The Effects of Temperature on the Growth and Heat Resistance of Cronobacter spp.

SHIGEKO UEDA

Laboratory of Hygiene, Kagawa Nutrition University, 3-9-1 Chiyoda, Sakado-shi, Saitama 350-0214, Japan

Received 18 November, 2016/Accepted 20 January, 2017

The growth and survival of Cronobacter isolates were examined under incubation at different temperatures, and their thermal death behavior was investigated at high temperature conditions of above 50°C. Seventy three strains isolated from fresh vegetables, dried foods and soil were tested: 28 of Cronobacter sakazakii, 5 of C. dublienensis, 27 of C. malonaticus and 13 of C. turicensis. All Cronobacter strains grew and multiplied predominantly at 35 and 44°C until 16 hours of incubation, but showed poor growth at 15°C, and no growth at 5°C. At 48°C, the bacteria grew slightly during 6 to 8 h-incubation but decreased or were inactivated after 16 h-incubation. The heat resistance of Cronobacter spp. was measured under the conditions of 50, 55, 60, 65 and 70°C. Cronobacter strains survived almost without decrement for 30 min at 50°C, but decreased suddenly and perished completely within 10 to 20 min at 55°C and within 2 - 5 min at above 60°C. Some food materials should be stored below 5°C until the preparation, and dried food including powdered infant milk formula should be utilized immediately after reconstitution and preparation.

Key words : Cronobacter spp. / Growth / Heat Resistance.

Cronobacter spp. have been isolated from a wide spectrum of food and food ingredients including infant food, dairy food, eggs and meat, cereal products, legumes, spices and herbs, seeds, and various dried foods (Hochel et al., 2012). Moreover, the bacteria are ubiquitously distributed in the environmental soil, water, and dust, as well as utensils used for the preparation of dried food. Cronobacter spp. are gram negative, facultative anaerobic, nonspore-forming, motile bacteria of the family Enterobacteriaceae, and are recognized as one of agents of foodborne illnesses. The main species of Cronobacter are C. sakazakii, C. dublienensis, C. malonaticus, C. muytjensii and C. turicensis (Iversen et al., 2008). Furthermore, C. condiment, C. uninera, C. helventicus, C. pulveris and C. zuricensensis have recently been added as novel species (Joseph et al., 2012; Brady et al., 2013). Ueda (2016) surveyed the occurrence of Cronobacter spp. in dried foods, fresh vegetables and soil, and found that C. sakazakii, C. malonaticus, C. dublienensis and C. turicensis were predominant among these materials. In this study, the growth and survival of the Cronobacter isolates were determined under incubation at different temperatures, and their thermal death behavior was investigated at high temperature conditions of above 50°C.

Seventy three bacterial strains were used in this study including 28 of C. sakazakii, 5 of C. dublienensis, 27 of C. malonaticus and 13 of C. turicensis. These had been isolated from fresh vegetables, dried foods and soil in a previous survey (Ueda, 2017) (Table 1). For the determination of the growth behavior of Cronobacter spp., each strain was inoculated into Brain Heart Infusion broth (Difco) at the level of 10^2 cfu/mL, and cultured under shaking until 24 h at temperatures ranging from 5 to 48°C. The bacterial plate counts were measured on the ENTEROBACTER SAKAZAKII agar plate (Merck) at 2, 4, 6, 8, 16 and 24 h after the incubation.

Similarly, several types of dried food including infant powdered milk formula prepared according to the manufacturer’s instructions were used for determining the growth of the bacteria. For the estimation of heat resistance and/or thermal death time, the cells of each of Cronobacter strain were suspended in nutrient broth (Difco) at the level of 10^2 cfu/mL, and the suspensions were heated for 1, 2, 5, 10, 15, 20 and 30 min under
the heating conditions of 50, 55, 60, 65 and 70°C, respectively. The survival bacterial counts were measured on the ENTEROBACTER SAKAZAKII agar plate (Merck) after the heated samples were immediately cooled.

The growth of Cronobacter strains from various sources was assessed at a temperature range of 5 to 48°C. As shown in Fig.1, several strains including C. sakazakii, C. malonaticus, C. turicensis and C. dublensis strains grew and multiplied predominantly at 35 and 44°C until 16 h-incubation, but showed poor growth at 15°C, and no growth at 5°C. At 48°C, the bacteria grew slightly during 6 to 8 h-incubation but decreased or were inactivated after 16 h-incubation. These growth patterns were almost identical in all tested strains from different sources. The growth patterns in powdered infant milk formula were similar to the growth curves in BHI broth, as shown in Fig.2.

The generation times (GT) of Cronobacter spp. from different sources were estimated on the basis of the changes in the bacterial counts in the log phase from each growth curve at the incubation temperature of 44°C, 35°C and 25°C. The GT of 28 strains of C. sakazakii were 18.2±4.2 min at 44°C, 22.4±5.1 min at 35°C and 42.6±10.7 min at 25°C. The GT of 5 C. dublensis strains were 16.9±1.9 min at 44°C, 27.9±5.8 min at 35°C and 45.1±5.6 min at 25°C. The GT of 27 C. malonaticus strains were 19.7±3.0 min at 44°C, 22.7±3.8 min at 35°C and 42.1±10.7 min at 25°C, and the GT of 13 C. turicensis strains were 20.3±2.9 min at 45°C, 24.7±5.4 min at 35°C and 48.2±12.9 min at 25°C, as shown Table 1. The generation times of each Cronobacter species were not found among the strains originated from different sources, and were commonly shorter at 44°C-incubation conditions than at 35 and 25°C regardless of species.

Fukuda et al. (2007) investigated the growth behavior of Cronobacter in infant formula at different temperatures and observed no growth at 4°C. The growth behavior of Cronobacter in this investigation was identical with the descriptions of the previous studies described above.

The heat resistance of Cronobacter spp. was measured under the conditions of 50, 55, 60, 65 and 70°C. The thermal death curves of five strains including C. sakazakii ATCC 29004 and C. sakazakii F11, C. malonaticus F12, C. dublensis F9 and C. turicensis F19 from dried foods are shown in Fig.3. Five Cronobacter strains survived almost without the decrement for 30 min at 50°C, but decreased suddenly and perished completely within 10 to 20 min at above 55°C. The numerical decrements of 72 strains of Cronobacter spp. after heating at 50, 55 and 60°C are shown in Table 2. The viable cells survived approximately in the original numbers until 15 min-heating at 50°C, but the cells of 4 species of Cronobacter perished completely during 10 - 15 min at 55°C. The decimal reduction time at 60°C (D₉₀ value) estimated from the thermal death curves was suggested to be within 60 s in all Cronobacter cells. Nakamura et al. (2011) described that cells of C. sakazakii were not eliminated and survived at low levels in powdered infant milk inoculated with 10⁵ cfu/mL, reconstituted with water at 60 and 70°C. Thus, some food materials should be stored below 5°C until preparation, and dried food including powdered infant milk formula should be utilized immediately after reconstitution and preparation.

### Table 1. Generation time of Cronobacter spp. isolated from fresh vegetables, dried foods and soil at different temperatures.

| Origin       | No. of strains | Generation time (min)¹ at: |
|--------------|----------------|----------------------------|
|              |                | 44°C | 35°C | 25°C |
| C. sakazakii | vegetables     | 9    | 16.8±2.0 | 24.1±6.4 | 42.9±13.1 |
|              | dried food     | 8    | 16.5±1.8 | 21.9±6.2 | 46.7±11.6 |
|              | soil           | 11   | 20.4±5.7 | 21.5±1.6 | 39.4±6.1 |
| total        |                | 28   | 18.2±4.2 | 22.4±5.1 | 42.6±10.7 |
| C. dublensis | vegetables     | 1    | 14.8     | 38.3     | 39.3     |
|              | dried food     | 1    | 15.6     | 22.3     | 51.8     |
|              | soil           | 3    | 18.0±1.6 | 26.2±2.6 | 44.8±5.1 |
| total        |                | 5    | 16.9±1.9 | 27.9±5.8 | 45.1±5.6 |
| C. malonaticus| vegetables   | 9    | 21.0±3.7 | 21.2±1.9 | 42.5±9.5 |
|              | dried food     | 9    | 18.7±1.5 | 23.1±3.7 | 39.6±5.7 |
|              | soil           | 9    | 19.4±2.8 | 23.9±4.7 | 44.3±14.5 |
| total        |                | 27   | 19.7±3.0 | 22.7±3.8 | 42.1±10.7 |
| C. turicensis | vegetables   | 6    | 20.6±3.5 | 24.1±4.4 | 44.6±5.1 |
|              | dried food     | 7    | 20.0±2.2 | 25.1±6.1 | 51.3±16.3 |
|              | soil           | 0    |          |          |          |
| total        |                | 13   | 20.3±2.9 | 24.7±5.4 | 48.2±12.9 |

¹ Mean±SD
FIG. 1. Growth curves of Cronobacter isolates at different incubation temperatures.

FIG. 2. Growth curves of C. sakazakii F1 at different temperatures in powdered infant milk formula inoculated at different levels.
TABLE 2. Numerical decrements of *Cronobacter* spp. from soil, vegetables and dried foods heated at different temperatures.

| Species tested (No. of strains) | Heat Temp. °C | Survival bacterial counts<sup>1</sup> after Heat Time (min) for: | 0 | 1 | 2 | 5 | 10 | 15 |
|---------------------------------|---------------|-----------------------------------------------------------------|----|---|---|----|-----|-----|
| *C. sakazakii* (27 strains)     | 50            |                                                                 | 7.2±0.34 | 7.2±0.35 | 7.2±0.35 | 7.1±0.41 | 6.9±0.51 | 6.8±0.63 |
|                                 | 55            |                                                                 | 7.2±0.34 | 7.1±0.38 | 6.6±0.50 | 3.0±0.68 | 0.4±0.80 | ND |
|                                 | 60            |                                                                 | 7.2±0.34 | 6.5±0.83 | 0.5±1.10 | ND | ND | ND |
| *C. malonaticus* (28 strains)   | 50            |                                                                 | 7.0±0.14 | 6.9±0.17 | 6.9±0.15 | 6.8±0.18 | 6.6±0.18 | 6.3±0.41 |
|                                 | 55            |                                                                 | 7.0±0.23 | 6.9±0.27 | 6.2±0.57 | 2.8±0.67 | 0.4±0.90 | ND |
|                                 | 60            |                                                                 | 7.0±0.23 | 6.4±0.49 | 0.6±1.40 | ND | ND | ND |
| *C. turicensis* (13 strains)    | 50            |                                                                 | 6.9±0.12 | 6.9±0.21 | 6.9±0.26 | 6.7±0.27 | 6.5±0.37 | 6.4±0.53 |
|                                 | 55            |                                                                 | 6.9±0.12 | 6.9±0.21 | 5.9±0.59 | 2.9±0.69 | ND | ND |
|                                 | 60            |                                                                 | 6.9±0.12 | 6.3±0.32 | 0.3±0.91 | ND | ND | ND |
| *C. dublinensis* (4 strains)    | 50            |                                                                 | 7.1±0.16 | 7.2±0.25 | 7.2±0.22 | 7.0±0.44 | 6.7±0.53 | 6.4±0.60 |
|                                 | 55            |                                                                 | 7.1±0.16 | 7.2±0.22 | 6.5±0.36 | 2.8±0.47 | 0.3±0.56 | ND |
|                                 | 60            |                                                                 | 7.1±0.16 | 6.5±0.28 | ND | ND | ND | ND |

<sup>1</sup> The bacterial counts were expressed as log of CFU/mL.

FIG. 3. Decrement curves of *Cronobacter* spp. after heating at different temperatures.
GROWTH AND HEAT RESISTANCE OF CRONOBACTER

REFERENCES

Brady, C., Cleenwerck, I., Venter, S., Coutinho, T., and De Vos, P. (2013) Taxonomic evaluation of the genus Enterobacter based on multilocus sequence analysis. Syst. Appl. Microbiol., 36, 309-319.

Fukuda, N., Arai, N., Fujiwara, A., Furukawa, S., and Ogawa, H. (2014) The effect of storage temperature on the growth of Cronobacter sakazakii in prepared infant milk. Jpn. J. Food Microbiol., 31, 204-208.

Gurtler, J. B., and Beuchat, L. R. (2007) Growth of Enterobacter sakazakii in reconstituted infant formula as affected by composition and temperature. J. Food Prot., 70, 2095-2103.

Hochel, I., Ruzickova, H., Krasny, L., and Demnerova, K. (2012) Occurrence of Cronobacter spp. in retail foods. J. Appl. Microbiol., 112, 1257-1265.

Iversen, C., Mullane, N., Cardell, B., Tall, B. D., Lehner, A., Faning, S., Stephan, R., and Joosten, H. (2008) Cronobacter gen. nov., a new genus to accommodate the biogroups of Enterobacter sakazakii, and proposal of Cronobacter sakazakii gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol., 58, 1442-1447.

Joseph, S., Centikaya, E., Drahovska, H., Levican, A., Figueras, M. J., and Forsthe, S. J., (2012) Cronobacter condiment sp. nov., isolated from spiced meat, and Cronobacter universalis sp. nov., a species designation for Cronobacter sp. genospecies 1, recovered from a leg infection, water and food ingredients. Int. J. Syst. Evol. Microbiol., 62, 1277-1283.

Nakamura, H., Yasufuku, K., Ogasawara, J., Oyama, M., Arikawa, K., and Hase, A. (2011) Survival and growth of Enterobacter sakazakii (Cronobacter spp.) in powdered infant foods. Report of the Osaka City Institute of Public Health and Environmental Science, 73, 15-22.

Nazarowec-White, M., and Farber, J. M. (1997) Incidence, survival, and growth of Enterobacter sakazakii in infant formula. J. Food Prot., 60, 226-230.

Ueda, S. (2017) Occurrence of Cronobacter spp. in dried foods, fresh vegetables and soil. Biocontrol Sci., 22, 55-59.