

Molecular Characterization of *Cronobacter* Lipopolysaccharide O-Antigen Gene Clusters and Development of Serotype-Specific PCR Assays[∇]

K. G. Jarvis,^{1,2*} C. J. Grim,^{1,2} A. A. Franco,¹ G. Gopinath,¹ V. Sathyamoorthy,¹ L. Hu,¹ J. A. Sadowski,¹ C. S. Lee,¹ and B. D. Tall¹

CFSAN, FDA, Laurel, Maryland,¹ and Oak Ridge Institute for Science and Education, Oak Ridge, Tennessee²

Received 24 January 2011/Accepted 18 April 2011

***Cronobacter* (formerly *Enterobacter sakazakii*) is a recently defined genus consisting of six species, *C. sakazakii*, *C. malonaticus*, *C. dublinensis*, *C. muytjensii*, *C. turicensis*, and *Cronobacter* genomspecies 1. In this study, MboII restriction fragment length polymorphism (RFLP) patterns of O-antigen gene clusters, located between *galF* and *gnd*, were used to identify serotypes in *Cronobacter* spp. Seven O-antigen RFLP clusters were generated, including three *C. sakazakii* clusters, previously identified as serotypes O1, O2, and O3. The O-antigen regions of six strains with unique RFLP patterns, including two *C. sakazakii* strains, two *C. malonaticus* strains, one *C. turicensis* strain, and one *C. muytjensii* strain, revealed three O-antigen gene clusters shared among *Cronobacter* species. PCR assays were developed, targeting the *wzx* O-antigen polymerase gene, and used to screen 231 *Cronobacter* strains to determine the frequency of these newly identified serotypes.**

Cronobacter spp. are a group of emerging opportunistic food-borne pathogens that causes rare cases of neonatal meningitis, septicemia, and enterocolitis (4, 9, 24, 32, 46). Typical vehicles of infection are temperature-abused, contaminated infant formula and dried food products, and these organisms have been cultured from a variety of food production environments (13, 17, 20). The *Cronobacter* genus consists of six species, *Cronobacter sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. dublinensis* (three subspecies, *Cronobacter dublinensis* subsp. *dublinensis*, *C. dublinensis* subsp. *lausannensis*, and *C. dublinensis* subsp. *lactaridi*), and *Cronobacter* genomspecies 1, that are distinguishable with the use of biochemical and molecular techniques (19, 41).

The O antigen is a highly variable constituent of the lipopolysaccharide (LPS) of Gram-negative bacteria. It consists of oligosaccharide repeats ranging in size from 2 to 8 sugar residues, and the variability comes from the different sugars present, their specific order in the O-antigen structure, and the chemical linkages between sugar residues (48). In the *Enterobacteriaceae*, O-antigen gene clusters are primarily located between *galF* and *gnd*, contain from 6 to 19 genes, and are usually 6 to 20 kb in length (39). O-antigen regions contain three classes of genes: (i) those encoding enzymes specific to biosynthetic pathways of nucleotide sugars, (ii) glycosyl transferase genes that provide unique linkages between sugar residues, and (iii) O-antigen-processing genes, namely, *wzx* and *wzy*, required for assembly and transport of the polysaccharide repeats (39). The conserved yet divergent nature of the glycosyl

transferase and O-antigen-processing genes has been utilized in developing PCR assays for serotyping.

Serotyping assays based on PCR specific to O-antigen genes have become acceptable methods for typing *Escherichia coli*, *Salmonella*, *Shigella*, and many other Gram-negative bacteria, and this method was successful in identifying *C. sakazakii* serotypes O1 and O2 (25, 26, 31, 49). In a recent study, serological typing was used to identify five additional *C. sakazakii* serotypes, bringing the total number of serotypes for this species to seven (42). Other information available about *Cronobacter* O antigens comes from chemical characterization studies that have identified the repeat polysaccharide sugar composition of five *C. sakazakii* isolates, one *C. malonaticus* isolate, and one *C. muytjensii* isolate (1, 2, 7, 27–30). Two of the *C. sakazakii* O-antigen polysaccharide structures were from *C. sakazakii* O1 and O2 strains, and the structural analyses were in agreement with the O-antigen gene cluster results (1, 2, 31). In addition, there is a third *C. sakazakii* O-polysaccharide structure that has the same composition but a different pentasaccharide arrangement from the analyzed O1 structure, suggesting a serological relationship (2, 27).

Traditional serological typing is expensive, time-consuming, and labor-intensive. Additionally, commercial antisera are not always available, as is the case with *Cronobacter* spp., making PCR assays that target serotype-specific genes useful alternatives to serological typing. In this study, we sequenced and characterized additional O-antigen gene clusters in *Cronobacter* spp. and developed PCR assays specific to these new serotypes.

MATERIALS AND METHODS

Bacterial strains used in this study. A collection of 231 strains representing the six *Cronobacter* species was used in this study. Species designations were determined according to the classification scheme suggested by Iversen et al.

* Corresponding author. Mailing address: Lab 3412, MOD 1 Facility, Virulence Mechanisms Branch (HFS-025), Division of Virulence Assessment, OARSA, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, 8301 Muirkirk Road, Laurel, MD 20708. Phone: (301) 210-5171. Fax: (301) 210-7976. E-mail: karen.jarvis@fda.hhs.gov.

[∇] Published ahead of print on 29 April 2011.

TABLE 1. *Cronobacter* strains used for O-antigen sequencing

Strain name	Synonym(s)	Species	Country	Sample type(s)
LMG 23827	z3032, E866, 3032, E3032, DSM 18703, CI3032	<i>C. turicensis</i>	Switzerland	Blood, neonatal meningitis
E615	CI615	<i>C. malonaticus</i>	Czech Republic	Clinical
CDC 1059-77		<i>C. sakazakii</i>	United States	Unknown
2156	2005-14-08	<i>C. sakazakii</i>	United States	Blood
E769	CI769	<i>C. muytjensii</i>	Denmark	Milk powder
LMG 23826	E825, CI825, CDC 1058-77, API 76-2121, DSM 18702	<i>C. malonaticus</i>	United States	Breast abscess

(19), using the *rpoB* PCR method developed by Stoop et al. (41). The collection represents isolates from clinical, food, and environmental sources from diverse geographic locations. All strain information and results were submitted to the Pathogen Annotated Tracking Resource Network (PATRN), located at <http://www.patrn.net/> (43), and is accessible to users after a free registration process. *Enterobacter helveticus* and *Citrobacter sedlakii*, which are closely related to *Cronobacter*, were negative-control strains for the O-antigen-specific PCR screening. The six *Cronobacter* strains used for O-antigen sequencing in this study are listed in Table 1.

Frozen bacterial cultures were stored at -80°C in Trypticase soy broth (BBL, Cockeysville, MD) supplemented with 1% NaCl (TSBS) and 50% glycerol. For propagation, frozen cultures were plated on Trypticase soy agar (TSA; BBL) supplemented with 1% NaCl (TSAS) or Luria-Bertani (LB) agar (LBA; BBL) and the plates incubated for 16 to 18 h at 37°C .

PCR of *Cronobacter* O-antigen gene clusters. Bacterial genomic DNA was purified from log-phase broth cultures grown in LB at 37°C using a Qiagen DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA). PCR primers specific to the *galF* and *gnd* genes of *C. sakazakii* ATCC BAA-894 (Table 2) were used in long PCR with AccuPrime *Taq* high-fidelity DNA polymerase (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions (23, 31). The PCR cycling conditions consisted of an initial denaturing step at 94°C for 30 s, followed by 35 cycles of 94°C for 10 s, 65°C for 35 s, and 68°C for 15 min, followed by a final 10-min extension step at 72°C .

Restriction fragment length polymorphism (RFLP) analysis of O-antigen amplicons by use of MboII. O-antigen PCR amplicons (1.5 μg) were digested with MboII (New England BioLabs, Ipswich, MA) according to the manufacturer's instructions. Restriction digests were subjected to gel electrophoresis using 1.5% agarose gels and were visualized with ethidium bromide. TIFF images were imported into Bionumerics software (Applied Maths, Inc., Austin, TX), and dendrograms were generated using the Dice similarity coefficient and the unweighted-pair group method with arithmetic means (UPGMA). Band tolerance and optimization coefficient settings of 1.5% were applied. *In silico* analysis of MboII restriction sites of O-antigen amplicons was performed with the NEBCutter program (47).

Subcloning and DNA sequencing of *Cronobacter* O-antigen amplicons. O-antigen amplicons from *C. sakazakii* 2156, *C. malonaticus* LMG 23826, and *C. turicensis* LMG 23827 isolates were subcloned using a TOPO shotgun subcloning kit (Invitrogen, Carlsbad, CA). Long PCR amplicons were generated in triplicate, pooled, and subcloned according to the manufacturer's instructions. Individual clones were screened using PCR with the T7 and M13 universal primers supplied in the kit to verify the presence of an insert. Positive clones were submitted to Macrogen (Rockville, MD) for sequencing using the T7 and M13 universal primers. The O-antigen regions of *C. sakazakii* CDC 1059-77, *C. malonaticus* E615, and *C. muytjensii* E769 were sequenced using the primer walking method (14).

Sequence analysis of putative O-antigen gene clusters. Sequencing data were assembled in Bionumerics, and BLAST analysis was used to search available databases for amino acid similarities. The Pfam database was used to search for conserved protein domains; cluster and phylogenetic analyses were done with the MEGA4 program (12, 44). Evolutionary relationships of concatenated *wzx* and *wzy* nucleotide sequences were determined with MEGA4 (44) using the neighbor-joining method (37), with distances computed using the maximum composite likelihood method (45). Transmembrane-spanning domains were identified using the THMMER program (22).

PCR screening of *Cronobacter* genomic DNA for serotype identification. PCR primers specific to the *wzx* genes of *C. turicensis* LMG 23827, *C. sakazakii* 2156, and *C. malonaticus* LMG 23826 were designed from the O-antigen regions sequenced in this study (Table 2). These PCR primers and those previously published by Mullane et al. (31) to detect *C. sakazakii* serotypes O1 and O2 were used to screen a collection of 231 *Cronobacter* strains. The PCR cycling conditions for all reactions included an initial denaturation step at 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 5 min.

Nucleotide sequence accession numbers. The nucleotide sequences for *C. turicensis* LMG 23827, *C. malonaticus* E615, *C. sakazakii* 2156, *C. sakazakii* CDC 1059-77, *C. muytjensii* E769, and *C. malonaticus* LMG 23826 were deposited in GenBank under accession numbers HQ646166, HQ646167, HQ646168, HQ646169, HQ646170, and HQ646171, respectively.

TABLE 2. PCR primers used in this study

Target gene	Serogroup(s)	Primer pair	Primer sequence	Amplicon size (bp)	Source or reference
<i>wehC</i>	<i>C. sakazakii</i> O1	EsLPS1F EsLPS1R	CACGTTTCGCCCTGCAAAAAT GCAAGCGGCCAGACTGGATA	341	31
<i>wehI</i>	<i>C. sakazakii</i> O2	EsLPS2F EsLPS2R	TCCTGCATTTGTGGATTTTTC AACGCATTGCGCTTGAGAAA	329	31
<i>wzx</i>	<i>C. turicensis</i> O1 and <i>C. malonaticus</i> O1	z3032-wzxF5 z3032-wzxR4	AGGGGCACGGCTTAGTTCTGG CCCGCTTGCCCTTCACCTAAC	323	This study
<i>wzx</i>	<i>C. sakazakii</i> O3 and <i>C. muytjensii</i> O1	2156-wzxF1 2156-wzxR1	TGGCTGTCATGGTTTTCTTGC TAGTTGGCACCATCAACGCC	258	This study
<i>wzx</i>	<i>C. malonaticus</i> O2	E825-wzxF3 E825-wzxR2	TGGCCCTTGTTAGCAAGACGTTTC ATCCACATGCCGTCCTTCATCTGT	394	This study
<i>galF</i> ^a <i>gnd</i> ^a		EsgalF-F2 gndSeqR1	TACCCACTCCTCCAAGAACG TTTGTACAGAGACGGTTGAATAC	~8,000–14,000	31 This study

^a Primers for amplification of O-antigen regions.

RESULTS

RFLP analysis of *Cronobacter* O-antigen amplicons with MboII restriction digestion. RFLP analysis of 44 *Cronobacter* strains discriminated seven species-specific *Cronobacter* clusters (Fig. 1). Two of the *C. sakazakii* clusters correspond to those identified as *C. sakazakii* serotypes O1 and O2 by Mullane et al. (31), and a third cluster, consisting of two distinct RFLP patterns, is similar to a *C. sakazakii* O3 cluster identified by Sun et al. (personal communication) (42). The *C. malonaticus* strains clustered into two RFLP groups, and there is a single cluster of two *C. muytjensii* strains (Fig. 1). Finally, the RFLP pattern of the single *C. turicensis* strain is distinct from those of all of the other strains.

These data prompted us to sequence the O-antigen regions from six strains with distinct RFLP patterns in order to define new serotypes for this genus. These included *C. sakazakii* 2156 and *C. sakazakii* CDC 1059-77 (from the same cluster but with distinct RFLP patterns), *C. malonaticus* E615, *C. malonaticus* LMG 23826, *C. muytjensii* E769, and *C. turicensis* LMG 23827 (Fig. 1). Open reading frames (ORFs) were identified and assigned functions based on comparisons with current databases and are summarized in Table 3. Gene names for serotype-specific amplicons were assigned according to the bacterial polysaccharide gene nomenclature scheme described by Reeves et al. (36).

Nucleotide sequence analysis and identification of new *Cronobacter* serotypes. PCR products using primers specific to the *galF* and *gnd* genes of *C. turicensis* LMG 23827, *C. malonaticus* E615, *C. sakazakii* 2156 and CDC 1059-77, *C. muytjensii* E769, and *C. malonaticus* LMG 23826 resulted in sequences of 13,995, 13,499, 13,741, 13,687, 13,688, and 8,415 bp, respectively. BLAST analysis revealed three unique O-antigen gene clusters among these six strains (Fig. 2).

The nucleotide sequences of *C. turicensis* LMG 23827 and *C. malonaticus* E615 (excluding *galF* and *gnd*) are 91% homologous and were designated *C. turicensis* serotype O1 and *C. malonaticus* O1, respectively. *In silico* digestion with MboII and BLAST analysis of the two gene clusters showed that despite vastly different RFLP patterns at the species level (Fig. 3), the predicted ORFs from these O-antigen regions encode the same proteins (Fig. 2 and Table 3). Furthermore, the predicted proteins in the *C. turicensis* and *C. malonaticus* O1 gene clusters are highly similar to those of *Shigella dysenteriae* D11 and *E. coli* O29 (71 to 91% identities); therefore, the nomenclature of the *S. dysenteriae* D11 and *E. coli* O29 (D11/O29) gene clusters was used in naming the genes contained in these two new *Cronobacter* O-antigen regions (Fig. 2 and Table 3) (25, 33).

The O-antigen gene clusters of *C. sakazakii* 2156 and CDC 1059-77 and *C. muytjensii* E769, designated *C. sakazakii* serotype O3 and *C. muytjensii* O1, respectively, carry 13 ORFs (Fig. 2). BLAST analysis revealed 99% nucleotide homology between the two *C. sakazakii* gene clusters and 91% homology between the *C. sakazakii* and *C. muytjensii* gene clusters. Even though the MboII RFLP patterns of the O-antigen amplicons from these three strains differed considerably (Fig. 1), *in silico* and BLAST analysis revealed that this variability is due to differences in the location of MboII sites that do not change the predicted translation of proteins in these gene clusters (Fig. 3 and Table 3).

The O-antigen gene cluster of *C. malonaticus* LMG 23826 consists of eight ORFs and was designated *C. malonaticus* serotype O2 (Fig. 2). The predicted proteins encoded by genes in the *C. malonaticus* O2 gene cluster possessed the lowest similarities to currently known O-antigen region proteins, with all of the amino acid alignments having several gaps (Table 3). The exception to this observation is the predicted product of ORF 5, which is 51% similar to the Core-2/I-Branching family of enzymes (Table 3).

Nucleotide sugar synthesis pathway genes. Five genes encode putative nucleotide sugar synthesis pathway enzymes with 83 to 99% identities to D11/O29 homologous gene products in the *C. turicensis* O1 and *C. malonaticus* O1 gene clusters (Fig. 2 and Table 3). These include WffW and WffX, which are part of a CDP-glycerol nucleotide sugar synthesis pathway that produces a glycerol-3-phosphate moiety, and the FnlABC proteins, known to produce 2-acetamido-2,6-dideoxy-hexose (UDP-L-FucNAc) in the D11/O29 O antigens (25, 33, 39).

The *C. sakazakii* O3 and *C. muytjensii* O1 gene clusters encode putative enzymes involved in two nucleotide sugar synthesis pathways (Fig. 2 and Table 3). The RmlBDAC proteins are known to function in a dTDP-sugar biosynthesis pathway that produces dTDP-L-rhamnose (39). ORFs 5, 6, and 7 share significant identity with FdtA, FdtC, and FdtB (76, 75, and 76%), respectively, a group of enzymes required for the synthesis of the dTDP-sugar 3-acetamido-3,6-dideoxy-D-galactose (dTDP-D-Fucp3NAc) (34, 39). They also share 73, 73, and 63% identities with QdtA, QdtC, and QdtB, respectively, of *E. coli* O71 and *Salmonella enterica* O28, which, based on studies with *Thermoanaerobacterium thermosaccharolyticum*, are part of a biosynthetic pathway that includes the RmlA and RmlB proteins. The product of this pathway is dTDP-3-acetamido-3,6-dideoxy- α -D-glucose (dTDP- α -D-Qui3NAc) (16, 35). ORFs 5, 6, and 7 of the *C. sakazakii* O3 and *C. muytjensii* O1 O-antigen regions were named *fdtA*, *fdtC*, and *fdtB*, respectively, because of their slightly higher similarities, with the caveat that these gene names may change if structural data that reveal the sugars present in these O-antigen gene clusters become available (Table 3).

Surprisingly, none of the putative proteins encoded by the ORFs in the *C. malonaticus* O2 gene cluster are common to the nucleotide sugar synthesis pathways typically found in O-antigen regions, suggesting that genes outside the O-antigen region encode the O repeat sugars in this serotype (39).

Sugar transferase genes. Glycosyl transferase genes have been categorized into 26 families based on amino acid sequence similarities (3). The *C. turicensis* O1 and *C. malonaticus* O1 gene clusters encode four putative glycosyl transferase family 1 proteins that share 78, 79, 81, and 76% identity with WffY, WffZ, WfgA, and WbuB, respectively, in the D11/O29 O-antigen regions (Fig. 2 and Table 3). Four genes in the *C. sakazakii* O3 and *C. muytjensii* O1 gene clusters encode predicted glycosyl transferase proteins, including one family 1 protein and two family 2 proteins. The predicted product of the fourth putative glycosyl transferase, encoded by ORF 9 of *C. sakazakii* O3 and *C. muytjensii* O1, is similar to WdaN, a glycosyl transferase recently identified in *E. coli* O71 and *S. enterica* O28, which does not share conserved domains with any other of the glycosyl transferase families (Fig. 2 and Table 3) (16). There are three predicted glycosyl transferase proteins in the *C. malonaticus* O2 gene cluster (two family 2 proteins and a third

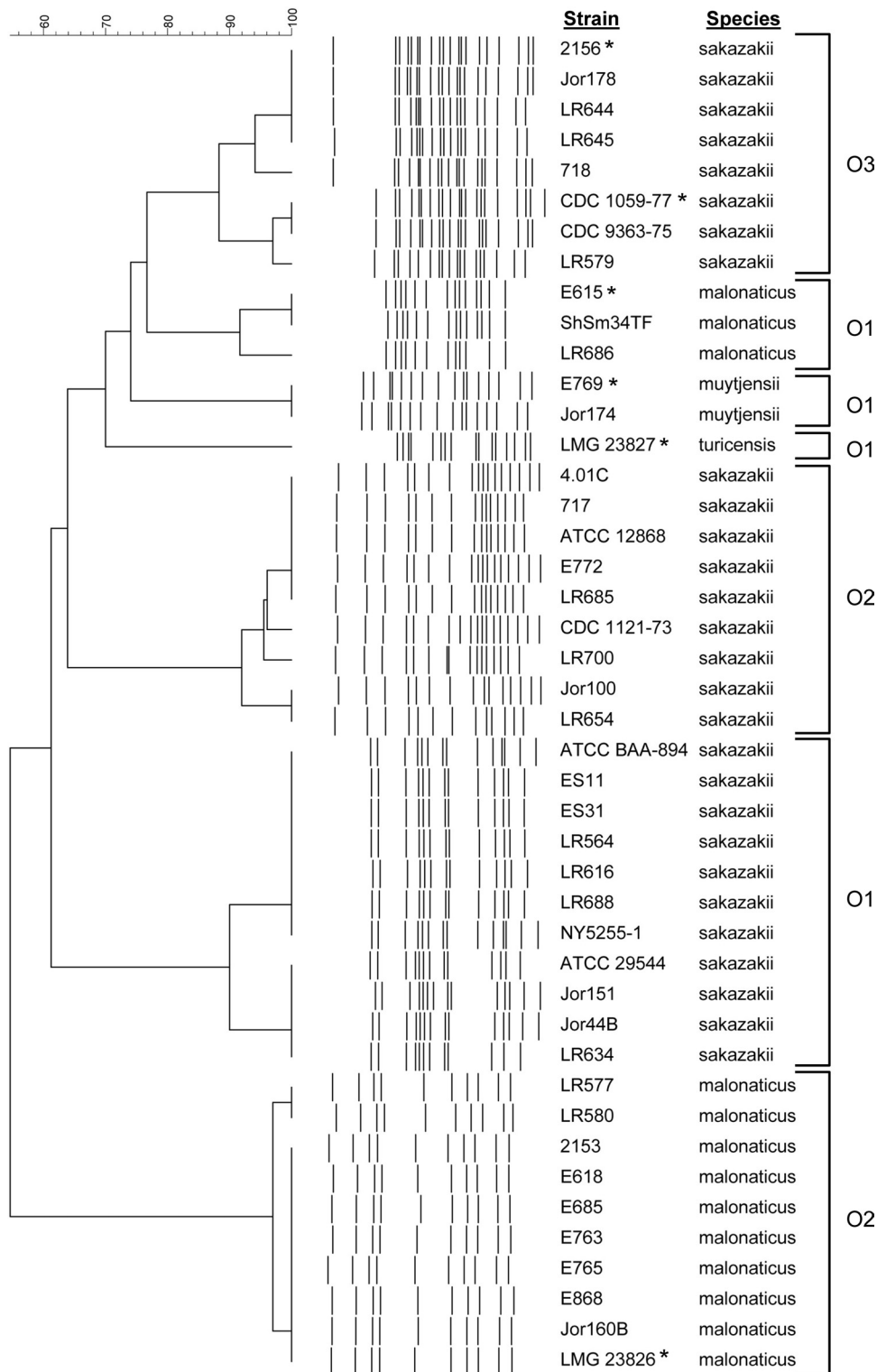


FIG. 1. Cluster analysis of MboII digests of *Cronobacter* O-antigen gene clusters digested with MboII. The dendrogram was generated using Bionumerics software, with an 80% similarity value used to distinguish serogroup-specific clusters. Asterisks (*) indicate the *Cronobacter* strains used for O-antigen sequencing in this study.

protein, which has conserved domains relative to glycosyl transferases in family 14/Core-2/I-Branching enzymes) (Table 3).

O-antigen-processing genes. Putative Wzx (O-antigen flip-pase) and Wzy (O-antigen polymerase) proteins were identi-

fied in all of the *Cronobacter* O-antigen gene clusters sequenced in this study (Fig. 2 and Table 3). O-antigen-processing proteins possess several well-pronounced transmembrane-spanning domains (6, 8). TMHMM analysis

TABLE 3. ORFs in new *Cronobacter* gene clusters^a

Gene cluster group and ORF	Gene	Position (nt)	Size (aa)	Species or strain with similar protein ^b (GenBank accession no.)	% identity/% similarity/size (aa) of gap (no. of overlapping amino acids) ^c	Putative function
<i>C. malonaticus</i> O1 and <i>C. turicensis</i> O1 gene clusters						
1	<i>wjfw</i>	1048-1443	131	(ACA24847, ACA24835)	94/97 (131)	Glycerol-3-phosphate cytidyltransferase
2	<i>wjfx</i>	1440-2600	386	(ACA24848, ACA24836)	83/92 (386)	Glycerophosphotransferase
3	<i>wjfy</i>	2597-3694	365	(ACA24849, ACA24837)	78/90 (365)	Glycosyl transferase group 1
4	<i>wjzz</i>	3684-4739	351	(ACA24850, ACA24838)	79/90 (351)	Glycosyl transferase group 1
5	<i>wzx</i>	4742-6142	466	(ACA24851, ACA24839)	83/93 (466)	O-antigen flipase
6	<i>wzy</i>	6145-7302	385	(ACA24852, ACA24840)	67/82 (385)	O-antigen polymerase
7	<i>wjg4</i>	7290-8408	372	(ACA24853, ACA24841)	81/91 (369)	Glycosyl transferase group 1
8	<i>fnl4</i>	8345-9469	374	(ACA24854, ACA24842)	99/99 (344)	4-6-Dehydratase, 3- and 5-epimerase
9	<i>fnlB</i>	9487-10575	362	(ACA24855, ACA24843)	85/93 (347)	Epimerase
10	<i>fnlC</i>	10575-11705	376	(ACA24856, ACA24844)	94/97 (376)	C-2 epimerase
11	<i>wbuB</i>	11705-12913	402	(ACA24857, ACA24845)	76/86 (398)	Glycosyl transferase group 1
12	<i>wbuC</i>	12904-13308	134	(ACA24858, ACA24846)	71/88 (131)	Unknown protein function
<i>C. sakazakii</i> O3 and <i>C. myjiensis</i> O1 gene clusters						
1	<i>mlb</i>	1119-2201	360	RmlB, <i>Cronobacter sakazakii</i> O2 (ABX51885)	99/99 (360)	dTDP-D-glucose-4,6-dehydratase
2	<i>mlD</i>	2198-3100	300	<i>Cronobacter sakazakii</i> O2 (ABX51886)	98/99 (300)	dTDP-6-deoxy-L-mannose dehydrogenase
3	<i>mlA</i>	3150-4028	292	<i>Cronobacter sakazakii</i> O2 (ABX51887)	94/98 (292)	Glucose-1-phosphate thymidyltransferase
4	<i>mlC</i>	4032-4586	184	<i>E. coli</i> (ZP_07502864)	72/83 (175)	Putative dTDP-6-deoxy-D-glucose-3,5 epimerase
5	<i>flhA</i>	4583-4999	139	<i>Pectobacterium carotovorum</i> (ACT12347)	76/93 (127)	WxCM domain protein isomerase
6	<i>flhC</i>	4923-5435	170	<i>Salmonella enterica</i> (AD139339)	75/83 (144)	dTDP-D-Fuc3N acetyltransferase
7	<i>flhB</i>	5437-6543	368	<i>Shewanella hallica</i> OS195 (ZP_07069428)	70/82 (365)	DegT/DntI/EryC1/SrS aminotransferase
8	<i>wzx</i>	6540-7811	423	<i>Pectobacterium carotovorum</i> (ZP_03826804)	52/71 (413)	O-antigen flipase
9	<i>wzy</i>	7801-9228	475	<i>E. coli</i> O71 (AD177032)	39/59 (422)	Glycosyltransferase
10	<i>wdaN</i>	9277-10548	423	<i>Pseudomonas aeruginosa</i> (AAM27646)	23/44 (412)	O-antigen polymerase
11	<i>wzh</i>	10556-11605	349	<i>Flavobacterium bacterium</i> (EAZ96080)	41/59 (342)	Glycosyl transferase family 2
12	<i>wzhK</i>	11608-12693	361	<i>Flavobacterium bacterium</i> (EAZ96080)	26/47 (378)	Glycosyl transferases group 1
13	<i>wzhL</i>	12695-13465	256	<i>Cronobacter sakazakii</i> O1 (ABX51882)	66/80 (246)	Glycosyl transferase family 2
<i>C. malonaticus</i> O2 gene cluster						
1	<i>wethM</i>	1009-1791	260	<i>Fusobacterium mortiferum</i> (EEO36671)	31/48/32 (265)	Glycosyl transferase family 2
2	<i>wethN</i>	1793-3034	413	<i>Marimononas</i> sp. (EAQ06012)	33/51/19 (346)	Capsule biosynthesis protein
3	<i>wzx</i>	3027-4232	401	<i>Geobacillus kaustophilus</i> (BAD77602)	23/47/18 (400)	O-antigen flipase
4	<i>wzy</i>	4222-5448	408	<i>Proteus mirabilis</i> (AD132282)	24/44/39 (286)	O-antigen polymerase
5	<i>wzhO</i>	5450-6289	279	<i>Actinobacillus mhov</i> (EEF16045)	32/51/8 (275)	Glycosyl transferase family 14; Core-2/I-Branching enzyme
6	<i>wehP</i>	6286-7122	278	<i>E. coli</i> (AAV28251)	51/66/0 (268)	Glycosyl transferase family 2
7	<i>wehQ</i>	7147-7824	225	<i>Erwinia</i> sp. (ADP10993)	41/61/5 (173)	Acyltransferase 3
8	<i>wehR</i>	7856-8188	110	<i>Burkholderia plymnum</i> (ACC71456)	37/60/8 (77)	Acyltransferase 3

^a nt, nucleotides; aa, amino acids.^b The GenBank accession numbers for the *C. malonaticus* O1 and *C. turicensis* O1 genes are all from *E. coli* O29 (GenBank accession no. EU294173) and *S. dysenteriae* (GenBank accession no. EU294172) predicted proteins.^c Size of gap is included for genes in the O5 gene cluster.

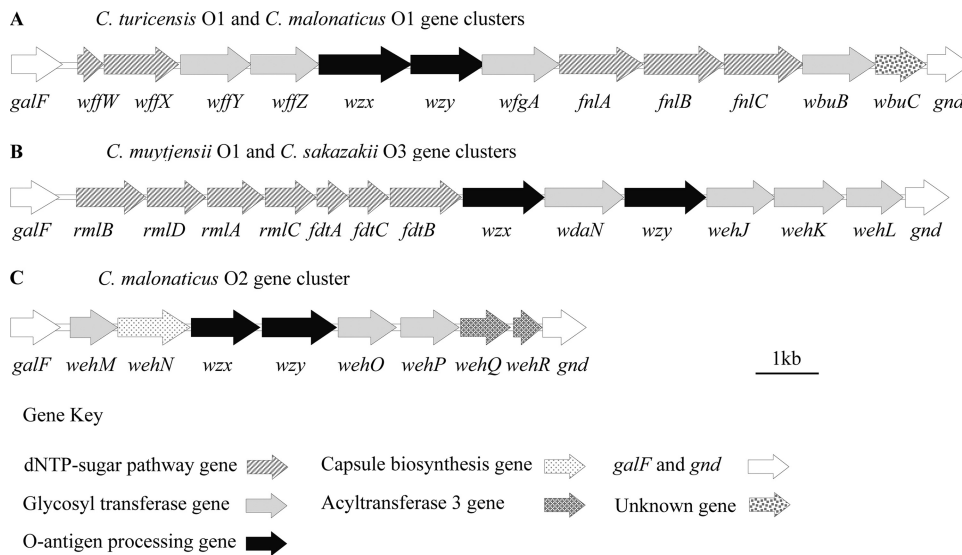


FIG. 2. O-antigen gene clusters of *Cronobacter* serotypes. (A) Predicted genes for the *C. turicensis* O1 and *C. malonaticus* O1 serotypes derived from the sequences of *C. turicensis* LMG 23827 and *C. malonaticus* E615. (B) Predicted genes for the *C. sakazakii* O3 and *C. muytjensii* O1 serotypes derived from the sequences of *C. sakazakii* 2156, *C. sakazakii* CDC 1059-77, and *C. muytjensii* E769. (C) Predicted genes for the *C. malonaticus* O2 serotype derived from the sequence of *C. malonaticus* LMG 23826.

of the serotype-specific *Cronobacter* Wzx proteins revealed 11 to 14 transmembrane-spanning domains. The Wzy proteins were predicted to have 10 or 11 transmembrane-spanning domains.

Other genes in *Cronobacter* O-antigen gene clusters. The last predicted ORF in the *C. turicensis* O1 and *C. malonaticus* O1 gene clusters, ORF 12, shares 71% identity with WbuC of D11/O29, which has no known function (Fig. 2 and Table 3).

This gene is also present in the O-antigen gene clusters of *E. coli* O26, O145, and O98 (5, 10, 11).

The putative ORF 2 protein of the *C. malonaticus* O2 gene cluster shares 33% identity with a predicted capsule polysaccharide biosynthesis protein of Pfam PF01943 (Table 3) (12). The conserved domains of the proteins in the PF01943 family are thought to be involved in the proper translocation and surface expression of capsule polymers (12). ORFs 7 and 8 of the *C. malonaticus* O2 gene cluster carry predicted acyltransferase genes associated with exopolysaccharide biosynthesis (Table 3). ORF 7 is 41% identical to the amino terminus of a predicted acyltransferase 3 in *Erwinia* sp. strain Ejp617, and the putative product of ORF 8 is 34% identical to the carboxyl terminus of a predicted acyltransferase 3 protein in *Burkholderia phymatum* (Table 3). Multiple rounds of sequencing through both DNA strands in this region confirmed that the two ORFs are in fact separate genes. The partial identities of ORFs 7 and 8 to opposite ends of the same type of protein suggest the presence of a frameshift mutation that resulted in the splitting of this coding region into 2 genes.

Comparison of the *C. turicensis* LMG 23827 sequence from this study to the published sequence of *C. turicensis* z3032. Our work with the O-antigen region of *C. turicensis* LMG 23827 coincided with the release of the whole-genome sequence from the synonymous *C. turicensis* strain, z3032, by Stephan et al. (GenBank accession no. NC_013282) (40). A comparison of the nucleotide sequence from the O-antigen gene cluster of LMG 23827 obtained in our study to that of z3032 revealed three nucleotide differences in z3032, including an extra thymine in the *wzx* gene, an extra thymine in the *wzy* gene, and a missing adenine in the *fnlA* gene. Each of these nucleotide differences result in frameshift mutations that affect the predicted translations of ORFs 5 to 12 of the z3032 O-antigen locus. We believe that the sequence generated in this study reconciles these discrepancies.

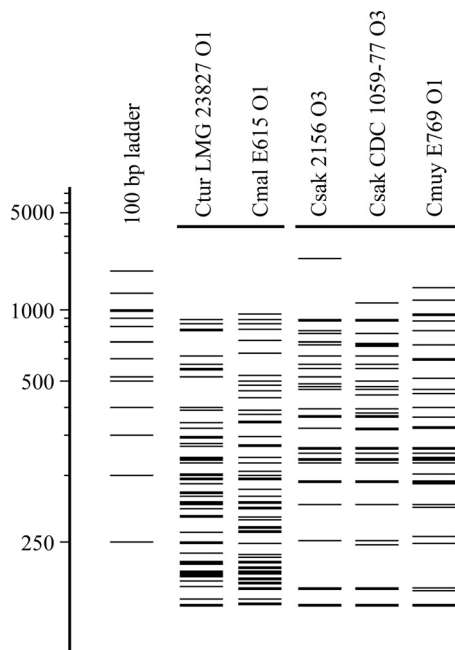


FIG. 3. *In silico* analysis, showing the variability in MboII restriction patterns of serogroups sequenced in this study using the NEBcuter program.

TABLE 4. Distribution of *Cronobacter* serogroups among 231 strains

Species	No. of strains								Total
	<i>C. sakazakii</i> O1 ^a	<i>C. sakazakii</i> O2 ^b	<i>C. sakazakii</i> O3 ^c	<i>C. malonaticus</i> O1 ^c	<i>C. malonaticus</i> O2 ^c	<i>C. muytjensii</i> O1 ^c	<i>C. turicensis</i> O1 ^c	ND ^d	
<i>C. dublinensis</i>								6	6
<i>C. malonaticus</i>				4	21				25
<i>C. muytjensii</i>						2		10	12
<i>C. sakazakii</i>	74	71	10					25	180
<i>C. turicensis</i>							1	5	6
<i>Cronobacter</i> genomospecies 1								2	2
Serogroup total (%)	32.0	30.7	4.3	1.7	9.1	0.9	0.4	20.8	

^a *Cronobacter* O1 serogroup PCR is specific to the *wehC* gene.

^b *Cronobacter* O2 serogroup PCR is specific to the *wehI* gene.

^c *Cronobacter* serogroup PCR is specific to the *wzx* gene.

^d ND, not determined (negative with *Cronobacter* PCR assays tested in this study).

PCR assays developed to detect *Cronobacter* serotypes. In a previous study, Mullane et al. (31) identified serotypes O1 and O2 of *C. sakazakii* using PCR primers specific to the O1 *wehC* gene and the O2 *wehI* gene. Analysis of the six *Cronobacter wzx* genes sequenced in this study led to the design of three O-antigen cluster-specific PCR primer pairs, which identify five new *Cronobacter* serotypes. Genomic DNA from 231 *Cronobacter* isolates in our collection was screened with these primer pairs, and 32% and 31% of our strains were identified as *C. sakazakii* serotypes O1 and O2, respectively (Table 4). Furthermore, all of the *C. sakazakii* serotype O1 and O2 strains cluster into two clades by MboII RFLP fingerprinting (Fig. 1).

Four *C. malonaticus* isolates were identified by the same *wzx* PCR primer set as the one used for the *C. turicensis* isolate from which the *wzx* PCR primers were designed, and these groups of isolates were designated *C. malonaticus* serotype O1 and *C. turicensis* serotype O1, respectively (Table 4). Three of the four *C. malonaticus* O1 strains form one RFLP clade, which has a different fingerprint from the *C. turicensis* O1 strain (Fig. 1). Additionally, 10 *C. sakazakii* and 2 *C. muytjensii* strains, which form two clades, were identified by the same *wzx* primer pair (Fig. 1 and Table 4). A third *wzx* primer pair identified a *C. malonaticus* serotype O2 clade, consisting of 21 (9%) isolates (Fig. 1 and Table 4). Twenty-one percent of the isolates in our collection were negative by the *Cronobacter* serotype-specific PCR assays developed to date, including all of the *C. dublinensis* and *C. genomospecies 1* strains (Table 4). *C. sakazakii* has the most diverse serotype profile of all the species in our collection, with 32% (74) of the strains positive by the O1 PCR assay, 31% (71) positive by the O2 PCR assay, 4% (10) positive by the O3 PCR assay, and 14% (25) not determined (ND) (Table 4). Similarly, most of the *C. muytjensii* and *C. turicensis* strains tested in this study were negative by all of the serotype PCR assays, suggesting that there are additional serotypes within the *Cronobacter* genus. *C. malonaticus* is the only species in which all isolates in our collection were assigned to a serotype (Table 4).

DISCUSSION

The O-antigen region is one of the most variable regions in the membranes of Gram-negative bacteria, and this variability

is routinely used to discriminate serotypes within species of bacteria. Within O-antigen gene clusters, the *wzx* and *wzy* genes have the most diverse nucleotide sequences, which has led to their wide use in serotype-specific PCR assays. The use of RFLP fingerprinting with MboII, combined with DNA sequence analysis of *Cronobacter* O-antigen regions, allowed us to develop serotype-specific PCR assays from *Cronobacter wzx* gene sequences, revealing similarities between *C. malonaticus* O1 and *C. turicensis* O1 as well as *C. sakazakii* O3 and *C. muytjensii* O1 strains, which had different RFLP fingerprints. Additionally, the *C. malonaticus* O1 and *C. turicensis* O1 O-antigen gene clusters are highly similar to those from *S. dysenteriae* D11 and *E. coli* O29. Analysis of the nucleotide sequences of concatenated *wzx* and *wzy* genes from *Cronobacter* spp. and D11/O29 confirmed these phylogenetic relationships (Fig. 4). In all cases, *Cronobacter wzx* and *wzy* sequences from different species with similar O-antigen regions clustered together, and as expected, *C. malonaticus* O1 and *C. turicensis* O1 *wzx* and *wzy* genes clustered with those from the O-antigen regions of D11/O29. Furthermore, O-antigen sequences from the same species clustered together, a pattern that was not apparent from the clustering of the RFLP fingerprints (Fig. 4).

The sequence similarities between *C. malonaticus* O1, *C. turicensis* O1, and O29/D11 suggests similar ancestries for the origination of the O-antigen regions from these four strains (25, 33). Liu et al. determined that 18 *Shigella* type strains share their O-antigen gene clusters with an *E. coli* gene cluster (25). Evolutionary relationships have also been suggested for the origins of O-antigen gene clusters in *E. coli* and *Salmonella*. Some examples include *E. coli* O103 and *Salmonella* O55, *E. coli* O157 and *Salmonella* O30, *E. coli* O55 and *Salmonella* O50, *E. coli* O77 and *Salmonella* O:6,14, and *E. coli* O71 and *S. enterica* O28, where all of the pairs of gene clusters share genetic and structural similarities (16, 26, 38, 49). The presence of *wbuC* in the *C. malonaticus* O1 and *C. turicensis* O1 gene clusters also suggests evolutionary ties to *E. coli*. In *E. coli* O29, O26, and O145, *S. dysenteriae* D11, *C. malonaticus* O1, and *C. turicensis* O1, *wbuC* is downstream of the genes *fnlA*, *fnlB*, *fnlC*, and *wbuB* (Fig. 2), which encode the proteins for the putative or, in some instances, known synthesis of UDP-L-FucNAc (10, 11, 25). In *E. coli* O98, *wbuC* is downstream of a similar group of genes, comprising *fnlA*, *qnlA*, *qnlB*, and *wbwW*, which syn-

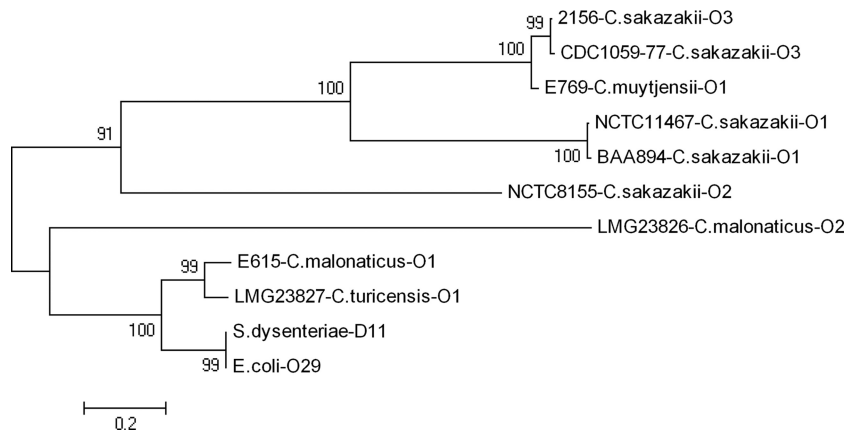


FIG. 4. Evolutionary relationships of concatenated *wxz* and *wzy* nucleotide sequences from *Cronobacter* species serotypes, *E. coli* O29 and *S. dysenteriae* D11. Relationships were analyzed with MEGA4 using the neighbor-joining method, with distances computed using the maximum composite likelihood method. Numbers on the branches denote bootstrap percentages from 1,000 bootstrap replicates, with a cutoff value of 50%. The GenBank accession numbers for NCTC 11467, BAA 894, NCTC 8155, *S. dysenteriae* D11, and *E. coli* O29 are EU076545, NC_009778, EU076546, EU294172, and EU294173, respectively.

thesize UDP-L-QuiNAc, and interestingly, the *E. coli* O98 gene cluster was acquired by *Yersinia kristensenii* O11 through lateral gene transfer without the *wbuC* gene (5). The function of *WbuC* is not known, and although it has been proposed to be a nonfunctional remnant gene product in *E. coli* O26 and O145 (10, 11), Cunneen and Reeves (5) suggested that its conservation in *E. coli* implies that *wbuC* must have some function specific to *E. coli* and now possibly in *C. malonaticus* and *C. turicensis*. The *C. malonaticus* O1 and *C. turicensis* O1 gene clusters are another example of the existence of related ancestors responsible for the evolution of antigenic variation among *Enterobacteriaceae*. It is likely that lateral gene transfer by homologous recombination between species is responsible for gene sharing since most of the clusters are located between *galF* and *gnd* genes. Additionally, it is probable that if it were available, *C. malonaticus* O1 and *C. turicensis* O1 antisera would cross-react between strains, as was the case with pairs of *E. coli* and *Shigella* strains, including D11/O29, which have identical or closely related O-antigen structures (25).

Our analysis of the *C. sakazakii* O3 and *C. mutytjensii* O1 gene clusters revealed additional similarities among the O-antigen regions from different *Cronobacter* species. Initial screening with the serotype-specific PCR assays developed by Mullane et al. (31) followed by cluster analysis of MboII RFLP fingerprints revealed a clade of *C. sakazakii* strains that were negative by both the *C. sakazakii* O1 and the *C. sakazakii* O2 assays (Fig. 1). We sequenced the O-antigen regions of *C. sakazakii* 2156 and CDC 1059-77 because although they were in the same clade, they had different RFLP fingerprints (Fig. 1). Surprisingly, the *wxz*-specific PCR assay designed from these sequences was positive for two *C. mutytjensii* isolates that form their own RFLP clade, in addition to all members of the subgroups of the *C. sakazakii* O3 antigen clade (Fig. 1). The *C. sakazakii* O3 and *C. mutytjensii* O1 O-antigen gene clusters contain the *rmlBDAC* genes, a highly conserved and well characterized set of genes known to encode the proteins for the production of L-rhamnose in the O-antigen regions of *E. coli* and *S. enterica* (39). The same genes, in the same order, are present at the 5' end of the *C. sakazakii* O2 gene cluster, and

it was recently reported that L-rhamnose is present in the O polysaccharide of *C. sakazakii* O2 by chemical composition analysis (1, 31). It is reasonable to assume that the *C. sakazakii* O3 and *C. mutytjensii* O1 O polysaccharides also contain L-rhamnose sugar residues.

In many O-antigen gene clusters, the *rmlA* and *rmlB* genes provide an intermediate sugar that serves as a branching point for other dTDP-sugar biosynthesis pathways (39). Two examples of this are in the S-layer glycoprotein of *Aneurinibacillus thermoaerophilus*, where studies have shown that *FdtA*, *FdtB*, and *FdtC* use the *rmlAB* branch point product to synthesize dTDP-D-Fuc3NAc, and in *Thermoanaerobacterium thermo-saccharolyticum*, where *QdtA*, *QdtB*, and *QdtC* are part of a biosynthetic pathway that includes *RmlA* and *RmlB* to produce dTDP-3-acetamido-3,6-dideoxy- α -D-glucose (dTDP-D-Qui3NAc) (34, 35). ORFs 5, 6, and 7 of the *C. sakazakii* O3 and *C. mutytjensii* O1 regions were named *FdtA*, *FdtC*, and *FdtB*, respectively, because of their similarities to proteins predicted to synthesize dTDP-D-Fuc3NAc (Table 3). However, these ORFs are also similar to the *E. coli* O71 *QdtA*, *QdtC*, and *QdtB* proteins, which are part of the sugar synthesis pathway for dTDP-D-Qui3NAc (16). A recent structural analysis study of the O polysaccharide from a *C. sakazakii* O1 isolate showed that it contains dTDP-D-Qui3NAc as part of the repeat sugars, whereas the molecular analysis of Mullane et al. (31) identified genes in the O1 cluster as *fdtA*, *fdtB*, and *fdtC*. The results of the structural analysis suggest that the *fdt* gene names originally given by Mullane et al. (31) be changed to *qdt* to account for this finding (2). Without knowing the chemical composition of the *C. sakazakii* O3 and *C. mutytjensii* O1 O polysaccharide, it is difficult to definitively assign gene names; therefore, we suggest the provisional names of *fdtA*, *fdtC*, and *fdtB* until structural data are available to confirm the sugars present in this O-antigen region.

The *C. malonaticus* O2 serotype accounts for 21% of our strain collection. Analysis of the O-antigen region showed that there are no genes for nucleotide sugar synthesis predicted from the sequence data, which accounts for the shorter length of this gene cluster (~8 kb, compared to ~12 to 14 kb for the

other *Cronobacter* O-antigen regions). There are at least 69 sugars known to be components of the repeat polysaccharide structures in Gram-negative bacteria, and some, such as glucose and galactose, provide housekeeping functions, and therefore, their genes are not duplicated in the O-antigen region (39). For example, the O-polysaccharide structure of *C. malonaticus* 3267 isolated from powdered infant formula in Australia is known, and the polymer consists of a branched pentasaccharide composed of D-glucose (D-Glc), D-galactose (D-Gal), 2-amino-2-deoxy-D-glucose (D-GlcN), 2-amino-2-deoxy-D-galactose (D-GalN), and 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) (29). All of these sugars provide other functions to bacterial cells, and therefore, it is plausible that their genes are not part of the O-antigen gene clusters. The presence of numerous glucose and galactose sugars in the O polysaccharide of *C. malonaticus* 3267 supports the absence of nucleotide sugar synthesis pathway genes in our sequence of the *C. malonaticus* O2 gene cluster, as they are probably carried elsewhere in the genome. Further, Kdo, a predicted protein in the *C. malonaticus* O-antigen region and a known component of the inner core oligosaccharide of LPS, is rarely found in O-antigen polysaccharide structures, with some exceptions, such as the O structure of *Providencia alcalifaciens* O36 (15, 21). It was not determined in this study whether the putative acyltransferase 3 genes carried by ORFs 7 and 8 of the *C. malonaticus* O2 gene cluster are functional; however, the structural analysis of *C. malonaticus* 3267 did not identify any O-acetylated residues in the O-antigen repeat unit, which suggests that the enzyme is inactive (29). Structural analysis of *C. malonaticus* LMG 23826 and genetic characterization of *C. malonaticus* 3267 are necessary for further investigation of these hypotheses.

In conclusion, we have identified five new serotypes of *Cronobacter* spp., using PCR assays with primers specific to the *wzx* genes of the O-antigen locus. The assays reported here will be valuable tools for identifying and typing *Cronobacter* species in clinical, environmental, and food samples. Cluster analysis of the RFLP patterns from isolates in our strain collection showed that there are additional serotypes in all of the *Cronobacter* species except *C. malonaticus* (data not shown). Additional studies are needed to define the serotypes for these strains.

ACKNOWLEDGMENTS

We thank Atin R. Datta, Barbara A. McCardell, and Marianna D. Solomotis for reviewing the manuscript. K. G. Jarvis, L. Hu, and C. J. Grim are Oak Ridge Institute for Science and Education fellows, and we thank the Department of Energy for their support.

REFERENCES

1. Arbatsky, N. P., et al. 2010. Structure of the O-polysaccharide of *Cronobacter sakazakii* O2 with a randomly O-acetylated l-rhamnose residue. *Carbohydr. Res.* **345**:2090–2094. doi:10.1016/j.carres.2010.07.014.
2. Arbatsky, N. P., et al. 2010. Structure of the O-polysaccharide of *Cronobacter sakazakii* O1 containing 3-(N-acetyl-L-alanyl)amino-3,6-dideoxy-D-glucose. *Carbohydr. Res.* **345**:2095–2098. doi:10.1016/j.carres.2010.07.013.
3. Campbell, J. A., G. J. Davies, V. Bulone, and B. Henrissat. 1997. A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem. J.* **326**(Pt. 3):929–939.
4. Caubilla-Barron, J., et al. 2007. Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. *J. Clin. Microbiol.* **45**:3979–3985. doi:10.1128/JCM.01075-07.
5. Cunneen, M. M., and P. R. Reeves. 2007. The *Yersinia kristensenii* O11 O-antigen gene cluster was acquired by lateral gene transfer and incorporated at a novel chromosomal locus. *Mol. Biol. Evol.* **24**:1355–1365. doi:10.1093/molbev/msm058.
6. Cunneen, M. M., and P. R. Reeves. 2008. Membrane topology of the *Salmonella enterica* serovar Typhimurium group B O-antigen translocase Wzx. *FEMS Microbiol. Lett.* **287**:76–84. doi:10.1111/j.1574-6968.2008.01295.x.
7. Czerwicka, M., et al. 2010. Structure of the O-polysaccharide isolated from *Cronobacter sakazakii* 767. *Carbohydr. Res.* **345**:908–913. doi:10.1016/j.carres.2010.01.020.
8. Daniels, C., C. Vindurampulle, and R. Morona. 1998. Overexpression and topology of the *Shigella flexneri* O-antigen polymerase (Rfc/Wzy). *Mol. Microbiol.* **28**:1211–1222.
9. Drudy, D., et al. 2006. Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. *Int. J. Food Microbiol.* **110**:127–134. doi:10.1016/j.ijfoodmicro.2006.02.008.
10. D'Souza, J. M., L. Wang, and P. Reeves. 2002. Sequence of the *Escherichia coli* O26 O antigen gene cluster and identification of O26 specific genes. *Gene* **297**:123–127.
11. Feng, L., et al. 2005. Structural and genetic characterization of enterohemorrhagic *Escherichia coli* O145 O antigen and development of an O145 serogroup-specific PCR assay. *J. Bacteriol.* **187**:758–764. doi:10.1128/JB.187.2.758-764.2005.
12. Finn, R. D., et al. 2008. The Pfam protein families database. *Nucleic Acids Res.* **36**:D281–D288. doi:10.1093/nar/gkm960.
13. Friedemann, M. 2007. *Enterobacter sakazakii* in food and beverages (other than infant formula and milk powder). *Int. J. Food Microbiol.* **116**:1–10. doi:10.1016/j.ijfoodmicro.2006.12.018.
14. Giesecke, H., B. Obermaier, H. Domdey, and W. J. Neubert. 1992. Rapid sequencing of the Sendai virus 6.8 kb large (L) gene through primer walking with an automated DNA sequencer. *J. Virol. Methods* **38**:47–60. doi:10.1016/0934(92)90168-D.
15. Holst, O. 2007. The structures of core regions from enterobacterial lipopolysaccharides—an update. *FEMS Microbiol. Lett.* **271**:3–11. doi:10.1111/j.1574-6968.2007.00708.x.
16. Hu, B., et al. 2010. Structural and genetic evidence for the close relationship between *Escherichia coli* O71 and *Salmonella enterica* O28 O-antigens. *FEMS Immunol. Med. Microbiol.* **59**:161–169. doi:10.1111/j.1574-695X.2010.00676.x.
17. Iversen, C., P. Druggan, and S. Forsythe. 2004. A selective differential medium for *Enterobacter sakazakii*, a preliminary study. *Int. J. Food Microbiol.* **96**:133–139. doi:10.1016/j.ijfoodmicro.2004.01.024.
18. Iversen, C., et al. 2007. Identification of “*Cronobacter*” spp. (*Enterobacter sakazakii*). *J. Clin. Microbiol.* **45**:3814–3816. doi:10.1128/JCM.01026-07.
19. Iversen, C., et al. 2008. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. *Int. J. Syst. Evol. Microbiol.* **58**:1442–1447. doi:10.1099/ijs.0.65577-0.
20. Kim, K., et al. 2008. Prevalence and genetic diversity of *Enterobacter sakazakii* in ingredients of infant foods. *Int. J. Food Microbiol.* **122**:196–203. doi:10.1016/j.ijfoodmicro.2007.11.072.
21. Kocharova, N. A., et al. 2007. The structure of the O-polysaccharide from the lipopolysaccharide of *Providencia alcalifaciens* O36 containing 3-deoxy-D-manno-oct-2-ulosonic acid. *Carbohydr. Res.* **342**:665–670. doi:10.1016/j.carres.2006.06.003.
22. Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305**:567–580. doi:10.1006/jmbi.2000.4315.
23. Kucerova, E., et al. 2010. Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other *Cronobacter* species. *PLoS One* **5**:e9556. doi:10.1371/journal.pone.0009556.
24. Lai, K. K. 2001. *Enterobacter sakazakii* infections among neonates, infants, children, and adults. Case reports and a review of the literature. *Medicine (Baltimore)* **80**:113–122.
25. Liu, B., et al. 2008. Structure and genetics of *Shigella* O antigens. *FEMS Microbiol. Rev.* **32**:627–653. doi:10.1111/j.1574-6976.2008.00114.x.
26. Liu, B., et al. 2010. Genetic and structural relationships of *Salmonella* O55 and *Escherichia coli* O103 O-antigens and identification of a 3-hydroxybutanoyltransferase gene involved in the synthesis of a Fuc3N derivative. *Glycobiology* **20**:679–688. doi:10.1093/glycob/cwq015.
27. MacLean, L. L., F. Pagotto, J. M. Farber, and M. B. Perry. 2009. Structure of the antigenic repeating pentasaccharide unit of the LPS O-polysaccharide of *Cronobacter sakazakii* implicated in the Tennessee outbreak. *Biochem. Cell Biol.* **87**:459–465. doi:10.1139/o09-004.
28. MacLean, L. L., F. Pagotto, J. M. Farber, and M. B. Perry. 2009. The structure of the O-antigen in the endotoxin of the emerging food pathogen *Cronobacter (Enterobacter) muytjensii* strain 3270. *Carbohydr. Res.* **344**:667–671. doi:10.1016/j.carres.2009.01.020.
29. MacLean, L. L., E. Vinogradov, F. Pagotto, J. M. Farber, and M. B. Perry. 2009. Characterization of the O-antigen in the lipopolysaccharide of *Cronobacter* spp.

- bacter (Enterobacter) malonaticus* 3267. *Biochem. Cell Biol.* **87**:927–932. doi:10.1139/o09-059.
30. MacLean, L. L., E. Vinogradov, F. Pagotto, J. M. Farber, and M. B. Perry. 2010. The structure of the O-antigen of *Cronobacter sakazakii* HPB 2855 isolate involved in a neonatal infection. *Carbohydr. Res.* **345**:1932–1937. doi:10.1016/j.carres.2010.06.020.
 31. Mullane, N., et al. 2008. Molecular analysis of the *Enterobacter sakazakii* O-antigen gene locus. *Appl. Environ. Microbiol.* **74**:3783–3794. doi:10.1128/AEM.02302-07.
 32. Muytjens, H. L., et al. 1983. Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii*. *J. Clin. Microbiol.* **18**:115–120.
 33. Perepelov, A. V., et al. 2006. Structure of a teichoic acid-like O-polysaccharide of *Escherichia coli* O29. *Carbohydr. Res.* **341**:2176–2180. doi:10.1016/j.carres.2006.05.016.
 34. Pfoestl, A., A. Hofinger, P. Kosma, and P. Messner. 2003. Biosynthesis of dTDP-3-acetamido-3,6-dideoxy-alpha-D-galactose in *Aneurinibacillus thermoaerophilus* L420-91T. *J. Biol. Chem.* **278**:26410–26417. doi:10.1074/jbc.M300858200.
 35. Pfoestl, A., et al. 2008. Biosynthesis of dTDP-3-acetamido-3,6-dideoxy-alpha-D-glucose. *Biochem. J.* **410**:187–194. doi:10.1042/BJ20071044.
 36. Reeves, P. R., et al. 1996. Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol.* **4**:495–503.
 37. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
 38. Samuel, G., J. P. Hogbin, L. Wang, and P. R. Reeves. 2004. Relationships of the *Escherichia coli* O157, O111, and O55 O-antigen gene clusters with those of *Salmonella enterica* and *Citrobacter freundii*, which express identical O antigens. *J. Bacteriol.* **186**:6536–6543. doi:10.1128/JB.186.19.6536-6543.2004.
 39. Samuel, G., and P. Reeves. 2003. Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. *Carbohydr. Res.* **338**:2503–2519.
 40. Stephan, R., A. Lehner, P. Tischler, and T. Rattei. 2011. Complete genome sequence of *Cronobacter turicensis* LMG 23827, a food-borne pathogen causing deaths in neonates. *J. Bacteriol.* **193**:309–310. doi:10.1128/JB.01162-10.
 41. Stoop, B., A. Lehner, C. Iversen, S. Fanning, and R. Stephan. 2009. Development and evaluation of rpoB based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. *Int. J. Food Microbiol.* **136**:165–168. doi:10.1016/j.ijfoodmicro.2009.04.023.
 42. Sun, Y., et al. 2011. Development of an O-antigen serotyping scheme for *Cronobacter sakazakii*. *Appl. Environ. Microbiol.* **77**:2209–2214. doi:10.1128/AEM.02229-10.
 43. Tall, B. D., et al. 2010. Development and application of a Pathogen Annotated Tracking Resource Network (PATRN) system to aid analysis of food-borne pathogens, abstr. R-2447. Abstr. 110th Gen. Meet. Am., Soc. Microbiol. American Society for Microbiology, Washington, DC.
 44. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**:1596–1599. doi:10.1093/molbev/msm092.
 45. Tamura, K., M. Nei, and S. Kumar. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. U. S. A.* **101**:11030–11035. doi:10.1073/pnas.0404206101.
 46. van Acker, J., et al. 2001. Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *J. Clin. Microbiol.* **39**:293–297. doi:10.1128/JCM.39.1.293-297.2001.
 47. Vincze, T., J. Posfai, and R. J. Roberts. 2003. NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Res.* **31**:3688–3691.
 48. Wang, L., Q. Wang, and P. R. Reeves. 2010. The variation of O antigens in gram-negative bacteria. *Subcell. Biochem.* **53**:123–152. doi:10.1007/978-90-481-9078-2_6.
 49. Wang, W., et al. 2007. A group of *Escherichia coli* and *Salmonella enterica* O antigens sharing a common backbone structure. *Microbiology* **153**:2159–2167. doi:10.1099/mic.0.2007/004192-0.