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Prevalence of curli genes among *Cronobacter* species and their roles in biofilm formation and cell-cell aggregation

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ABSTRACT

Cronobacter species are food-borne opportunistic pathogens that cause sepsis, meningitis, and necrotizing enterocolitis in neonates. Bacterial pathogens such as pathogenic *Escherichia coli* and *Salmonella* species express extracellular curli fimbriae that are involved in rugosity, biofilm formation, and host cell adherence. *csgBAC* operon encodes the major curli structural subunit CsgA and the nucleator protein CsgB. *csgDEFG* operon encodes the regulatory protein CsgD and putative assembly factors. In this study, the curli operons were analyzed in the sequences of 13 *Cronobacter* strains and other enteric bacterial pathogens. Isogenic mutants of *csgA* and *csgB* were generated in *C. turicensis* LMG23827 (z3032). *csgA* and *csgB* mutants did not express curli fimbriae as indicated by a lack of Congo red binding and absence of curli by electron microscopic evaluation. Compared to the wild type strain, biofilm formation and cell-cell aggregation of *csgA* and *csgB* mutants were remarkably decreased. The prevalence of curli operons were investigated in 231 *Cronobacter* strains isolated from different sources using polymerase chain reaction (PCR) assay. The results of the PCR analysis showed that *csgA* and *csgG* were present in 30% clinical isolates, 8% food, and 11% environmental isolates. These genes were present in *C. dublinensis*, *C. malonaticus*, *C. turicensis*, and *C. universalis*, but not in *C. muytjensii* and *C. sakazakii*. Our data indicate that *csgBAC* and *csgDEFG* operons were present about three fold higher in clinical isolates than in isolates from other sources. The *csgA* and *csgB* genes were shown to be involved in the early stages of biofilm development and cell-cell aggregation in *Cronobacter*.

1. Introduction

Cronobacter species are motile, rod-shaped, Gram-negative bacilli and opportunistic foodborne pathogens that cause sepsis, meningitis, and necrotizing enterocolitis in infants (children < 1 year old), especially in premature neonates (children < 28 days old and born before 34 week gestation) (Friedemann, 2009; Tall et al., 2015). While the incidences of these illnesses are low, the mortality rate has been reported to be as high as 40–80% (Drudy et al., 2006; Friedemann, 2009; Healy et al., 2010; Iversen et al., 2004). Most cases of *Cronobacter* infections have occurred among infants who consumed contaminated powdered infant formula that were produced, stored or reconstituted incorrectly (Beuchat et al., 2009; FAO/WHO, 2007; Himelright et al., 2002; Kim and Loessner, 2008). *Cronobacter* also cause urinary tract infections, septicemia, pneumonia, and catheter-associated wound infections in people of all ages, especially in the elderly and those with other diseases or immune-compromised individuals (Joseph et al., 2012; Patrick et al., 2014; Tamigniau et al., 2015). The genus *Cronobacter* consists of seven species including *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis*, *C. universalis*, and *C. condimenti* (Iversen et al., 2008).

Cronobacter spp. have been isolated from a variety of food, food substances, food manufacturing environments, and the digestive tracts of humans and animals (Friedemann, 2007; Muytjens et al., 1988). It has been reported that controling *Cronobacter* spp. is very difficult in a processing environment because of the high resistance of these organisms to heat, dryness, osmotic, UV light, and antimicrobial reagents (Kim et al., 2006; Lehner et al., 2005). This resistant phenotype may result from *Cronobacter* attaching to and forming biofilms on silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride, which are commonly used in food process, storage, and reconstitution equipment and environments (Iversen et al., 2006; Kim et al., 2006; Lehner et al., 2005). The possible mechanisms as to why biofilm provides the resistance are complex, and may come from the rich extracellular polysaccharide matrix, high cell density, slow growth, and a novel efflux system in biofilms (Zhang and Mah, 2008). Biofilm formation by *Salmonella* spp., pathogenic *E. coli* and other members of the *Enterobacteriaceae* is associated with the expression of curli and extracellular cellulose (Römling et al., 1998).

Curli fimbriae (called thin aggregative fimbriae in *Salmonella*) is the

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major proteinaceous component of the complex extracellular matrix produced by many *Enterobacteriaceae* (Collinson et al., 1991; Olsén et al., 1989). Curli belong to a class of thin, highly aggregated surface fibers (6–8 nm in diameter and 1 μm in length) known as amyloids and are involve in adhesion to cell or material surfaces, cell-cell aggregation, and biofilm development. Amyloid proteins are abundant in bacterial biofilms, and make up to approximately 40% of the biomass in an activated sludge community (Larsen et al., 2007, 2008). Among adherence factors produced by bacteria, curli are distinguished by their unusual resistance to proteinase digestion, insoluble property when boiled in 1% SDS or 8 M urea (Collinson et al., 1991), and the ability to bind Congo red and thioflavin T (Chapman et al., 2002). The biosynthesis of curli is encoded by two operons, *csgBAC* and *csgDEFG* (*csg*, curli-specific genes in *E. coli*) (Hammar et al., 1995; Römling et al., 1998). Curli genes can be found in several members of *Enterobacteriaceae* including *Salmonella* spp., *Escherichia* spp., *Shigella* spp., and *Citrobacter* spp. (Zogaj et al., 2003). *csgA* encodes the major curli structural subunit and *csgB* encodes a nuclear protein subunit while *csgC* may have accessory function in the formation of curli fimbriae (Hammar et al., 1995, 1996). CsgA and CsgB are the proteins of identical predicted size, share 30% sequence identity, including similar repeated motifs (Hammar et al., 1996). Curli are not assembled if CsgB is absent, and in that case, CsgA is secreted from the cell in an unpolymerized form (Chapman et al., 2002; Hammar et al., 1996). CsgD is a transcriptional activator of the *csgBAC* operon, and is also a critical activator of several other operons that are necessary for biofilm formation (Brombacher et al., 2006; Chirwa and Herrington, 2003). CsgE, CsgF, and CsgG are putative assembly factors, and CsgG is a lipoprotein located in periplasm (Loferer et al., 1997). The efficient secretion and polymerization of CsgA and CsgB require *csgE*, *csgF*, and *csgG* (Hammar et al., 1995; Römling et al., 1998).

In both *Salmonella* species and pathogenic *E. coli*, the extracellular matrix is composed of curli and cellulose (Römling et al., 1998). Curli gene homologues have been reported in *Cronobacter* spp. (Grimm et al., 2008; Zogaj et al., 2003). The expression of curli fimbriae can lead to different multicellular colony morphotypes, such as rdar (red, dry, and rough colony), and pdar (pink, dry, and rough colony) when grown on medium contained Congo red (Tall et al., 2008). Curli have also been reported to be involved in biofilm formation in *Cronobacter* (Hartmann et al., 2010). Currently, little information is available on the role of the curli *csgA* and *csgB* in *Cronobacter*. Thus, understanding the presence and distribution of curli genes and their role in rugose and biofilm formation is important to understand the pathogenesis of *Cronobacter*.

In this study, the nucleotide sequences of curli in 13 *Cronobacter* strains were analyzed. To understand curli function in *Cronobacter*, *csgA* and *csgB* isogenic mutants were constructed in *C. turicensis* LMG23827 (z3032). Biofilm formation and cell-cell aggregation were compared between the wild type and *csgA* and *csgB* mutants. The presence and distribution of *csgBAC* and *csgDEFG* in 231 *Cronobacter* isolates were investigated by using PCR assay targeting *csgA* and *csgG* genes.

2. Materials and methods

2.1. Bacterial growth condition

231 *Cronobacter* strains isolated from clinical, food, and environmental sources analyzed in the study consisted of six *C. dublinensis*, 25 *C. malonaticus*, 12 *C. muytjensii*, 180 *C. sakazakii*, six *C. turicensis*, and two *C. universalis* strains. All strains were identified to the species level using the species-specific *rpoB and cgcA* PCR assays described by Carter et al. (2013), Lehner et al. (2012), and Stoop et al. (2009). *Cronobacter* strains were stored at − 80 °C in Trypticase Soy Broth (BBL, Cockeysville, MD) supplemented with 1% NaCl (TSBS) and 50% glycerol. Bacterial cultures were streaked onto Trypticase Soy Agar (TSA) or Luria-Bertani (LB) (10 gl tryptone, 5 gl yeast extract, 5 gl NaCl, 15 gl agar) agar, or 10 × diluted LB without salt (LBNS) agar or inoculated into tubes contained LB or 10 × diluted LBNS broth, and incubated at 37 °C or 28 °C. When needed, ampicillin (100 μg/ml) and/or nalidixic acid (250 μg/ml) were added into media.

2.2. In silico sequence analysis

The curli *csgBAC* and *csgDEFG* operons were analyzed in 13 *Cronobacter* strains. *C. sakazakii* strain BAA894 and *C. turicensis* strain LMG23827 (z3032) were obtained from the GenBank database (http://www.ncbi.nlm.nih./gov/Genbank/index.html; Accession numbers: FN_543093, NC_009778) (Kucerova et al., 2010). Sequences of the other eleven strains include three strains of *C. dublinensis* subsp. *dublinensis* (strain LMG 23823, AJKZ01000000), *lausanensis* (strain LMG23824, AJKY01000000), and *lactaridii* (strain LMG23825, AJKX01000000), four strains of *C. sakazakii* [2151 (BioProject: PRJNA89093, INSDC: AJKT01000000.1), 713 (BioProject: PRJNA68023, INSDC: AJLB00000000.1), E764 (BioProject: PRJNA89095, INSDC: AJLA00000000.1) and E35 (BioProject: PRJNA89113, INSDC: AJLC00000000.1)], and single strains of *C. malonaticus* (LMG23826, AJKV01000000), *C. muytjensii* (51329, AJKU01000000), *C. universalis* (CI797, AJKW01000000), and *C. condimenti* (1330, CAKW00000000). These strains include 8 clinical, 4 food or water, and 1 unknown isolates. The *csgBAC* and *csgDEFG* sequences of these strains and those of *S.* Typhimurium LT2, *S.* Typhi Ty2, *Shigella flexneri* 2a strain 301, *E. coli* K12, *E. coli* O157:H7 strain EDL933, *Klebsiella pneumoniae* strain MGH 78578, and other strains obtained from NCBI were analyzed using SEED Viewer (Aziz et al., 2008). The evolutionary relatedness among the *csgBAC* and *csgDEFG* operons was inferred using the Neighbor-Joining method. The revolutionary distances were computed using the Maximum Composite Likelihood Method.

2.3. Construction of isogenic csgA and csgB mutants

Sequences of *csgA*, *csgB* and associated flanking regions of *C. turicensis* z3032 were used to design primers. *csgA* and *csgB* isogenic mutants were created in *C. turicensis* z3032 according to mutagenesis protocol described by Franco et al. (2011) and Rhee et al. (2009). The primers for gene mutation of *csgA* and *csgB* are shown in Table 1. All primers used in the PCR assay were prepared by Integrated DNA Technologies (Coralville, IA). PCR assays were performed with AccuPrime Tag DNA polymerase High Fidelity PCR kit (Invitrogen, Inc., Carlsbad, CA). PCR reactions were run in an Applied Biosystems Models 2720 Thermal Cycler (Applied Biosystems, Foster City, CL). Plasmid pCVD442::Δ*csgA* and pCVD442::Δ*csgB* were selected by PCR removing ~76% of the 417 bp of *csgA*, and ~41% of 456 bp of *csgB*. Then, pCVD442::Δ*csgA* and pCVD442::Δ*csgB* were transformed into *E. coli* SM10 λpir and mobilized into a spontaneous nalidixic resistant strain of z3032. In-frame deletion of *csgA* and *csgB* were confirmed by PCR and gene sequencing with primers located in the flanking genes of the deleted open reading frames.

2.4. Gene complementation

For complementation of *csgA* and *csgB* mutants, the *csgA* and *csgB* genes were amplified by PCR using primers digested with EcoRI and HindIII (*csgA*) or EcoRI and PstI (*csgB*) and cloned into the Ptac expression vector pMMB66EH (Franco et al., 2011; Fürste et al., 1986). The resultant plasmid pMMB66EH::*csgA* and pMMB66EH::*csgB* were transformed into *E. coli* SM10 λpir, and then mobilized into z3032Δ*csgA*, z3032Δ*csgB* by conjugation. Expression of CsgA and CsgB by pMMB66EH::*csgA* and pMMB66EH::*csgB* using z3032Δ*csgA* and z3032Δ*csgB* or DH5 as hosts was induced by 1 mM IPTG, and was confirmed by Congo red assay.