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Diversity of O Antigens within the Genus *Cronobacter*: from Disorder to Order

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***Cronobacter* species are Gram-negative opportunistic pathogens that can cause serious infections in neonates. The lipopolysaccharides (LPSs) that form part of the outer membrane of such bacteria are possibly related to the virulence of particular bacterial strains. However, currently there is no clear overview of O-antigen diversity within the various *Cronobacter* strains and links with virulence. In this study, we tested a total of 82 strains, covering each of the *Cronobacter* species. The nucleotide variability of the O-antigen gene cluster was determined by restriction fragment length polymorphism (RFLP) analysis. As a result, the 82 strains were distributed into 11 previously published serotypes and 6 new serotypes, each defined by its characteristic restriction profile. These new serotypes were confirmed using genomic analysis of strains available in public databases: GenBank and PubMLST *Cronobacter*. Laboratory strains were then tested using the current serotype-specific PCR probes. The results show that the current PCR probes did not always correspond to genomic O-antigen gene cluster variation. In addition, we analyzed the LPS phenotype of the reference strains of all distinguishable serotypes. The identified serotypes were compared with data from the literature and the MLST database (www.pubmlst.org/cronobacter/). Based on the findings, we systematically classified a total of 24 serotypes for the *Cronobacter* genus. Moreover, we evaluated the clinical history of these strains and show that *Cronobacter sakazakii* O2, O1, and O4, *C. turicensis* O1, and *C. malonaticus* O2 serotypes are particularly predominant in clinical cases.**

The bacterial genus *Cronobacter* has received considerable interest because of its association with rare but severe cases of neonatal infections. The genus was first defined in 2007 and currently contains seven species (1, 2). Not all species have been linked to human infections, and only *Cronobacter sakazakii*, *C. turicensis*, and *C. malonaticus* have been associated with fatal neonatal infections (3, 4). The severity of illness varies among strains, which suggested the existence of different types of pathogenicity. Although numerous virulence factors have already been identified within *Cronobacter* strains (5), we are still far from understanding the pathogenesis of the organism. Better knowledge of the infection process would contribute to more-effective control and neonatal risk reduction.

As with other Gram-negative bacteria, lipopolysaccharides (LPSs) are probably among the most important virulence factors of *Cronobacter*. Lipopolysaccharides are the major components of the outer surface of Gram-negative bacteria and typically comprise three parts: lipid A, an oligosaccharide core, and an O antigen (6). The O antigen is a polysaccharide (O-PS) that extends from the cell surface and consists of repetitive oligosaccharide units (O units) generally composed of 3 to 6 sugars. It interacts with environmental factors and helps in the effective colonization of host tissues and resistance to a host immune response. These effects have played a significant role in the diversification of outer LPS structures. In the majority of studied species, the O antigen has been found to be highly polymorphic. Even closely related species have few or no O-antigen types in common (7). The differences in O-antigen structures have given rise to serotyping classification methods (8). Serotyping is a useful strategy for the characterization and risk assessment of strains and is widely used to monitor outbreaks (9). Although traditional antiserum-based serotyping technology is still routinely used, this approach is both laborious and time-intensive. Therefore, molecular methods based on PCR probes and restriction fragment length polymorphism (RFLP) have been developed (10). These methods are directed to an O-antigen gene cluster located between *galF* and *gnd*: the coding sequence containing genes for sugar biosynthesis, sugar transfer, and O-antigen processing. There have been several molecular serotyping studies associated with most *Cronobacter* species (10–15). However, because most researchers have focused on particular strains, the findings are inconsistent and incomplete. In addition, a number of key strains were classified into the wrong species by the use of certain defined serotypes (11, 12), a portion of strains do not give PCR amplicons with current primer sets (12), and *C. condimenti* has not been included in any studies. This study therefore focused on identifying further serotypes across the entire *Cronobacter* genus. We combined molecular methods with phenotype observations and whole-genome analysis to confirm the currently known serotypes. These experiments resulted in the correction of the misclassified serotypes as well as the detection of new serotypes, in each case linking the serotype to the correct

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TABLE 1 Summary of strains used in this study

Bacterial species	No. of strains	*fusA* allele(s)	No. of clinical isolates
C. condimenti	1	86	0
C. dublinensis	12	20, 21, 30, 46, 48	1
C. malonaticus	9	7, 13	4
C. muytjensii	7	24, 35, 64	1
C. sakazakii	42	1, 3, 8, 9, 15, 17, 18	15
C. turicensis	8	22, 28	2
C. universalis	3	19, 32, 34	1
E. cloacae	1		
E. aerogenes	1		

species. As a conclusion, we propose a revised systematic classification of the *Cronobacter* serotypes. We believe that our results also unify current *Cronobacter* lipopolysaccharide and O-PS chemical structure research.

MATERIALS AND METHODS

Bacterial strains. The bacterial cultures used in this study were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA) and The Czech Collection of Microorganisms (CCM; Brno, Czech Republic). Some isolates of *Cronobacter* spp. were kindly donated by Carol Iversen (University of Dundee), Hana Drahovská (Comenius University, Bratislava, Slovakia), and Igor Hochel (University of Chemistry and Technology, Prague, Czech Republic). Details of the strains are summarized in Table 1, and the strains are characterized in Table S1 in the supplemental material.

The provisional *Cronobacter* species identity was based on the species-specific PCR system targeted to the *rpoB* gene (16) and confirmed using partial sequence analysis of the elongation factor G gene (*fusA*) (17, 18). DNA was extracted using a GenElute bacterial genomic DNA kit (Sigma-Aldrich), via the Gram-negative bacterial protocol. In addition, the biochemical characterization (data not shown) was performed by the use of an ID 32E test system (bioMérieux), and more-precise biochemical testing was accomplished using conventional tube methods as described previously (1, 19).

Genomes. *In silico* analyses were carried out using ($n = 30$) *Cronobacter* genomes accessible at GenBank (http://www.ncbi.nlm.nih.gov/genome/?term=cronobacter) (20) and the PubMLST *Cronobacter* database (www.pubmlst.org/cronobacter/) (21). The latter hosts newer assemblies of the GenBank entries. The *Cronobacter* genomes included in the study were *C. sakazakii* 680, 696, 701, 2151, 8399, HPB 5174, NCIMB 8272, SP291, NBRC 102416, ATCC BAA-894, ES15, ES713, ES35, E764, and E899; *C. malonaticus* 507, 681, LMG 23826, and CMCC 45402; *C. turicensis* z3032, 564, and z610; *C. condimenti* 1330; *C. dublinensis* 582 and 1210; *C. dublinensis* subsp. *dublinensis* LMG 23823; *C. dublinensis* subsp. *lactaridi* LMG 23825; *C. dublinensis* subsp. *lausannensis* LMG 23824; *C. muytjensii* ATCC 51329; and *C. universalis* NCTC 9529 (Table 2).

Restriction fragment length polymorphism analysis of O-antigen gene cluster amplicons by use of MboII (OAg-RFLP). Three pairs of primers (see Table S2 in the supplemental material) for amplification of the O-antigen gene cluster (10, 13, 15) were evaluated *in silico* using Fast PCR software (PrimerDigital Ltd.). Their complementarity was tested on a set of available genomes (Table 2).

RFLP analysis of the O-antigen gene cluster was performed with all 82 strains (10). Primers were targeted to the JUMPStart site and *gnd* gene. The PCR products were digested with MboII. The resulting mixtures were separated using a 1.5% agarose gel and analyzed by BioNumerics 6.4 (Applied Maths) (Table 3).

Serotyping PCRs. Eleven PCR protocols for *Cronobacter* serotyping were applied. These methods were targeted to different serotypes and performed according to previously published protocols as follows: (i) *C. sakazakii* serotypes O1 to O7 (heptaplex, S_HO1 to S_HO7) (12), (ii) *C. sakazakii* O4 (SO4) (14), (iii) *C. malonaticus* O2 (MaO2) (13), (iv) *C. muytjensii* O2 (MuO2) (14), (v) *C. turicensis* O2 (TO2) (11), (vi) *C. turicensis* O3 (TO3) (14), (vii) *C. dublinensis* O1 (DO1) (14), (viii) *C. dublinensis* O2 (DO2) (14), (ix) *C. universalis* O1 (UO1) (14), (x) *C. turicensis* O1 and *C. malonaticus* O1 combined (TO1/MaO1) (13), and (xi) *C. sakazakii* O3 and *C. muytjensii* O1 combined (SO3/MuO1) (13) (see Table S2 in the supplemental material).

Lipopolysaccharide structures. In this work, lipopolysaccharides are compared with their assigned serogroups. Structures of lipopolysaccharides described in the literature were discussed. The structures of the available serotypes were as follows: *C. sakazakii* O1 (22), O2 (23–25), O3 (26), O4 (27, 28), O6 (28), and O7 (29); *C. malonaticus* O1 (27), O2 (30), and O4 (11); *C. turicensis* O4 (31); *C. dublinensis* O1 (32) and O3 (33); *C. muytjensii* O1 (34); *C. universalis* O1 (35); and *E. asburiae* (36) (see Table S3 in the supplemental material).

Isolation of lipopolysaccharides. LPS was prepared following the method described by Jaradat et al. (37) with minor modifications. Briefly, cells (250 ml) were harvested in the exponential phase of growth by centrifugation (5,000 × *g*, 10 min), washed 3 times, and resuspended in 5 ml of 50 mM sodium phosphate buffer (pH 7.0). The cells were sonicated for 30-s intervals for 5 min at 9 to 12 W. The suspension was incubated first with 20 μg/ml of proteinase K in 0.1 M Tris-HCl (pH 8.0) at 60°C for 1 h and second with pancreatic RNase and DNase (0.1 μg ml^{-1}) in 20 mM $MgCl_2$ at 37°C for 10 min. Inactivation of enzymes was performed at 60°C for 10 min, and the obtained suspension was mixed with an equal volume of preheated phenol. After incubation (70°C for 15 min) with occasional mixing was performed, the mixture was centrifuged (18,000 × *g*, 1 h) and the resulting aqueous layer was collected and dialyzed using dialysis tubing with molecular weight (MW) cutoff values of 6,000 to 8,000 at room temperature against distilled water until no detectable phenol odor remained. The samples were then lyophilized and stored at −20°C until used. The extracted LPSs from *Cronobacter* were separated using both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and sodium deoxycholate-polyacrylamide gel electrophoresis (SDS-PAGE and DOC-PAGE, respectively).

Software. For the *in silico* analyses, FastPCR 6.1 (27) and Genetyx 5.2 (Genetyx Corporation) were used. RFLP profiles were analyzed using the TotalLab TL100 software (Nonlinear USA Inc.).

RESULTS

Restriction fragment length polymorphism of O-antigen gene cluster. The primers (see Table S2 in the supplemental material) previously described for the amplification of complete O-antigen clusters (sequencing primers) (10, 13, 15) of *Cronobacter* strains were tested *in silico*. The emphasis was put on the sensitivity of sequencing primers. The primers were compared to the full-genome sequences of 30 *Cronobacter* strains representing the whole genus (Tables 2 and 4).

From three pairs of the sequencing primers (10, 13, 15) (see Table S2 in the supplemental material), the primer pair described by Sun et al. (10) was predicted to amplify the targeted gene cluster from most genomes (73%) (Table 2). However, seven strains of *C. sakazakii* and one of *C. condimenti* were predicted to produce no amplicon with this primer pair.

Subsequently, this set of primers was chosen for the restriction fragment length polymorphism analysis of 82 *Cronobacter* strains. Amplified O-antigen gene products, with a size range of from 7 to 12.5 kbp, were detected in all strains (Table 3; see also Fig. S1 in the supplemental material). Amplicons were digested with MboII and separated by gel electrophoresis. The restriction digest profiles produced from the tested strains were clear and reproducible, giving from 8 to 18 restriction DNA fragments with sizes ranging from 150 to 2,800 bp (Table 3; see also Fig. S1). The obtained profiles were grouped into 17 distinguishable genotypes desig-

TABLE 2 *In silico* analysis of sequencing and serotyping primers carried out using publically available *Cronobacter* genome sequences

Bacterial species	Isolate	GenBank accession no.	MLST sequence type (ST/CC)	Size (bp) of amplified O-antigen cluster according to:			Serotyping PCR result(s)		
				Mullane et al.[a]	Sun et al.[b]	Jarvis et al.[c]	Heptaplex[d]	Duplex[e]	Individual[f]
C. sakazakii	ES15	NC_017933	125	12,660	10,684	11,418	S_HO1		
	680	NZ_CALG01000025	8				S_HO1		
	NBRC 102416	NZ_BAWU00000000	8	12,370	10,395	11,418	S_HO1		
	ATCC BAA-894	NC_009778	1	12,371	10,395	11,771	S_HO1		
	ES35	NZ_AJLC00000000	8				S_HO1		
	NCIMB 8272	NZ_AWFW00000000	4	12,660	10,684	11,708	S_HO2		
	SP291	NC_020260	4	12,660	10,684	11,708	S_HO2		
	701	NZ_CALE01000616	4	12,723	10,716	11,771	S_HO2		
	8399	NZ_AWSP00000000	4	12,660	10,684	11,708	S_HO2		
	2151	NZ_AJKT00000000	4	12,660	10,684	11,708	S_HO2		
	ES713	AJLB00000000	4						
	E764	NZ_AJLA00000000	73				S_HO4		SO4
	696	NZ_CALF00000000	12			22,276	S_HO4		SO4
	HPB5174	NZ_JNBN00000000	45				S_HO4		SO4
	E899	NZ_AFMO00000000	4						
C. malonaticus	507	NZ_CALD00000000	11	14,356	12,378	13,404	S_HO5		
	681	NZ_CALC00000000	7	9,608	7,614	8,656	S_HO6		MaO2
	CMCC 45402[g]	NC_023032	7	9,396	7,386	8,444	S_HO6		MaO2
	LMG 23826	NZ_AJKV00000000	7		7,387		S_HO6		MaO2
C. turicensis	z3032	NC_013282	24	14,484	12,463	13,532		TO1/MaO1	
	564	NZ_CALB00000000	5	10,792	8,814	9,840			
C. condimenti	1330	NZ_CAKW00000000	98						
C. dublinensis	582	NZ_CALA00000000	80		7,641				DO2
	1210	NZ_CAKZ00000000	106	13,357	11,380				DO1
C. dublinensis subsp. *dublinensis*	LMG 23823	NZ_AJKZ00000000	106	13,362	11,383	12,410			DO1
C. dulinensis subsp. *lactaridi*	LMG 23825	NZ_AJKX00000000	79	12,374	10,396	11,423			DO1
C. dublinensis subsp. *lausannensis*	LMG 23824	NZ_AJKY00000000	80		7,423	11,423			DO2
C. muytjensii	ATCC 51329	NZ_AJKU00000000	81		12,305	13,328			MuO2
C. universalis	NCTC 9529	NZ_CAKX00000000	54	11,781	9,828	10,829			UO1
	NCTC 9529/2	NZ_AJKW00000000	54	11,782	9,829	10,830			UO1

[a] 2008 (15).
[b] 2011 (10).
[c] 2011 (13).
[d] Probe targeted to SHO1 to SHO7 (heptaplex), Sun et al., 2011 and 2012 (10, 12).
[e] Probe targeted to *C. turicensis* and *C. malonaticus* (TO1/MaO1) (duplex) and *C. sakazakii* and *C. muytjensii* (SO3/MuO1) (duplex), Jarvis et al., 2011 (13).
[f] Probe targeted to *C. malonaticus* (MaO2), Jarvis et al., 2011 (13), and to *C. dublinensis* (DO1 and DO2), *C. sakazakii* (SO4), *C. turicensis* (TO3), *C. muytjensii* (MuO2), and *C. universalis* (UO1), Jarvis et al., 2013 (14), and Sun et al., 2012 (11).
[g] Originally identified as *C. sakazakii*.

nated A to Q and Y (Tables 3 and 4; see also Table S1). Some of the characteristic profiles (A, B, C, D, E, G, H, I, J, K, and M) were identical to those previously reported (10, 13–15) (Table 4). Four previously described profiles (*C. sakazakii* O5 and O7, *C. malonaticus* O1, and *C. muytjensii* O1) were not detected within our strain collection. In contrast, we detected six new profiles, five of which were as follows: *C. turicensis* (F), *C. dublinensis* (L and Q), *C. universalis* (P), and *C. condimenti* (N). The sixth profile, that of strain Cb43 (CDC9369-75), is very similar to that of *C. sakazakii* O2 (B), with few significant differences (lacking 1,665-bp and 975-bp bands and possessing 1,545-bp and 870-bp bands). This was probably caused by a mutation within the O-antigen gene cluster and was therefore given a different profile designation (Y).

Serotype-specific PCRs. The primers (see Table S2 in the supplemental material) previously described for the amplification of the genes from the O-antigen cluster (10–14) of *Cronobacter* were

TABLE 3 Results of serotyping PCR and characteristic MboII restriction profiles of amplified O-antigen gene clusters

Profile[a]	Species/strain	PCR serotyping[b]					Fragments (bp)
		O1	O2	O3	O4	Heptaplex	2800 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 600 500 400 300 200 100
D	*C. sakazakii*					O1	
B	*C. sakazakii*					O2	
Y	***C. sakazakii* CB 43**					O2[c]	
I	*C. sakazakii*			S		O3	
C	*C. sakazakii*				S	O4	
M	*C. malonaticus*		Ma			O6	
K	*C. muytjensii*		Mu			-	
J	*C. dublinensis* subsp. *lactaridi*	D				-	
L	***C. dublinensis* subsp. *dublinensis***	D				-	
E	*C. dublinensis* subsp. *lausannensis*		D			-	
Q	***C. dublinensis* CB 13**						
A	*C. turicensis*			T		+	
G	*C. turicensis*	T				-	
F	***C. turicensis***					-	
H	*C. universalis*	U				-	
P	***C. universalis***					-	
N	***C. condimenti***					-	
Z	***C. malonaticus* G2706[d]**					O5	

[a] Serotypes in bold represent newly identified RFLP profiles.
[b] PCR protocols were based on the literature as follows: *C. sakazakii* serotypes 1 to 7 (heptaplex, O1-SHO7) (12), *C. sakazakii* O4 (14), *C. malonaticus* O2 (13), *C. muytjensii* O2 (14), *C. turicensis* O2 (11), *C. turicensis* O3 (14), *C. dublinensis* O1 (14), *C. dublinensis* O2 (14), *C. universalis* O1 (UO1) (14), *C. turicensis* O1 and *C. malonaticus* O1 together (13), and *C. sakazakii* O3 and *C. muytjensii* O1 together (13). S, *sakazakii*; Ma, *malonaticus*; Mu, *muytjensii*; D, *dublinensis*, T, *turicensis*, U, *universalis*.
[c] Unexpected size of the PCR product (890 bp).
[d] The restriction profile was created by Genetyx software using the sequence of *C. malonaticus* strain G2706.

tested *in silico* using the full sequences of 30 *Cronobacter* genomes (Tables 2 and 4). Twenty-six genomes gave predicted positive results. However, primers specific for *C. sakazakii* O3 and O7, *C. turicensis* O2, and *C. sakazakii* O3/*C. muytjensii* O1 were predicted to give negative results for all tested genomes. The published serogroup-specific assays (11–15) (see Table S2) were then applied to 82 *Cronobacter* strains (see Table S1). The specificity of each of the primer pairs was cross-tested with strains in the corresponding serotype (Table 3 and 4). Briefly, the majority (78/82) of strains gave positive results, with only primers specific for *C. sakazakii* O5 and O7 and *C. turicensis* O2 giving negative results. The data from the *in silico* analysis and experimental results were collated and are discussed below.

Analysis of LPS patterns. O-antigen variations are responsible for the well-known ladder-like patterns of LPS molecules on polyacrylamide gels. The LPS patterns were determined for 37 selected strains, including representatives of all serogroups that were distinguishable with respect to RFLP profiles (Fig. 1). Each group of strains defined by RFLP profiles demonstrated one characteristic LPS pattern. All strains showed typical smooth LPS patterns containing lipid A and core (located toward the end of each gel) and O-antigen sugar repeats, with the exception of one: strain Cb 43 produced two bands only in the lower part of the gel that were characteristic of the lipid A-oligosaccharide core (lipid A-core) and of the lipid A-core with only a short O-antigen polysaccharide or single O-unit molecule (lipid A-core plus one O unit) without any repetitive subunits in the upper part of the gel (Fig. 1).

For a polyphasic analysis of *Cronobacter* serotype groups, the

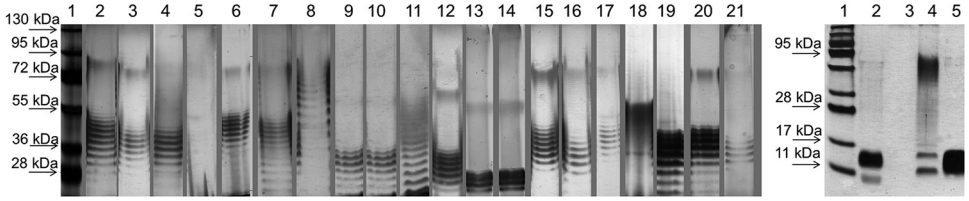

FIG 1 Electrophoretic patterns of LPS from *Cronobacter* strains. (Left) Silver-stained DOC-PAGE of LPS patterns of the strains of genus *Cronobacter*. Lane 1, marker (Page Ruler Plus prestained protein ladder); lane 2, Cb28 (*C. sakazakii* O1, D); lane 3, Cb24 (*C. sakazakii* O2, B); lane 4, Cb05 (*C. sakazakii* O2, B); lane 5, Cb43 (Y); lane 6, Cb08 (*C. sakazakii* O3, I); lane 7, Cb26 (*C. sakazakii* O4, C); lane 8, Cb01 (*C. malonaticus* O2, M); lane 9, Cb55 (*C. muytjensii* O2, K); lane 10, Cb79 (*C. muytjensii* O2, K); lane 11, Cb78 (*C. condimenti* O1, N); lane 12, Cb53 (*C. turicensis* O1, G); lane 13, Cb73 (*C. turicensis* O5, F); lane 14, Cb75 (*C. turicensis* O5, F); lane 15, Cb71 (*C. turicensis* O3, A); lane 16, Cb59 (*C. universalis* O1, H); lane 17, Cb70 (*C. universalis* O2, P); lane 18, Cb69 (*C. dublinensis* O2, E); lane 19, Cb62 (*C. dublinensis* O1b, L); lane 20, Cb56 (*C. dublinensis* O1a, J); lane 21, Cb13 (*C. dublinensis* O4, Q). Corresponding serotypes and restriction profiles are presented in the brackets. (Right) Silver-stained SDS-PAGE of LPS patterns of the strains of genus *Cronobacter*. Lane 1, marker (Page Ruler Plus prestained protein ladder); lane 2, Cb43 (rough type of *C. sakazakii* O2); lane 3, empty; lane 4, standard of smooth type of LPS (*E. coli* E 0111); lane 5, standard of rough type of LPS (*E. coli* EH 100).

TABLE 4 Summary of the genus *Cronobacter* serotyping

Species and serotype	Result(s) of PCR positivity analysis[a]			Profile(s) detected by OAg-RFLP	No. of strains with the indicated serotype				Proposed classification			Characteristic(s) reported in the literature[g]						
		Detected interaction(s)					This study							Structure				
	Expected interaction(s)	*In silico*[b]	Collection strains[c]		MLST database[d]	Other studies[e]	Known serotype	Unknown serotype	Serotype[f]	Abbreviation	Reference strain isolate	Strain(s) subjected to whole-genome sequencing	Strain(s) subjected to *rfb* cluster gene mapping	Strain	qty$_S$[h]	Type[p]	Bond	qty$_B$[i]
C. sakazakii																		
O1	S_HO1	S_HO1	S_HO1	D	48 (6)	158	18 (8)		O1	**S O1**	**ATCC 29544**	ES15, 680, NBRC 102416, ATCC BAA-894, ES35	ATCC 29544, NCTC 11467	ATCC 29544	5	B	1→4	2
O2	S_HO2	S_HO2	S_HO2	B, (Y)[l]	116 (55)	152	17 (7)		O2	**S O2**	**NCTC 8155**	NCIMB 8272, sp291, 701, 8399, 2151	NCTC 8155	ATCC 29004	6	B	1→2	2
														767	7	B	1→2 1→4	2 1
														2855	6	B	1→2	2
O3	S_HO3 SO3/MuO1	S_HO3 SO3/MuO1	S_HO3 SO3/MuO1	I	10 (0)	26	4 (0)		O3	**S O3**	**G2726**			G2726	6	B	1→4	1
O4	S_HO4 SO4	S_HO4 SO4	S_HO4 SO4	C	17 (6)	24	4 (1)		O4	**S O4**	**G2594**	E764, 696, HPB5174	G2594	G2594	7	B	1→6 1→4	1 1
O5	S_HO5				0 (0)	3	0 (0)		**X**[m]		**G2706**[n]							
O6	S_HO6				3 (0)	7	0 (0)		S O6	**S O6**	**G2704**		G2704	G2704	5	B	1→2	1
O7	S_HO7				6 (0)	8	0 (0)		O7	**S O7**	**G2592**		G2592	G2592	5	B	1→2	1
C. malonaticus																		
O1	TO1/MaO1				0 (0)	12	0 (0)		O1	**Ma O1**	**E615**		E615	G3864	6	B	1→3	1
O2	S_HO6 MaO2	S_HO6 MaO2	S_HO6 MaO2	M	31 (18)	35	9 (4)		O2	**Ma O2**	**LMG 23826**	681, CMCC 45402, LMG 23826		HPB-3267	5	B	1→2	1
On[j]		S_HO5		**Z**[o]	1 (1)	1 (0)	0 (0)		**S O5 → Ma O3**	**Ma O3**	**G2706**	507	G2706	G2706	5	L		
On[j]	TO2				0 (0)	1	0 (0)		**T O2 → Ma O4**	**Ma O4**	**G3882**		G3882	G3882	4	L		
C. turicensis																		
O1	TO1/MaO1	TO1/MaO1	TO1/MaO1	G	1 (1)	3	3 (2)		O1	**T O1**	**LMG 23827**	z3032/LMG 23827	z3032/LMG 23827					
O2	TO2				0 (0)	1	0		**X**[m]				G3882	G3882[e]	4	L		
O3	TO3		TO3 S_HO5	A	0 (0)	6	3 (0)		O3	**T O3**	**E609**		E609					
On[j]									**O4**	**T O4**	**564**	564		57, 564, 566	4	B	1→2	1
On[j]				**F**				2	**O5**	**T O5**	**E676**							
C. dublinensis																		
O1	DO1	DO1	DO1	J	5 (0)	6	3		O1a	**D O1a**	**LMG 23823**	LMG 23825	LMG 23825, 23823, G3983, G3977	G3983	5	B	1→2	1
			DO1	**L**	0 (0)	0	2		**O1b**	**D O1b**	**LMG 23825**	1210, LMG 23823		G3977	6	B	1→2 1→4	1 1
O2	DO2	DO2	DO2	E	1 (0)	4	6 (1)		O2	**D O2**	**LMG 23824**	LMG 23824, 582	LMG 23824					
On[j]					0 (0)	0			**O3**	**D O3**	**HPB 3169**			HPB 3169	5	L		
O?[k]				**Q**				1	**O4**	**D O4?**[k]	**CB 13**							
C. muytjensii																		
O1	MuO1/SO3				2 (0)	5	0 (0)		O1	**Mu O1**	**7692**		7692	HPB 3270	5	L		
O2	MuO2	MuO2	MuO2	K	1 (0)	4	7 (1)		O2	**Mu O2**	**ATCC 51329**	ATCC 51329	ATCC 51329					
C. universalis																		
O1	UO1	UO1	UO1	H	1 (0)	1	2 (1)		O1	**U O1**	**NCTC 9529**	NCTC 9529	NCTC 9529	NCTC 9529	4	L		
On[j]				**P**				1	**O2**	**U O2**	**E680**							
C. condimenti																		
On[j]				**N**				1	**O1**	**C O1**	**1330**	1330						
Previously misidentified strains																		
C. turicensis									*E. asburiae*		**HPB 3287**			HPB 3287				

laboratory results and additional information from the literature have been collated. The RFLP restriction digestion profiles were compared with LPS patterns and PCR profiling results. These laboratory results were then evaluated with existing metadata about the strains: the incidence in the *Cronobacter* PubMLST database, source, O-antigen chemical structures, genome sequences, and O-antigen gene cluster description. On the basis of this polyphasic investigation, we proposed a systematic classification of the currently known serotypes within the genus *Cronobacter* (Table 4).

DISCUSSION

Serotyping is a useful method for characterization of Gram-negative bacteria. Several studies focused on the O-antigen classification within the *Cronobacter* genus have been reported, including molecular methods (12–15) as well as structural studies (12, 22, 25, 34). Until now, there have been 17 recognized serotypes among the *Cronobacter* species. However, some serotypes were defined using culture collections with strains identified by 16S rRNA and biochemical profiling, neither of which can accurate identify *Cronobacter* species (10, 17). In contrast, the multilocus sequence type (MLST) analysis of *Cronobacter* strains is supported by whole-genome phylogeny (39–41).

In this study, different serotyping techniques were used to analyze 82 different *Cronobacter* strains representing all currently known species which have been identified based on DNA sequencing and reference to the open-access PubMLST *Cronobacter* database (http://pubmlst.org/cronobacter/ [accessed 31 December 2014]) with over 1,000 described strains and metadata (39). The genotypes of the O-antigen gene clusters of the strains were evaluated by restriction analysis and PCRs using the O-antigen processing genes, and LPS patterns were determined in chosen strains (Fig. 1). The obtained results were compared with published data, including the open-access *Cronobacter* PubMLST database (Table 4).

Serotyping of *C. sakazakii*. Within the *C. sakazakii* species, *C. sakazakii* O1 (SO1) and SO2 were the predominant serotypes; SO3 and SO4 were seen less often. Furthermore, the percentage of clinical isolates within particular serotypes was considered by reference to the *Cronobacter* PubMLST database. Almost 50% of *C. sakazakii* O2 strains from the database originated in clinical samples, and 90% are sequence type (ST) 4. This particular sequence type is of importance as it is the predominant *C. sakazakii* neonatal meningitis pathovar (42). This implies the association of this serotype with neonatal health risk. *C. sakazakii* O4 strains had a higher incidence of clinical samples (35%) than *C. sakazakii* O1 strains (12%) and *C. sakazakii* O3 strains (0%).

SO5, SO6, and SO7 were not detected in the identified *C. sakazakii* strains in the DNA sequence base.

The structures of O antigen have been determined for *C. sakazakii* serotypes O1, O2, O3, O4, and O7 (Table 4). These contain between five and seven saccharides in each O-antigen unit (12, 22, 24–26, 28, 29). They all produce a branched form of O antigen; the most frequent serotypes, *C. sakazakii* O1 and O2, have two saccharides in the branching fragment of the O antigen, whereas the rest have only one.

Serotyping of *C. malonaticus*. Within the *C. malonaticus* species, two serotypes (MaO1 and MaO2) were previously described and we propose two new ones: *C. malonaticus* O3 (MaO3) and O4 (MaO4). *C. malonaticus* O1 shares sequence similarities with *C. turicensis* O1, *Shigella dysenteriae* D11, and *Escherichia coli* O29,

[a] PCR protocols were based on the literature as follows: *C. sakazakii* serotypes 1 to 7 (heptaplex, S_HO1 to S_HO7) (12), *C. sakazakii* O4 (SO4) (14), *C. malonaticus* O2 (MaO2) (13), *C. muytjensii* O2 (MuO2) (14), *C. turicensis* O2 (TO2) (11), *C. turicensis* O3 (TO3) (14), *C. dublinensis* O1 (DO1) (14), *C. dublinensis* O2 (DO2) (14), *C. universalis* O1 (UO1) (14), *C. turicensis* O1 and *C. malonaticus* O1 together (TO1/MaO1) (13), and *C. sakazakii* O3 and *C. muytjensii* O1 together (SO3/MuO1) (13).
[b] Analysis was performed on the published genomes (38).
[c] Analysis was performed on all 82 strains of *Cronobacter*.
[d] The numbers of the strains of clinical origin are shown in parentheses.
[e] Characteristics from the literature (10–15).
[f] RFLP profiles and serotypes in bold are newly identified.
[g] Data represent characteristics reported in the literature (10–14, 22–31, 34–36). The structures highlighted in gray belong to *C. dublinensis* serotype O1 without recognition between D O1a and D O1b.
[h] qty$_S$, quantity of sugars in O-antigen unit.
[i] qty$_B$, quantity of sugars in the branching part of the O-antigen unit.
[j] On, previously unknown serotype.
[k] O?, previously unknown serotype, proposed serotype.
[l] RFLP profile of the strain with rough type of lipopolysaccharides.
[m] X, cancelled serotype.
[n] Incorrectly identified strain; the MLST database identified it as *C. malonaticus*.
[o] RFLP profile created by the use of Genetyx software.
[p] L, linear, B, branched.

suggesting related ancestries (31). The branched type of O-antigen structure has been previously reported for the *C. malonaticus* O2 serotype (27). Approximately half of the *C. malonaticus* O2 strains are of clinical origin ($n = 58$) (39).

Discrepant results were observed between the species designation and serotype *C. sakazakii* O6. Only three strains in the PubMLST database are categorized as *C. sakazakii* O6. All three are *C. sakazakii* isolates from deli meat in China. *In silico* analysis showed positive results with two genomes from the species *C. malonaticus* (LMG 23826 and 681), which was consistent with obtained experimental data. All of our *C. sakazakii* strains gave negative results with *C. sakazakii* O6-specific primers; moreover, all of our *C. malonaticus* strains (identified reliably as *C. malonaticus* O2) showed the same O-antigen gene cluster profile (M) and gave positive results with this serotyping PCR. Hence, *C. sakazakii* serotype O6 should be regarded as identical to *C. malonaticus* O2.

The reassignment of *C. sakazakii* O5 and O6 to *C. malonaticus* is due to the apparent initial incorrect species identification by Sun et al. (10). The original G2706 and G2708 strains used to define the *C. sakazakii* O5 serotype were originally identified to the species level using 16S rRNA sequencing and biochemical profiling; both procedures are now recognized as being prone to errors (17). According to MLST analysis, both strains are *C. malonaticus*. With respect to these findings, we propose to reclassify these *C. sakazakii* O5 strains as *C. malonaticus* O3 based on the *C. malonaticus* 507 reference strain (restriction profile Z generated *in silico*; *Cronobacter* PubMLST genome sequenced strain ID74).

Furthermore, we propose the new serotype *C. malonaticus* O4 (MaO4), as specified by the G3882 reference strain. This strain was previously assigned as *C. turicensis* O2 (11); however, according to the *Cronobacter* PubMLST database, strain G3882 is *C. malonaticus* (ID949, sequence type 371). The O-antigen gene sequence, specific PCR results, and even the structure of the O-polysaccharide for this strain have already been described (11) (http://pubmlst.org/cronobacter/). Both *C. malonaticus* O3 and O4 have the linear type of O antigen (11, 12).

Serotyping of *C. turicensis*. The species *C. turicensis* contains at least three serotypes. It is noteworthy that the LMG 23827^T reference strain for *C. turicensis* O1 (TO1) was isolated from blood from the fatal infant meningitis case and also that two of our three *C. turicensis* O1 strains were clinical isolates. *C. turicensis* O3 (TO3) was detected in three strains, which were also positive with *C. sakazakii* S_HO5. This can be explained by the reclassification of strain G4105 (identified by Sun et al. [10] as *C. sakazakii* O5) as a *C. turicensis* strain as given on the MLST database (http://pubmlst.org/cronobacter/). Though the described O-antigen gene clusters of *C. malonaticus* O3 and *C. turicensis* O3 share high sequence similarity (12, 14), they have different restriction profiles (Z and A, respectively) (10). To make this clear, the chemical structures of the corresponding O-antigens would be supportive, but unfortunately they have not been reported as yet.

Recently, Czerwicka et al. (31) published the only O-antigen structure of three strains of *C. turicensis*. This is a branched type of O-antigen with only four saccharides. Based on their results, the new serotype *C. turicensis* O4 (TO4) should be recognized. The new serotype is specified by the reference strain *C. turicensis* 564 (genome sequenced; *Cronobacter* PubMLST strain ID82) and has the structure published by Czerwicka et al. (31). A further *C. turicensis* serotype (O5) is proposed for reference strain E676, with a defined RFLP profile (profile F) and LPS pattern (Table 3 and Fig. 1).

In the literature, one more O-PS structure was reported for *C. turicensis* HPB 3287 (36). It was, however, later identified as *Enterobacter asburiae*, as given in the *Cronobacter* PubMLST database (http://pubmlst.org/cronobacter/).

Serotyping of *C. dublinensis*. For *C. dublinensis*, two serotypes, *C. dublinensis* O1 (DO1) and DO2, have already been reported (14). Both serotypes were identified among our *C. dublinensis* strains. The *C. dublinensis* O1 serotyping PCR recognized strains with two types of O-antigen gene profiles (profiles J and L). Since those strains produced different lipopolysaccharide profiles (Fig. 1, lanes 20 and 19), this serotype should be subdivided as DO1a and DO1b, respectively. Two similar O-PS structures have been published for *C. dublinensis* O1 (32). Interestingly, the *C. dublinensis* serotypes mentioned above corresponded to the previous distinction of *C. dublinensis* subspecies: *C. dublinensis* O1a correlates with *C. dublinensis* subsp. *lactaridi*, *C. dublinensis* O1b with *C. dublinensis* subsp. *dublinensis*, and *C. dublinensis* O2 with *C. dublinensis* subsp. *lausannensis* (1).

MacLean et al. characterized the O-antigen structure of *C. dublinensis* strain HPB 3169 (33). This O antigen is structurally similar to that recently reported for *Cronobacter malonaticus* O3 strain G2706 (28). Due to the significant differences in the O-antigen gene sequence, it should be a new serotype, *C. dublinensis* O3 (DO3), specified by its O-antigen structure (33).

One of our *C. dublinensis* strains (Cb13) did not give a positive result in any serotyping PCR and had its own restriction profile (Q) and LPS pattern. This suggests that it could be a member of another *C. dublinensis* serotype, provisionally designated DO4 here; however, we do not have enough information to further classify this serotype.

Serotyping of *C. muytjensii*. Two serotypes (13, 14) have been described within the *C. muytjensii* species: O1 (MuO1) and O2 (MuO2). All *C. muytjensii* strains in this study were MuO2 and were assigned RFLP profile K.

Serotyping of *C. universalis* and *C. condimenti*. There were two RFLP restriction profiles (profiles H and P) for *C. universalis*. The two strains had previously been reported as *C. universalis* O1 (UO1) (35). The new serotype was classified as *C. universalis* O2 (UO2). The only strain of *C. condimenti* has its specific O-antigen gene profile (N) and LPS pattern, supporting assignment of its own serotype, *C. condimenti* O1 (CO1).

Occurrence of complete and incomplete LPSs within *Cronobacter* strains. In the literature, there is limited information on the occurrence of strain variants in the genus *Cronobacter*. Mullane et al. (15) reported two rough-colony strains: strain CFS1001 of *C. sakazakii* O1 and strain E892 of *C. sakazakii* O2. These strains produced restriction fragments of the O-antigen gene cluster identical to those of other (smooth) strains, namely, *C. sakazakii* O1 and O2, respectively. The mutations responsible for the rough LPS phenotype are often localized outside the O-antigen gene cluster (30, 38). Therefore, the RFLP of the O-antigen gene cluster may not indicate whether the isolate exhibits smooth or rough LPS, as was the case with strains CFS1001 and E892 (both exhibiting rough LPS) reported by Mullane et al. (15).

In our study, all strains except one (CDC9369-75) had full-length LPSs (Fig. 1). The LPS pattern indicated a defect in the O-antigen polymerization in this strain (43, 44). Correspondingly, its O-antigen RFLP profile (Y) was very similar but not

identical (see Results and Table 3) to the profile characteristic of *C. sakazakii* O2 (B), suggesting the existence of a mutation in the O-antigen cluster. This finding was supported also by the results from serotyping heptaplex PCR. The Cb43 strain gave positive results with the primer pair specific to *C. sakazakii* heptaplex O2 (S_HO2); however, the size of the product was 900 bp instead of 152 bp (specific size for all other *C. sakazakii* O2 strains). Remarkably, this primer pair is targeted to the *wzy* gene for the O-antigen polymerase that mediates the transfer of nascent polymer to the new O unit. Strains with mutations in this gene are observed to produce semirough LPS (38). It is therefore probable that the Cb43 strain shows a semirough LPS phenotype. To confirm our observations, molecular studies of the genes involved in the LPS biosynthesis, especially studies of the O antigen and the core, would be appropriate.

Conclusion. To the best of our knowledge, this report provides the first polyphasic description of serotype diversity across the entire *Cronobacter* genus. Several genotyping and phenotyping techniques were successfully combined to separate *Cronobacter* strains into serotype clusters. We compared our results with those reported in the literature and with the data from the curated open-access *Cronobacter* PubMLST database. From the information obtained, 24 *Cronobacter* serotypes are now described. This includes 7 new serotypes. These serotypes have been characterized by the RFLP profiles of the O-antigen gene clusters and LPS patterns.

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