

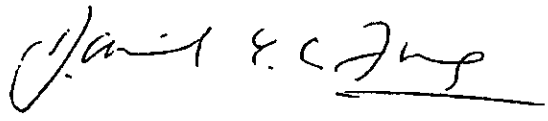
subspecies *niger*, on a suitable grade of paper carrier, individually packaged in a container readily penetrable by dry heat, and characterized for predictable resistance to dry heat sterilization." The species is further defined as "The biological indicator organism complies substantially with the morphological, cultural and biochemical characteristics of the strain of *Bacillus subtilis*, ATCC No. 9372, designated subspecies *niger*,... " This USP Monograph also provides D-value performance requirements for the production of certified carriers to be used to validate dry heat sterilization processes.

The International Standards Organization (ISO) has also formally recognized *Bacillus subtilis* var. *niger* organism as *the preferred and most appropriate* indicator organism for the validation of dry heat sterilization technologies. ISO is a network of national standards institutes of 155 countries and is the world's largest developer of standards publishing more than 16,000 diverse fields. ISO 11138-4:2006 provides specific requirements for test organisms, suspensions, inoculated carriers, biological indicators, and test methods intended for use in assessing the performance of sterilization processes employing dry heat as the sterilizing agent within the range of 120°C and 180°C. This standard has specifically found that *Bacillus atrophaeus* (known as CIP 77.18, NCIMB 8058, DSM 675, NRRL B-4418, and ATCC 9372) or *Bacillis subtilis* (DSM 13019 isolated from Hay in Denmark) are preferred biological indicator organisms for the validation of dry heat processes. The standard further defines manufacturer quality and test requirements to certify the D-value and z-value performance of given spore populations. Note the American National Standards Institute (ANSI) has also formally adopted the ISO 11138-4:2006 standard for the selection of the preferred and most appropriate biological indicator organism for the validation of dry heat sterilization technologies.

Based on the overwhelming evidence, it is my expert opinion that *Bacillus subtilis var. niger* (ATCC 9372, also known as *Bacillus atrophaeus)* is the *most appropriate* biological indicator organism for the validation of dry heat sterilization technologies. This specific subspecies of *Bacillus subtilis* demonstrates excellent growth and dry heat resistance characteristics. Standards for performance have been established by USP, ISO, and others to ensure that certified biological indicators for dry heat sterilization deliver predictable and standardized resistance.

The Demolizer® technology is an alternative infectious waste treatment system that employs dry heat as the sterilization agent. As such, the most appropriate biological indicator organism for the validation of the efficacy of the Demolizer® technology is the ISO and USP recognized standard. *Bacillus subtilis* var. *niger* (also known as *Bacillus atrophaeus*). Further, certified carriers manufactured under rigorous quality standards should be used, wherever possible, since such carriers are tested for purity and performance meeting defined D-value and z-value performance criteria.

Respectfully submitted,



Daniel Y. C. Fung, M.S.P.H., Ph.D.

Professor of Food Science, Professor of Animal Science and Industry, Kansas State University

Distinguished Professor, Universitat Autonoma de Barcelona, Spain

Food Microbiologist, Environmental and Public Health Microbiologist

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Dr. Daniel Y. C. Fung

Biographical Background Information

Dr. Daniel Y. C. Fung, internationally recognized Food, Environmental and Public Health Microbiologist, has published extensively in Food Microbiology, Applied Microbiology and Rapid Methods with more than 700 Journal articles, meeting abstracts, proceeding papers, book chapters and books in his career. He has served as the major professor for more than 90 M.S. and Ph.D. graduate students. The Kansas State University Rapid Methods and Automation in Microbiology Workshop, directed by Dr. Fung, has attracted more than 3,500 participants from 60 countries and 46 states to the program in the past 27 years.

Dr. Fung is a Fellow in the American Academy of Microbiology, Institute of Food Technologists (IFT), International Academy of Food Science and Technology and Institute for Food Science and Technology (UK). He has won more than 30 professional awards which included the International Award from IFT (1997), Waksman Outstanding Educator Award from The Society of Industrial Microbiology (2001), KSU College of Agriculture Excellence in Graduate Teaching Award (2005), and the Exceptional Achievement and Founder of the KSU International Workshop on Rapid Methods and Automation in Microbiology Award given by the Director of the Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, 2005.

Dr. Fung received the B.A. degree from International Christian University, Tokyo, Japan in 1965, M.S.P.H. at University of North Carolina-Chapel Hill in 1967, and the Ph.D. in Food Technology from Iowa State University in 1969. He is currently a Professor of Food Science, Professor of Animal Sciences and Industry and Ancillary Professor of Biology at Kansas State University and Distinguished Professo,r Universitat Autonama de Barcelona, Spain.

Exhibit G

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 H

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

Exhibit I

BMTS
BioMedical Technology Solutions, Inc.

September 25, 2007

Mr. Neal H. Weinfield, J.D.
Greenberg Traurig
77 West Wacker Drive, Suite 2500
Chicago, IL 60601

Re: Estimate for Repeating Demolizer® Validation Study using B. subtilis ATCC 19659

Dear Neal:

As you requested, we have obtained a firm estimate from Dr. James Marsden consistent with the verbal estimate provided in June 2007 for repeating the Demolizer® Validation using *B. subtilis* ATCC 19659 instead of *B. subtilis (B. atrophaeus)* ATCC 9372, the most appropriate and preferred *B. subtilis* organism for the validation of dry heat sterilization processes. A copy of the formal estimate from Dr. Marsden is included with this letter, as requested.

The first phase involves stabilizing a culture population of the *B. subtilis* spores and "certifying" its resistance properties through exhaustive D-value studies. Dr. Marsden would use standard protocols for validating the resistance of the culture similar to those used by industry. It is very possible that this study would need to be repeated several times until a population is grown to the standards comparable to those obtained from certified manufactures. Manufacturers such as STERIS Corporation have a complete research unit dedicated to such efforts. 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 2 years.

The second phase 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 results comparable to those already reported could be obtained. The estimate provided by Dr. Marsden for the validation study using ATCC 19659 is $40,000.

In addition to these costs, BMTS would incur direct costs totally more than $30,000 which includes the cost of three dedicated systems, supplies and other consumables, and the cost of BMTS staff time to be onsite at Kansas State University to facilitate and witness the trial. This practice is necessary to support representations BMTS makes in relation to the efficacy of its products.

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Therefore the total cost for repeating the study using a different, non-certified, *B. subtilis* isolate is estimated between $130,000 and $320,000 dollars. The timeframe to complete this work would range from 9-12 months to as much as 2.5 years.

Sincerely,

Diane R. Gorder
Director of Regulatory Compliance

Cc: Mr. Don Cox, BMTS

K-STATE
Kansas State University
Food Science Institute
148 Waters Hall
Manhattan, KS 66506-4010
785-532-2202
Fax: 785-532-5861
E-mail: foodsci@k-state.edu
http://foodsci.k-state.edu

September 23, 2007

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

Re: Proposal for additional spore validation testing on the Demolizer® technology

Dear Ms. Gorder,

As requested, this letter provides a formal proposal for the validation of the Demolizer technology using *Bacillus subtilis* ATCC 19659 consistent with the verbal estimates provided earlier this year.

The new validation study would be carried out using the same protocol utilized in the original trial conducted in 2006. The objective would be to perform an equivalent validation trial using the Illinois designated spore organism, an organism that is not recognized for the validation of dry heat sterilization technologies. The certified USP ,internationally-recognized and most appropriate *B. subtilis* substrain was used in the original study and is well understood to deliver superior resistance properties in dry heat applications.

The spores used in the July 2006 study were comprehensively tested and certified by Steris Corporation for purity and performance as represented by D-value resistance consistent with FDA, USP and other international standards requirements. Since the ATCC 19659 spore is not available in a certified form, we would need to complete an exhaustive D-value trial to replicate the standards achieved in the original study. This type of a study is extensive and is typical of the level of effort for a Ph.D research initiative. As is the nature for microbiological studies, it is possible the growth of the population and D-value certification may need to be repeated several times if satisfactory results cannot be easily obtained.

Upon acceptance of a given population, the trial would then be conducted. All experiments would be conducted in triplicate to support meaningful findings. This effort would be led by either a Ph.D. candidate or a post-doctoral research associate. The work would be supervised by me along with the remainder of our scientific team. Please find below an estimate of this scope of work. There is substantial effort involved in coordinating such an effort. It is reasonable to expect that the study could involve a

fsi

minimum of 6 months up to 2 years should the culture prove difficult to grown to the standards obtained in the original study. As with the previous study, BMTS is responsible for delivery of three Demolizer II Systems and associated supplies for representative waste loads. We would also recommend that you provide a member of the BMTS engineering team be onsite in support of the effort particularly during the completion of Phase II of this effort.

Phase 1 – Growth and stabilization of *B. subtlis* ATCC 19659 to a minimum population density of 2.0×10^6 to a maximum population density of 5.0×10^6; D-value study reporting the heat resistance of the population and comparisons with published standards (may need to be repeated up to 3 times prior to accepting a given population)	$60,000 per repetition for up to $180,000
Phase 2 – Validation study of the Demolizer technology against *B. subtilis* ATCC 19659 using representative waste loads	$40,000
Total Estimated Cost	$100,000 to $220,000 Lead time: minimum 6 months up to 2 years

As discussed with the Illinois representatives in our recent conference call, this effort is not only expensive in both money and time, it is not scientifically warranted because the *Bacillus subtilis* certified spore used in the original study is the most appropriate spore organism for the Demolizer® sterilization process. Please refer to Dr. Daniel Y.C. Fung's letter of authority on this matter for additional details.

Please advise if additional information is required.

Sincerely,

James L. Marsden, Ph.D.
Regent's Distinguished Professor
Kansas State University

Exhibit J

Journal of Applied Bacteriology 1980, 48, 231–247 582/04/79

Thermal Activation and Dry-heat Inactivation of Spores of *Bacillus subtilis* MD2 and *Bacillus subtilis* var. *niger*

TESSA R. GURNEY* AND L. B. QUESNEL

Department of Bacteriology and Virology, University of Manchester, Manchester M13 9PT, U.K.

Received 12 April 1979 and accepted 10 October 1979

Spores of *Bacillus subtilis* MD2 and *Bacillus subtilis* var. *niger* were heat activated for different times at 60° and 80°C. Strain MD2 required considerable heat activation while *B. subtilis* var. *niger* did not. Maximum germination rates increased with heat activation dose and declined subsequently without loss of germinability. Germination rates and percentages were considerably greater in tryptone glucose extract (TGE) than in nutrient broth. The addition of 2% dimethyl sulphoxide did not increase germination in nutrient broth. The spores of var. *niger* are more resistant to dry-heat than MD2 although they are less resistant to moist heat. Survivor curves in the dry-heat range 140°–170°C gave D-values from 4·123 to 0·106 min for MD2 and 5·679 to 0·233 min for var. *niger* recovered on TGE agar. D-values were lower on poorer media. The z-values for MD2 and var. *niger* on TGE were 18·7°C and 21·25°C respectively.

THE LITERATURE on moist heat sterilization has been vast; that on dry-heat sterilization less so but is increasing, partly because of interest in its use for sterilization of space-craft components. There has also been considerable interest in recent years in the biochemical nature of the lesions caused by heating bacteria under sublethal and lethal time/temperature conditions. The nature of heat damage to bacterial spores (Keynan 1969; Gould 1970; Brown & Melling 1971; Russell 1971) and vegetative cells (Allwood & Russell 1970; Ingram 1971; Corry 1973; Mossel & Corry 1977) has been reviewed at various times and damage to membrane, protein, DNA and RNA are recorded. Most of the records relate to moist heat damage.

The indicator organism for moist heat sterilization is *Bacillus stearothermophilus*; the organism commonly used for the evaluation of dry-heat killing procedures is *B. subtilis* var. *niger*. For the spores of both organisms a wide variety of D-values has been reported, varying with the experimental procedures used. Results for dry-heat conditions have been notoriously variable, largely because of the lack of attention to the thermal lags encountered in heating-up times.

On the basis of results obtained by H.M. Darlow and W.R. Bale (unpublished report: 'Observations on a high speed hot air instrument sterilizer') who used an equal mixture of *B. subtilis* var. *niger* and *B. subtilis* MD2 and found the var. *niger* strain to be 'somewhat less resistant', Quesnel *et al.* (1967) used the same organisms for the evaluation of dry-heat sterilization at 200°C. The study reported in this paper was designed to compare the dry-heat resistance of the *B. subtilis* MD2 and var. *niger* strains using a simple but efficient method of dry-heat treatment. A subsequent paper will report on the nature of dry-heat damage to these spores.

* Present address: Department of Science, Salford College of Technology, Salford 6, U.K.

0021–8847/80/020231+17$01.00/0

Materials and Methods

Organisms

Two strains of *Bacillus subtilis* selected for their high resistance to dry-heat killing were originally supplied by Dr H.M. Darlow, Microbiological Research Establishment, Porton. These strains, *B. subtilis* MD2 and *B. subtilis* var. *niger* were biochemically similar in activity to *B. subtilis* as characterized by Cowan & Steel (1974) except that neither strain fermented xylose, and the *niger* strain did not reduce nitrate. Strain MD2 gave a circular, buff-white, matt textured colony with irregular margin on nutrient agar at 36°C, while the *niger* strain gave a smaller circular, orange-chestnut, shiny, raised colony. Morphology and colour varied quite considerably on different media, especially after heat treatment.

Preparation of spore suspensions

The sporulation medium of Ohye & Murrell (1962) was used, solidified with 1·2% Oxoid Agar No. 3. Each of 30 plates were spread with 0·2 ml overnight Nutrient Broth (Oxoid CM 67) culture of test organism grown at 36°C, in each case, and the plates were incubated at 36°C for 4 d. Samples from plates were removed daily for visual evaluation of sporulation by phase-contrast microscopy. After 2 d strain MD2 showed 98% sporulation, while strain *niger*, even after 4 d, was only 80% sporulated.

Spores were washed from the plates with three rinses of distilled water, centrifuged and washed once in distilled water then stored at 4°C for 2d, when the suspensions were treated with 50 μg lysozyme/ml to remove remaining cells. Microscopic examination showed that after 1 h all cells had lysed in the case of strain MD2; 2 h treatment was required for var. *niger*. Spores were then centrifuged and washed six times in distilled water before storing at 4°C. From these stock suspensions working suspensions were made by diluting in sterile distilled water to give suspensions of absorbance 1·0 at 660 nm (Cambridge Unicam SP 600 spectrophotometer). Spore viable counts were performed on both Nutrient Agar (NA, Oxoid) and Tryptone Glucose Extract Agar (TGE, Difco) using a surface spread method with unactivated and heat-activated (60°C for 10 min) samples.

Germination studies

Germination and outgrowth was tested on four different media: Nutrient Broth (NB, Oxoid CM 67), Nutrient Broth + 1% glucose; Nutrient Broth + 2% dimethylsulphoxide (NB + 2% DMSO) and Tryptone Glucose Extract Broth (TGE, Difco).

Germination was followed by measuring loss of refractility as a decrease in optical density (OD) at 660 nm (Unicam SP 600 spectrophotometer). For the experiments 1·4 ml of working spore suspension was added to 10 ml of the appropriate medium in a side-arm flask equilibrated at 37°C. OD readings were taken at zero time and at 5 min intervals using sterile medium as the blank. Between readings flasks were returned to a water bath at 37°C and shaken at 50 strokes/min.

For activation, 1·4 ml of spore suspension was added to 2 ml of medium in a sterile test tube held at 60 or 80°C for the required activation time, after which the spores were

cooled in iced water for 2 min before adding to 8 ml of medium in a flask pre-warmed to 37°C.

Germination is recorded in the graphs as per cent fall in OD against time. Rates of germination were derived as per cent fall in OD/min from the straight line portions of the curves.

Before and after heat activation, the percentage germination of spores was derived by direct observation under phase contrast, scoring 100–200 spores on each occasion. The final per cent germination was always taken when OD ceased to fall.

Dry-heat inactivation

Preparation of spore samples

Squares of side 6 mm were cut from aluminium foil and sterilized by dry-heat at 160°C for 1 h. Standard spore suspensions were distributed by calibrated Microtiter pipette (Dynatech Laboratories, Billingshurst, Sussex), allowing one drop (0·0254 ml) to dry on the dull side of each square at 37°C for 1 h. The inoculated foil squares were equilibrated to an RH value of 32% in a sealed cabinet containing saturated calcium chloride solution at 20°C for 14 d before use.

For use the foil squares were enclosed in a foil envelope formed from a 30 mm square of sterile aluminium foil, by folding as illustrated in Fig. 1 with the dull surface inside.

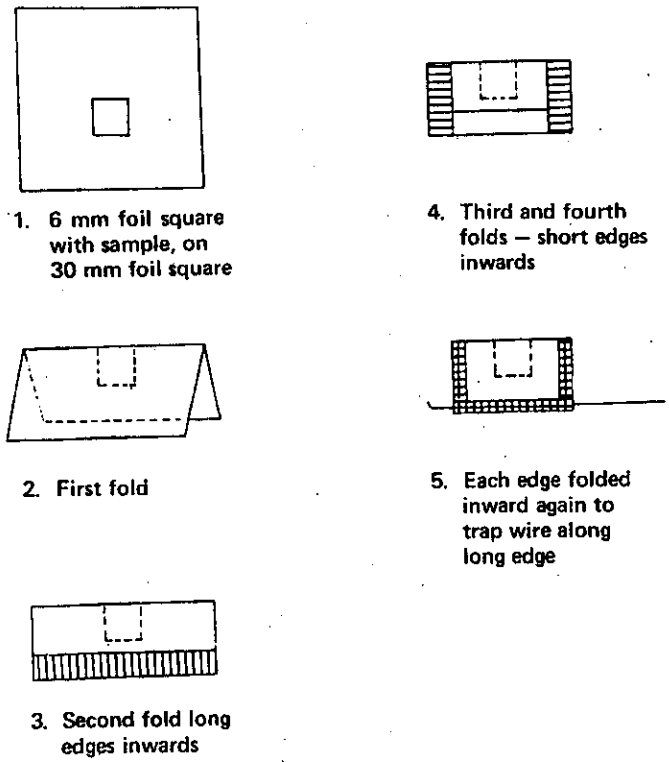


Fig. 1. Method of enclosing spore sample in aluminium foil envelope.

The foil enclosure was flattened with the side of a pair of forceps to minimize the amount of air trapped inside the foil envelope. A 120 mm length of nichrome wire was inserted under the fold along one edge of the foil envelope to enable transfer to and from the hot oil bath. The entire wrapping procedure was carried out in the humidity cabinet with the gloves provided and the wrapped spore samples were removed from cabinet to oil-bath in the minimum amount of time.

Heating procedure

The wrapped spore samples were treated for the required times in hot oil carefully maintained at the required temperature. The oil (Edwards High Vacuum oil) was filled to a depth of *ca.* 45 mm in 16 × 95 mm Pyrex test tubes held in the aluminium heating block of a Techne Dri-Block DB3-H heater (Techne Ltd., Cambridge, England). The block was loaded with 12 such tubes in holes of 16·75 mm diameter, and was placed between two other aluminium heating blocks in the same apparatus.

The apparatus was calibrated (by adjustment of two controls in the electronic control unit) so that the temperature on a thermometer placed in the oil was also recorded on the instrument meter. Slight adjustment to the level of oil in some tubes was needed to achieve the same temperature in each tube (indicating that heating was not even throughout the block). The minimum temperature available was 90°C and the maximum 170°C, and the heating-up time from ambient to 170°C was 40 min.

Recovery media

The three media used were: (1) Tryptone Glucose Extract Agar (TGE, Difco); (2) casamino acids medium (CAA) (g/l): NH_4Cl,4; Na_2HPO_4, 12; KH_2PO_4, 6; NaCl, 6; $MgSO_4.7H_2O$, 0·1; Difco vitamin-free casamino acids, 5; dextrose, 10; Oxoid agar No. 1, 10; pH 7–7·2; dextrose was added as a sterile solution to the other sterilized ingredients cooled to 50°C; (3) minimal growth requirement agar (MGR) as for CAA medium except that casamino acids are replaced by alanine, 100 μg/ml; aspartic acid, 50 μg/ml; glycine, 100 μg/ml; methionine 100 μg/ml. L-isomer amino acids were used unless otherwise stated.

Recovery of spores

Spore 'envelopes' removed from hot oil were plunged immediately into a mixture of ice and water for *ca.* 5 s and dried on a sterile paper towel. Two sides of the envelope were then cut with sterile scissors and the spore sample removed with sterile forceps to 10 ml of sterile distilled water in a screw-capped bottle.

Spores were removed from their foil support by placing the sealed screw-capped bottle in a Megason ultrasonic cleaning bath (Shuco Scientific, London) for 5 min which was shown by experiment to be an adequate procedure for removal of spores. The 10 ml suspension of spores this produced was called 'neat' and suitable dilution for plating were made in distilled water. Duplicate samples of 0·2 ml were spread on one or more of three recovery media: TGE, CAA or MGR. All plates were incubated at 30°C for 3 d before counting. (A 4% increase in count was found between days 2 and 3, but no increase after 3 d.)

The thermal death curves and values derived from them are based on the mean values obtained by performing each determination between 3 and 6 times.

Measurement of thermal lag

To measure the time taken for heat penetration through the aluminium envelope surrounding the spore samples a fast-response fine-wire (0·139 mm diam.) thermocouple, type K76 P1 (Comark, Rustington, Sussex) was used with a Comark Electronic Thermometer (160 series). The thermocouple was enclosed in the foil envelope which was then placed in the oil bath under the experimental conditions used and readings of temperature and time taken for Dri-Block instrument settings of 90°C rising in 10°C steps to 170°C.

The response time lag of the electronic thermometer was 0·2 s and any remaining lag was assumed to be due to time required for heat transfer from oil and through the aluminium envelope. The temperature rise with time followed a logarithmic curve asymtoting to the set maximum. The heat transfer time was found to be *ca.* 15 s at 90°C and *ca.* 20 s at 170°C. At each set temperature 90% of the maximum was reached in *ca.* 2 s. For the values plotted and used to calculate killing rates, no allowance was made, therefore, for heating-up time.

Derivation of data

The data from the thermal inactivation experiments were subjected to a computer analysis to derive regression lines of best fit and from these the D-values at different temperatures on different media were derived. Similarly z-values and *Y* intercepts were also calculated from the computer determinations.

Results

Typical germination curves for *B. subtilis* MD2 and var. *niger* strains are shown in Figs 2 and 3. Table 1 gives the rates of germination (calculated as per cent loss of OD/min) and final germination (as per cent phase dark when OD ceased to fall) for the two strains after various activation treatments and germination in several media.

The data show that the *B. subtilis* var. *niger* strain germinates more readily and gives a higher percentage germination than *B. subtilis* MD2 on all media and under all the conditions tried. In both cases TGE was a far superior medium to the others and was necessary for germination approaching 100%. Increasing the time of activation at 60°C increased the rate of germination to a maximum at 10–15 min, with concomitant increases in percentage germination on TGE. On the other hand increasing the activation time beyond 5 min at 80°C reduced the germination rate of MD2 in all three media with a decrease in percentage germination except in TGE, which was clearly the superior medium. It is interesting that the germination of 99% obtained after 80°C for 15 min was obtained at a slower rate which presumably reflects the need to repair a damaged germination system. The thermal death curves resulting from dry-heat treatment of MD2 and *niger* spores at 160°C are given in Figs 4 and 5. The curves are of greatly different form, MD2 curves having pronounced shoulders which were absent in the case of *niger* spores. Table 2 gives the D-values obtained for the strains at temperatures of 140, 150, 160 and 170°C on several media. Clearly the highest *D*-values were obtained for recovery on TGE medium indicating the ability of TGE to allow repair of heat-damaged spores. At all temperatures var. *niger* showed greater dry-heat resistance than strain MD2. Table 3 shows that the *Y* axis intercepts, obtained by

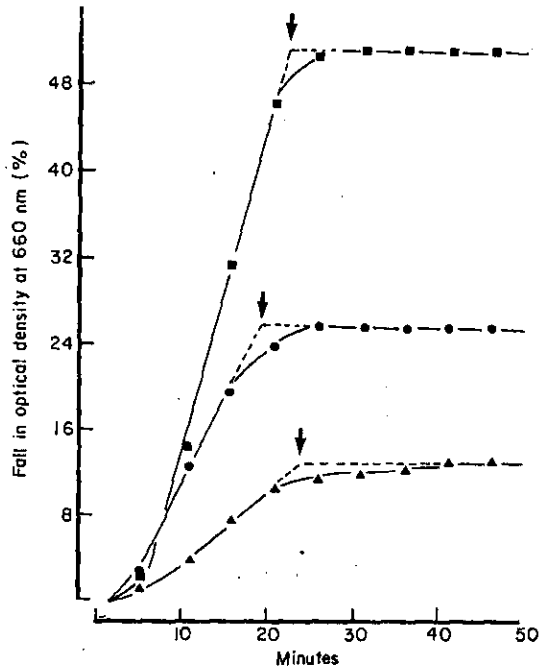


Fig. 2. Germination of *Bacillus subtilis* MD2 spores activated in various media at 60°C for 5 min and germinated to completion in the media at 37°C. ●, nutrient broth; ▲, nutrient broth + 2% DMSO; ■, tryptone glucose extract broth. Extrapolations (dashed lines) intersect at points arrowed to give 'completion times' (see Table 1).

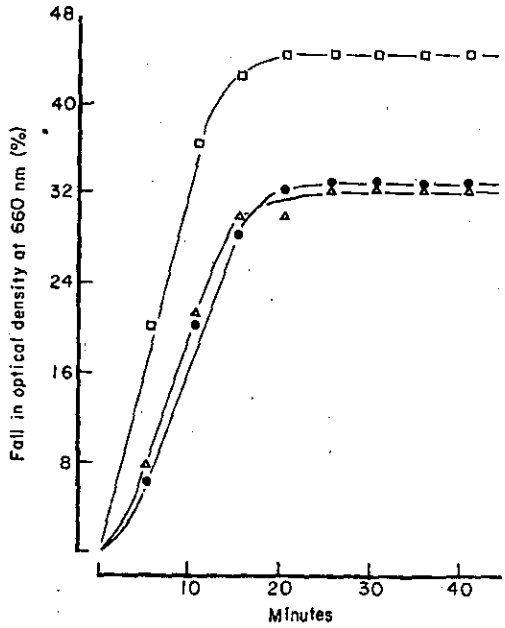


Fig. 3. Germination of *Bacillus subtilis* var. *niger* spores activated in various media at 60°C for 5 min and germinated to completion in the media at 37°C: ●, nutrient broth; ▲, nutrient broth + 2% DMSO; □, tryptone glucose extract broth (see Table 1).

°C for 5
nutrient

°C for 5 min
it broth+2%
ect at points

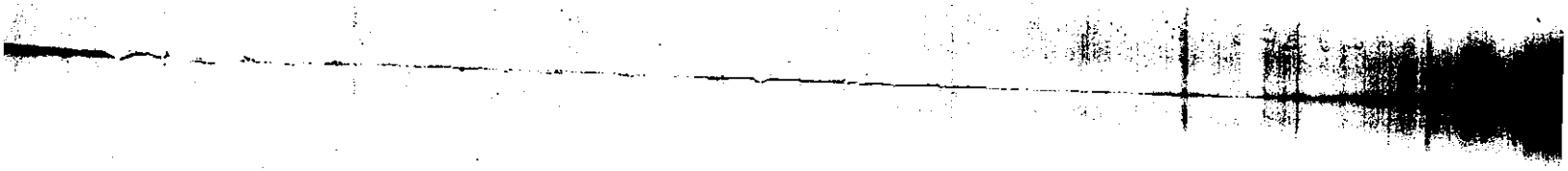


TABLE 1

The effect of heat activation and medium composition on the germination of spores of Bacillus subtilis MD2 *and* Bacillus subtilis *var.* niger

	TGE			NB+1% glucose			NB			NB+2% DMSO		
	Max. germ. rate*	% Germ.†	Completion time‡	Max. germ. rate	% Germ.	Completion time	Max. germ. rate	% Germ.	Completion time	Max. germ. rate	% Germ.	Completion time
Bacillus subtilis MD2												
unactivated	1·54	80	38	1·0	54	40	0·69	49	39·5	0·48	28	43
60°/5 min	2·94	89	21·4				1·43	52	18·2	0·66	24	23·2
60°/10 min	4·0	93	18				0·91	34	19·5	0·87	26	22
60°/15 min	4·0	96	18·6				N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
80°/5 min	3·57	94	21				1·66	60	19	0·87	35	27
80°/10 min	3·13	97	19·5				0·83	30	24	0·83	25	23
80°/15 min	2·94	99	24				0·34	24	42·5	0·42	17	25·5
B. subtilis var. *niger*												
unactivated	2·38	95	18	2·34	85	20·5	1·64	76	22	1·43	69	26
60°/5 min	4·0	93	12				2·27	70	15·6	2·27	66	15·2

* Maximum germination rate measured as per cent OD fall/min, calculated from the straight line region of the curve.
† Per cent germination recorded as per cent phase-dark spores present when OD ceased to fall.
‡ Completion time obtained by finding the intersect of extrapolations of straight line 'fall' and terminal OD values (see Fig. 1). Times in minutes from end of activation time.
N.D. = not determined.

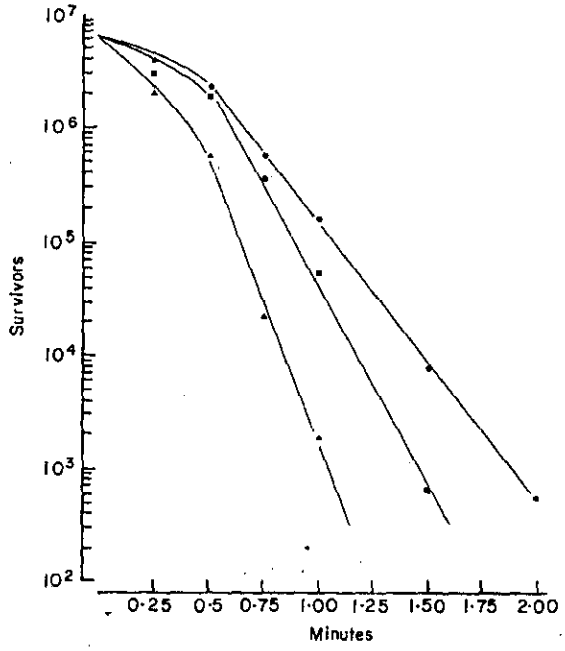


Fig. 4. Thermal death curves of *Bacillus subtilis* MD2 spores dry-heated at 160°C and recovered on various media: ●, tryptone glucose extract agar; ■, casamino acids agar; ▲, minimal growth requirement agar. D-values are given in Table 2.

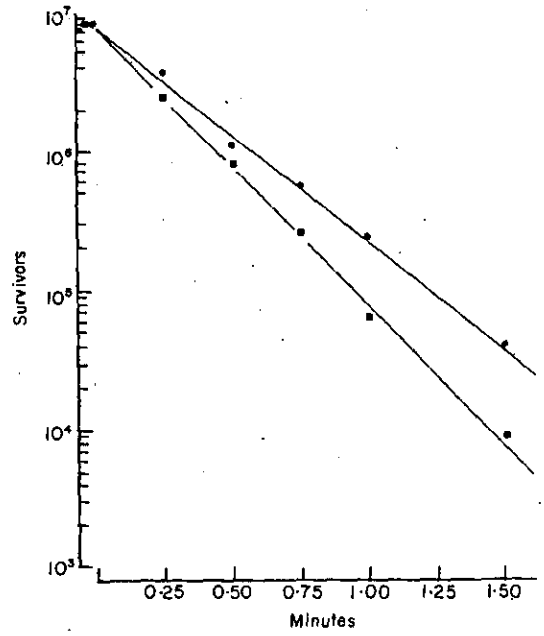


Fig. 5. Thermal death curves of *Bacillus subtilis* var. *niger* spores dry-heated at 160°C and recovered on: ●, tryptone glucose extract agar; ■, casamino acids agar (see Table 2).

TABLE 2

Decimal reduction times for spores of Bacillus subtilis MD2 *and* Bacillus subtilis *var.* niger *heated at several temperatures and recovered on different media*

Temperature (°C)	*Bacillus subtilis* MD2, D-value (min)			*B. subtilis* var. *niger* D-value min)	
	TGE	CAA	MGR	TGE	CAA
140	4·123	3·493	2·190	5·679	4·791
150	1·307	0·889	0·511	1·600	1·259
160	0·412	0·284	0·201	0·654	0·501
170	0·106	0·081	N.D.	0·233	0·166

TABLE 3

Y-axis intercepts for* Bacillus subtilis MD2 *dry-heat death curves, and* Y_o/N_o† *ratios, at various temperatures for different media*

Temperature (°C)	TGE		CAA		MGR	
	Y intercept	Y_o/N_o	*Y* intercept	Y_o/N_o	*Y* intercept	Y_o/N_o
140	$9·45 \times 10^6$	1·43	$9·89 \times 10^6$	1·49	$1·22 \times 10^7$	1·84
150	$1·43 \times 10^7$	2·16	$3·06 \times 10^7$	4·62	$9·23 \times 10^7$	13·94
160	$3·94 \times 10^7$	3·35	$9·58 \times 10^7$	14·47	$1·57 \times 10^8$	23·72
170	$2·28 \times 10^8$	34·44	$7·82 \times 10^8$	118·13	N.D.	

* Recorded as the hypothetical original population of spores, Y_o.
† N_o is the actual number of spores in the sample (value derived from viable counts and direct microscopic determination of germination percentages).

TABLE 4

z-values for Bacillus subtilis MD2 *and* Bacillus subtilis *var.* niger *spores recovered on different media*

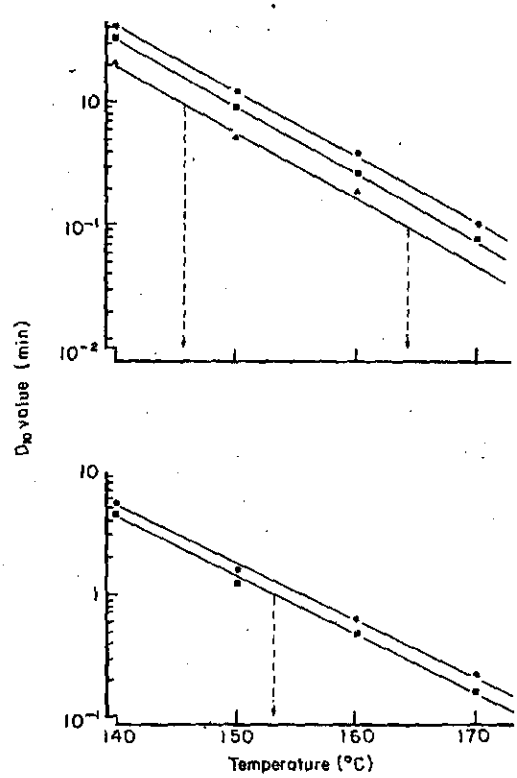
Medium	z-value (°C)	
	B. subtilis MD2	*B. subtilis* var. *niger*
TGE	18·75	21·25
CAA	18·25	21·0
MGR	18·25	N.D.

extrapolation of the logarithmic regions of the death curves, increased significantly as the temperature of inactivation was increased.

From Table 4 it can be seen that the z-values for var. *niger* are some 2·5°C higher than the MD2 strain, while the *z*-values on different media differ but the curves are essentially parallel in both strains (Figs 6 and 7).

To our knowledge this method of dry-heat treatment has not been used before and it was important to assess the accuracy and precision of the techniques. From the prepared stock suspensions of spores, one drop (as distributed to foil squares) was suspended in 10 ml distilled water and a 5×10^{-4} dilution in water made in ten fold and two fold steps. This was done three times and duplicate 0·2 ml samples were spread on TGE agar plates on each occasion for each strain; colonies were counted after incubation at 36°C for 24 h. Table 5 gives the estimated number of spores per drop.

Table 6 shows the number of spores recovered after sonication of sample-loaded aluminium foil squares as described above, using the same methods for diluting and plating. Sonication times of 2, 4, 6, 8 and 10 min were used and yielded essentially the same number of viable spores as obtained from an equivalent drop of suspension not sonicated.



Figs 6 (above) and 7 (below). Derivation of z-values for *Bacillus subtilis* MD2 spores (above) and *Bacillus subtilis* var. *niger* spores (below) from survivor curves of cells recovered on: ●, tryptone glucose extract agar; ■, casamino acids agar; ▲, minimal growth requirement agar. Dashed lines denote intercepts at each log unit of ordinate (see Table 4).

TABLE 5

Calibration of spore count per drop of stock suspension

	B. subtilis MD2		*B. subtilis* var. *niger*	
Sample	count	mean	count	mean
a	$5{\cdot}35 \times 10^6$		$7{\cdot}70 \times 10^6$	
b	$5{\cdot}40 \times 10^6$	$5{\cdot}35 \times 10^6$	$7{\cdot}90 \times 10^6$	$7{\cdot}77 \times 10^6$
c	$5{\cdot}30 \times 10^6$		$7{\cdot}70 \times 10^6$	

TABLE 6

Effect of sonication on the recovery of spores dried on to aluminium foil

Exposure time (min)	*B. subtilis* MD2 recovered count ($\times 10^6$)	*B. subtilis* var. *niger* recovered count ($\times 10^6$)
2	5·45	7·95
4	5·20	8·15
6	5·60	7·8
8	5·40	7·8
10	5·25	7·95

Discussion

While both of these strains have been recommended for use in dry-heat sterilization tests they are clearly different in their characteristics. These differences can be seen both in their germination and thermal inactivation behaviour. The var. *niger* spores germinated in the three germination media without appreciable lag while there was a significant lag for MD2 spores (Table 1). The difference is typified by the data for germination in TGE broth. Activation of 60°C for 5 min gave a germination rate of 2·94, a germination percentage of 89 and a completion time of 21·4 min for strain MD2, while the corresponding values for *niger* spores were 4·0, 93 and 12. The difference in germination mechanism between these strains is further shown by the fact that var. *niger*, but not MD2, may be germinated by subtilisin alone (Quesnel *et al.* 1977).

The increase in germination rate with increased activation dose (*e.g.* at 60°C, Table 1) followed by a decrease in germination rate as the thermal dose is increased (*e.g.* at 80°C) has been found for other species as well (Curran & Evans 1945; Powell 1955; Keynan 1969; Levinson & Hyatt 1970; Hashimoto *et al.* 1972). It is significant, however, that the increased dose at the higher temperature gives decreased rates and lower percentages of germination on the poorer media, but an increased percentage germination on the richer medium.

Hashimoto *et al.* (1972) have shown that the kinetics of germination of individual *B. cereus* spores are biphasic and that after about a 42% loss of refractility, individual heat-damaged spores exhibited a secondary microlag period related to the heat dose, before the second phase of microgermination produced the phase dark spore. Such heat-damaged spores showed no loss of viability until a lethal dose of heat was applied. Lethal heating at 90°C for 30 min did not, however, prevent germination as measured by loss in OD of a spore suspension, although outgrowth and colony formation was impossible on trypticase soy agar.

In this study phase-grey spores were classed as ungerminated (as indeed they are) but to judge from their number many of the phase-grey (damaged) spores found in nutrient broth after treatment at 80°C were arrested in the secondary microlag and would have proceeded to the second phase of microgermination in TGE medium. Clearly some heat-damage is reversible, or such damage can be ignored in the presence of additional germinant compounds present in the richer medium, and germination then proceed by an alternative mechanism as suggested by Sogin *et al.* (1972).

While the specific nature of the biochemical lesion which inhibits progress to the second phase of germination is not known, Schacter & Hashimoto (1975) have suggested that extended heat treatment may cause the alteration of the structural components of spores in such a manner that the rapid degradation of these structures may become difficult. These structures would not involve those responsible for heat resistance or dipicolinic acid retention as both these properties have been lost by the time of secondary microlag (Schacter & Hashimoto 1975). Dring & Gould (1975) have provided convincing evidence for the initiation of endogenous metabolism during germination and shown the importance of the membrane-linked electron-transport chain in germination. They suggest this as the motive force for bound ion translocation necessary for germination, but leave open the question whether germinative amino acids are themselves metabolized, or function allosterically to trigger metabolism of endogenous reserves.

Although Quesnel *et al.* (1971) found that low concentrations of DMSO enhanced recovery in a simple glucose-peptone medium this result was not confirmed here, although it can be seen that the adverse effect of DMSO at non-damaging temperatures was eliminated when damaging conditions were used (Table 1).

The method of dry-heating devised for this study allowed fairly accurate measurement and great ease of handling. The numbers of spores in each sample showed slight but not significant differences and recovery by sonication in distilled water proved to be adequately reproducible (Table 6).

While every effort was made to control accurately the temperature of the oil baths used to immerse foil-wrapped spore samples it was found that increasing the level of oil (*e.g.* by immersion of a thermometer) caused a slight lowering of the temperature of 2–3°C. However, the foil samples raised the level of oil to a much lower extent than did a thermometer and thermocouple measurements indicated that the heating temperatures were accurate to within < 1°C, when the oil level was maintained below the top of the Dri-Block.

At temperatures below 140°C, where holding times in excess of 15 min were used, it was found that oil sometimes entered the foil envelope. While experiments indicated that the oil was non-toxic even to heated spores, it did appear to interfere with the precision of the sonication technique used to release the spores from thin foil supports.

only treatments at or above 140°C, where no oil seepage was experienced, were recorded here. Sonication has been used successfully to remove spores from films dried on glass coverslips (Molin & Ostlund 1975, 1976; Molin & Svensson 1976; Molin 1977*a*,*b*). The use of an ultrasonic probe inserted into the liquid was found to be totally unsatisfactory presumably because of aerosolization on to the cotton plug surrounding the probe at the neck of the container.

A preliminary inactivation experiment gave the same thermal death times (TDT) of 4·34 min at 150°C, 3·0 min at 160°C and 1·5 min at 170°C for spore populations of $5{\cdot}53 \times 10^6$ MD2 spores and $7{\cdot}77 \times 10^6$ var. *niger* spores, which might indicate a greater dry-heat resistance for MD2 spores which was previously reported for MD2 (Quesnel *et al.* 1967). In fact the thermal death curves showed the opposite to be true although MD2 is more resistant to moist heat (Fig. 8). The fact that var. *niger* spores are less resistant to moist heat but more resistant to dry-heat might indicate different killing mechanisms under the two types of condition. More experiments would be needed to confirm this.

The recovery medium greatly influenced the survival of the organisms. Decimal reduction times for MD2 spores recovered on TGE were almost twice as great as for spores recovered on MGR (Table 2). Independent experiments (not reported) showed that 100 μg L-alanine/ml caused complete germination of both strains of spores. While germination of >97% was obtained in liquid MGR medium, the count on solidified MGR medium was only 85% of that on TGE medium for undamaged spores, and the greater the amount of heat-damage the smaller was the fraction of spores recovered on MGR relative to TGE.

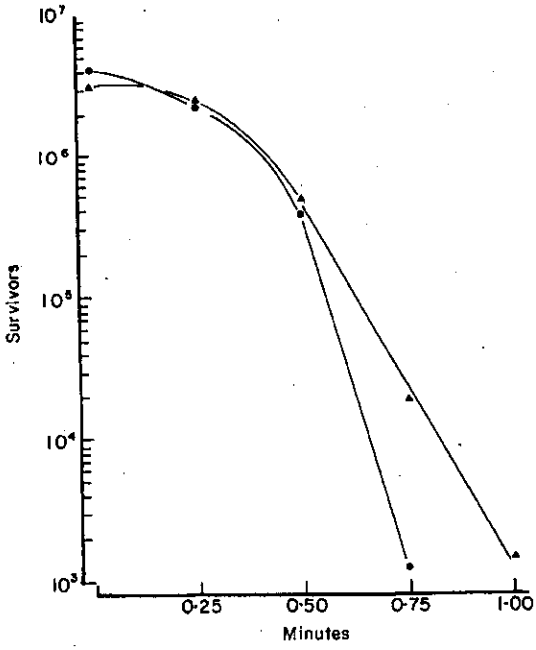


Fig. 8. Thermal death curves for *Bacillus subtilis* MD2 (▲) and var. *niger* (●) spores in moist heat at 110°C. Survivors were recovered on tryptone glucose extract agar.

The death curves derived by using CAA medium lay between those for MGR and TGE (Figs 4, 5 and Table 2). As CAA contains amino acids not present in MGR, and TGE contains amino acids and vitamins not present in CAA it is possible to identify requirements for specific growth factors consequent upon dry-heat damage to these spores. (The nature of these growth factors has been investigated and will be reported separately.)

Obviously, the magnitude of D-values at any temperature, and of z-values, depends on the medium used for recovery (Table 3) and comparison with published data should compare like with like. Data for *B. subtilis* MD2 spores are not available, but Table 7 lists data for var. *niger* spores for D_{160} values obtained under several different conditions. The values range from 0·33 to 3·17 min, which compares with the value of 0·654 min obtained in this study. The value for MD2 recovered on TGE is $D_{160}=0{\cdot}412$ min. Similarly the z-value (var.*niger*) obtained by various workers using TGE range from 12·9°C to 32°C compared with 21.25°C obtained by our methods (Table 8). The z-value graphs are linear (Figs 6 and 7) and in good agreement with those published by Molin (1977*b*) using the same temperature range. Molin & Ostlund (1976) have shown that spore density affects the rate of kill of spores dried on to glass surfaces heated by infra-red radiation and this is an additional cause of variation between dry-heat killing data. The relative humidity of the spores at time of treatment also markedly affects inactivation rates and the driest conditions do not give the lowest D-values. Brannen & Garst (1972) found that as the r.h. was raised from 0·003 to 1·67% the D-values rose from 1·4 to 2·5 min at 105°C for *B. subtilis* var *niger* spores. According to Murrell & Scott (1966) spores are most heat resistant in the range of 0·20–0·40 water activity and 0·32 was chosen for equilibration of the spores in this study. During the heating process changes in water activity relationships would occur, but no attempt was made to control this during heat treatment.

TABLE 7

Published D_{160} *values for dry-heated* Bacillus subtilis *var.* niger *spores recovered on TGE medium*

Supporting material	D_{160} value (min)	Reference
Glass coverslip	0·37	Molin (1977*a*)
Glass coverslip +		
soybean oil	0·45	Molin (1977*a*)
serum albumin	1·07	Molin (1977*a*)
sucrose	1·00	Molin (1977*a*)
starch	0·417	Molin (1977*a*)
Glass coverslip– (spores produced on different media)	0·33–7·17	Molin & Svensson (1976)
Aluminium foil	0·654	Gurney & Quesnel (this paper)

TABLE 8

Published z-values for dry-heated Bacillus subtilis *var.* niger *spores recovered on TGE medium*

Supporting material	z-value (°C)	Reference
Paper strips	12·9	Angelotti *et al.* (1968)
Stainless steel strips	20·8	Angelotti *et al.* (1968)
In lucite rods	20·7	Angelotti *et al.* (1968)
In epoxy rods	21·4	Angelotti *et al.* (1968)
On steel washers (under 150 in -1b torque)	32·0	Angelotti *et al.* (1968)
Glass coverslips	23·0	Molin (1977*b*)
Glass coverslips	25·0	Molin & Svensson (1976)
Glass coverslips	22·0	Molin & Ostlund (1976)
Filter paper strips	27·22	Bruch *et al.* (1963)
Aluminium foil	21·25	Gurney & Quesnel (this paper)

The difference in shape of the thermal death curves is also significant. Inactivation curves for strain MD2 are all shouldered while those for var. *niger* are not, in spite of the fact that unsonicated samples of the stock suspension showed significant clumping while MD2 spores did not. The difference is due to the facts that MD2 spore germination is considerably enhanced by heat activation, while *niger* spores germinate readily and rapidly with little or no heat activation (Table 1). Table 3 gives the increase in *Y*-intercept values with temperature rise shown by MD2 in all three recovery media. Similarly, for any given temperature *Y*-intercepts increase as the medium gets poorer, indicating that the number of 'targets' inactivated in rich media are lower than for poorer media. This difference is a measure of the ability to repair damage, or, more probably, to ignore damaged molecules because of the availability of growth factors in the recovery medium. Interestingly enough both strains yielded shouldered curves for moist heat inactivation at 110°C (Fig. 8), but only one such experiment was performed.

Adams & Busta (1972) provide strong evidence that the germination inactivation of a strain of *B. subtilis* spores stimulated by L-alanine was due to protein denaturation, and drew attention to the difference between germination inactivation and thermal injury inhibiting outgrowth. Similar differences apply here and the nature of the injury in these two strains under similar conditions may, moreover, be different. From this study the var. *niger* strain is clearly the organism of choice as an indicator of dry-heat sterilization in the temperature range 140–170°C.

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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:

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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*, *rrnA*, 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

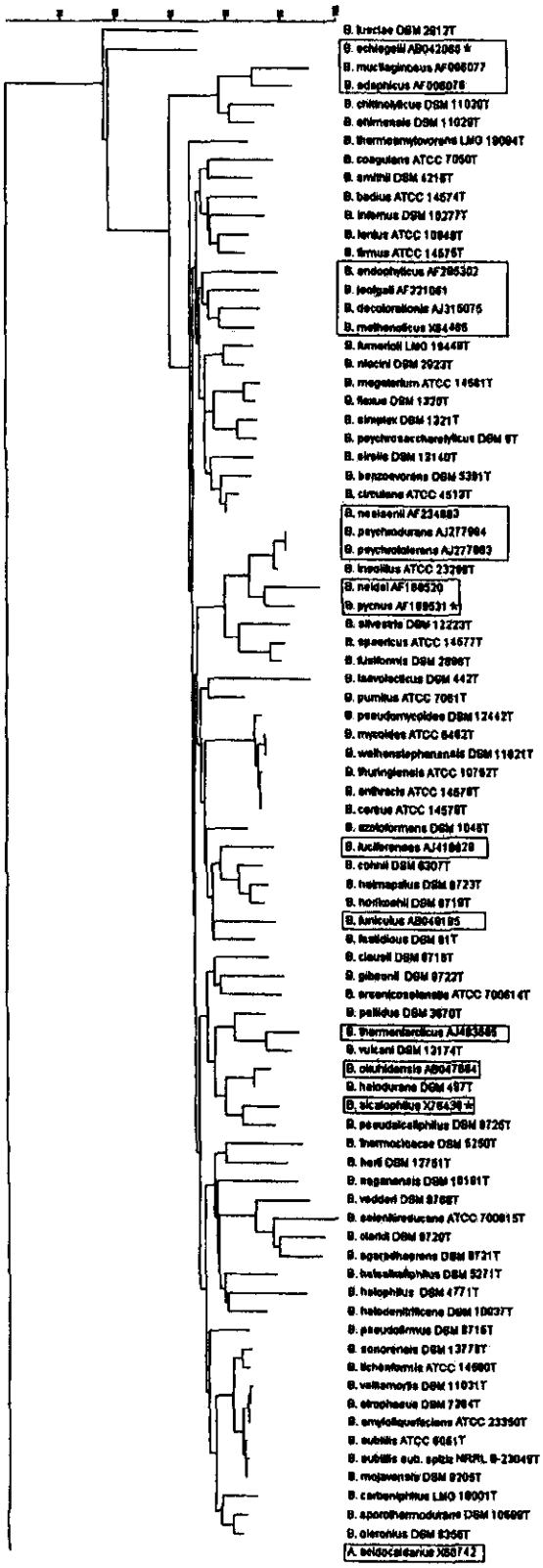

FIG. 1. Neighbor-joining phylogenetic tree based on the V1-V3 region of the 16S rRNA gene (*E. coli* nt 54 to 510) of *Bacillus* species used in this study. Sequences we were unable to obtain in this study were taken from GenBank (boxed). Three strains (*) had one ambiguous base pair (n). The branching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

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

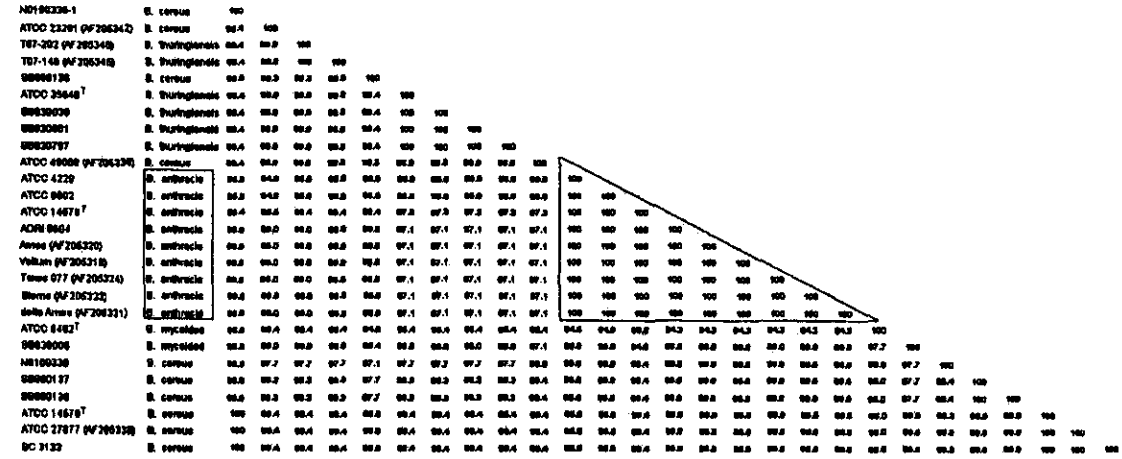

FIG. 2. *B. cereus* group members (clinical as well as type strains) used for *rpoB* gene analysis. The similarity matrix (pairwise comparison) and corresponding phylogenetic tree (neighbor joining) were created with Bionumerics (version 2.5).

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 pre-existing 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).

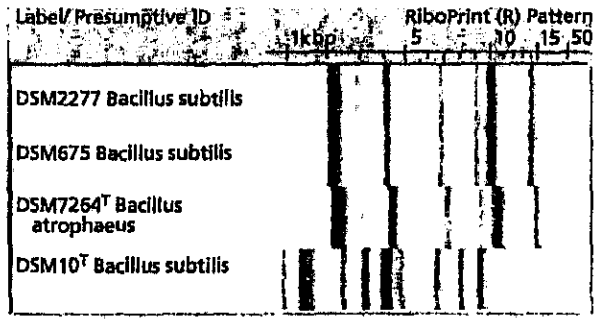

Fig. 1. Normalized RiboPrint pattern found within '*Bacillus subtilis*' strains DSM 675 and DSM 2277, related to the type strain of *Bacillus atrophaeus*, compared with the ribotype pattern of the type strain of *B. subtilis*.

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. *nlger*' 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 (Mollinckrodt, 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-

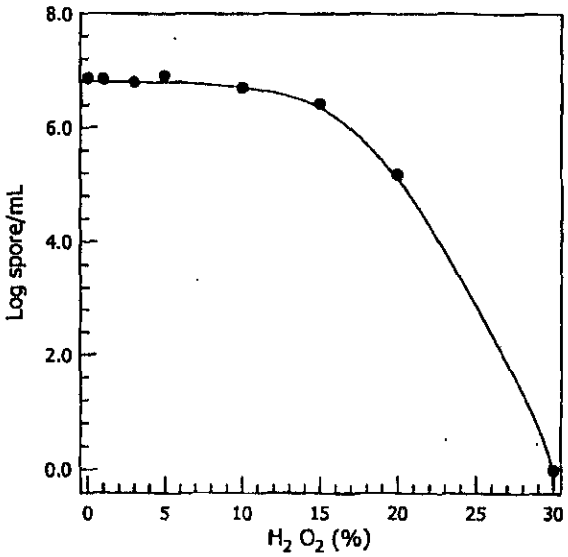

Fig. 1. Inactivation of spores of *B. subtilis* OSU494, 7.3×10^6 initially, when treated with a varying concentration of hydrogen peroxide (1% to 30%) at 22 °C for 1 min.

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-

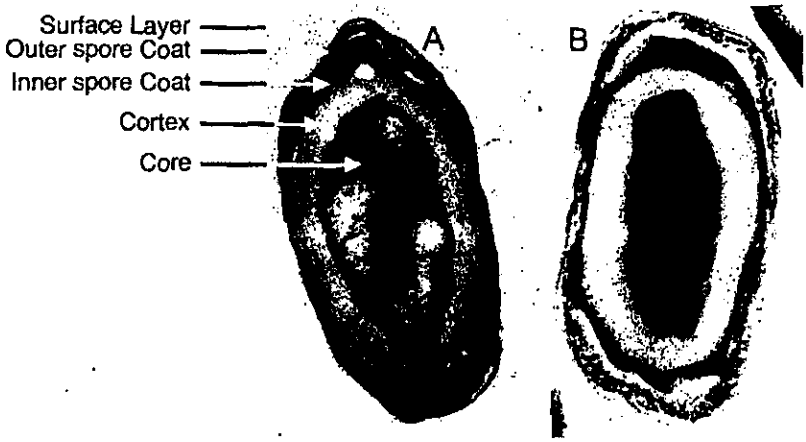

Fig. 2. Transmission electron microscopic micrograph of *B. subtilis* OSU494 spores, untreated (A), or treated (B) with ozone. Ozone-treated spores were exposed to aqueous ozone (10 μg/ml) at 22 °C for 1 min followed by neutralization with sodium thiosulfate. Note that the surface layer and the outer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

[a] 1, N. R. Smith, U. S. Department of Agriculture Research Center, Beltsville, Md.; 2, C. E. Georgi, University of Nebraska, Lincoln; 3, J. Naghski, Eastern Regional Research Laboratory, Chestnut Hill, Pa.; 4, L. J. Wickerham, Northern Regional Research Laboratory, Peoria, Ill.; 5, American Type Culture Collection, Rockville, Md.; 6, P. Stansly, American Cyanamid Co., Pearl River, N.Y.; 7, R. Gillis, Amsco Co., Erie Pa.

[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%.

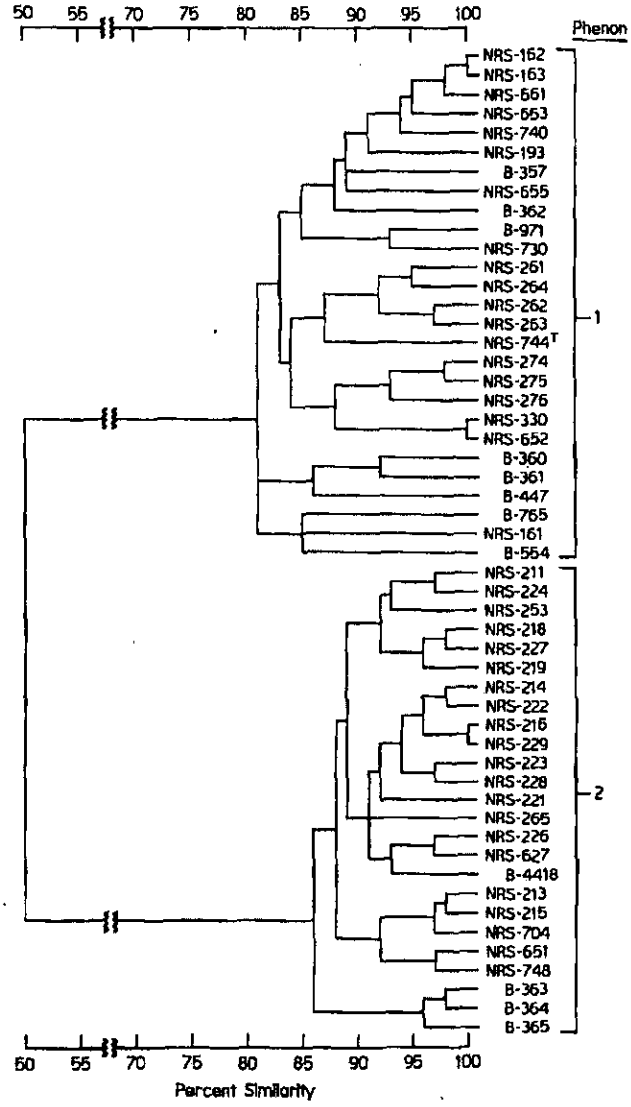

FIG. 1. Relationships of pigmented *B. subtilis* strains. The dendrogram was generated by unweighted average linkage clustering from a matrix of simple matching coefficients based on 12 enzyme loci.

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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August 2004

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	BI	S1	S2	S3	BI
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
BI = 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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Keywords
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

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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-

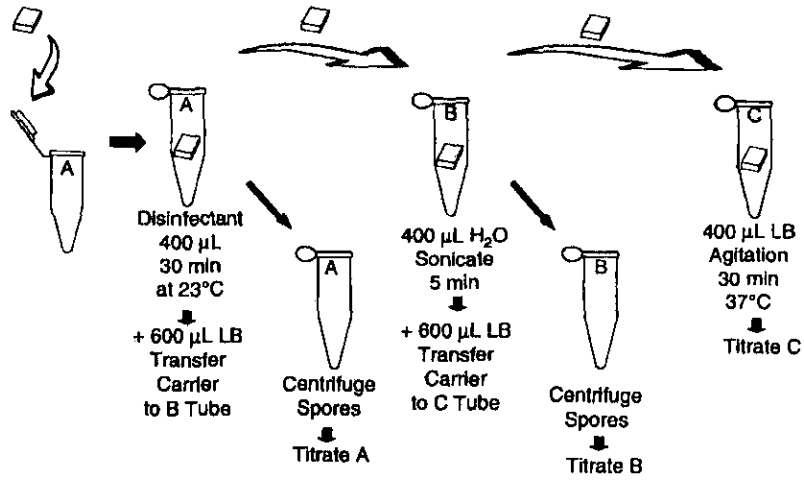

Figure 1 Schematics of the three-step sporicidal method used in this work. Reprinted, with permission, from E2414-05 Standard Test Method for Quantitative Sporicidal Three-Step Method to Determine Sporicidal Efficacy of Liquids, Liquid Sprays, and Vapour or Gases on Contaminated Carrier Surfaces, copyright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

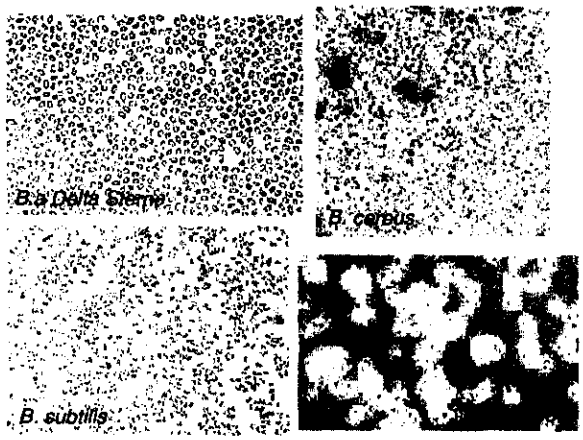

Figure 2 Quality of spores. Phase contrast microscopy at 1000× (total magnification) of *Bacillus anthracis* delta Sterne, *B. anthracis* ba 1087, *Bacillus cereus*, and *Bacillus subtilis* showing more than 95% of phase bright dormant spores.

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

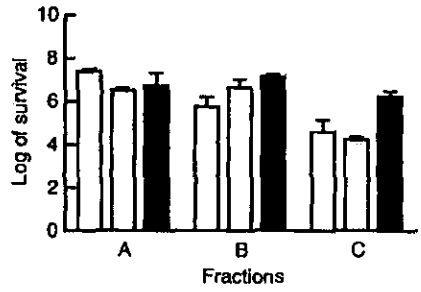

Figure 3 Effect of surface materials. *Bacillus subtilis* spores dried onto glass (empty bars), metal (grey bars), or rubber (black bars) carriers were treated with water (in absence of disinfectants) and eluted in three steps of increasing stringency. Each fraction A, B and C was titrated separately 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-

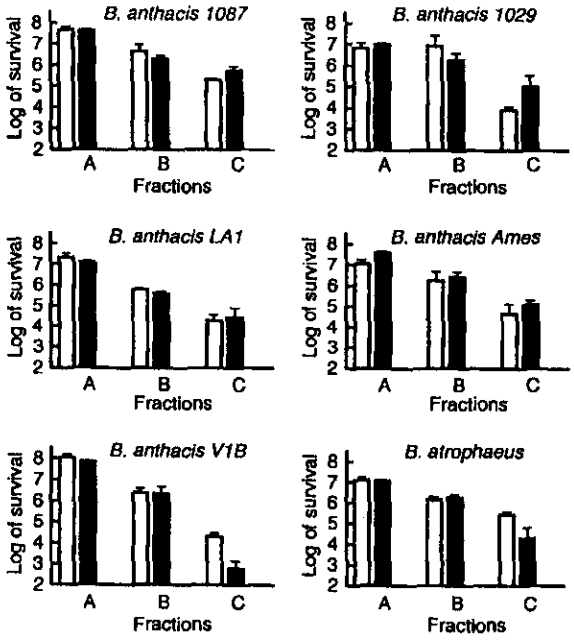

Figure 4 Elution of *Bacillus anthracis* spores from metal or rubber. The different strains of virulent *B. anthracis* indicated in the graphs were tested on metal carriers (grey bars) or rubber carriers (black bars). The elution profile of *Bacillus atrophaeus* is included for comparison. Bar height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

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, $n \geqslant 3$).

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

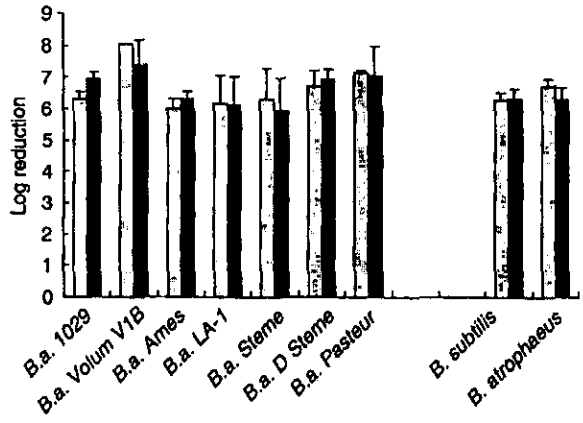

Figure 5 Comparison of *Bacillus anthracis* and its simulants. Bar height represents the log reduction of the number of spores deposited either on metal carriers (grey bars) or rubber carriers (black bars). Log reduction was calculated by subtracting the total number of spores surviving treatment with 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* *ue* under *Biological Indicators—Resistance Performance Tests* 5). 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*.

Survival 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⟩. requirements of the test are met if all of the specimens subjected the steam sterilization for the survival time show evidence of wth, 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 tional 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 ival time test or meet the kill requirement for the kill time test, ichever is applicable, the requirements of the test are met.

Total viable spore count—Proceed as directed for *Total Viable ore 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—

Presence 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 Steriliion, Paper Carrier individually packaged in a suitable tainer readily penetrable by steam and designed to an appropriate bacteriological culture medium, so o enable the packaged carrier, after subjection to ated steam sterilization conditions, to be incubated he 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- ained system is such that, after exposure to the ified sterilization conditions and inoculation of the ium under closed conditions as stated in the eling, there is no loss of medium and inoculum ing 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-

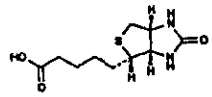
mens under test show growth. If one or more of the specimens do not show growth, repeat the test with 20 additional units. The additional units all show growth.

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.

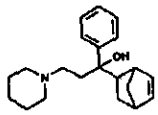
Organic volatile impurities, *Method V* ⟨467⟩: meets the requirements.

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 precipi formed. To a second 5-mL portion of the solution add bromi dropwise: a yellow precipitate forms which redissolves on sh and finally, upon the addition of more bromine TS, a perm 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 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 hyd (100 : 1.5).

Visualization: 17.

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

(Official until July 1,

Assay—Dissolve about 500 mg of Biperiden, accurately weig 20 mL of benzene, add 2 drops of crystal violet TS, and titr 0.1 N perchloric acid VS to a blue endpoint. Perform a determination, and make any necessary correction. Each mL 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-phen drochloride.

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