...Pathogenesis of *Cronobacter*...

Enterotoxin Production, Adherence and Invasion of the Blood-brain Barrier

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Possibility of cross-contamination during manipulation in hospitals or households

Expressed human breast milk
Expressed fortified human breast milk

Storage at room temperature (23°C) for a prolonged period (>4 h); or enteral feeding at temperature of incubator (>23°C) for longer than the recommended hang time

C. sakazakii growth characteristics from our work (inoculum 10-100 cfu):
Average generation time at 23°C (0.82 h) and 37°C (0.40 h)
Average lag time at 23°C (4.09 h) and 37°C (2.45 h)

C. sakazakii crossing the blood-brain barrier leads to meningitis, ventriculitis, brain abscess, infarction, and/or cyst formation causing neurological sequelae in many cases

Stomach of neonates has a higher pH (>4.0); this may facilitate survival of C. sakazakii

Permeable intestine: C. sakazakii crossing the GI-barrier leads to sepsis and bacteremia

Gastrointestinal symptoms of C. sakazakii infection: necrotizing enterocolitis and diarrhoea

At-risk population: neonates, pre-term infants and very low-birth-weight infants
What we know so far…

- **Adhesion & Invasion**
  - Adhesion to and invasion of intestinal epithelial cells has been demonstrated several times (Mange et al. 2006; Kim & Loessner 2007; Mohan Nair & Venkitanarayanan 2007)
  - Adhesion to and invasion of brain endothelial cells has also been shown (Townsend et al. 2007)

- Conditions leading to the formation of an exopolysaccharide have been shown in *C. sakazakii* (Gurtler et al. 2005)
  - Capsule formation has not yet been demonstrated

- Has not been determined whether or not the source of the *C. sakazakii* strain influences the ability to adhere to or invade human cells
Enterobacter sakazakii: Infectivity and Enterotoxin Production
In Vitro and In Vivo

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Enterobacter sakazakii, the etiological agent of enterobacter sakazakii gastroenteritis (ESGE), is a rare cause of neonatal infection, having been reported to produce enterotoxin. The antibiotic susceptibility and virulence characteristics of this organism have been reported recently. This study characterizes the enterotoxin produced by E. sakazakii strain 3287. The toxin was active at pH 3 and stable for 30 min at 90 °C. This stability combined with the potent activity of the toxin (LD50 = 36 pg) emphasizes the potential risk to neonates fed infant milk formula contaminated with E. sakazakii. Further detailed molecular biological studies on the toxin are warranted in view of its stability and activity.
Phenotypic study: Congo red

Left: *E. coli* K12 (5039); Right: *K. pneumoniae* (385)

Left: *C. sakazakii* (3434); Right: *C. turicensis* (3287)

- 70% of clinical isolates
- 40% of food isolates
- 30% of environmental isolates
Adhesion Assay: (MOI 100:1)

3404 (Env; C. sakazakii)

No. adherent bacteria (CFU/mL X10^4)

Strain

Canada
Invasion Assays

3267 (Food; C. malonaticus)
Transposon Screening

- Over 1000 isogenic mutants screened
- Approximately 200 were identified, and will be tested further using the standard adhesion/invasion assay
- 24 mutants found “interesting”
  - Adhere less
  - Invade less or not at all
  - No obvious correlation to enterotoxin production
  - Some were found to adhere and/or invade more
Crystal Violet Assay

- Crystal violet stains the DNA of each living cell
- Provides quantitative information about the density of cells adhering to each well

- The ability of *C. sakazakii* to produce toxin can be evaluated by comparing the shade or degree of purple colour in each well
- Less purple = more enterotoxin made
Treatment of Vero cells with *C. sakazakii* strains 2871, 2878, 3267, 3287, 3234, and 3436 produced the greatest amount of cell death.

22% of strains tested by Pagotto et al. were positive for enterotoxin production.
5 of the 6 most toxic strains were isolated from food samples, while the other strain that produced the most cell death was isolated from the environment.

Many of the less toxic strains (2855, 3231, 3234, 3290, and 3295) were clinical isolates.
**Alamar Blue Assay**

- Detects cell viability by using a blue, nonfluorescent dye, resazurin, which is converted to a pink, fluorescent dye, resorufin, upon cell growth

- Reduction of resazurin occurs with continued cell growth following treatment, while the inhibition of growth maintains an oxidized environment

- The degree of colour change can be determined visually, or more accurately with a spectrophotometer

- “blue = death”; “red = health”
Large band present at 66 kD in strain 2878 that is not present in the Casaminoacids broth

Protein Sequencing

- Not the typical A-B toxin
- Monomer and polymer versions available
- Mode of action versus intracellular interference

Band Excised from Gel
Collectively, toxin, adhesion/invasion work to help address how pathogen binding may trigger different host responses:

- alteration in intracellular signalling
- chemokine and cytokine release
- induction of apoptosis
Current work

- Continue / finish screening isogenic mutants
  - Includes + / - enterotoxin production

- Rescue cloning / sequencing of transposon insertion site

- Identify the receptor(s) on host cells of any putative adhesins/invasins identified through rescue cloning

- Intracellular survival
  - Location
  - Duration
  - Gene regulation
Growth of bacteria

Kim *et al.* 2007 noticed a different pattern of bacterial adherence when overnight cultures were grown in broth supplemented with horse blood (what should “we” recommend? Type strains?)

Enterotoxin expression and mutagenesis

- Active site
- Mechanism(s) of action
- Purification

- Surprisingly, 5 of the 6 most virulent (and presumably pathogenic) strains were food isolates, while the other strain was from the environment
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