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Development of a Novel Screening Method for the Isolation of "*Cronobacter*" spp. (*Enterobacter sakazakii*)[▽]

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A differential medium, "*Cronobacter*" screening broth, has been designed to complement agars based on hydrolysis of chromogenic α-glucopyranoside substrates. The broth was evaluated using 329 *Enterobacteriaceae* strains (229 target isolates), spiked/naturally contaminated samples, and a parallel comparison with current methods for raw materials, line/end products, and factory environment samples.

Enterobacter sakazakii is an opportunistic neonatal pathogen that has been reported as an occasional contaminant of powdered infant formula (PIF) (2, 3). Recent polyphasic analysis led to the proposal of a reclassification of *E. sakazakii* as several novel genomospecies within a novel genus, "*Cronobacter*," with the novel genus being synonymous with *E. sakazakii* (11, 13). Various chromogenic and fluorogenic agar media have been described for detection of *Cronobacter* (7, 17, 19, 20). These are based mainly on the enzyme α-glucosidase, which is constitutively expressed in *Cronobacter* but in few other *Enterobacteriaceae* (18, 21, 22). Assessment of several of these media has shown that they provide comparable sensitivities and specificities (5, 12, 16). However, it has been established that some isolates of *Cronobacter* do not grow well in currently proposed enrichment broths, such as modified lauryl sulfate tryptose broth (mLST) and *Enterobacteriaceae* enrichment broth (6, 9, 16). Samples containing only such strains could give false-negative results; therefore, the enrichment procedure should be improved. Fermentation of sucrose and metabolism of 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside have been shown to be useful distinguishing tests for *Cronobacter* spp. (12, 18). Therefore, a differential broth has been developed that enables samples to be screened for potential *Cronobacter* contamination without incorporating selective agents that may affect the recovery of sensitive strains (9).

CSB. *Cronobacter* screening broth (CSB) comprises 10.0 g liter^{-1} peptone, 3.0 g liter^{-1} meat extract, 5.0 g liter^{-1} NaCl (7647-14-5), 0.04 g liter^{-1} bromocresol purple (115-40-2), 10 g liter^{-1} sucrose (57-50-1), and 10 mg liter^{-1} vancomycin hydrochloride (1404-93-9). (All chemicals are described by their Chemical Abstract Service numbers to allow an exact identification of materials independent of supplier.) The final pH value is 7.4 ± 0.1, and the optimum incubation temperature is 42°C.

Microbiological strains. Inclusivity and exclusivity of CSB were assessed using 329 strains, including 229 target *Cronobacter* isolates, covering a diversity of global distribution, species, and biogroups (11, 13). The 100 nontarget *Enterobacteriaceae* strains included 27 species from nine genera. After 24 h at 42°C, all 229 *Cronobacter* strains tested were able to grow in CSB and ferment the sucrose, thus lowering the pH and effecting a change in the color of the broth from purple to yellow. The minimum inoculum required to observe a color change in CSB within 24 h was 10^2 CFU ml^{-1}, as determined with the slowest-growing strain (E770). This corresponds to a final concentration of 10^4 CFU ml^{-1} after preenrichment of the sample in buffered peptone water (BPW) (CM0509; Oxoid AG, Pratteln, Switzerland). The level of competitive flora in PIF is generally low (8), the lag time for desiccated cells to recover after rehydration of PIF at 37°C has been estimated as 0.254 h (15), and the approximate doubling time at 37°C in infant formula for *Cronobacter* spp. is 20 min (10). Therefore, theoretically an initial contamination of only 1 CFU in a 300-g sample should be able to multiply to exceed a final concentration of 10^4 CFU ml^{-1} well within an 18-h incubation time. The sensitivity and negative predictive value (NPV) of CSB were 100% when CSB was used in conjunction with a nonselective chromogenic medium (X-TSA) comprised of tryptone soya agar (TSA) (CM0131; Oxoid AG, Pratteln, Switzerland) containing 0.15 g liter^{-1} 5-bromo-4-chloro-3-indolyl-α-D-glucoside (108789-36-2). The specificity was 94%, and the positive predictive value (PPV) was 97.4%. The nontarget strains yielding presumptively positive reactions (6/100) were all isolates of the novel species *Enterobacter pulveris* (22), which ferment sucrose and metabolize 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside. However, they can be distinguished from *Cronobacter* spp. by using phenotypic tests (11, 22). Caution needs to be used with interpretation of commercial biochemical galleries, as these species are not yet incorporated in the corresponding databases and can be designated a nearest match to *E. sakazakii*. Based on data from our culture collection, all *E. pulveris* strains are negative for arginine dihydrolase, ornithine decarboxylase, and Voges-Proskauer tests, whereas *Cronobacter* strains are, respectively, 97%, 92%, and 99.5% positive for these tests. Additionally, all *E. pulveris* strains are positive for fermentation of D-arabitol and the methyl red test,

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whereas *Cronobacter* strains are 100% negative for fermentation of D-arabitol and 99.5% negative for the methyl red test.

Recovery from spiked samples. To mimic the recovery of low numbers of cells from PIF, 100 g of PIF was diluted 1/10 with BPW. The rehydrated PIF was spiked with 20 target *Cronobacter* strains and 10 nontarget *Enterobacteriaceae* strains to achieve for each strain a final concentration of 1 to 10 CFU liter^{-1}. The resulting spiked 1,000 ml of rehydrated formula was divided into 200 5-ml analytical samples; this gave an estimated average of 0.75 CFU 5 ml^{-1}. The strains included representatives of the different *Cronobacter* species (13) as well as target strains that were previously found difficult to grow in *Cronobacter* isolation media (9). Nontarget organisms included the recently described species *Enterobacter helveticus*, *E. turicensis*, and *E. pulveris* (21, 22), which can be found in the same ecological niches as *Cronobacter*, such as dried food products and factory environments, and also yield presumptively positive colonies on chromogenic agar. The 200 spiked analytical samples were incubated for 24 h at 37°C. Each analytical sample was then diluted 1/100 into both CSB and mLST (4) and incubated for 24 h at 42°C and 44°C, respectively. All analytical samples were streaked onto X-TSA and the plates incubated at 37°C for 24 h to detect presumptively positive *Cronobacter* colonies. The presumptively positive colonies were distinguished using the methyl red and Voges-Proskauer tests. Bacteria were recovered from 147/200 spiked 5-ml analytical samples, with multiple isolates obtained from 17 samples. Fermentation of CSB occurred in 134 samples, and 105 gave presumptively positive colonies on chromogenic agar. *Cronobacter* was detected in a total of 91 of the spiked 5-ml samples, all of which were positive in CSB and produced presumptively positive colonies on the agar (Table 1). Of the 56 samples from which only nontarget strains were recovered, 43 were positive in CSB and 14 of these gave presumptively positive colonies on X-TSA. Fewer samples were found positive for *Cronobacter* when using mLST than when using CSB.

Recovery from factory samples. A total of 80 10-g samples (41 PIF, two dried infant cereals, 15 vitamin-mineral premixes, and 22 factory environmental samples) were preenriched for 24 h at 37°C in BPW at a 1/10 dilution. Each preenriched sample homogenate was then inoculated (100 μl) into 10 ml CSB, giving a 1/100 dilution of homogenate, and incubated at 42°C for 24 h, after which 10 μl was streaked onto X-TSA and incubated at 37°C for 24 h. The presumptively positive isolates were identified using ID 32E biochemical galleries (bioMérieux Industries, Marcy l'Etoile, France). No *Cronobacter* strains were detected in any of the PIF, infant food, or vitamin-mineral premixes, with only one nontarget presumptively positive (i.e., positive in CSB) out of 58 samples tested. Therefore, 98.3% (57/58) of these samples would correctly be identified as negative after 48 h and a decision to release could be made. For the environmental samples, 12/22 showed fermentation of CSB; nine of these produced presumptively positive colonies on chromogenic agar, of which eight were confirmed as *Cronobacter*.

Comparison with current methods. Samples were obtained from multiple factory sites and divided into 322 finished products (PIF), 146 ingredients, and 482 environmental samples (950 test samples in total, comprising a comprehensive survey of the infant formula manufacturing process). All samples were preenriched for 24 h at 37°C in BPW. Sample size varied depending on the nature of the material, and dilution in BPW was 1/10. For some samples, such as starches, chocolate powders, and vitamin-mineral premixes, a dilution of 1/100 was also used. Where feasible, raw materials were tested as 25-g aliquots, PIF were tested as 30 10-g, 10 30-g, or 2 150-g aliquots, and environmental swabs were preenriched in 10 ml BPW. Preenriched samples were analyzed using the FDA method (1) and the ISO/TS 22964 method (4) as well as the CSB method. For the last method, 0.1 ml of the preenriched sample was transferred to 10 ml CSB and incubated at 42°C for 24 h. In practice, only samples in which fermentation of the carbohydrate occurs, resulting in a color change from purple to yellow, need to be streaked onto chromogenic agar. However, in this study all broths were streaked onto a modified version of chromogenic *E. sakazakii* agar, DFI (Druggan, Forsythe, and Iverson) formulation (mDFI) (Oxoid Ltd., Basingstoke, United Kingdom). This medium comprised 7.0 g liter^{-1} tryptone, 3.0 g liter^{-1} yeast extract (8013-01-2), 5.0 g liter^{-1} NaCl (7647-14-5), 0.15 g liter^{-1} 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (108789-36-2), 0.25 g liter^{-1} sodium desoxycholate (302-95-4), 1.0 g liter^{-1} sodium thiosulfate (7772-98-7), 1.0 g liter^{-1} ammonium iron(III) citrate (1185-57-5), and 13.0 g liter^{-1} agar-agar (9002-18-0).

TABLE 1. Performance of mLST with chromogenic agar, CSB alone, and CSB with chromogenic agar for isolation of *Cronobacter* from spiked samples

Organism or result detected (total no. of strains)	No. of strains with results typical of *Cronobacter* spp.		
	mLST with X-TSA	CSB	CSB with X-TSA
Cronobacter (88)	84	88	88
Cronobacter plus nontarget strains (3)	2	3	3
Nontarget strains (56)	2	43	14
Uncontaminated aliquots (53)	0	0	0

Although the FDA and ISO/TS 22964 methods cite the use of yellow pigmentation and biochemical tests as indicative of positive *Cronobacter* isolates (1, 4), these criteria have been shown to be unreliable (5, 9, 12). Therefore, in this study presumptive *Cronobacter* isolates obtained using the three methods were confirmed as positive by use of a *Cronobacter*-specific α-glucosidase PCR assay (12). Biochemical tests and 16S rRNA gene sequencing (Fasteris S.A., Plan-les-Ouates, Switzerland) were used to indicate *Cronobacter* species identity (11, 13).

All three methods recovered *Cronobacter* from 2.17% (7/322) of end product samples. The CSB and ISO/TS 22964 methods both recovered target strains from 10.96% (16/146) of raw materials; however, the FDA method detected *Cronobacter* in only 3.42% (5/146) of raw materials. As these ingredients and products were from infant food production facilities, the sensitivity of the methods for detecting *Cronobacter* in these samples is of particular importance; both the ISO/TS 22964 and CSB methods were found to have 100% sensitivity and 100% NPV (Table 2). The advantage of using the CSB screening method is that a release decision can be made on negative samples within 48 h; also, the costs of materials and labor are considerably reduced, as only positive broths need to be streaked onto the chromogenic agars.

TABLE 2. Recovery of *Cronobacter* spp. from product, raw materials, and environment by use of CSB in combination with mDFI agar and comparison with the FDA and ISO/TS 22964 methods[a]

Method	No. of confirmed *Cronobacter* isolates			Result for product and RM samples				Result for ENV samples			
	Product	RM	ENV	SE (%)	SP (%)	PPV (%)	NPV (%)	SE (%)	SP (%)	PPV (%)	NPV (%)
FDA	7	5	13	52.2	73.5	9	96.8	26	35.6	4.6	80.2
ISO/TS 22964	7	16	46	100	94.2	46.9	100	92	99.3	93.9	99.1
CSB with mDFI agar	7	16	47	100	93.9	46	100	94	99.3	94	99.3

[a] RM, raw materials; ENV, environmental samples; SE, sensitivity; SP, specificity.

The testing of environmental samples in production facilities is performed to verify the effectiveness of hygiene control measures aimed at minimizing product recontamination. Due to the higher level of bacterial flora in these samples, the specificity of a detection method becomes of increased importance. In this study, *Cronobacter* was recovered from a total of 10.37% (50/482) of the environmental samples by one or more methods. The CSB method recovered *Cronobacter* from 9.75% (47/482), the ISO/TS 22964 from 9.54% (46/482), and the FDA method from only 2.7% (13/482) of environmental samples. The strains that were recovered using the CSB method but not the ISO/TS 22964 method and vice versa were tested for the ability to grow in the respective media. All seven of these strains grew to $>10^9$ CFU ml^{-1} in CSB after 24 h at 42°C and fermented the sucrose, yielding a distinct yellow coloration. Also, all of these strains were able to grow when incubated for 24 h on mDFI and *Enterobacter sakazakii* identification agar at 42°C and 44°C, respectively, producing distinct blue-green colonies. However, only three of these strains were able to grow to $>10^9$ CFU ml^{-1} in mLST when incubated for 24 h at 44°C. The other four strains did not produce turbid cultures, and plate counts on TSA incubated for 24 h at 37°C showed that the final concentrations reached in the mLST ranged from 2.2×10^7 to 7.3×10^7 CFU ml^{-1}, an increase of approximately 2 log from the inoculum. Therefore, the inability of the methods to recover strains from some samples is probably due to a combination of competition from other bacterial species in the samples and reduced growth rate in mLST. McNemar's test for marginal homogeneity with continuity correction showed there was no significant difference in performance of the CSB and ISO/TS 22964 methods (two-tailed P value of 1.0 and odds ratio of 0.750, with a 95% confidence interval extending from 0.11 to 4.43). For the environmental samples, both methods showed a high specificity and high NPVs and PPVs (Table 2). The sensitivity of the CSB method (94%) was slightly higher than that of the ISO/TS 22964 method (92%), demonstrating that despite the differential rather than selective nature of CSB, this method is equally suited for testing environmental samples, ingredients, and end products. Moreover, examples of all proposed *Cronobacter* species were isolated during this study, indicating that this method is suitable for detection of all members of this genus.

Conclusions. A differential screening medium, CSB, has been developed to identify samples potentially contaminated with *Cronobacter* spp. The broth is designed to circumvent the problems encountered with selective enrichment media for these organisms and to be complementary to currently available chromogenic media in order to improve overall sensitivity and selectivity of *Cronobacter* detection. The CSB screening method was able to detect *Cronobacter* in spiked and in naturally contaminated samples. Potentially, this screening method can enable the decision to release uncontaminated product after 48 h. As CSB is a differential rather than selective enrichment broth, all *Cronobacter* strains are able to grow in CSB. This differential screening broth is complementary to any agar medium that incorporates a test for metabolism of α-glucopyranoside. However, while CSB has been shown to be 100% sensitive for *Cronobacter* species, the overall effectiveness of the method is dependent on the sensitivity and specificity of the chromogenic agar used. The numbers of positive samples found from ingredients and the environment are in line with previous findings that *Cronobacter* spp. are ubiquitous environmental organisms that can be isolated from various food products as well as from households (8, 14).

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REFERENCES

1. **Anonymous.** 2002. Isolation and enumeration of *Enterobacter sakazakii* from dehydrated powdered infant formula. U.S. Food and Drug Administration, Rockville, MD. http://www.cfsan.fda.gov/~comm/mmesakaz.html.
2. **Anonymous.** 2004. *Enterobacter sakazakii* and other microorganisms in powdered infant formula: meeting report, MRA Series 6. FAO/WHO, Geneva, Switzerland.
3. **Anonymous.** 2006. *Enterobacter sakazakii* and *Salmonella* in powdered infant formula. Second Risk Assessment Workshop, 16 to 20 January 2006. FAO/WHO, Rome, Italy.
4. **Anonymous.** 2006. Milk and milk products—detection of *Enterobacter sakazakii*. Technical specification ISO/TS 22964. ISO/TS 22964:2006(E) and IDF/RM 210:2006(E), 1st ed. International Organization for Standardization, Geneva, Switzerland.
5. **Gnanou Besse, N., A. Leclercq, V. Maladen, C. Tyburski, and B. Lombard.** 2006. Evaluation of the International Organization for Standardization-International Dairy Federation (ISO-IDF) draft standard method for detection of *Enterobacter sakazakii* in powdered infant food formulas. J. AOAC Int. **89:**1309–1316.
6. **Guillaume-Gentil, O., V. Sonnard, M. C. Kandhai, J. D. Marugg, and H. Joosten.** 2005. A simple and rapid cultural method for detection of *Enterobacter sakazakii* in environmental samples. J. Food Prot. **68:**64–69.
7. **Iversen, C., P. Druggan, and S. J. Forsythe.** 2004. A selective differential medium for *Enterobacter sakazakii*. Int. J. Food Microbiol. **96:**133–139.
8. **Iversen, C., and S. Forsythe.** 2004. Isolation of *Enterobacter sakazakii* and

other *Enterobacteriaceae* from powdered infant formula milk and related products. Food Microbiol. **21:**771–777.
9. **Iversen, C., and S. Forsythe.** 2007. Comparison of media for the isolation of *Enterobacter sakazakii*. Appl. Environ. Microbiol. **73:**48–52.
10. **Iversen, C., M. Lane, and S. Forsythe.** 2004. The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. Lett. Appl. Microbiol. **38:**378–382.
11. **Iversen, C., A. Lehner, N. Mullane, E. Bidlas, I. Cleenwerck, J. Marugg, S. Fanning, R. Stephan, and H. Joosten.** 2007. The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies 1. BMC Evol. Biol. **7:**64.
12. **Iversen, C., A. Lehner, N. Mullane, J. Marugg, S. Fanning, R. Stephan, and H. Joosten.** 2007. The identification of *Cronobacter* spp. (*Enterobacter sakazakii*). J. Clin. Microbiol. **45:**3814–3816.
13. **Iversen, C., N. Mullane, B. McCardell, B. D. Tall, A. Lehner, S. Fanning, R. Stephan, and H. Joosten.** *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov. comb. nov., *C. malonaticus* sp. nov., *C. turicensis* sp. nov., *C. muytjensii* sp. nov., *C. dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *C. dublinensis* sp. nov. subsp. *dublinensis* subsp. nov., *C. dublinensis* sp. nov. subsp. *lausannensis* subsp. nov., and *C. dublinensis* sp. nov. subsp. *lactaridi* subsp. nov. Int. J. Syst. Evol. Microbiol., in press.
14. **Kandhai, M., M. Reij, L. Gorris, O. Guillaume-Gentil, and M. van Schothorst.** 2004. Occurrence of *Enterobacter sakazakii* in food production environments and households. Lancet **363:**39–40.
15. **Kandhai, M., M. Reij, C. Grognou, M. van Schothorst, L. Gorris, and M. H. Zwietering.** 2006. Effects of preculturing conditions on lag time and specific growth rate of *Enterobacter sakazakii* in reconstituted powdered infant formula. Appl. Environ. Microbiol. **72:**2721–2729.
16. **Lehner, A., S. Nitzsche, P. Breeuwer, B. Diep, K. Thelen, and R. Stephan.** 2006. Comparison of two chromogenic media and evaluation of two molecular based identification systems for *Enterobacter sakazakii* detection. BMC Microbiol. **6:**15.
17. **Leuschner, R. G. K., and J. Bew.** 2004. A medium for the presumptive detection of *Enterobacter sakazakii* in infant formula: interlaboratory study. J. AOAC Int. **87:**604–613.
18. **Muytjens, H. L., J. van der Ros-van de Repe, and H. A. M. van Druten.** 1984. Enzymatic profiles of *Enterobacter sakazakii* and related species with special reference to the alpha glucosidase reaction and reproducibility of the test system. J. Clin. Microbiol. **20:**684–686.
19. **Oh, S. W., and D. H. Kang.** 2004. Fluorogenic selective and differential medium for isolation of *Enterobacter sakazakii*. Appl. Environ. Microbiol. **70:**5692–5694.
20. **Restaino, L., E. W. Frampton, W. C. Lionberg, and R. J. Becker.** 2006. A chromogenic plating medium for the isolation and identification of *Enterobacter sakazakii* from foods, food ingredients and environmental sources. J. Food Prot. **69:**315–322.
21. **Stephan, R., S. Van Trappen, I. Cleenwerck, M. Vancanneyt, P. De Vos, and A. Lehner.** 2007. *Enterobacter turicensis* sp. nov. and *Enterobacter helveticus* sp. nov., isolated from fruit powder. Int. J. Syst. Evol. Microbiol. **57:**820–826.
22. **Stephan, R., S. Van Trappen, I. Cleenwerck, C. Iversen, H. Joosten, P. De Vos, and A. Lehner.** 2008. *Enterobacter pulveris* sp. nov., isolated from fruit powder, infant formula and an infant formula production environment. Int. J. Syst. Evol. Microbiol. **58:**237–241.