Identification of “Cronobacter” spp. (Enterobacter sakazakii)\textsuperscript{V}

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A taxonomic reclassification of the neonatal pathogen Enterobacter sakazakii to consist of five species within a new genus, “Cronobacter,” has recently been proposed. The correct identification of these organisms is important to clinicians. Therefore, using 312 Enterobacteriaceae, including 210 “Cronobacter” strains, the reliabilities of biochemical and genetic confirmation tests were investigated. All “Cronobacter” isolates were positive using dnaG and gluA PCR protocols, and all expressed α-glucosidase activity. ID32E v3.0 identified 99.5% of “Cronobacter” isolates (as the nearest match to E. sakazakii).

Updating the original taxonomy of Enterobacter sakazakii has resulted in the clear definition of at least five new species based on extensive geno- and phenotypic evaluations (7). These pathogenic species are etiological agents in rare cases of meningitis, necrotizing enterocolitis, and bacteremia in neonates (11, 13, 15, 21). In order to facilitate their continued inclusion in schemata for the diagnosis of infection and the microbiological monitoring of food products, it has been proposed that these species be moved to a novel genus, “Cronobacter” (7). Current culture-based isolation methods for these species include a test for α-glucosidase activity (5, 6, 12, 14, 16, 17). However, the reliability of this test has been questioned (9, 17). The observation of yellow pigment for presumptive identification and the efficacy of biochemical test kits have also been placed in doubt (1, 4, 8). Identification results obtained using the ID32E biochemical gallery (bioMérieux), with updated software, v3.0, have recently been reported using a limited number of “Cronobacter sakazakii” isolates (3).

In this study 102 Enterobacteriaceae from 27 species in nine genera, as well as 210 “Cronobacter” strains covering the five species (7), were obtained from food, environmental, and clinical sources. The reliabilities of PCR amplification assays targeting the dnaG (18) and gluA (10) genes, biochemical galleries (API20E and ID32E), α-glucosidase assays, and yellow pigmentation and the diagnostic value of chromogenic isolation media were investigated.

Phenotypic tests. Acid production from methyl-α-D-glucopyranoside (AMG) was tested in phenol red broth containing 0.5% filter-sterilized carbohydrate solution. Use of 4-methylumbelliferyl-α-D-glucopyranoside (4-MU-α-Glc; 44051; Glycosynth, United Kingdom) was evaluated by observing fluorescence under UV light of cultures previously cultured for 24 h in buffered peptone-water containing 0.1 g liter\textsuperscript{-1} 4-MU-α-Glc and of individual colonies grown for 24 h on tryptic soy agar containing 0.1 g liter\textsuperscript{-1} 4-MU-α-Glc. Constitutive metabolism of 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (X-α-Glc; 70051; Glycosynth, United Kingdom) was determined by observing the formation of an indigo pigment on nonselective medium containing peptone (7 g), yeast extract (3 g), NaCl (5 g), agar (15 g), X-α-Glc (0.15 g), and H2O (1,000 ml), incubated at 37°C for 24 h. Growth on selective, differential media including E. sakazakii isolation agar (ESIA; AEB520010; AES Laboratoire, France), E. sakazakii chromogenic agar (DFIA; CM1055; Oxoid Ltd., United Kingdom), E. sakazakii plating medium (ESPm; R&F Products Inc.), and E. sakazakii screening plate (ESSP; R&F Products Inc.) was determined using a 10-μl inoculum from turbid cultures grown in brain heart infusion for 18 to 24 h. Incubation of DFIA, ESPm, and ESSP was at 37°C, and that of ESIA was at 44°C. All plates were incubated for 24 h; the ESSP was also read at 6 h, and a positive result at either time point was recorded. Production of yellow pigment was determined on tryptic soy agar incubated at 25°C for 72 h, followed by exposure to natural light for 6 h at room temperature. Commercial identification biochemical galleries, API20E and ID32E (bioMérieux, France), were used according to the manufacturers’ instructions.

dnaG. A real-time PCR assay targeting the dnaG gene, a component of the macromolecular synthesis operon, was developed previously by Seo and Brackett (18) and modified by Drudy et al. (2) using the TaqMan probe (6-carboxyfluorescein–ACA GAG TAG TAG TTG TAG AGG CCG TGC TTC C–) to increase the discriminatory power of the assay.

gluA. The α-glucosidase (gluA) gene was amplified using the following primers: EsAgf, 5′-TGA AAG CAA TCG ACA-3′ and EsAg5_5r (5′-TTG ATG CCA AGC TGT-3′), generating a product of 1,680 bp in size. The amplification reaction mixture and the thermal cycling conditions were described previously (10). The gluA short fragment was amplified using primers EsAg5f (5′-TAT CAG ATC TAC CCG CGC C3′) and EsAg5_5r (5′-TTG ATG CCA AGC TGT TGC-3′), resulting in a 105-bp amplicon. The PCR conditions were as described previously (10) except that annealing was at 62°C for 30 s and extension was at 72°C for 30 s. The amplification products were analyzed in a 2% agarose gel.

In this study 193/210 “Cronobacter” strains (92%) exhibited...
yellow or pale yellow colony pigmentation with the remaining 17 strains (8%) appearing cream-white. Also, 34% of the other Enterobacteriaceae in this data set produced yellow-pigmented colonies, clearly illustrating the lack of confidence in this feature as a reliable identification criterion.

All “Cronobacter” strains demonstrated biochemical activity consistent with the constitutive expression of functional α-glucosidase and metabolized the substrates X-α-Glc, 4-MU-α-Glc, and 4-nitrophenyl-α-D-glucopyranoside (4-NP-α-Glc) (Table 1). All were positive for the presence of the α-glucosidase gene (gluA) using both primer sets. Unlike other “Cronobacter” species, “Cronobacter muytjensii” was negative for acid production from AMG. This may indicate a difference between “Cronobacter” spp. in either specificity or inducibility of the α-glucosidase enzyme and/or transport mechanisms for the different glucopyranosides. The fluorogenic compound 4-MU-α-Glc was not as specific as the other substrates for the presumptive identification of “Cronobacter” spp., with 46% of other Enterobacteriaceae in this data set displaying a positive reaction. One strain (E770) did not grow on any of the commercial chromogenic media. Additionally, 4.3% of “Cronobacter” strains were sensitive to crystal violet and/or the elevated incubation temperature (44°C) and failed to grow on ESIA. Interestingly 3.8% of “Cronobacter” strains grew but did not produce blue-green-pigmented colonies on DFIA containing X-α-Glc at a final concentration of 0.1 g liter$^{-1}$. However, when this substrate was used at a concentration of 0.15 g liter$^{-1}$ in nonselective medium, these strains produced blue-green colonies. For the non-Cronobacter strains in this data set, the proportion producing typical colonies on the chromogenic media ranged from 24.5% (ESIA) to 38.2% (ESPM). Screening the presumptive positive colonies for fermentation of sucrose on ESSP did not reduce the number of positive “Cronobacter” strains but did successfully reduce the number of false-presumptive-positive Enterobacteriaceae. The melibiose fermentation test reduced false positives on ESPM by one additional strain; melibiose fermentation also appeared transient for some strains with positive results at 6 h but not at 24 h. The false-positive strains on ESIA and DFIA were identified as Enterobacter helveticus and Enterobacter turicensis (19) and a proposed novel species, Enterobacter pulveris (20). In addition, the false-positive strains from ESPM included Enterobacter asburiae, Enterobacter amnigenus, Enterobacter cloacae, and Enterobacter hormaechei.

The API20E gallery positively identified only 70% of the “Cronobacter” strains to species level (as E. sakazakii) with 28% of strains identified to the genus Enterobacter. No false identifications were obtained for “Cronobacter” strains; however, seven strains previously identified as E. cloacae, E. hormaechei, or E. asburiae using 16S rRNA gene sequencing (7) and/or the ID32E gallery were identified as E. sakazakii by using API20E. These strains were among those positive on ESPM and ESSP but negative on ESIA and DFIA. They were also negative for α-glucosidase activity using the different substrates and negative in the gluA and dnaG PCR assays. Mis-identification of these types of Enterobacter strains using limited biochemical galleries may have contributed to the reports of α-glucosidase-negative E. sakazakii isolates (9, 17). Using the ID32E gallery with version 3.0 of the apiweb database, 189/210 “Cronobacter” strains (90%) were identified to the species level (as E. sakazakii) with “good-excellent” identification. The nearest significant taxon for all but one of the remaining strains (20/21) was E. sakazakii, and none of the other Enterobacteriaceae were misidentified as E. sakazakii with this gallery.

In conclusion, using this extensive data set of “Cronobacter” strains, all target isolates demonstrated α-glucosidase activity, the chromogenic media were 95.7 to 99.5% sensitive, and the ID32E v3.0 gallery was 99.5% successful for identifying target strains to a correct nearest match. Notably for the purposes of confident identification, the dnaG and gluA gene PCR assays were 100% sensitive and specific.

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REFERENCES

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>α-Glucosidase substrate$^a$</th>
<th>% Positive strains by assay</th>
<th>Chromogenic agar$^b$</th>
<th>gluA PCR</th>
<th>dnaG reverse transcriptase PCR</th>
<th>% Strains identified as a match by biochemical gallery$^d$</th>
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<tbody>
<tr>
<td>“C. sakazakii”</td>
<td>185</td>
<td>4-NP-α-Glc</td>
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<tr>
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<td>100</td>
<td>7</td>
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$^a$ Substrate assays were for yellow pigment (4-NP-α-Glc), fluorescence (4-MU-α-Glc), blue-green pigment (X-α-Glc), and acid production (AMG).

$^b$ Chromogenic agar assays were for blue-green colonies (ESIA and DFIA), blue-black or blue-gray colonies (ESPM), and fermentation of sucrose and melibiose (ESSP).

$^d$ Percent strains identified as a good to excellent match with E. sakazakii.


