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Insights into virulence factors determining the pathogenicity of *Cronobacter sakazakii*

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Cronobacter sakazakii is an opportunistic pathogen associated with outbreaks of life-threatening necrotizing enterocolitis, meningitis and sepsis in neonates and infants. The pathogen possesses an array of virulence factors which aid in tissue adhesion, invasion and host cell injury. Although the identification and validation of *C. sakazakii* virulence factors has been hindered by availability of suitable neonatal animal model, various studies has reported outer membrane protein A (*omp*A) as a potential virulence marker. Various other plasmid associated genes such as filamentous hemagglutinin (*fhaBC*), *Cronobacter* plasminogen activator (*cpa*) and genes responsible for iron acquisition (*eit*CBAD and *iuc*ABD/*iut*A) have been reported in different strains of *C. sakazakii*. Besides these proposed virulence factors, several biophysical growth factors such as formation of biofilms and resistance to various environmental stresses also contributes to the pathogenic potential of this pathogen. This review provides an update on virulence determinants associated with the pathogenesis of *C. sakazakii*. The potential reservoirs of the pathogen, mode of transmission and epidemiology are also discussed.

Introduction

The genus *Cronobacter* (previously known as *Enterobacter sakazakii*) is recognized as an emerging opportunistic pathogen causing life-threatening infections in neonates and immune-compromised infants.[1–5] Urmenyi and Franklin[6] firstly, reported severe *C. sakazakii* induced systemic infection in neonates in England. Since then, there have been around 150 reported cases of this contagion with 26 deaths worldwide.[7-9] The pathogen received world-wide attention after an outbreak of meningitis in Tennessee in 2001.[10] It was Farmer et al.[11] who foremost established the taxonomic position of novel species named *C. sakazakii* which was initially referred to as Gram negative, facultative anaerobe yellow-pigmented *Enterobacter cloacae*. Till the date, the *Cronobacter* consists of 11 species that include *C. sakazakii, C. malonaticus, C. dublinensis, C. turicensis, C. muytjensii, C. condimenti, C. universalis, C. helveticus, C. zurichenesis, C. pulveris* and *C. colletis*.[12-15] The species *C. sakazakii, C. turicensis* and *C. malonaticus* are reported to cause neonatal infections.[12] Although the incidence of disease is very low, the fatality rates range between 40 to 80% and survivors often had severe neurological and developmental disorders.[8,16,17,18] It is worth mentioning that premature birth and/or low birth weight are often cited as highest risk individuals for *Cronobacter* infection because they lack normal gut microflora and established gut epithelial lining which makes them more susceptible to increased mucosal permeability.[19] Reconstituted powdered infant formula (PIF) is reported to be the most associated vehicle for transmission of pathogen. Voluntary recalls of PIF contaminated with *Cronobacter* in the United States, Europe and Asia-Pacific region suggested the need of a collective effort among PIF manufacturers, health-care facilities and governing bodies to develop hygienic practices and maintain higher microbiological standards.[20]

The application of genome sequencing data and multilocus sequence typing (MLST) validated the revision of the taxonomic position of the 11 identified *Cronobacter* species.[21-31] The MLST utilizing 7 (atpD, fusA, glnS, gltB, gyrB, infB, ppsA) is a more successful typing method for the *Cronobacter* genus and has exhibited a high caliber of discrimination between the isolates.[32-36] The *Cronobacter* PubMLST database curated by Stephen Forsythe comprised the enteries for 1193 *Cronobacter* isolates reported worldwide. The MLST database of 739 *C. sakazakii* isolates indicates clonal complex 4 (CC4) as stable and predominantly coupled with neonatal meningitis. The ribosomal-MLST (53-loci) and Clusters of Orthologous Groups–core genome (COG-cg) MLST (1865 loci) has also confirmed CC4 as dominant lineage.[37] However, due to limited information on the virulence characteristics of CC4, its association with neonatal meningitis is unclear.[34] Therefore, unveiling the virulence characteristics of this pathogen would contribute toward underpinning the association of the pathogen to infant foods and to develop mitigation strategies.

The little information on the ecology, pathogenesis and virulence of *C. sakazakii* warrants an update on this enteric pathogen with special emphasis on virulence factors associated with the pathogenesis of *C. sakazakii*.

Reservoir and mode of transmission

The bacterium is ubiquitous and has been isolated from a wide variety of foods, including cheese products, infant cereal, dried foods, fruits, vegetables, meats, water, medicinal plants, herbs and spices, bread, rice and PIF.[38-50] Moreover, the *Cronobacter* spp. has also been reported from clinical sources, including

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Table 1. Powdered Infant Formula (PIF) implicated in worldwide outbreaks of *Cronobacter* infection

Country	Year	No. of cases / No. of deaths	Reference(s)
New Mexico	2008	2/2	106
France	2004	4/2	107
USA	2004	1/0	108
New Zealand	2004	1/1	109
Tennessee	2001	10/1	55
Israel	1999–2000	2/0	2
Belgium	1998	12/2	16
India	1992	1/1	110
Maryland	1990	1/0	111
Tennessee	1988	4/0	112,113
Iceland	1986–1987	3/1	36
Denmark	1983	1/1	114

cerebrospinal fluid, blood, intestinal and respiratory tracts, bone marrow and skin wounds.[51] The pathogen has also been detected from domestic vacuum cleaner bags, river water, the gut of a Mexican fruit fly and stable fly and faecal sample of animals.[19,52-54] Reconstituted PIF is the most associated vehicle for transmission of the pathogen, being an intrinsic or extrinsic contaminant during manufacturing under poor good manufacturing practices (GMP) or reconstitution of PIF (**Table 1**).[2,16,38,55-64] The presence of the pathogen as vaginal microflora has been neglected by several studies however, babies delivered through birth canal or Caesarean section (C-section) have been contracted with the pathogen few days after birth.[4,22,65]

Epidemiology

The epidemiology of *Cronobacter* species is incomplete and poorly described because of its rare infections and often underreported cases due to missing or different reporting criteria in developed and some developing countries.[66] Feeding with reconstituted PIF has been epidemiologically implicated in numerous clinical cases (**Table 2**). Cases are somewhat sporadic, but epidemics are not unusual; the utmost-risk group is neonates (<28 days old) that have low birth weights (<2,000 to 2,500 g) or the premature (<37 weeks of gestation stage). Friedemann[8] reported the lethality of *Cronobacter* meningitis, bacteraemia and NEC to be 41.9% ($P < 0.0001$), <10% and 19.0% ($P < 0.05$), respectively, for 120–150 microbiologically *Cronobacter* confirmed neonatal infections occurred between 2000 and 2008. The annual occurrence rate among the premature and underweight infant is reported to a figure of 8.7 per 100,000 low-birth weight neonates in the USA, and one *Cronobacter* infection per 10,660 every low-birth neonates.[59,67] Hunter and Bean[65] demonstrated the worldwide distribution of reported cases; the majority of them are within the developed countries (approx. 45%). This distribution may be underestimated since not all clinical analysis laboratories carry out research on the pathogen and not all countries have a system for reporting diseases. The Food and Drug Administration (FDA) has accounted a series of neonatal disease outbreaks in Florida, Missouri Illinois and Oklahoma in December 2011.[68] The limited information on its epidemiology necessitates that the researchers should record consistent and sufficiently informative data of invasive neonatal Cronobacter infections as developed under PubMLST database.

Pathogenicity and virulence factors

The high mortality and fatality rate caused by *C. sakazakii* is still poorly understood, and the list of virulence factors (**Table 2**) is probably far from complete. The specific virulence factors associated with the pathogenesis are discussed in this section.

Outer membrane proteins (OMPs)

Outer membrane proteins (OMPs) are of peculiar interest, owing to their cell-surface exposure and contribution in export of extracellular virulence factors, and in anchoring the structures that mediate adhesion and motility.

Several studies put forwarded that outer membrane protein A (*ompA*), contributes significantly to the virulence potential of *Cronobacter* spp by invading various epithelial and endothelial cells of human and animal origin. The invasion studies with human intestinal (INT407) cells showed the contribution of

Table 2. Characteristics of major known virulence factors of *Cronobacter sakazakii*

Factors	Genes	Potential role	Reference(s)
Outer membrane proteins (OMPs)	*ompXompA*	Involved in the basolateral invasion of enterocyte-like human epithelial cells	59,60–62
Enterotoxin	Not known yet	Heat stable toxin elaborated by the pathogen	63,64
Outer membrane protease	*cpa*	Provides resistance against bactericidal activity of serum; activates plasminogen and inactivates α2-AP	65,66
Sialic acid utilization	*nanAKT*	Confers in pathogenesis	71
Iron acquisition system	*iuc*	Encodes an iron-uptake system mediated by the active siderophorethat plays a role in iron transport and regulation	67,73
Efflux system	*ibeB*	Encodes copper and silver resistance cation efflux system facilitating invasion of brain microvascular endothelial cells (BMEC)	23
Proteolytic enzymes	*zpx*	Cause cell deformation and rounding of cells	83
Lipopoysaccarides	Chromosomal encoded genes	Disrupt epithelial tight junctions	91,102
Type III hemolysin	*hly*	Hemolytic activity	66,90

both microfilaments and microtubules from host and bacterial *ompA*.[69–71] Mittal and co-workers[72] reported that *OmpA*-positive isolates breach blood-brain barrier and invade central nervous system (CNS) causing clinical manifestations. In addition to *ompA*, Kim et al.[73] reported that *ompX* also played vital roles in the invasion not only the apical side, but also the basolateral side of the host cells and can translocate into the deeper organs (spleen and liver) of rats (**Fig. 1**).

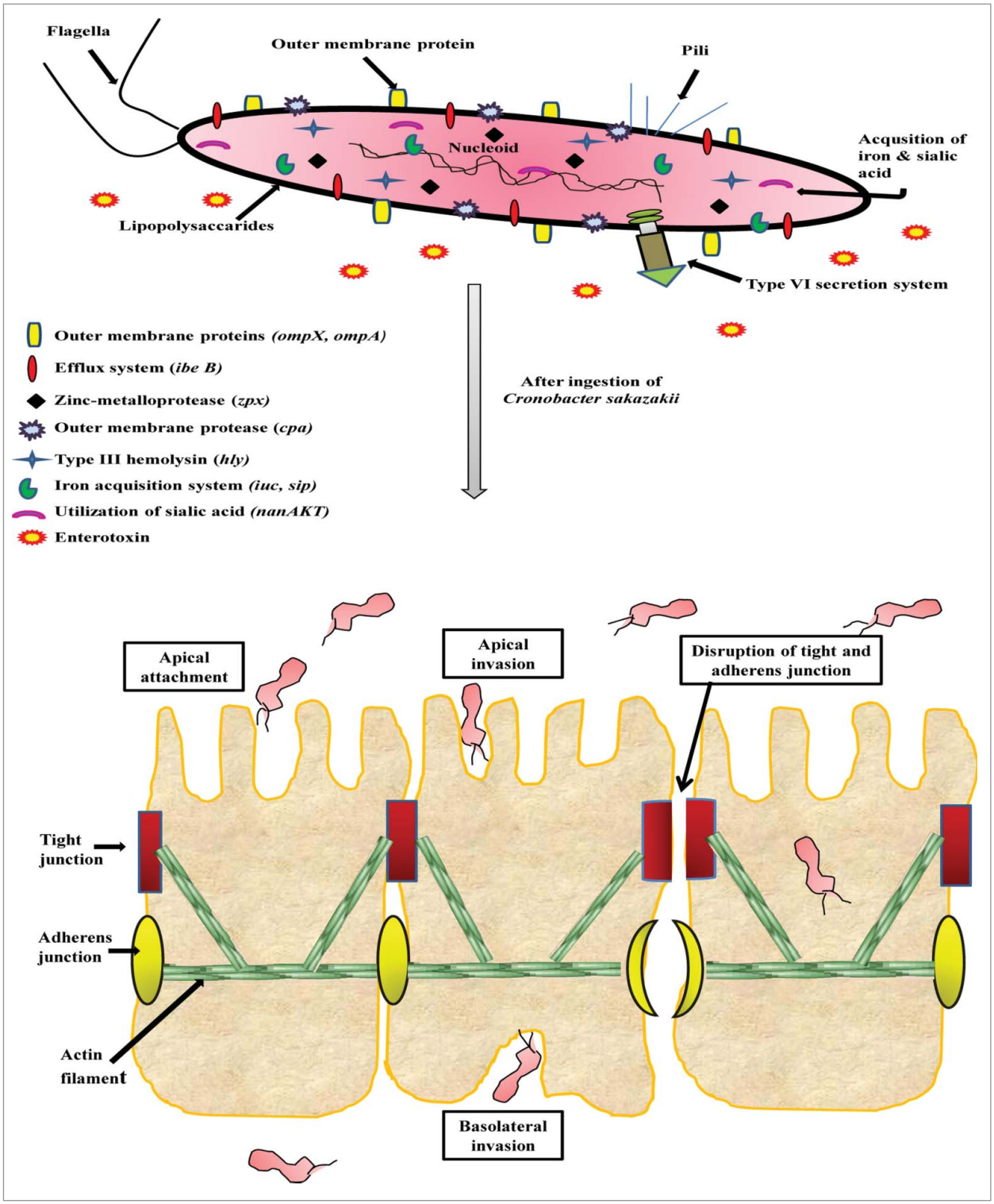

Figure 1. Proposed model for *Cronobacter sakazakii* infection and pathogenesis. The pathogen encodes several illustrated pathogenicity-associated factors engaged in imperative processes including adhere to host surfaces, transmigration across, invasion into and disrupt the intestinal barrier within intestinal epithelial cells.

Enterotoxin

Pagotto et al.[74] were the first to study the dose-response of *C. sakazakii* in suckling mouse and reported a minimum lethal dose of 10^8 colony-forming units (cfu) in neonatal mouse suggesting the possibility of enterotoxin analog in infections. The function of this toxin may act in a parallel fashion to lipopolysaccaride (LPS), mediating toll-like receptor 4 (TLR4) activation and stimulating a host inflammatory response.[74] However, later it was Raghav and Aggarwal[75] who identified a thermostable putative toxin with molecular mass of 66 kDa. The potent activity of the toxin ($LD_{50} = 56$ pg) emphasizes the emerging risk to neonates fed reconstituted PIF contaminated with *C. sakazakii*. The implication of the enterotoxin is still blurred as the genes encoding the putative toxin and the protein itself remain unidentified. Further studies using functional genomics and system biology might help in characterization of toxin-related genes.

***Cronobacter* plasminogen activator (*cpa*)**

Recently, the study on *C. sakazakii* BAA-894 reported presence of a plasmid (pESA3) encoding an outer membrane proteases (*cpa*) that has significant identity to proteins that belong to Pla subfamily of omptins. This protease has an ability to render serum resistance by cleaving complement components, activating plasminogen and inactivating the plasmin inhibitor α2-AP.[76] Franco et al. and Cruz et al. also portray the prevalence and distribution of plasmid-encoded virulence genes *cpa*, a type 6 secretion system (T6SS, also encoded on pESA3) and a filamentous haemaggltunin/adhesion (FHA) gene locus (located on pCTU1) among 231 *Cronobacter* strains.[77,78]

Sialic acid utilization

Sialic acid is found in human milk and in infant formulae in the form of sialyloligosaccharides.[79] These oligosaccharides remain undigested in neonates and infants, therefore the intestinal microvilli of neonates have increased sialic acid and N-acetylglucosamine residues leading to proliferation of gut microbiota.[80,81] Recently, Joseph et al.[82] explained a plausible linkage between sialic acid metabolism and the pathogenicity of *C. sakazakii* as it is the only *Cronobacter* species possessing the *nanAKT* gene cluster encoding for sialic acid utilization.

Iron acquisition gene system

Iron is an essential micro element for bacterial growth and metabolism and a vital factor for bacterial pathogenesis.[83] In *Cronobacter*, Franco et al.[78] reported that plasmid pESA3 contain 2 clusters of genes, a homolog of an ABC transport-mediated iron uptake siderophore system (*eitCBAD* operon) and a siderophore-mediated iron acquisition system (*iucABCD/iutA* operon). This characteristic may contribute to the systemic survival of *C. sakazakii* and subsequent invasion of the CNS to cause diseases. It was later, Grim et al.[84] who identified both the *feo* and *efe* systems for acquisition of ferrous iron. They confirmed that 98% of the plasmid-harboring *Cronobacter* strains have the aerobactin-like siderophore, cronobactin, for transport of ferric iron in *Cronobacter*. Cruz et al.[77] have also revealed that *C. sakazakii* isolates harbour siderophore-interacting protein (*sip*) gene. The *sip* gene has a ferrodoxin-reductase domain with binding sites to FAD and NAD(P), capable of transfer an electron from reduced ferrodoxin to FAD and then convert $NADP^+$ to NADPH.[85]

Efflux system

Active efflux system is a recognized virulence mechanism contributing to survival of members of Enterobacteriaceae in the host's gastrointestinal tract.[86] Interestingly, *ibeB* (a gene synonymous with *cusC*) in *C. sakazakii* has been reported, belonging to constellate of genes encoding a copper and silver resistance cation efflux system, ultimately allowing the invasion to brain microvascular endothelial cells (BMEC) cells.[23,87] When assessed by Kucerova et al.[23] it was discovered that the entire cation efflux operon (*cusA, cusB and cusC*) and its regulatory gene *cusR* were present in isolates colligated with neonatal infections (including *C. sakazakii* ATCC 29544^T, 696, 701, 767, *C. malonaticus* and *C. turicensis*) and absent in the other strains evaluated (*C. sakazakii* B894, ATCC 12868, 20, *C. dublinensis* and *C. muytjensii*).

Biofilm formation

Biofilms are interface-associated consortia of microorganisms embedded in an endogenous slimy matrix referred to as extracellular polysaccharides (EPS) and are well-known to contribute to survival and increased resistance to antimicrobial treatments.[88–90] Two hypothetical proteins have been newly described as possible adhesins engaged in biofilm formation in *Cronobacter* (ESA_00281 and ESA_00282).[91] Iverson et al.[92] reported that *Cronobacter* was able to adhere to silicon, stainless steel, polycarbonate and latex with apparently greater attachment occurring with EPS producing bacteria. Colanic acid (CA) was identified as an EPS component in *Cronobacter* spp. contributing to adherence to various surfaces and increased resistance to environmental stresses thermal, desiccation and pH.[93]

Other potential factors

Among the minor but important virulent factors, the proteolytic enzymes of *Cronobacter* strains have been found to cause deformation of the tissue cells in mice.[74] Kothary et al.[94] isolated and characterized a cell-bound zinc-containing metalloprotease encoded by a nucleotide sequence (*zpx*), unique among all the 135 *Cronobacter* strains tested. The protease was active in against azocasein, caused rounding of Chinese's hamster ovarian cells. It is hypothesized that proteolytic enzymes may permit the organism to cross the blood–brain barrier or cause extensive cellular destruction in neonates with NEC.

Recently, Hamby et al.[95] investigated the genomes of *C.sakazakii* and *C.turicensis* and reported that the gene for inositol monophosphatase is also associated with virulence of this pathogen.

Current studies revealed that the plasmid-encoded methyl-accepting chemotaxis protein (MCP) sequences present in *C. sakazakii* sequence type 8 (ST8) lineage are involved in virulence, invasion/adhesion, motility and biofilm formation.[96] It was also observed that this sequence was not found in any other lineages, implying that the MCP association with virulence is probably specific to the ST8 lineage.

LysR-type transcriptional regulator (LTTRs) are known to regulate a range of regulons involved in quorum sensing and virulence of bacteria.[97,98] Recently, Choi et al. (2012) characterized LysR-type transcriptional regulator (LTTR) gene (ESA_01081 homolog) as a potential regulator for *C. sakazakii* ATCC 29544 pathogenesis. They reported that the putative LysR-type protein plays a role in regulating genes involved in a host cell invasion, but not in adhesion.[99]

In another study, Cruz et al.[77] in addition to *sip* and *cpa* identified putative virulence genes, including type III haemolysin (*hly*) in *Cronobacter* isolated from human and non-human sources. The type III hemolysin, a virulence factor in numerous pathogenesis, is an integral outer membrane protein with hemolytic activity.[100,101]

Lipopolysaccarides (LPS) is an outer membrane virulence factor of *C. sakazakii*, which interacts with enterocytes through LPS mediated binding to TLR4 inducing NEC in animals.[102-105] In the NEC patients, the elevated level of LPS in serum and stools has been reported.[106-111] Altogether, these findings raise the intriguing possibility that LPS may engage in the pathogenesis of NEC and the role of TLR4 within the intestinal epithelium seeks detailed consideration. It has been also reported that PIF is frequently contaminated with elevated levels of LPS, which disrupts tight junctions thereby increasing the permeability of the host cell membrane.[112,113]

The genome study of *Cronobacter* has revealed the presence of gene for type IV pili in addition to a P pilus homologous to other pathogens such as *E.coli*.[28] Additionally the role of fibronectin, a glycoprotein in an extracellular matrix of *Cronobacter* has been postulated in the adherence to intestinal epithelial or endothelial cells.[69,72,114] However, the implications from these findings in pathogenesis and virulence have not been fully understood.

Recently, the role of *hfq* in pathogenesis of *C. sakazakii* ATCC 29544 has been demonstrated by generating the mutants using lambda red recombination where the mutants indicated defects in survival and invasion within host cells and exhibited low resistance to hydrogen peroxide.[115] *Hfq*, identified as RNA chaperone, is considered as a post-transcriptional regulator engaged in the biogenesis of quorum sensing, OMPs and various stress responses.[116–118] The studies in other Gram negative pathogens i.e. *Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Yersinia pseudotuberculosis*, and *Francisella tularensis* have also expressed the importance of *Hfq* in the pathogenesis.[119-123]

Limited studies regarding the effect of *Cronobacter* invasion on immune response have been done. The pathogen is reported to persist within human macrophages indicating that the *Cronobacter* possessed virulence properties that make it allows to tolerate the intracellular environment of macrophages.[124,125]

Conclusions and future perspectives

Cronobacter spp is a newly classified genus and more research is yet to be completed for better understanding this unique group of organism. As a virulent species, it causes high mortalities in the neonates, therefore, it is important to understand which gene products are responsible for the pathogenicity of the bacteria and how the expression of these virulence factors is regulated. Work is therefore required to address better understanding of the progression and pathogenesis of *Cronobacter* spp. related diseases, particularly using *in vitro* cell-based assays combined with animal models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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oryzae comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis, E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. Syst Appl Microbiol 2013; 36:309-319; PMID:23632228; http://dx.doi.org/10.1016/j.syapm.2013.03.005

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