Single Spore Mass Spectrometric Analysis for Microbial Forensics

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Abstract

- Microbial attribution generally requires fundamental knowledge of the natural variation or mutation rate across organisms in varied environments
- Genomic data not providing all answers to single spore attribution
  - Simultaneously develop genomic *and* non-genomic measurements
  - Spore population measurements are unable to distinguish rare pathogenic spores (either nefariously produced or naturally occurring) present within a population of hundreds to thousands of normal spores
- Single spore differentiation requires a measurement to have:
  - Appropriate specificity and sensitivity for relevant and quantifiable signatures
  - Enough precision to correlate signatures with different environmental origins
  - Enough individual spore analyses to place the natural variability of a single spore in context for establishing attribution confidence
- Large geometry secondary ion mass spectrometry (LG-SIMS) was used to obtain single *Bacillus thuringiensis* spore profiles
  - For common elemental signatures Mg, Ca, and Fe at a dynamic signal range of > 4 orders of magnitude
  - Removal of Ca from the sporulation conditions resulted in a significant reduction in Ca signal with respect to Mg
- Efforts underway to establish measurement/spore variability to provide context for measurement values of unknown elemental concentrations within spores

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Spore Preparation

B. thuringiensis **sporulation procedure:**

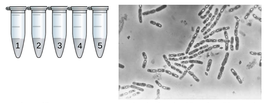
- PGSM solution prepared by combining Bacto-peptone, glucose, KH_2PO_4, K_2HPO_4 in 1 L DI water (7.2 pH-adjusted)
- Resulting solution autoclaved for 20 min at 121 °C.
- $ZnSO_4$, $MnSO_4$, $MgSO_4$, $FeSO_4$ and $CaCl_2$ prepared separately to supplement PGSM (Table 1)
- 200 µL of the inoculum (*B. thuringiensis* cultured overnight in LB broth at 30 °C) placed into each tube containing the different ion compositions (Table 1) and shaken at 30 °C for 5 days
- Cells harvested, spun down at 4000x g for 15 min at 4 °C
- Debris removal and washing with NaCl and SDS at 4 °C[1]
- Major debris removed with 5 µm filter at 1000x g for 30 sec
- Spores stored in sterile DI water; Working solutions prepared in 0.2 µm filtered ethanol
- 2 µL to 4 µL of the working solution were deposited onto clean silicon wafers

[1]PANDEY, N. K. & ARONSON, A. I. 1979. Properties of the Bacillus-Subtilis Spore Coat. *Journal of Bacteriology,* 137, 1208-1218.

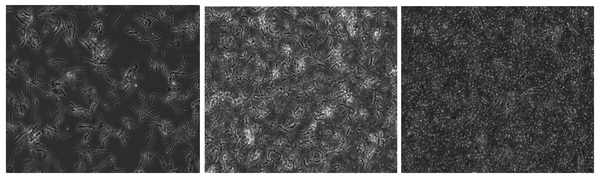
Table 1: ***B. thuringiensis*** were induced to sporulation in PGSM media supplemented with five different ion compositions as described below

1 (+All)	2 (-Mg)	3 (-Ca)	4 (-Fe)	5 (-All)
Mg^{2+}	x	Mg^{2+}	Mg^{2+}	x
Ca^{2+}	Ca^{2+}	x	Ca^{2+}	x
Fe^{2+}	Fe^{2+}	Fe^{2+}	x	x

x = suppressed ions



http://home.comcast.net/~pholowko/OnLineShows/Soil/MicroBio/BioBacteriaDescription.html



plusALL-24 hours plusALL-72 hours plusALL-96 hours

Vegetative cells → Spores

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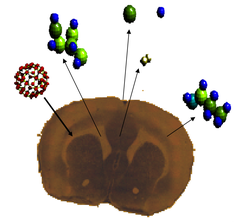
Mass Spectrometry

Large Geometry Secondary Ion Mass Spectrometry (LG-SIMS)

- Focused primary ion beam is directed at sample
- Material is removed (sputtered) in form of neutrals, (+) ions, (-) ions, and electrons
- Secondary ions are focused and separated by an electrostatic analyzer (ESA) and a magnetic sector before impinging on an electron multiplier for ion counting
- Instrument is specially designed for high mass resolution at high transmission
- Net 23 keV O^- primary ions collimated into 50 µm area containing single *B. thuringiensis* spore
- Each spore sputtered like a particle until all material removed from silicon substrate
- Individual positive ion isotopes of ^{24}Mg, ^{44}Ca, and ^{56}Fe monitored along with $^{28}Si^{29}Si$ dimer (background)
- Elemental ratios of monitored species calculated



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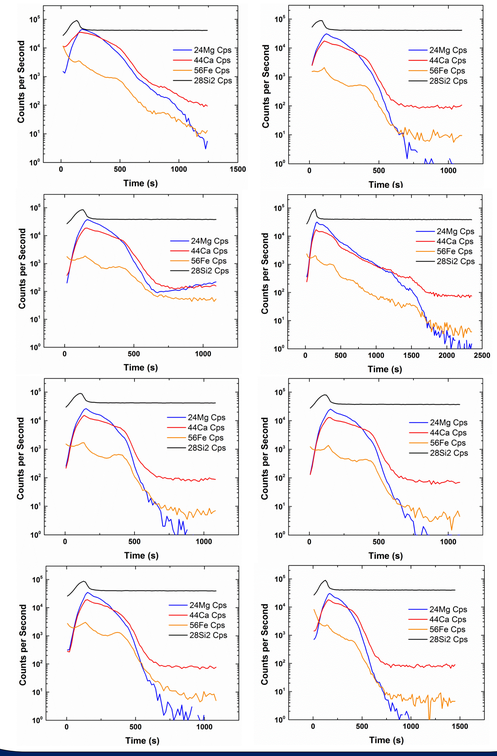
Single Spore Profiles

- Single 1-µm bacterial spores consistently analyzed with LG-SIMS
- Elemental signatures detected over 4 orders of signal magnitude
- Key developments towards establishing natural spore variability



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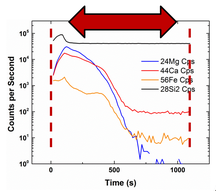
Elemental Ratios

- Elemental ratios measured using total counts throughout full profiles
  - Ca/Mg ratios consistent; a bit less so for Ca/Fe and Mg/Fe

n = 7	$^{44}Ca/^{24}Mg$	$^{44}Ca/^{56}Fe$	$^{24}Mg/^{56}Fe$
Average	0.713	8.08	11.48
1 σ	0.051	1.88	3.08
1 σ (%)	7.08	23.3	26.9
2 σ	0.101	3.77	6.17



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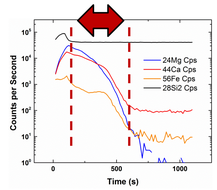
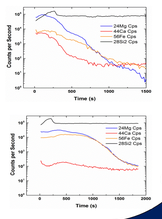
- Elemental ratios measured using counts from equilibrium through spore
  - Ca/Mg ratios slightly less consistent; Ca/Fe and Mg/Fe both improved
    - Overall improved quantification and natural variability
  - Compare with case where Ca excluded from sporulation media below:

n = 7	$^{44}Ca/^{24}Mg$	$^{44}Ca/^{56}Fe$	$^{24}Mg/^{56}Fe$
Average	0.770	12.12	15.87
1 σ	0.065	2.22	3.39
1 σ (%)	8.49	18.4	21.4
2 σ	0.131	4.45	6.79



- Dramatic difference in Ca ratios when Ca not in sporulation media
- Otherwise, more statistics needed with cleaner, more dilute solutions

n = 2	$^{44}Ca/^{24}Mg$	$^{44}Ca/^{56}Fe$	$^{24}Mg/^{56}Fe$
Average	0.023	0.079	2.59
1 σ	0.024	0.097	1.55
1 σ (%)	100	123	59.9
2 σ	0.047	0.194	3.10



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Conclusions

- LG-SIMS method being established for single bacterial spore analysis
- Natural variability of elemental ratios being measured to provide context
- Preliminary data gives confidence for quantification
- Work towards single spore analysis in dirtier samples; other elements

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