MASTER'S ORAL PRESENTATION (June 19th, 2015):

« Inhibiting inosine hydrolase and alanine racemase to enhance the germination of Bacillus anthracis Sterne spores: potential spore decontamination strategies »

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The original document contains color images.
Anthrax Background

- Caused by a gram-positive spore forming rod.

- Important veterinary disease as herbivores may be prone to the disease if they feed in ‘anthrax zones’

- Accidentally in Humans

- Natural reservoir is soil

- Anthrax Disease Cycle:
  - animals infected by soilborne spores in food and water or bites from certain insects
  - Humans can be infected when in contact with flesh, bones, hides, hair or excrement
Anthrax Background

- **4 forms**: cutaneous and inhalational most common.
  
  ![Images of cutaneous, inhalation, ingestion, and injection pathways]

- **Concern for Biodefense Community: Intentional or Accidental release of spores**
  - **Why?** Anthrax spores are easily found in nature, can be produced in a lab, and can last for a long time in the environment.
  - **How?** Can be released easily and quietly. Nobody is able to see, smell, or taste them. Signs and symptoms are non-descript flu-like symptoms making rapid diagnosis difficult.
  - Decontamination difficult, expensive and with toxic/corrosive effects to the environment and other sensitive materials.
    - 22 cases, 11 inhalational, 5 deaths
    - $650 million and took more than three years
Decontamination

- US Government Priority after 2001
- Current decontamination methods include:
  - Burn or bury animal carcasses
  - Treat soil with 5% lye, quicklime, or bleach (sodium hypochlorite)
  - High-efficiency particulate arrestance vacuuming (source reduction)
  - Liquid antimicrobials (non-porous surfaces)
  - Fumigation (chlorine dioxide, vaporous hydrogen peroxide)

- Decontamination objectives: be EASIER, SAFER, and CHEAPER
  Inducing spore germination should make resulting bacteria much more susceptible to decontamination methods and will be less hazardous to first responders.
Bacillus anthracis Cycle

- Sufficient nutrients
- Germination
- Germinated spores
- Ungerminated spores
- Anthrax cycle
- Sporulation
- Bacilli
- Growth
Bacillus anthracis Cycle

Germination

Germinated spores

REQUIRED FOR BACTERIAL REPLICATION, HOWEVER NOT NORMALLY INFECTIOUS

INFECTION PARTICLE THAT IS INTRODUCED INTO THE HOST

Sporulation

Bacilli

Produce toxin and capsule; Ultimately kill host

Growth

Anthrax cycle

UNCLASSIFIED
In vitro germination induction by AI

- Simple
- Effective
- Safe
- Will not support replication and subsequent sporulation
- Will germinate spores and then "stop"
Interest of AI induced germination

Ungerminated spores

Germination

Germinated spores

Resistant
- to desiccation
- to most of desinfectant
- to antibiotics
- to heating
- to host immune response

Sensitive
- to desiccation
- to most of desinfectant
- to heating
- to antibiotics
- to host immune response
In vitro alanine and inosine germination pathways

- **L-alanine**
  - L-amino acid
  - Can acts alone
  - Action on specific germinant receptors (*gerR*)
  - Action on enzyme alanine racemase (*Alr*)

  ![L-alanine to D-alanine diagram](image)

  - *Alr* inhibits by the antibiotic D-cycloserine (Gould 1968, Omotade et al., 2013)

- **Inosine**
  - Purine nucleoside
  - Co-germinant only in *Bacillus anthracis*
  - Action on specific germinant receptors (*gerl*, *gerQ* and *gerR*)
  - Action on Inosine uridine nucleoside Hydrolase (*lunH*)

  ![Inosine to hypoxanthine + ribose diagram](image)

UNCLASSIFIED
Localization of enzymes

- Alanine racemase
- Inosine uridine nucleotide hydrolase
- Germinant receptor
Objectives

- Test the impact of the inactivation of two germination-inhibiting enzymes, alanine racemase and inosine hydrolase on the alanine and inosine induced germination:
  - using a $iunH$ gene deletion
  - by D-cycloserine treatment
- in order to identify new strategies for an efficient decontamination.
Material

- Attenuated *B. anthracis* strain Sterne (pXO1+, pXO2-): veterinarian vaccinal strain. Lost its ability to produce a capsule.

- Inosine hydrolase (IunH) defective mutant of Sterne strain with kanamycin insertion (Sterne *iunh::Ω-kan-2*) from Biology Department at Louisiana Tech University, Ruston, LA.
Methods

In vitro detection of spores germination induced by AI

- **Heat resistance assay**
  Once spores germinate they become sensitive to elevated temperatures, thus a difference in viable colony forming unit/ml (cfu/ml) in samples that were heated versus samples that were not subjected to heat treatment, reflects the amount of germination induced.

- **Loss of optical density**: spectrophotometric determination of germination rate based on alterations in spore refractility. During the process of germination, spore releases its large pool of Ca\(^2+\)-dipicolinate stored in the core, and becomes partially rehydrated through an influx of water.

- **Fluorescence spectrophotometry** (Welkos et al., 2004): increase in fluorescence of spores with time during their incubation in germination medium containing a fluorescent nucleic acid-binding dye which stained germinated *B. anthracis* but not ungerminated spores.
Results: inosine hydrolase inhibition

(a) Heat resistance assay

(b) Spectrophotometer assay

(c) Fluorescent assay

Spores deficient in the inosine hydrolase (encoded by \textit{iunH}) germinate more rapidly than wild-type spores
Results: both enzymes inhibition

Germination rate of *iunH* mutant spores initiated by L-alanine and inosine in presence of DCS 10 mmol l$^{-1}$ was significantly greater than the germination rate of the wild-type spores under same conditions ($p=0.0001$)
**Results: interest of a 24h DCS pretreatment in Sterne**

- Previously demonstrated that DCS is dose and time dependant (Omotade *et al.*, 2013)

Concomitant delivery of DCS with germinant solutions is more beneficial to wide-area decontamination efforts that pretreatment with DCS followed by germinant solutions.
Conclusion

Interest in context of novel decontamination strategies

- Increase of the germination rate induce:
  - By inhibiting Alr and IunH separately
  - By inhibiting the both concommitantly (*iunH* mutant spores positively affected by the block of Alr)

- Better understanding and manipulating spore germination.

- Induction of the transition from highly resistant ungerminated spores to much more susceptible and less virulent germinated spores.

- Strengthens the early work published in 2013 and 2014 showing that spore germination rates are augmented potentially improving decontamination strategies.
Prospect for the future

- Optimize the L-alanine concentration in addition to the inosine concentration in presence of DCS

- Test potential inhibitors of *B. anthracis* inosine hydrolase and prove that such enzymatic inhibitors could be used in conjunction with DCS to facilitate more efficient and environmentally friendly surface decontamination of *B. anthracis* spores.

- Test current decontamination methods after germination induction by inhibiting both Air and lunH
USAMRIID BACTERIOLOGY DIVISION

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