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FACULTY
OF BIOTECHNOLOGY

PORTO

Spore forming pathogenic bacteria

Paul Gibbs

Paula Teixeira

Spore forming bacteria

- Basic characteristics
  - All species produce endospores that are heat, chemical and radiation resistant to different degrees.
  - Some species cause food poisoning / intoxication.
  - Some species cause specific types of food spoilage.
  - Several species cause disease of man and domestic animals.

Bacterial spores

- Dehydrated
  - therefore heat resistant.
- Dormant core containing essentials of a new cell (e.g. DNA, etc.).
- Cysteine rich proteinaceous outer spore coat that absorbs ionising radiation and is chemically resistant.
- Germination may require heat shock and specific compounds, e.g. alanine, lactate

Sporulation

1. Division of nuclear material
2. Septation of pre-spore nucleus
3. New spore membrane around nucleus
4. Synthesis of spore cortex, UV & chemical resistance, core dehydration
5. Coat synthesis, radiation resistance
6. DPA & divalent ion uptake, spore dehydration, refractility & dormancy completed; cell lysis to release spore.

Spore forming bacteria

- Species of concern to food microbiologists:
  - *Bacillus* spp; aerobic - facultative, sporulate only aerobically
  - *Clostridium* spp; anaerobic - aerotolerant, sporulate only anaerobically
  - *Desulfotomaculum* spp.

Clostridium species

- Gram positive spore forming rods.
- Anaerobic to aerotolerant.
- Sporulate anaerobically only
- Species of concern:
  - *Clostridium botulinum.*
  - *Clostridium perfringens (welchii).*
  - *Clostridium difficile*?

Clostridium botulinum

- Seven toxin types
  - A - G.
- A, B, E, F
  - associated with human botulism.
- C, D
  - Group III -associated with animal botulism only (NP)

- A, B, F
  - Group I - Proteolytic, mesophilic
- B, E, F
  - Group II - Non-proteolytic, psychrotrophic

Clostridium botulinum - botulism

- Most severe form of food poisoning.
- Illness due to ingestion of toxin.
- Incubation period
  - 8 hours to 8 days.
- Lethal dose
  - 0.005 - 0.1 µg (proteolytic).
  - 0.1 - 0.5 µg (non-proteolytic).
- Mortality rate
  - 10% (if prompt treatment).

Clostridium botulinum - botulism

- Symptoms
  - generalised muscular weakness.
  - headache.
  - dizziness.
  - visual disturbances.
  - nausea.
  - vomiting.
  - difficulties with speech and swallowing.
  - descending paralysis.
  - respiratory failure.

Clostridium botulinum - foods involved

- Meat and meat products.
- Dairy products.
- Fish.
- Vegetables.
- Infant foods.
- Canned foods.

Examples of recent outbreaks

Year	Location	No. of cases	No. of deaths	Implicated food
2016	Germany and Spain	6	?	Dried salted fish
2015	Ohio	29	2	Potato salad prepared with home-canned potatoes
2015	Portugal	4	0	Smoked sausages

A recent recall…

Illnesses reported; Loblaw expands recall of organic baby food

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‘Botulinum cook’

- A heat process giving a 12 log cycle kill of the spores of the most heat resistant *C. botulinum* strain.

- Commercially
  - equivalent to at least 3 minutes at 121° C at the slowest heating point in the container.

Characteristics of *C. botulinum*

	Group I	Group II
Toxin types	A, B, F	B, E, F
Proteolytic	+	-
Inhibitory pH	4.6	5.0
Inhibitory NaCl conc^{n}	10%	5%
Minimal a_w	0.94	0.97
Temperature range	10 - 48 °C	3.3 – 45 °C
D_{100} of spores	25 min	<0.1 min

Characteristics of botulinal toxin

- Proteins; MWt *ca* 150kDa; heavy & light chains
- Neurotoxins
- H-chain binds to neurons; L-chain internalised; blocks acetylcholine release
- Specific peptidase activities on synaptic vesicle proteins

C. botulinum - control of psychrotrophic strains

- A heat treatment of 90 ° C for 10 min or equivalent lethality.
- A pH of 5 or less throughout the food and throughout components of complex foods.
- A minimum salt level of 3.5% in the aqueous phase throughout the food and throughout the components of complex foods.
- An Aw of 0.97 or less throughout the food and throughout the components of complex foods.
- A combination of heat and preservative factors which can be shown consistently to prevent growth and toxin production by psychrotrophic *C. botulinum* at temperatures up to 10 ° C.

ACMSF; 1992

C. botulinum - plating media

- Horse Blood Agar.
- Reinforced Clostridial Agar.
- Egg Yolk Agar.

C. botulinum - isolation from foods

- Enrichment
  - self enrichment
    - vacuum pack & incubate at 5-30 ° C for >10 days if food will support good growth of the organism.
  - enrichment media
    - Robertson's cooked meat medium.

Clostridium perfringens Type A food poisoning

- Incubation period
  - 8 - 22 hours.
- Symptoms
  - diarrhoea, severe abdominal pain, nausea (occasionally)
- Infective dose
  - $>10^5$ cells/g
- Mortality
  - very rare.

Clostridium perfringens - foods involved

- Meat and meat products.
- Milk and dairy products.
- Fish and fish products.

Characteristics of *C. perfringens*

Temperature range	15 – 50 °C (optimum 43 – 45 °C)
Inhibitory pH	5.0
Minimal aw	0.95
Inhibitory NaCl conc^{n}	5%
D_{95} of spores	1.3 – 6.4 minutes

Characteristics of *C. perfringens* enterotoxin (cpe)

- Cpe formed on sporulation in intestine
- Cpe a protein of *ca* **35kDa**
- **Heat sensitive (60 ° C / 5 mins)**
- Resistant to some proteases
- Initial binding to plasma membrane intestinal cells;
- Interaction with plasma membrane proteins ➲ leakage cell contents

Recent outbreaks…

Year	Location	No. of cases	No. of deaths	Implicated food
2016	USA	>20	3	Turkey and mashed potatoes
2013	UK	150	0	Chicken balti
2012	Norway	>43	0	Beef stew

Clostridium perfringens - media and methods

- Direct plating
  - if suspected high numbers.

- Most probable number technique
  - if suspected low numbers.

C. perfringens - plating media

- Egg yolk free tryptose sulphite cycloserine agar (EYFTSC).
- Oleandomycin polymyxin sulphadiazine perfringens agar (OPSP).
- Shahidi Ferguson perfringens agar (SFP).
- Neomycin blood agar.
- Sulphite polymyxin sulphadiazine agar (SPS).

C. perfringens - plating media

- EYFTSC
  - Selective agents - Cycloserine.
  - Indicator system - Sulphide blackening.
- OPSP
  - Selective agents - Oleandomycin, polymyxin, sulphadiazine.
  - Indicator system - Sulphide blackening.
- SFP
  - Selective agents - Sulphadiazine, polymyxin, kanamycin.
  - Indicator system - Sulphide blackening, egg yolk.

C. perfringens - plating media

- Neomycin blood
  - Selective agents - Neomycin.
  - Indicator system - Haemolysis.

- SPS
  - Selective agents - Polymyxin, sulphadiazine.
  - Indicator system - Sulphide blackening.

C. perfringens - MPN media

- Differential Reinforced Clostridia medium (DRCM).
- LS medium.
- Iron-milk medium.

C. perfringens - confirmation

- Motility (non-motile).
- Nitrate reduction.
- Gelatine liquefaction.
- Lactose fermentation.
- LEYM agar
- Lactose gelatine medium.
- Nitrate, indole motility medium

Clostridium difficile: Foodborne Transmission?

- *Clostridium difficile* is a major cause of illness
- Initially recognized as an hospital pathogen
- Now recognized as an important cause of severe community acquired infections
- The source of community acquired *C. difficile* yet to be established
- Foodborne being one route considered

C. difficile in foods

- High prevalence of *C. difficile* in animals
- Identified in food products:
  - Meat
  - Fish
  - Produce
  - Water
- No foodborne illness outbreaks have been directly linked to *C. difficile*

C. difficile in foods: research needed…

- Available data cannot be considered a true prevalence
- Low levels of the pathogen in foods may require improved detection methods
- Knowledge gaps with respect to growth ranges of *C. difficile* in foods

Bacillus species

- Gram positive spore forming rod.
- Aerobic and facultatively anaerobic.
- Species of concern
  - *Bacillus cereus and closely related species e.g. B. thuringiensis.*
  - *Bacillus subtilis.*
  - *Bacillus licheniformis.*

Bacillus cereus - two types of food poisoning

	Emetic	**Diarrhoeagenic**
Incubation time	1 – 5 hours	8 – 16 hours
Symptoms	Nausea / vomiting	Abdominal pain / diarrhoea
Mortality rate	Nil	Nil
Infective dose	$>10^5$	$>10^5$

Characteristics *B. cereus* toxins; (1) emetic toxin, cereulide

- Ring-form peptide; hydrophobic; 3 repeats of 4 amino acids; MWt 1.2kDa.
- Heat stable 121˚ C / 90 mins.
- Acid stable pH 2 - 11.
- Protease stable (trypsin, pepsin).
- Binds to 5-HT_3 receptor (vagus nerve stimulated).
- Non-immunogenic

Characteristics *B. cereus* toxins; (2) diarrhoeagenic enterotoxin

- **Excreted with a signal peptide**
- **May be at least two active moieties**
- **Protein sub-unit of haemolysin, MWt *ca* 38 kDa**
- **Mode of action unknown - binds weakly to ileal cells**
- **Immunogenic**

B. cereus - foods involved

- Emetic
  - farinaceous materials especially
    - boiled rice.
    - cooked pasta.
    - noodle dishes.
- Diarrhoeagenic
  - wide variety of foods but commonly
    - meat and vegetable dishes.
    - soups.
    - sauces.
    - puddings.

Characteristics of *B. cereus*

Temperature range	4 – 50 °C (optimum 28 – 35 °C)
Minimum pH growth (Stability)	4.3 (Diarrhoeagenic toxin 4 – 11) (Emetic toxin 2 – 11)
D_{95} spores	1.2 – 3.6 minutes
Minimum a_w	0.95 (0.91 – rice)

B. cereus - plating media

- Mannitol egg yolk polymyxin agar (MEYP).

- Polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA)

B. cereus - plating media
Selective and diagnostic components

- Polymyxin B
  - Selective agent.
- Mannitol and indicator
  - Differentiation.
- Egg yolk
  - Differentiation.
- Low level of peptone / absence of meat extract
  - Encourage sporulation
  - Inhibit lecithinase production by *B. polymyxa*.

Bacillus cereus - rapid stain

- Prepare films from centre of 1 day old colony or edge of 2 day old colony.
- Air-dry and heat fix.
- Stain with 0.3% Sudan Black in 70% ethanol for 15 minutes.
- Wash slide with xylene for 5 seconds. Blot dry.
- Counter stain with 0.5% w/v Safranin for 20 seconds.

Bacillus cereus - rapid stain

- Appearance
  - Cells are 4-5 µm long by 1-1.5 µm wide.
  - Square ends with rounded corners.
  - Spores stain pale to mid green
  - Spores are central or para-central in position.
  - Spores do not swell sporangium.
  - Lipid globules are black.
  - Vegetative cytoplasm is red.

Detection of *B. cereus* toxins

- Diarrhoeagenic
  - RPLA, TECRA.
- Emetic
  - Primate feeding tests.
  - HEp-2 cell assay.

Summarizing
‘Spore forming bacteria’

- **Basic characteristics**
  - All species produce endospores that are heat, chemical and radiation resistant to different degrees.
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  - Several species cause diseases of man, insects and domestic animals.