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Statistical Approach for Comparison of the Growth Rates of Five Strains of *Staphylococcus aureus*

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The interaction of temperature (10, 14, 25, 31, and 37°C), pH (pH 5, 5.6, 6.5, 7.4, and 8), and NaCl (0, 2, 5, 8, and 10%) in a laboratory medium affects the specific growth rate of *Staphylococcus aureus*. From growth curves obtained by the turbidimetric technique, a nonlinear model in which the specific growth rate (μ) is fitted directly, without data transformation and with the residual error variations taken into account, is proposed. This model correctly fits experimental data and gives more biological information than the quadratic polynomial model. Moreover, the comparison of five strains of *S. aureus* was performed by a principal-component analysis in which the specific growth rate was the identifying characteristic for *S. aureus* strains. The results obtained from model coefficient comparison among the five strains and from multivariate data analysis allow the same classification of strains to be performed. Two of them have similar behaviors during food spoilage, two others could be distinguished by their capacity to grow at a low temperature, whereas the last one was markedly different from the others.

Staphylococcus aureus is recognized as a cause of food poisoning which occurs after an initial contamination of food with a toxigenic strain. The growth may occur in the absence of enterotoxin synthesis (17) and could have a role in the pathogenicity of some other staphylococcal diseases.

In order to quantify the growth of *S. aureus* during storage at low temperature and to include the influence of pH and NaCl concentration on the shelf life of a food product, we have chosen a predictive microbiological approach. This method uses mathematical equations to estimate the specific growth rate (μ) as affected by storage conditions (3). Among the various models proposed, the response surface methodology technique is widely employed in food microbiology (4, 7). This method allows the estimation of μ by modelling growth curves and leads to the establishment of a linear model which describes log μ as a function of temperature, pH, and water activity. Recently, Sutherland et al. (15) proposed a polynomial model to predict the growth of *S. aureus*, but they noted that there was an important lack of fit between the published results and those predicted by the model. In order to predict *S. aureus* food spoilage, Broughall and Brown (2) used the nonlinear Arrhenius equation with constants describing the enthalpy of activation for microbial growth and low-temperature inactivation, which are linear functions of pH and water activity. Moreover, a nonlinear model was developed (20) and applied to predict μ when several factors act in combination for several microorganisms, but not for *S. aureus*. Basing our work upon this approach, we propose a nonlinear model in this paper. The contribution of this model in comparison with that of a polynomial function is presented.

Furthermore, the predictive microbiological technique has been employed, up to now, to describe the behavior of one strain—or a mixture of strains—as a function of environmental factors. It seemed interesting to attempt to generalize its use to the comparative study of several strains. This method allows the contamination of a specific food product by various microorganisms to be appreciated. In order to test whether predictive microbiological models were a suitable statistical tool to carry out comparisons, results obtained with five different strains of *S. aureus* were reported and compared with those obtained by a multivariate data analysis. This method allows strains to be classified according to their behaviors during food spoilage. Cluster analysis is a statistical method widely employed in taxonomic study, and its contribution to predictive microbiology is discussed.

MATERIALS AND METHODS

Organism, media, and culture conditions. Five enterotoxigenic strains of *S. aureus*, A, B, C, D, and E, were obtained from foods. Culture stocks were maintained on nutrient agar at 4°C and then subcultured on Trypticase soy agar (TSA) at 37°C on three successive days. The culture media were based upon Trypticase soy broth (TSB) supplemented with the appropriate level of NaCl and were pH adjusted with NaOH or lactic acid, transferred in 100-ml portions into 125-ml Erlenmeyer flasks, and autoclaved at 121°C for 15 min. Serial dilutions in salt-tryptone were carried out to obtain an inoculum concentration of 10^7 CFU · ml^{-1}, and the experiments were performed with a multiwell analyzer, Bioscreen C (Labsystems, Helsinki, Finland), with 400-μl volumes of medium in each well, at a 620-nm absorbance.

Firstly, bacterial growth curves were fitted by a sigmoidal growth model (modified from the Gompertz model) with three parameters (19):

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

where A is the asymptotic level of $\ln\left(\frac{N}{N_0}\right)$, N is cell density, μ is the maximum specific growth rate ($hour^{-1}$), λ is the lag phase duration (hours), t is time (hours), and e is exp(1).

Experimental design. Experiments were designed to assess the effects of temperature, initial pH, and salt concentration on *S. aureus* growth. Levels of factors and nature of salt were chosen by following processes widely used in the food industry. A factorial design was implemented with five levels of temperature (10, 14, 25, 31, and 37°C), five levels of pH (pH 5, 5.6, 6.5, 7.4, and 8), and five levels of NaCl concentration (0, 2, 5, 8, and 10% [wt/vol]). Therefore, 625 experimental growth kinetics and toxin assays were studied.

Statistical analyses. Statistical analyses (nonlinear and linear regression, anal-

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TABLE 1. Coefficients of linear regression

Strain	a_0	a_1	a_2	a_3	a_{33}	a_{12}	a_{13}	a_{23}
A	8.05	−5,901	−0.86	4.67	−0.19	272	−527	−0.013
B	30.16	−12,303	−0.38	2.69	−0.17	135	−17	−0.016
C	8.91	−5,454	−0.014	4.84	−0.15	17.8	−771	−0.015
D	−0.36	−2,331	0.37	5.17	−0.14	−107	−946	−0.006
E	1.8	−5,567	0.35	2.02	0.01	−105	−555	−0.007

ysis of variance, and cluster analysis) were computed by using Splus (AT&T Bell Laboratories, Murray Hill, N.J.) and SAS (SAS Institute Inc., Cary, N.C.) software. For the nonlinear regression, the parameters of the regression and variance functions were estimated by the maximum-likelihood method. To test significative term and to select the accurate submodel, a χ^2 test was performed. The Gauss-Marquardt algorithm was used in the numerical process (9). For the multivariate data analysis, the first stage was the reduction of the data by principal-component analysis by keeping only the principal component whose eigenvalues accounted for more than 1% of the total variance. Hierarchical cluster analysis was employed to produce a dendrogram by using average linkage clustering.

RESULTS

Modelling growth rate. (i) Linear model. Response surface methodology is widely employed to predict growth rate as a function of several factors (3, 7). In this case, linear, quadratic, and interactive effects of factors are tested in a general linear regression model. First, we decided to describe the effects of the three factors in a general model which takes interactive effects of the variables into account. Response surface methodology was employed with the factors $\frac{1}{T}$ (inverse of absolute temperature), pH, and NaCl concentration. Only significant terms (t test, $P < 0.05$) were written in the final equation. The equation of specific growth rate is expressed as follows:

$$\ln \mu_i = \ln \hat{\mu}_i + \varepsilon_i \quad (2)$$

with

$$\ln \hat{\mu}_i = a_0 + a_1 \cdot \frac{1}{T} + a_2 \cdot \mathrm{NaCl} + a_3 \cdot \mathrm{pH} + a_{33} \cdot \mathrm{pH}^2 + a_{12} \cdot \frac{1}{T} \cdot \mathrm{NaCl} + a_{13} \cdot \frac{1}{T} \cdot \mathrm{pH} + a_{23} \cdot \mathrm{pH} \cdot \mathrm{NaCl}$$

and

$$\varepsilon_i \sim N\left(0, \sigma_i^2\right)$$

where $\ln \mu_i$ is the observed value of logarithmic specific growth rate, $\ln \hat{\mu}_i$ is the predicted value, ε_i is the residual error, and σ_i is the standard error—assumed as constant—for the ith observation.

The analysis of variance indicated that the model was significant ($P < 0.05$) and had an r^2 value of 0.90. The coefficients of the regression for the five strains are given in Table 1. This growth model equation with eight estimated parameters is similar to the Davey equation (6), but here, salt interactions with temperature and pH are taken into account. It has previously been applied to vegetable spoilage by a *Pseudomonas* sp. (10) and has allowed an accurate fit to be obtained. In order to compare the fitness of this model with that of the nonlinear model, we calculated the sum of squares of the residual errors ($\mathrm{SCE_{lin}}$) as follows:

$$\mathrm{SCE_{lin}} = \Sigma(\mu - \hat{\mu}_{\mathrm{lin}})^2 \quad (3)$$

For the linear model, $\mathrm{SCE_{lin}}$ was 9.47. In Fig. 1A, predicted values are plotted against observed values.

(ii) Nonlinear model. The Ratkowsky equation is often proposed to describe the relationship between growth rate and temperature (1). It can be employed with a large range of temperatures and is as follows:

$$\mu_T = \left[b \cdot (T - T_{\min}) \cdot \{1 - \exp[c \cdot (T - T_{\max})]\}\right]^2 \quad (4)$$

where T is the actual temperature, $T_{\min}$ and $T_{\max}$ are the minimal and maximal temperatures of growth for the microorganism, respectively, and b and c are the Ratkowsky parameters.

In order to introduce the combined effect of water activity, pH, and temperature, Zwietering et al. (20) proposed use of a generalized Ratkowsky model in which the pH effect is written as the temperature effect:

$$\mu_{\mathrm{pH}} = \left[b \cdot (\mathrm{pH} - \mathrm{pH_{min}}) \cdot \{1 - \exp[c \cdot (\mathrm{pH} - \mathrm{pH_{max}})]\}\right]^2 \quad (5)$$

For the water activity (a_w), a linear relationship is assumed:

$$\mu_{a_w} = b \cdot (a_w - a_{w\min}) \quad (6)$$

Therefore, Zwietering et al. (20) calculate the general specific growth rate μ as follows:

$$\mu = \mu_T \cdot \mu_{\mathrm{pH}} \cdot \mu_{a_w} \quad (7)$$

Moreover, they decided to introduce the optimum conditions of growth and to solve the nonlinear equation system with equations 4 to 6.

In our work, we attempted to predict the behavior of *S. aureus* within the scope of our study. The effect of NaCl was not found to be linear; therefore, we decided to use the same mathematical expression for NaCl as for pH and temperature. Only significant terms (χ^2 test, $P < 0.05$) were written in the final equation. With this procedure, the final nonlinear model is written as follows:

$$\mu_i = \hat{\mu}_i + \varepsilon_i \quad (8)$$

with

$$\hat{\mu}_i = \left[b_0 \cdot (T_i - T_{\min}) \cdot (\mathrm{pH}_i - \mathrm{pH_{min}}) \cdot \{1 - \exp[b_1 \cdot (\mathrm{pH}_i - \mathrm{pH_{max}})]\} \cdot (\mathrm{NaCl_{min}} - \mathrm{NaCl}_i) \cdot \{1 - \exp[b_2 \cdot (\mathrm{NaCl_{max}} - \mathrm{NaCl}_i)]\}\right]^2$$

and

$$\varepsilon_i \rightarrow N\left(0, \sigma_i^2\right)$$

$$\hat{\sigma}_i^2 = h_0 + \hat{\mu}_i^h i$$

where μ_i is the specific growth rate, ε_i is the residual error, and σ_i is the standard error for the ith observation. It is assumed that the residual error leads asymptotically to a normal distri-

TABLE 2. Coefficients of nonlinear regression of the general model

Strain	T_{min}	pH_{min}	pH_{max}	$NaCl_{min}$	$NaCl_{max}$	b_0	b_1	b_2	h_0	h_1
A	5.46	2.8	9.8	26.4	−5	0.000449	0.59	0.34	0.4	1.2
B	9.7	3.0	9.8	26.0	−5	0.000743	0.37	0.28	0.4	1.6
C	9.9	2.8	9.8	30.1	−5	0.00052	0.39	0.38	0.4	0.7
D	7.1	3.3	9.8	33.0	−5	0.00077	0.21	0.29	0.4	1.7
E	5.6	2.8	9.8	36.7	−5	0.00033	0.50	0.42	0.4	0.5

bution, with a heterogeneous variance. This hypothesis allows the experimental error increase with the large specific growth rate value to be taken into account. This method avoids the introduction of a transformation of the response (for instance, $\sqrt{\mu}$, ln μ) before estimation of parameter values.

The estimated values of the ten parameters–eight for the regression function and two for the variance model—for the five strains are given in Table 2. The pH_{max} value was considered as being equal for all the strains. It was fixed at 9.8, referring to the upper limit of pH for growth (17). The maxi-

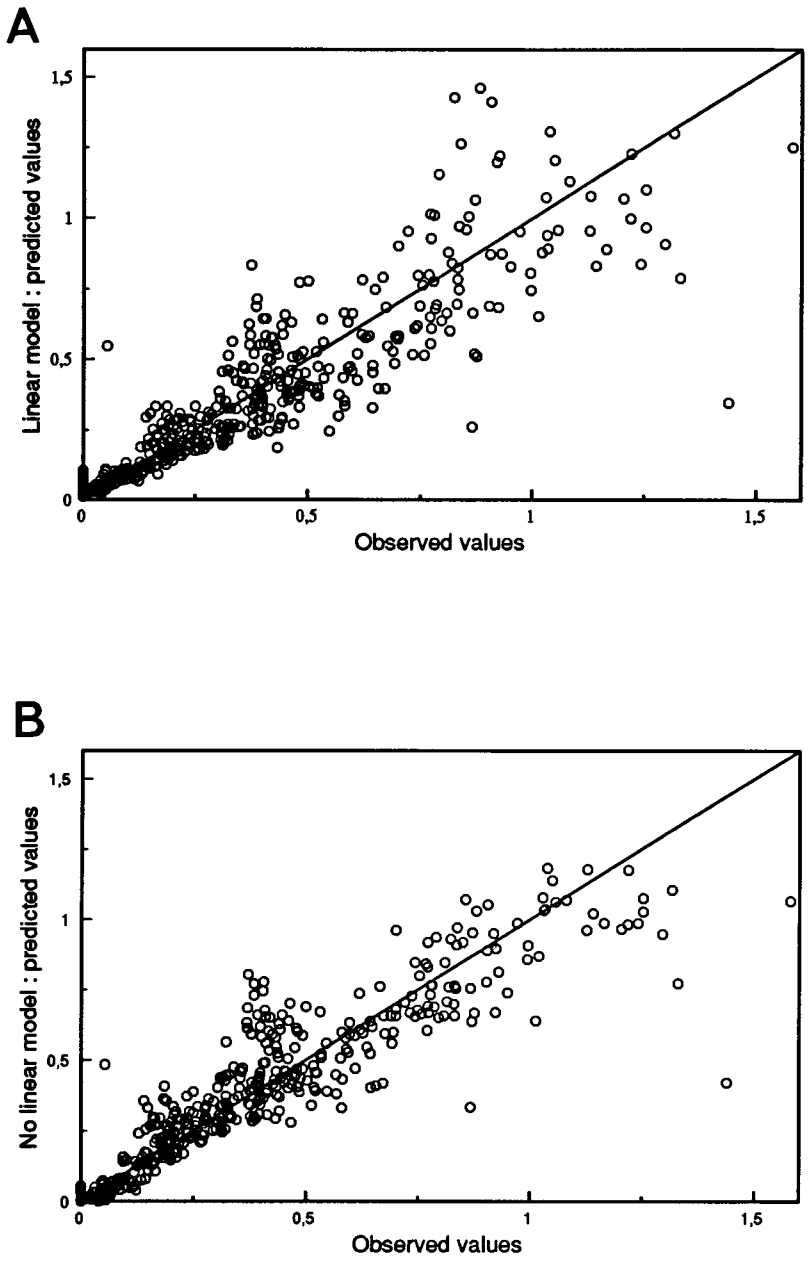

FIG. 1. Predicted and observed specific growth rates. (A) Linear model; (B) nonlinear model.

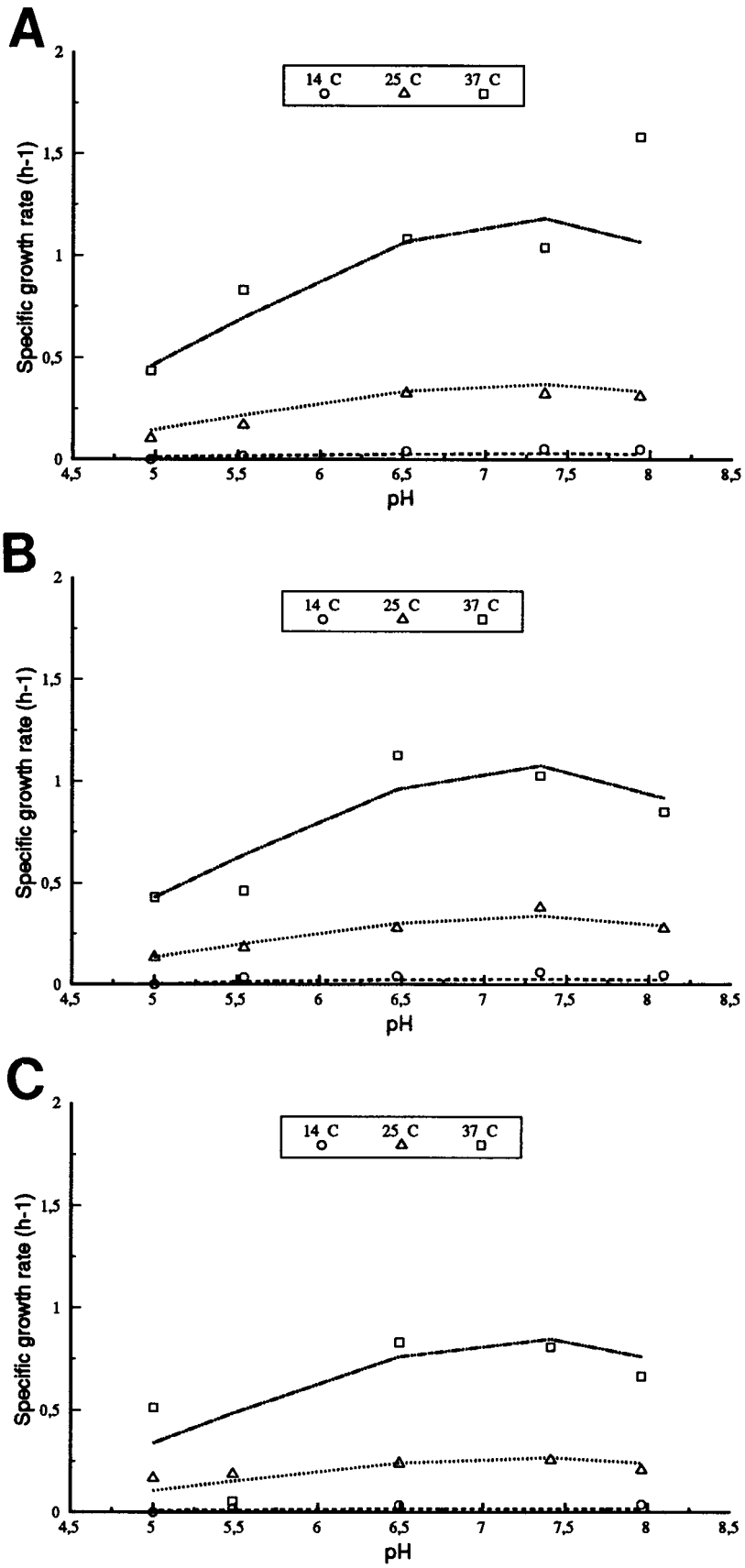

FIG. 2. Predicted and observed specific growth rates as functions of pH and temperature for strain B. (A) 2% NaCl; (B) 5% NaCl; (C) 8% NaCl.

TABLE 3. Coefficients of nonlinear regression of the submodel

Strain	T_{min}	pH_{min}	pH_{max}	$NaCl_{min}$	$NaCl_{max}$	b_0	b_1	b_2	h_0	h_1
A	5.46	2.8	9.8	26.4	−5	0.000449	0.59	0.34	0.4	1.2
B	8.6	3.2	9.8	29.1	−5	0.00074	0.29	0.29	0.4	1.7
C	8.7	2.8	9.8	33.3	−5	0.00040	0.45	0.40	0.4	0.6
D	8.6	3.2	9.8	29.1	−5	0.00074	0.29	0.29	0.4	1.7
E	6.7	2.8	9.8	33.3	−5	0.00040	0.45	0.40	0.4	0.6

mum salt tolerance, $NaCl_{max} = -5$, was chosen in order to obtain a model accurately describing the data without deviation. This parameter did not have a biological interpretation. It was only used in equation 7 to take into account the capacity of *S. aureus* to grow in a salted culture medium, NaCl not having a strictly linear inhibitory effect on growth.

In Fig. 1B, predicted values are plotted as functions of observed values. The sum of squares for the nonlinear model (SCE_{nl}) is 6.9. Modelling the specific growth rate with a nonlinear-model equation system allows a good estimation of the experimental data to be obtained. In Fig. 2, the results of modelling are plotted versus pH for three experimental conditions of temperature and NaCl concentration for strain B.

Comparison of the five strains. In order to compare the behaviors of the five strains during food storage, a comparison of the coefficient estimation values was performed. As nonlinear regression leads to correct fitting of the data, it was chosen as the reference for comparison. The maximum-likelihood method with a χ^2 test was used to find a restricted model including equality between parameters for at least two strains. The smallest submodel, in which the strain parameters are equal, was rejected. The five strains studied did not have the same behavior according to experimental conditions. After several trials, a restricted model was elaborated. The growth rate of strains B and D was predicted by equation 7 with the same values for all the parameters, while for strains C and E, the hypothesis of equality of the parameters was assumed, except for the T_{min} parameter. The specific growth rate for strain A was described by equation 7 with various parameters. The new values of the parameters are given in Table 3. With these model parameters, the sum of squares was 7.1.

Furthermore, the comparison of strains was performed by multivariate data analysis. For the five strains of *S. aureus*, the value of μ at each combination of temperature, pH, and NaCl level was reported and used as the identification characteristic. The classification of the five strains is represented by a dendrogram (Fig. 3). Two cluster groups were obtained at a correlation level of 59%; one contained strains B and D, and the other contained strains C and E. At a correlation level of 65%, strains C and E formed a small subcluster. Similarly, strains B and D were grouped into subclusters at 93% correlation. Finally, strain A was different from the other strains.

DISCUSSION

Nonlinear model. In Fig. 4, the histogram of residual errors is plotted for the nonlinear model and for the linear model. The fact that the values predicted from the nonlinear model closely agreed with the observed values is represented by the residual error: the better the fitness, the smaller the residual error. Since the number of estimated parameters was the same, the sum of squares of residual errors could be considered as a criterion of comparison. The latter was smaller for the nonlinear model—even for the submodel—than for the linear one. Moreover, the nonlinear regression allows μ to be directly taken into account as the response instead of $\sqrt{\mu}$ or $\ln \mu$. In addition, the effects of temperature, pH, and salt are described by equations in which each parameter keeps a biological interpretation (with the exception of the negative value for the low boundary of the salt tolerance term). Zwietering et al. (20) have already used a nonlinear model in order to establish a decision support system with an application to milk spoilage, but in their case, a linear mathematical expression is used to include the negative effect of water activity (equation 5). This simplified term can be employed only with microorganisms which are not salt tolerant at all. With *S. aureus*, equation 5 was no longer accurate.

Comparison of strains. The results obtained from model coefficient comparison and from multivariate data analysis allow the same remarks about strain behavior under storage conditions to be deduced. These two methods could be employed in predictive microbiology in order to complete the description of microorganism growth in food. The strong similarity (93%) between strains B and D indicates that these two strains would adopt the same behavior during food spoilage. On the other hand, the fairly close similarity of strains C and E (65%) is due to pH and NaCl having the same effects on these two strains. The difference is for the temperature effect: temperature has a greater inhibitory effect on strain C than on strain E. The two groups, strains B and D and strains C and E, presented a difference because strains B and D grow faster ($b_0 = 7.4 \cdot 10^{-4}$) than strains C and E ($b_0 = 4.0 \cdot 10^{-4}$). Finally, strain A is easily distinguishable from the others. The differentiation key is salt tolerance. Strain A is not able to grow as well as the other strains in a salted culture medium.

Food spoilage by *S. aureus*. The model equation parameter values are in agreement with the physiological characteristics

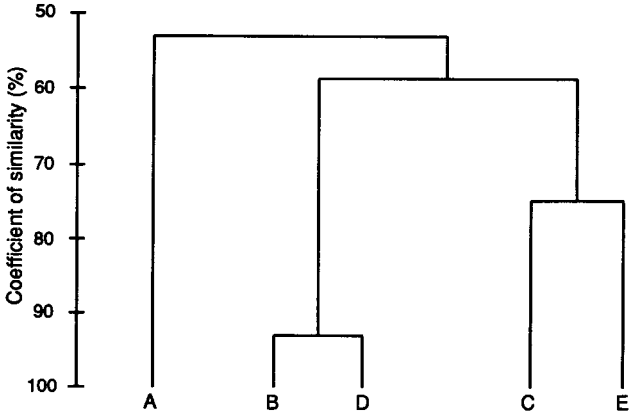

FIG. 3. Dendrogram of *S. aureus* strains A, B, C, D, and E based on growth rate at various temperatures, pH values, and NaCl concentrations.

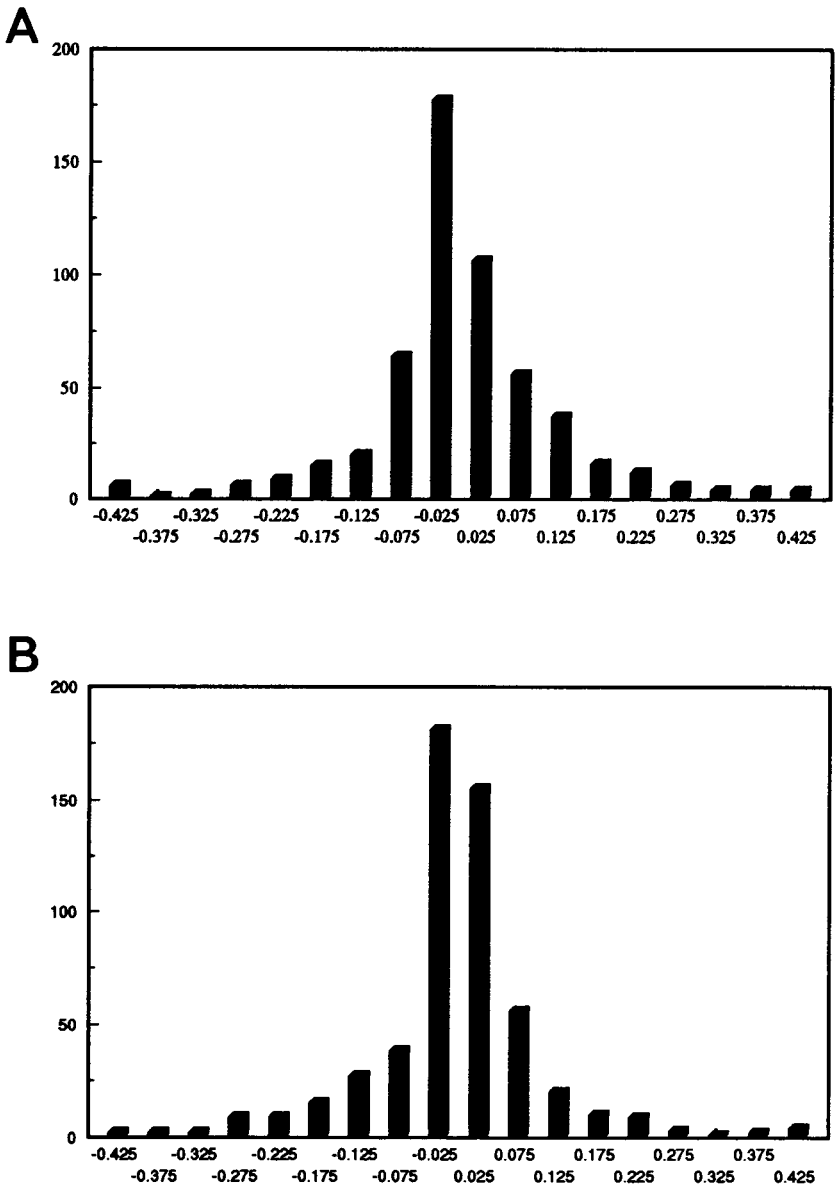

FIG. 4. Histogram of residual errors. (A) Linear model; (B) nonlinear model.

known for *S. aureus* strains. The low-temperature-limit estimation (T_{min} from 5 to 10) closely corresponds to the value given in the literature. Tatini (16) reviewed the environmental factors in food that influence the growth of this bacterium and proposed growth between 7 and 48°C, while Notermans and Heuvelman (11) did not observe growth at 8°C. We did not observe growth of *S. aureus* after 1 week on culture medium at a temperature of 8°C, except for strains D and E. For all the strains, temperature had a strong inhibitory effect on growth rate. For instance, strain B grew on synthetic culture medium at a low temperature of 14°C with a specific growth rate of 0.053 h^{-1} (generation time of 13 h) at pH 7.4, 0% NaCl, whereas at 37°C at the same pH and salt concentration, μ was 1.05 h^{-1} (generation time of 0.66 h).

Concentrations of NaCl above 5% decreased growth whatever the pH or temperature, while 2% NaCl had a positive effect on growth. μ increased with low quantities of NaCl in the culture. For instance, for strain C at 25°C, pH 7.4, μ increased from 0.44 h^{-1} (generation time of 1.6 h) with 0% NaCl to 0.87 h^{-1} (generation time of 0.8 h) with 2% NaCl. The term of the model describing the NaCl effect, a nonlinear effect with an optimum of about 2% according to the strains, corresponds well to the salt tolerance characteristic often described for *S. aureus* (17). According to Smith et al. (14), NaCl would exert an inhibitory effect on transport of substrates into cells of *S. aureus*, while the inhibitory action of NaCl in foods is often associated with a decrease in water activity with concomitant decreases in the growth and biochemical activities (5). Globally, the optimum growth rate was observed for pH values in a range of 6.5 to 7.4 whatever the temperature and NaCl concentration. The low-limit-of-growth pH found in our study, pH_{min} from 2.8 to 3.3, is lower than the minimum pH given in the literature (8, 13, 16, 17), but generally, studies are performed with a pH value of above 4. Therefore, the proposed nonlinear model is valid for the whole range of current studies of *S. aureus* growth at pH 4 to 10.

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