A Sensitive Real-Time Spore Detection Assay

Fei Chen[1], Shigeya Suzuki[2], Gregory Kuhlman[1], Kasthuri Venkateswaran[1], Roger Kern[1]

[1]Biotechnology and Planetary Protection Group,
Jet Propulsion Laboratory, California Institute of Technology,
4800 Oak Grove Drive, Pasadena, CA 91109, USA
[2]Kikkoman Corp., Noda City, Chiba Prefecture, Japan

National Aeronautics and Space Administration
Jet Propulsion Laboratory
California Institute of Technology
Pasadena, California

Abstract

NASA monitors the cleanliness of spacecraft to limit terrestrial microbes being transferred to other planetary bodies. Spore-forming organisms are of particular concern. The current NASA standard procedure involves collecting spacecraft surface sample with swab, and detecting spores using a culture based assay. This traditional method is laborious and requires a long cultivation time. A sensitive, rapid, and real-time spore detection (RSD) assay is needed to meet demanding spacecraft assembly schedules. A firefly luciferase ATP bioluminescence assay has been developed to determine the microbial cleanliness of spacecraft and associated- environments. This ATP assay is rapid and simple. However, the level of ATP is very low in spores, and the ATP assay is not very sensitive for spore detection. As the AMP level is much higher than the ATP level in spores, AMP may be a better biomarker for spore detection. In an AMP-targeted bioluminescence assay, AMP is converted to ATP using pyruvate orthophosphate diakinase, and ATP is subsequently detected by luciferase. In this present study, we measured the AMP content of several spores of *Bacillus* species using AMP bioluminescence assay and optimized conditions suitable for rapid spore detection. Spores of several *Bacillus* type strains as well as spacecraft-associated environmental isolates of the same species were purified and compared for AMP content. For the *Bacillus* strains tested, the mean AMP content of a bacterial spore is 10^{-18} moles per colony forming units. Several environmental samples were collected from the spacecraft assembly facility and the sensitivity of this advanced rapid spore detection method was evaluated. The ability of using RSD method to distinguish live vs dead spores was also investigated. In summary, this RSD spore assay is rapid and sensitive and detects spores in real time. The instrument required for the analysis is portable, which make the RSD method suitable for field-deployment and can be integrated. Once integrated this RSD spore detection method may have wide applications in homeland security, defense against bio-warfare agents, environmental monitoring, as well as in the food and pharmaceutical industries.

Introduction

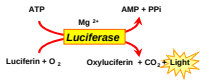
Current Spore Detection Methods

Methods	Sensitivity	Speed	Simplicity
Culture	Very High	Very slow	Complicated
Tb-DPA	Low	Rapid	Simple
PCR	High	Rapid	Complicated
Biochip	Low	Rapid	Complicated

Objective: To develop a new sensitive, rapid, and simple spore bioburden detection method.

A Rapid Method for Bio-cleanliness Monitoring

Enzyme Based ATP Assay



Advantages: Very simple, Very rapid, and Sensitive
Disadvantage: Does not detect spores.

AMP Level is much higher than ATP Level in Spores

Adenine Nucleotide Levels in Spores of Various Species

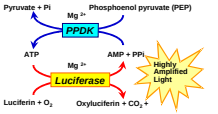
	ATP	ADP	AMP
B. cereus	0.005	0.2	1.3
B. megaterium	0.005	0.2	1.2
B. subtilis	< 0.005	0.2	1.2
C. bifermentans	0.006	0.6	2.3
C. perfringens	< 0.05	0.2	1.7
S. halophila	< 0.005	0.1	0.8
S.ureae	< 0.005	0.05	0.5

* Values are reported in micromoles per gram of dry spores. (Loshon, C. A. and Setlow, P. 1993)

AMP may be a better biomarker for spore detection.

AMP Based Spore Detection

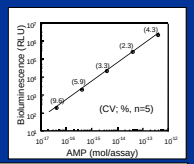
Principle of Bioluminescent Cycling Reaction Using PPDK and Luciferase



Advantages: Very rapid, more sensitive, CAN detect spores.

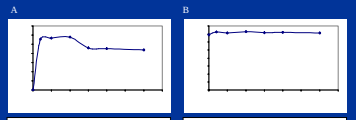
Typical Standard Curve for AMP

Using PPDK Bioluminescent Reagent



Sensitivity:	AMP Detection limit is 10^{-17} mol/assay
Linear Range:	over four magnitudes
Reproducibility:	coefficient of variation less than 10%

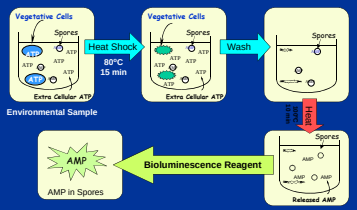
Rapid Release of AMP from Spores by Heat



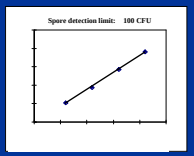
(A) AMP released rapidly from spores by heat at 100°C.
100 µl spore solutions (~ 10^4 CFU/ml) were heated at 100°C for various lengths of time (0, 1, 2.5, 5, 7.5, 10, and 15 min). The amount of AMP released from spores at each time point was measured using PPDK bioluminescence reagent.

(B) AMP is stable under heating condition.
100 µl AMP standard solutions (10^{-10} M) were heated at 100°C for various length of time (0, 1, 2.5, 5, 7.5, 10, and 15 min). The amount of AMP in each samples were measured at different time points using PPDK bioluminescence reagent.

Procedure for AMP Spore Detection Assay



Sensitivity of AMP Spore Detection Method



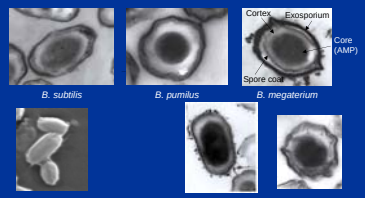
100 µl spore solutions (10^3~10^6 CFU/ml) were heated at 100°C for 10 min. The amount of AMP released from spores in each sample was measured using PPDK bioluminescence reagent.

Preparation of wild type stain spores

Sample collection:
Wild type strain spores were collected from various cleanroom facilities from year 2000 to 2002. The isolates were classified using Biolog system (Biolog, Hayward, CA) and identified by 16s ribosomal DNA sequencing.

Sporalation and purification:
Overnight culture was inoculated on NSM° agar-plates and grown at 37°C for two days. The plate was placed at room temperature for an additional day before purification procedure.
Spores were purified using water purification method

Spore Images of Various Bacillus Species

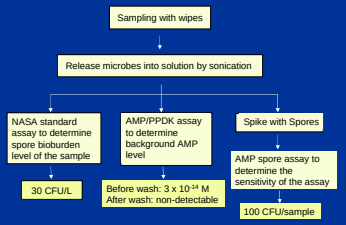


AMP Levels in Various Strains of Spores

Species	Strain		Moles AMP	CFU/assay	AMP/CFU
B. subtilis	ATCC 6051	Type Strain	1.76E-15	220	8.00E-18
	ATCC 168-2	Type Strain	2.82E-16	290	9.74E-19
B. pumilus	FO 36b	Wild Type	3.30E-16	140	2.36E-18
B. cereus	FO-11	Wild Type	8.00E-16	96	8.34E-18
B. megaterium	ATCC 14581	Type Strain	1.45E-15	830	1.75E-18
B. mojavensis	ATCC51516	Type Strain	3.34E-16	80	4.18E-18
	KL-154	Wild Type	2.07E-16	450	4.60E-19
B. odysseyi	34hs1	Wild Type	2.80E-16	200	1.40E-18
B. licheniformis	ATCC14580	Type Strain	1.76E-16	68	2.59E-18
	KL-196	Wild Type	1.75E-15	840	2.08E-18
				Mean	3.21E-18
				STDEV	2.80E-18

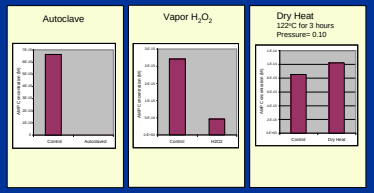
Spores of several *Bacillus* type strains as well as spacecraft-associated environmental isolates of the same species were purified and compared for AMP content. AMP content in a sample was determined from a standard curve that was established with purified chemical AMP. For the *Bacillus* strains tested, the mean AMP content of a bacteria spore is 10^{-18} moles per colony forming unit.

Environmental sample



Live vs Dead

Spores were killed by three procedures: autoclave, vapor H2O2, and dry heat. AMP levels in dead spores and control live spores were compared.



Summary

This method is very rapid and sensitive.

Detection time:	10 seconds
Assay time:	30 min
Sensitivity (for AMP):	10^{-17} moles/assay
Sensitivity (for Spores):	100 CFU

Rapid release of spore biomarker by heat using PCR machine enabled the HTP (high-through-put) processing of large amount of samples in 96 well format.

The instrument required for the analysis is portable, which makes this method suitable for field-applications.

This method can be used to evaluate the sterilization quality of autoclave and H_2O_2 procedure.