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# Effects of Preculturing Conditions on Lag Time and Specific Growth Rate of *Enterobacter sakazakii* in Reconstituted Powdered Infant Formula

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***Enterobacter sakazakii*** **can be present, although in low levels, in dry powdered infant formulae, and it has been linked to cases of meningitis in neonates, especially those born prematurely. In order to prevent illness, product contamination at manufacture and during preparation, as well as growth after reconstitution, must be minimized by appropriate control measures. In this publication, several determinants of the growth of *E. sakazakii* in reconstituted infant formula are reported. The following key growth parameters were determined: lag time, specific growth rate, and maximum population density. Cells were harvested at different phases of growth and spiked into powdered infant formula. After reconstitution in sterile water, *E. sakazakii* was able to grow at temperatures between 8 and 47°C. The estimated optimal growth temperature was 39.4°C, whereas the optimal specific growth rate was 2.31 $h^{-1}$. The effect of temperature on the specific growth rate was described with two secondary growth models. The resulting minimum and maximum temperatures estimated with the secondary Rosso equation were 3.6°C and 47.6°C, respectively. The estimated lag time varied from 83.3 ± 18.7 h at 10°C to 1.73 ± 0.43 h at 37°C and could be described with the hyperbolic model and reciprocal square root relation. Cells harvested at different phases of growth did not exhibit significant differences in either specific growth rate or lag time. Strains did not have different lag times, and lag times were short given that the cells had spent several (3 to 10) days in dry powdered infant formula. The growth rates and lag times at various temperatures obtained in this study may help in calculations of the period for which reconstituted infant formula can be stored at a specific temperature without detrimental impact on health.**

*Enterobacter sakazakii* is a motile, peritrichous, gram-negative rod that occasionally causes neonatal meningitis and sepsis, with mortality rates of 40 to 80% (3). The recovery of *E. sakazakii* from samples of commercially available dry powdered infant formulae has been reported (4, 8, 9). *E. sakazakii* organisms in infant formula have been associated with outbreaks of meningitis, sepsis, and necrotizing enterocolitis in premature and full-term infants, particularly those with predisposing medical conditions (17). Although the levels of *E. sakazakii* occurring in dry powdered infant formula are generally very low, reconstituted infant formula is a good medium for growth. When present in dry formula, *E. sakazakii* may grow during preparation, cooling, storage, and holding of the bottles, increasing the probability of illness. Occasional contamination of dried infant formula during manufacture is a source of the microorganism's occurrence in reconstituted product. However, as *E. sakazakii* has been detected in various other dry environments (7), contamination may also occur during reconstitution of dried infant formula in hospitals or at home.

In order to prevent illness, product contamination at manufacture and/or during preparation and growth after reconstitution must be minimized by appropriate control measures. Mathematical models are useful tools for evaluating the effectiveness of control measures. Depending on the source and the history of contaminating bacterial cells, which influence their physiological state, and the suitability of the product to sustain their growth, i.e., the product's (intrinsic) conditions and the environmental (extrinsic) conditions, microbial cells will either start to grow immediately or show a distinct phase of no apparent growth (the lag phase). In the case of *E. sakazakii* cells, reconstituted infant formulae offer rich growth environments that allow immediate proliferation provided that the cells are in a sound physiological state, that the external conditions (mainly temperature) are favorable, and that there is sufficient time for growth. Should lag times be apparent before growth, this may be a result of an injury to the *E. sakazakii* cells, from which they may gradually recover, as is evidenced by the start of cell proliferation (16). Baranyi and Roberts (1) emphasized that the lag time is a period of adjustment to a new environment, during which only intracellular conditions change. Growth models can simulate growth after reconstitution, and the effects of key intrinsic or extrinsic conditions can be determined. To develop growth models, insight into parameters describing growth of the microorganism, such as lag time and specific growth rate, is required.

This study describes the effects of a number of preculturing conditions on key growth parameters for *E. sakazakii* growing in reconstituted (with sterile water) powdered infant formula. Furthermore, the effects of temperature on specific growth rate and lag time were quantified and compared with literature values. Viable counts were used to construct growth curves that were used to derive the key growth parameters by curve fitting with the modified Gompertz equation as the primary growth model (19). During the secondary modeling step, the square

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root Ratkowsky model (12) and the secondary Rosso model (13) were fitted to the estimates of the specific growth rates at various temperatures. Likewise, the lag time data were fitted with the logarithm of the inverse of the Ratkowsky model and the hyperbolic equation (20). The resulting parameters permit useful predictions of the growth of *E. sakazakii* in reconstituted infant formula and aid in the design of effective control measures to reduce exposure of susceptible consumers in both hospital and household settings.

TABLE 1. Initial estimates of parameters for growth of *E. sakazakii* in reconstituted infant formula, based on published data

| Parameter | Initial estimate | Reference(s) |
|---|---|---|
| $T_{min}$ | 5.5°C | 9 |
| $T_{max}$ | 47°C | 3, 5 |
| $T_{opt}$ | 37°C | 5 |
| $\mu_{opt}$ | 2.5 $h^{-1}$ | 5 |
| $k$ | 3.75 | 6 |

## MATERIALS AND METHODS

**Organisms.** Four *Enterobacter sakazakii* isolates were used in this study. Strain MM9 was isolated from milk powder, and strain MC10 was isolated from a patient. Both isolates were obtained from H. L. Muytjens, University Medical Centre, St. Radboud, Nijmegen, The Netherlands. Strain 94 (S94) was isolated from a vacuum cleaner bag sample obtained from a domestic environment (7). *E. sakazakii* ATCC 29544 (3) was used as the reference strain. Stock cultures were maintained at −20°C in cryogenic vials (Greiner Bio-one GmbH, Frickenhausen, Germany) containing 0.7 ml of a stationary-phase culture suspension in brain heart infusion broth (BHI) (Becton Dickinson and Co., Le Pont de Claix, France) with 0.3 ml of 87% glycerol (Fluka-Chemica GmbH, Buchs, Switzerland). Characterization of these isolates, together with 70 other *E. sakazakii* isolates, by the pulsed-field electrophoresis technique (14) revealed large variability in molecular fingerprints. Specific groups could not be distinguished from the dendrograms (results not shown).

**Preparation of the bacterial suspension.** Strain ATCC 29544 was cultured by transferring 250 μl of the stock culture into 250 ml of BHI, followed by incubation at 37°C. Cells were incubated for 3, 6, 16, 24, and 72 h to obtain, respectively, exponential-phase, early-stationary-phase, mid-stationary-phase, stationary-phase, and late-stationary-phase cells. Strains MC10, MM9, and S94 were incubated at 37°C for 18 h to obtain mid-stationary-phase cells. In order to obtain enough cells in the lag phase, 1 ml of a stock culture of ATCC 29544 that had been maintained at −20°C was diluted in 2 ml of glycerol, whereupon the suspension was centrifuged (Hermle Z 231 M; B. Hermle GmbH & Co., Gosheim, Germany) for 10 min at 10,000 × *g*. Harvested cells were transferred into 30 ml of BHI and incubated for 1.5 h at 37°C.

In order to obtain cells for spiking of the dry infant formula, these BHI-grown cultures were centrifuged for 5 min at 20°C at 2,958 × *g* (Mistral 3000i; MSE, Leicester, United Kingdom). Cells were washed twice in 1% physiological salt solution and subsequently suspended in 30 ml of 1% physiological salt solution, for the lag-phase cells, or 250 ml of 1% physiological salt solution, resulting in a cell suspension of about $10^4$ CFU/ml for lag-phase cells and between $10^5$ and $10^7$ CFU/ml for the other growth phases.

**Spiking of the infant formula.** Infant formula was bought in local shops; the numbers of viable bacteria in the infant formula were sufficiently low to prevent them from influencing the growth of the spiked cells (data not shown). The obtained bacterial suspension was sprayed on commercial dry infant formula (1:50, wt/wt) with a perfume sprayer (designed by Gérard Brinard, DA Drogisterij, Leusden, The Netherlands). The final bacterial concentration at 3 to 4 days after spiking was $10^2$ to $10^4$ CFU/g of dry powder, and the infant formula maintained a water activity of <0.22. All growth experiments were performed within 10 days after spiking the infant formula.

**Design.** The initial estimates of the lowest temperature supporting growth ($T_{min}$), the highest temperature supporting growth ($T_{max}$), and the specific growth rate ($\mu_{opt}$) at the optimal growth temperature ($T_{opt}$), shown in Table 1, were used to predict the specific growth rates at various temperatures. The lag time at each temperature was initially estimated by the $k$ value, which is the product of the specific growth rate ($\mu_m$) and lag time ($\lambda$) and which is known to have a large variability but can be considered constant over a wide range of temperatures (18).

In order to determine appropriate sampling times and dilutions for plate counting, the number of microorganisms in each sample and at every sampling time was roughly predicted by using the exponential growth function ($N_t = N_0 e^{\mu_m \cdot t}$) and the secondary Rosso equation (equation 3), with the design values shown in Table 1. Here $N_t$ is the number of microorganisms (CFU/ml) at time $t$ (h), $N_0$ is the number of microorganisms at the time of inoculation, $\mu_m$ is the specific growth rate ($h^{-1}$), and $t$ is the time (h).

**Growth experiments.** To prepare samples for growth experiments, 10-g portions of spiked infant formula were reconstituted in 100 ml of sterilized tap water. In experiments to determine the effects of various growth phases, bottles with strain ATCC 29544 were incubated as follows (the number of bottles is given in parentheses): 10°C ($n$ = 9), 21°C ($n$ = 11), 29°C ($n$ = 14), 37°C ($n$ = 14). Mid-stationary-phase cells of strain ATCC 29544 were additionally used to assess (in duplicate) growth in reconstituted infant formula at the following temperatures: 8, 14, 38, 39, 41, 43, 45, 46, 47, 48, 49, and 50°C. Mid-stationary-phase cells of strains MM9, MC10, and S94 were incubated only at 29 and 37°C.

Growth of *E. sakazakii* was measured at various time intervals, depending on the temperature of incubation; appropriate dilutions were made in peptone saline solution (NaCl [8.5 g/liter] and neutralized Bacteriological Peptone [1g/liter]; Oxoid, Basingstoke, England). Samples were surface plated onto tryptone soy agar (Oxoid, Basingstoke, England) with a spiral plater (Eddy Jet; IUL Instruments, I.K.S., Leerdam, The Netherlands). Inoculated plates were incubated for 20 to 24 h at 37°C before manual counting.

**Data analysis.** For describing the evolution of the microbial count with time, a primary model was used. The three kinetic parameters, namely, lag time, specific growth rate, and maximum population density, were estimated by fitting with the modified Gompertz equation (equation 1):

$$\ln\left(\frac{N_t}{N_0}\right) = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\} \quad (1)$$

This resulted in estimates for the lag time ($\lambda$ [h]), the specific growth rate ($\mu_m$ [$h^{-1}$]), and the dimensionless asymptotic value ($A$) (19) at the tested temperature for each growth curve.

In order to obtain reliable estimates for the growth parameters, experimental growth curves had to meet the following requirements: (i) at least two data points should fall within the lag time, unless the lag time was shorter than 2 h; (ii) for data points within the exponential phase, there should be at least three data points over a range of 3 h and 3 logs; and (iii) three data points should fall within the stationary phase, at least 1 h apart. Growth curves that did not meet these requirements were excluded.

A Bélehrádek-type model (equation 2), also known as the (expanded) square root model of Ratkowsky (12), was used to describe the relation between the specific growth rate and the temperature. This model contains four parameters, of which two are easily interpretable, $T_{min}$ and $T_{max}$. If $T_{min} < T < T_{max}$, then

$$\mu_m(T) = (b(T - T_{min})\{1 - \exp[c(T - T_{max})]\})^2 \quad (2)$$

and if $T \leq T_{min}$ or $T \geq T_{max}$, then $\mu_m = 0$, where $T_{min}$ is the extrapolated minimum temperature (°C) at which the specific growth rate ($\mu_m$ [$h^{-1}$]) is zero, $T_{max}$ is the extrapolated maximum temperature at which $\mu_m$ is zero, and $b$ ($°C^{-1}\ h^{-0.5}$) and $c$ ($°C^{-1}$) are the so-called Ratkowsky parameters (12).

The secondary growth model of Rosso et al. (13) (equation 3) was used as well to describe the effect of temperature on growth rate. This model contains all four interpretable parameters: $\mu_{opt}$, $T_{min}$, $T_{max}$, and $T_{opt}$. If $T_{min} < T < T_{max}$, then

$$\mu_m(T) = \left(\frac{(T - T_{max})(T - T_{min})^2}{\{[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)](T_{opt} - T_{min})\}}\right) \cdot \mu_{opt} \quad (3)$$

and if $T \leq T_{min}$ or $T \geq T_{max}$, then $\mu_m = 0$, where $T_{min}$ and $T_{max}$ are defined as in equation 2, $T_{opt}$ is the temperature (°C) at which the specific growth rate $\mu_m$ ($h^{-1}$) is optimal, and $\mu_{opt}$ is the $\mu_m$ at the optimal temperature.

The logarithm of the inverse of the secondary Ratkowsky model (equation 4) and the hyperbolic equation (equation 5) were used (20) to describe the lag time/temperature relation.

$$\ln(\lambda) = \ln[(b(T - T_{min})\{1 - \exp[c(T - T_{max})]\})^{-2}] \quad (4)$$

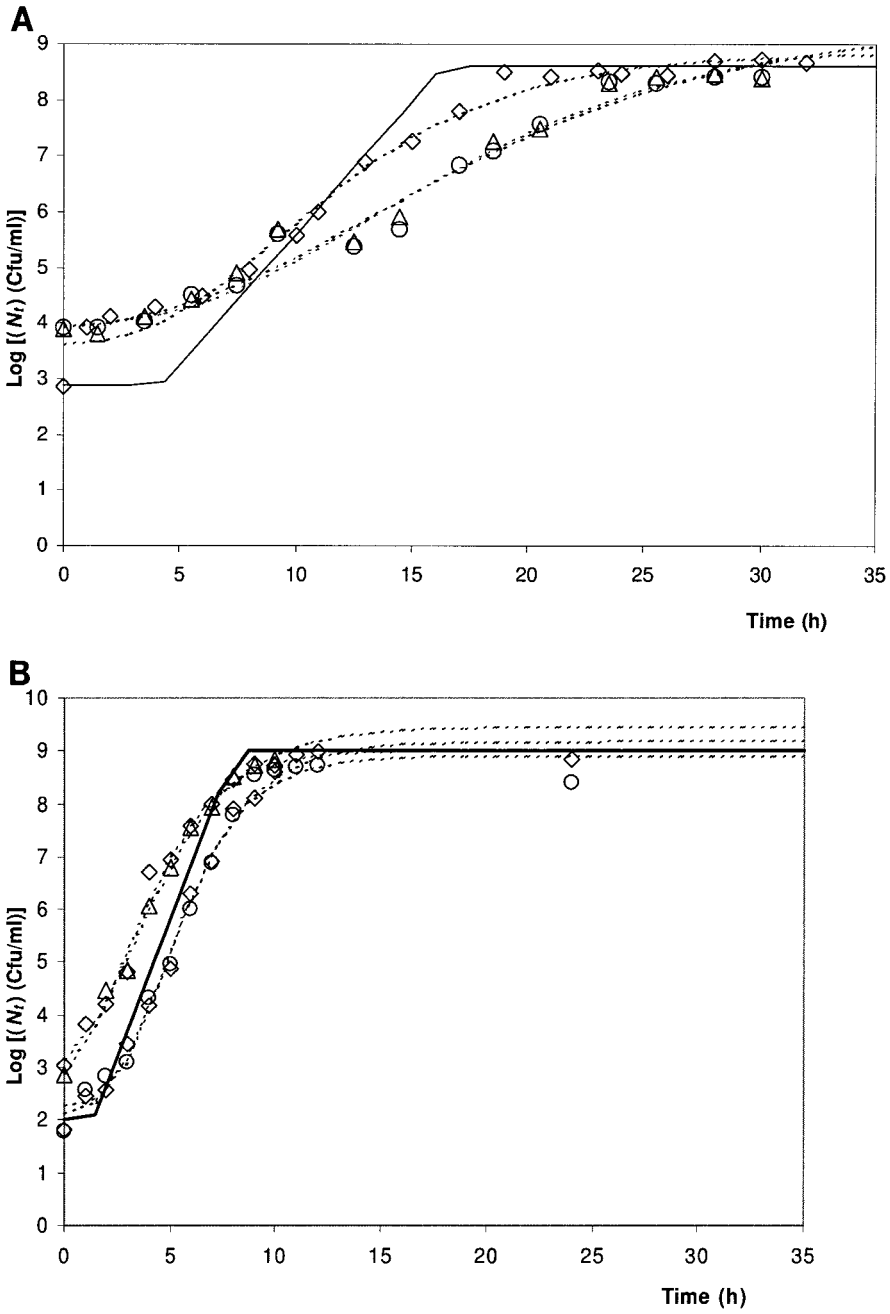


FIG. 1. (A) Predicted and measured growth curves of *E. sakazakii* ATCC 29544 at 21°C, precultured to mid-stationary phase. The different symbols indicate different replicate experiments. The solid line is the design growth curve at 21°C, calculated with the cardinal values shown in Table 1 under "initial estimate"; dotted lines represent fits of the modified Gompertz equation to each single experiment. (B) Predicted and measured growth curves of *E. sakazakii* ATCC 29544 at 37°C, precultured to mid-stationary phase. The different symbols indicate different replicate experiments. The solid line is the design growth curve at 37°C, calculated with the cardinal values shown in Table 1; dotted lines represent fits of the modified Gompertz equation to each single experiment.

where $b$ ($°C^{-1}\ h^{-0.5}$) and $c$ ($°C^{-1}$) are the so-called Ratkowsky parameters. The $T_{min}$ and $T_{max}$ values were assumed to be equal to the $T_{min}$ and $T_{max}$ of equation 3 (secondary Rosso growth model), describing the specific growth rate.

$$\ln(\lambda) = \frac{p}{(T - q)} \quad (5)$$

where $p$ is a measure of the decrease in the lag time when the temperature is increased and $q$ is the temperature at which the lag time is infinite (no growth). The $q$ value is comparable to $T_{min}$.

**Statistical analysis.** In order to determine whether preculturing conditions have a significant effect on lag times and/or specific growth rates, the data obtained with strain ATCC 29544 at 10, 21, 29, and 37°C were subjected to univariate analysis of variance. Lag time data were log transformed and specific growth rate data were square root transformed, in order to obtain homogeneity of variance. A significance level of 5% was used. All data analyses were performed with SPSS (SPSS, release 11.5, for Microsoft Windows 95/98/NT/2000; SPSS Inc., Chicago, Ill.).

Fitting was done by minimizing the residual sum of squares (RSS) with both

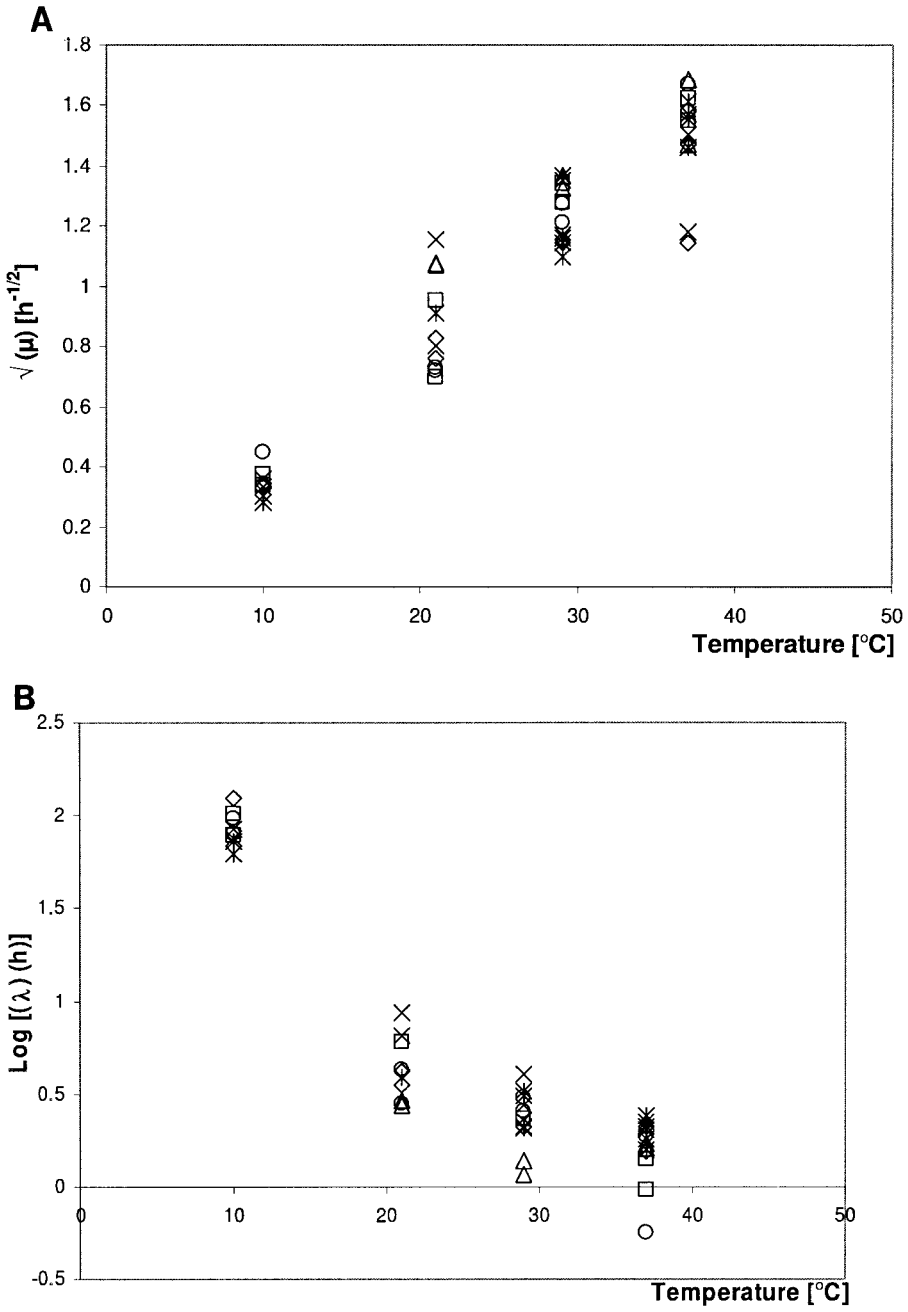


FIG. 2. (A) Square roots of specific growth rate data for various physiological growth phases of strain ATCC 29544 as a function of temperature. ×, lag-phase cells; △, exponential-phase cells; ◇, early-stationary-phase cells; ✳, mid-stationary-phase cells; ○, stationary-phase cells; □, late-stationary-phase cells. (B) Log lag times for various physiological growth states of ATCC 29544 as a function of temperature. ×, lag-phase cells; △, exponential-phase cells; ◇, early-stationary-phase cells; ✳, mid-stationary-phase cells; ○, stationary-phase cells; □, late-stationary-phase cells.

the solver function in Excel and Table Curve 2D for Windows, version 2.03, for verification.

Standard deviations were calculated with Excel and are reported as plus or minus the means.

## RESULTS

**Determination of growth parameters with precultured mid-stationary-phase ATCC 29544 cells.** In order to design the experiments optimally and to obtain growth curves meeting the specified requirements, the course of each individual growth curve of *E. sakazakii* at 10, 21, 29, and 37°C was predicted based on published growth parameters (Table 1, initial estimates). Examples of the design growth curve at 21°C and 37°C are shown in Fig. 1A and B, respectively. These figures show, furthermore, the resulting experimental count data of growth in reconstituted, contaminated infant formula. The modified Gompertz equation (equation 1) was fitted to the observed number of microorganisms in time, resulting in estimates for the lag time, spe-

TABLE 2. Statistical evaluation of univariate analyses of variance for the effects of the physiological growth phase of *E. sakazakii* strain ATCC 29544 cells on lag time, specific growth rate, and the product of both ($k$) at 10, 21, 29, and 37°C

| Parameter | Value at: | | | |
|---|---|---|---|---|
| | 10°C | 21°C | 29°C | 37°C |
| Lag time (λ) (h) | 0.092 | **0.048**[a] | 0.629 | 0.254 |
| Specific growth rate ($\mu_m$) ($h^{-1}$) | 0.461 | 0.295 | 0.314 | 0.166 |
| $k$ value ($\lambda \cdot \mu_m$) | 0.407 | 0.408 | 0.539 | 0.512 |

[a] Boldface type indicates a $P$ value of <0.05, i.e., a significant effect of physiological state on the variable.

cific growth rate, asymptote, and initial number of organisms (20). For the growth curves at 10°C and 29°C, comparable results were obtained (data not shown). Variations in the initial numbers of microorganisms at time zero may have been due to a gradual decline in cell numbers in the dry infant formula.

**Effects of physiological growth phase on $\mu_m$ and λ.** Six different precultures of the reference strain, ATCC 29544, were prepared to yield a variety of physiological growth phases at the moment of spiking of the powdered infant formula. After reconstitution with sterile tap water, all six types were incubated with various replications at 10, 21, 29, and 37°C to represent a temperature range relevant for reconstituted infant formulae. Growth was observed in all cases and at every temperature. Temperature had a marked effect on both the specific growth rate ($\mu_m$) and the lag phase (λ) (Fig. 2A and B). With increasing temperature, $\mu_m$ strongly increased. Values for specific growth rates varied from $0.12 \pm 0.04\ h^{-1}$ at 10°C to $2.29 \pm 0.45\ h^{-1}$ at 37°C, and no apparent effect of the physiological state was found. In Fig. 2B, it is shown that the lag time decreased with increasing temperature and that there was also no apparent effect of the various physiological growth phases on the lag time.

Estimates of specific growth rates and lag times for the various preculturing conditions (lag phase, exponential phase, early-stationary phase, mid-stationary phase, and late-stationary phase) of strain ATCC 29544, as shown in Fig. 2A and B, were analyzed by univariate analysis of variance. As can be seen in Table 2, this resulted in $P$ values of >0.05, except for the lag time at 21°C ($P = 0.048$). However, this value can be considered borderline significance. This test corroborates the visual observation (Fig. 2A and B) that cell history had no significant effect on either the specific growth rate or the lag time during subsequent cultivation in reconstituted infant formula. Furthermore, statistical analysis showed that the $k$ value (the product of $\lambda \cdot \mu_m$) was also not significantly influenced by cell history (Table 2).

**Effects of strain variability on $\mu_m$ and λ.** The effects of strain variability on growth parameters were studied by preculturing three other strains, MC10, MM9, and S94, to mid-stationary phase at 29 and 37°C. It is shown in Fig. 3 and 4 that neither the lag times nor the maximum specific growth rates were significantly different for strains of different origin.

**Estimations of growth parameters.** Since statistical analysis showed that the physiological growth phases of strain ATCC 29544 at 10, 21, 29, and 37°C did not significantly influence the specific growth rates and lag times, further experiments were performed over a wide range of temperatures, from 8 to 50°C, with mid-stationary-phase precultured cells of strain ATCC 29544 only. Although growth was observed up to 47°C, no

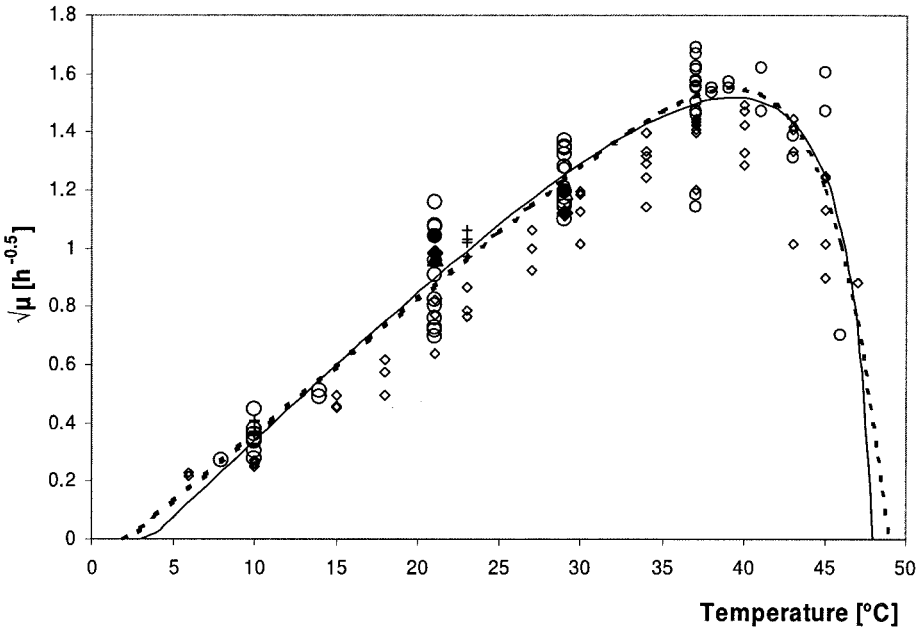


FIG. 3. Square roots of measured and fitted specific growth rates as a function of temperature. Shown are growth rates of ATCC 29544 precultures to various growth phases, as estimated by the fit of the modified Gompertz model to each individual growth curve ○, and growth rates for mid-stationary-phase cells of MM9 ●, S94 ◆, and MC10 ▲. Other symbols: +, growth rates published by Nazarowec-White and Farber (9); ◇: growth rates published by Iversen et al. (5); dashed line, fit by the secondary growth model of Ratkowsky; dotted line, fit by the secondary growth model of Rosso (fitted to square root transformed data of the present study only).

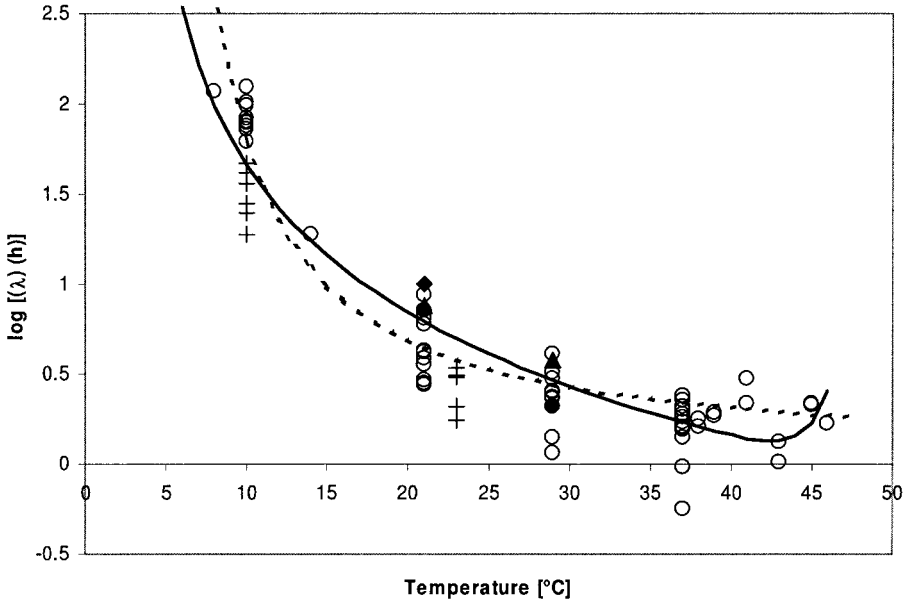


FIG. 4. Log lag times as a function of temperature. ○, ATCC 29544 precultures to various growth phases. Also shown are data for mid-stationary-phase cells of MM9 ●, S94 ◆, and MC10 ▲; lag times at 10 and 23°C as published by Nazarowec-White and Farber (9) (+); and logarithmic transformed lag time data from the present study, modeled with the hyperbolic model (dashed line) and the reciprocal Ratkowsky model (solid line).

reliable estimates of the specific growth rate and lag time could be derived from the models at that temperature, as the growth curves did not meet the requirements stated in Materials and Methods.

All of the estimated specific growth rates were combined and modeled with equations 2 and 3, as shown in Fig. 3, where the square root of the specific growth rate ($\sqrt{\mu_m}$) is plotted as a function of temperature. The secondary growth parameters derived from optimal fits are shown in Tables 3 and 4. From Fig. 3 it is apparent that the differences between the fits of these models are smaller than the experimental variability. The RSS for the secondary Rosso model (equation 3) was 1.31, and the RSS for the square root Ratkowsky model (equation 2) was 1.28. Although its RSS was slightly higher, the secondary Rosso model was used for further evaluation because it consists of four parameters that all have biological meaning and can be interpreted as such. Transforming the square root $\mu_m$ data from Fig. 3 back to $\mu_m$, it can be seen that the specific growth rate of *E. sakazakii* varied from 0.115 $h^{-1}$ ($\sqrt{\mu_m}$ = 0.339 $h^{-0.5}$) at 10°C to 1.113 $h^{-1}$ ($\sqrt{\mu_m}$ = 1.063 $h^{-0.5}$) at 46°C and 2.242 $h^{-1}$ ($\sqrt{\mu_m}$ = 1.498 $h^{-0.5}$) at 37°C. For comparison, the experimental specific growth data measured by Iversen et al. (5) and Nazarowec-White and Farber (9) have been transformed and included in Fig. 3.

The estimated lag times resulting from the fit by the modified Gompertz model were plotted against temperature and are presented in Fig. 4. The longest lag time observed was 83.3 ± 18.7 h, at temperatures around 10°C. The temperature at which the lag time was minimal was between 37 and 39°C, with an estimated minimal lag time of 1.73 ± 0.44 h.

The effect of temperature on lag time was described by fitting the reciprocal square root relation (equation 4) and the hyperbolic model (equation 5) to the logarithmic transformation of the data. It is assumed that the $T_{min}$ and $T_{max}$ values are equal to the $T_{min}$ and $T_{max}$ values of equation 3, describing the specific growth rate. Parameters estimated with both models are given in Table 5. The logarithmic transformed lag times, as fitted by the reciprocal square root relation and the hyperbolic model, are represented in Fig. 4. From this graph it can be concluded that both models fit the data reasonably well. The RSS for the hyperbolic model was 2.11, and the RSS for the reciprocal square root relation was 2.29. Figure 5

TABLE 3. Parameter values for the effects of temperature on the specific growth rate ($\mu_m$) of *E. sakazakii* in reconstituted infant formula resulting from fits by the Ratkowsky secondary growth model (equation 2)

| Parameter | Estimate | 95% confidence interval | Dimension |
|---|---|---|---|
| $T_{min}$ | 2.19 | −0.26–4.64 | °C |
| $T_{max}$ | 48.9 | 47.9–49.9 | °C |
| $b$ | 0.047 | 0.0407–0.0521 | $°C^{-1}\ h^{-0.5}$ |
| $c$ | 0.239 | 0.144–0.335 | $°C^{-1}$ |
| RSS | 1.28 | | |

TABLE 4. Parameter values for the effects of temperature on the specific growth rate ($\mu_m$) of *E. sakazakii* in reconstituted infant formula resulting from fits by the Rosso secondary growth model (equation 3)

| Parameter | Estimate | 95% confidence interval | Dimension |
|---|---|---|---|
| $T_{min}$ | 3.60 | 1.42–5.79 | °C |
| $T_{max}$ | 47.6 | 47.1–48.2 | °C |
| $T_{opt}$ | 39.4 | 38.1–40.7 | °C |
| $\mu_{opt}$ | 2.31 | 2.13–2.48 | $h^{-1}$ |
| RSS | 1.31 | | |

TABLE 5. Parameter values for the effects of temperature on the lag time (λ) of *E. sakazakii* in reconstituted infant formula

| Model | Parameter | Estimate | 95% confidence interval |
|---|---|---|---|
| Inverse Ratkowsky (equation 4) | $T_{min}$ | 3.60 | —[a] |
| | $T_{max}$ | 47.6 | — |
| | $b$ | 0.023 | 0.021–0.024 |
| | $c$ | 0.645 | 0.275–1.176 |
| | RSS | 2.29 | |
| Hyperbolic (equation 5) | $p$ | 25.8 | 22.3–29.3 |
| | $q$ | 3.75 | 2.88–4.62 |
| | RSS | 2.11 | |

[a] —, fixed (from Table 4) by using the values estimated with the secondary Rosso model for growth (equation 3).

shows the dimensionless parameter $k$, the product of the lag time and the specific growth rate ($\mu_m \cdot \lambda$), as a function of temperature. The $k$ value between 8 and 47°C was 5.08 ± 3.37. At temperatures ranging from 20 to 46°C, values for $k$ were between 0.82 and 11.6, with an average of 4.05 ± 1.92. Below 20°C, it increased to an average of 10.06 ± 4.49. Use of a $k$ value of 5.08 to predict the lag time at any temperature and equation 3 to determine the specific growth rate resulted in an RSS of 16.7. Based on the RSS values for the models describing the lag time, it can be concluded that the hyperbolic model and reciprocal square root model best convey the experimental lag times. The reciprocal square root model is recommended, since it has the ability to increase the lag time at higher temperatures and it contains more interpretable parameters.

The maximum numbers of cells of strain ATCC 29544 reached at the various incubation temperatures between 8 and 46°C varied between approximately $10^7$ and $10^9$ CFU/ml, with an average of $10^{8.2}$ CFU/ml (Fig. 6). At tested temperatures above 45°C, the maximum number of cells reached was $\pm 10^6$ CFU/ml. The initial inoculum level, which varied between $\pm 10^2$ and $10^5$ CFU/ml, did not seem to affect the maximum population density.

## DISCUSSION

*E. sakazakii* may contaminate infant formulae either during production or during bottle preparation. In factory environments, *E. sakazakii* may grow in wet spots and survive in dust containing residues of infant formulae and contaminate the product after the drying process. In hospital and household kitchens, infant formulae may likewise be contaminated with dry or wet residues by utensils and via environmental vectors. In all situations, the physiological state of the contaminating cells is not known. However, it is known that the duration of the lag time may depend not only on the growth environment but also on the previous history of the cells (11, 15). It was envisaged that the time of preculturing, i.e., the physiological growth phase of the inoculum, could have an effect on the lag time but probably not on the specific growth rate (15). In our study, based on visual observation of growth curves and on statistical analysis of growth data, no effect on either parameter was found. A possible explanation may be that the cells were spiked into dry powdered infant formula prior to the growth experiments and that their metabolic activities had changed in all cases to comparable levels. The period (3 to 10 days) for which the cells were in the powdered infant formulae before a growth experiment was carried out was found to not influence the growth parameters. Cells that had been in powdered infant formula for up to 4 weeks did not show a longer lag phase either (data not shown). Another explanation for the lack of measurable differences in resuscitation times may be the rich growth medium (reconstituted powdered infant formula). In

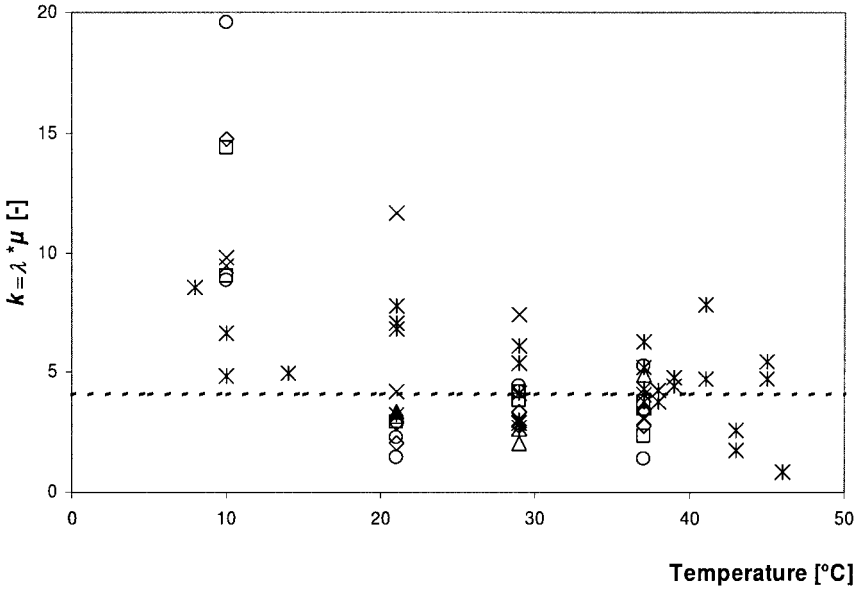


FIG. 5. Parameter $k$ (the product of λ and $\mu_m$) as a function of temperature for each growth experiment with strain ATCC 29544 precultured to various growth phases and grown at temperatures from 8 to 47°C. ×, lag-phase cells; △, exponential-phase cells; ◇, early-stationary-phase cells; ✳, mid-stationary-phase cells; ○, stationary-phase cells; □, late-stationary-phase cells. The dotted line represents the average value for the data from 20 to 46°C.

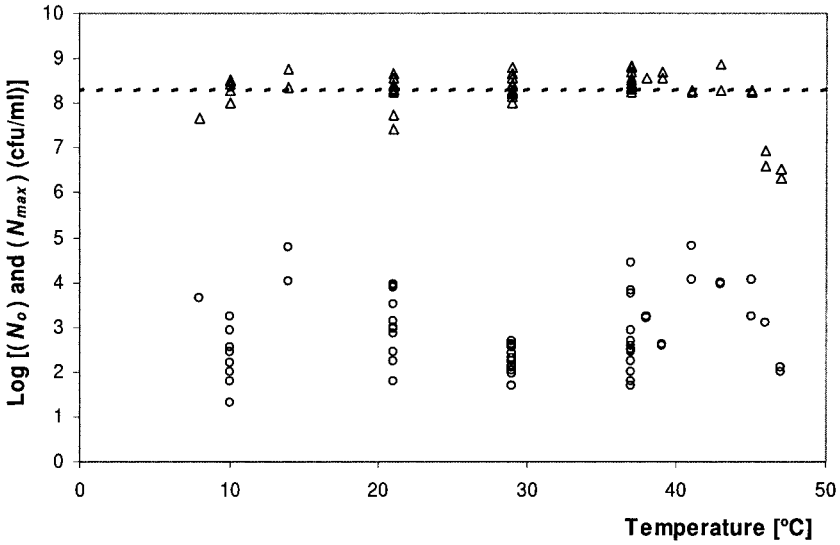


FIG. 6. Initial inoculum of strain ATCC 29544 precultured to various physiological states and maximum population densities at various temperatures. △, maximum population density; ○, initial number of cells. The dotted line represents the average value for the maximum population density data from 8 to 46°C.

this medium, *E. sakazakii* cells had a rather short lag time, 1.71 ± 0.50 h at 37°C, and small differences in lag phase may not have become apparent.

Our specific growth rate data were compared to specific growth rates published by Nazarowec-White and Farber (9) and Iversen et al. (5), as shown in Fig. 3. In our study, the specific growth rates of *E. sakazakii* cells spiked into dry infant formula were comparable to those of Nazarowec-White and Farber (9), who reported similar specific growth rates. The specific growth rates reported by Iversen et al. (5) were consistently over 10% lower than ours. One cause for the difference with the latter study may be differences in growth conditions: in our experiments, spiked dry powdered infant formula samples reconstituted with sterile tap water were used as growth medium, whereas Iversen et al. measured growth in other media by inoculating them with an overnight tryptic soy broth culture. Another difference is the use by Iversen et al. of the rapid automated bacterial impedance technique, which measures growth only at much higher levels ($>10^6$ CFU/ml) than the plate count method used in our study (15). In the present study, the plate count technique was used and growth was measured in a more representative range and over multiple logs of bacterial counts; thus, the estimates for lag time and specific growth rate can be considered more accurate (2).

The experimental design, based on initial estimates of specific growth rate and lag time, allowed prediction of growth with enough accuracy to determine the sampling times and dilutions appropriate for measuring growth curves, such that our quality requirements were met for 95% of the experimental growth curves. The modified Gompertz model (equation 1), the secondary Rosso model (equation 3), and the reciprocal square root model (equation 4) successfully estimated the lag times and specific growth rates for the whole growth temperature range (Tables 4 and 5). The theoretical minimal and maximal growth temperatures, as fitted with both secondary growth models, were 2.19°C and 48.9°C (equation 2) and 3.60°C and 47.6°C (equation 3), respectively. In practice, however, growth of *E. sakazakii* strains has been observed between 5.5°C and 47°C (3).

The fact that small numbers of *E. sakazakii* cells have occurred in dry infant formulae (4, 8, 9), in combination with the relatively short lag times and high specific growth rates found in this study, underscores the need for careful preparation and use of dry infant formulae. A study by Pagotto et al., however, showed that $10^5$ CFU/ml of certain *E. sakazakii* strains could be lethal to suckling mice after ingestion, though not all strains appeared to be pathogenic (10). As there is a lack of information about virulence factors, not all *E. sakazakii* strains necessarily need to be regarded as potential pathogens. Nevertheless, the results described in this paper (Tables 4 and 5) permit predictions of the growth of *E. sakazakii* in reconstituted infant formulae under conditions that closely mimic conditions in the actual food product in both hospital and household settings, and they will be useful in designing effective control measures.

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