Effect of NaCl, pH, Temperature, and Atmosphere on Growth of *Salmonella typhimurium* in Glucose-Mineral Salts Medium

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The interactions of pH (5.0, 6.0, and 7.0), temperature (19, 28, and 37°C), and atmosphere (aerobic versus anaerobic) with NaCl (0, 1, 2, 3, 4, and 5%) on the growth of *Salmonella typhimurium* ATCC 14028 in defined glucose-mineral salts culture medium were evaluated. Response surface methodology was used to develop equations describing the response of *S. typhimurium* to environmental changes. The response to an increasing concentration of NaCl at any temperature tested was nonlinear. The maximum growth was predicted to occur at an NaCl concentration of 0.5%, a temperature of 19°C, and an initial pH of 7.0 under aerobic growth conditions. The relative amounts of aerobic growth at 19°C, pH 7.0, and NaCl concentrations of 0, 0.5, 1, 2, 3, 4, and 5% were predicted to be 99.2, 100.0, 98.8, 90.2, 73.5, 48.6, and 15.6%, respectively. Anaerobic growth conditions repressed the amount of growth relative to that under aerobic conditions, and the effects of NaCl and pH were additive at low salt concentrations; however, at higher salt levels anaerobiosis provided protection against the effects of NaCl.

Recent reports and conferences have indicated the need to reduce salt (NaCl) intake by humans (2, 5, 13, 14). One way this might be accomplished is by reduction of the salt content in processed meats, but this could alter the survival and growth of both the normal and pathogenic flora associated with that product (6, 15). Sodium chloride can also alter the response of the microbial flora to other food additives, pH, temperature, and atmosphere (4, 9, 15). In addition, salts affect the texture of meat products, which in turn may influence the growth and survival of the microbial flora (7). Since processed meats have a large variety of possible formulations, processing temperatures, packaging conditions, and possible abuse conditions, investigation of even a small percentage of commercial products for effects of interactions of product components, pH, NaCl concentrations, and atmosphere with abuse temperature on the survival, growth, and toxigenicity of food-borne pathogens would be difficult. It is possible, however, to take the more limited approach of systematically investigating the effect(s) of changes in each environmental parameter in a defined medium on the growth or survival of the normal flora and selected pathogens associated with meat products. Such studies could include the interactions of at least major food components and additives other than sodium chloride and would result in a series of equations allowing prediction of the response of a pathogen to change in an environmental parameter. The difference in the responses of pathogens to altered conditions in defined medium as opposed to conditions within a real food product is large. Thus, if such a study were to have any validity, the predictions of the defined-medium studies must be tested in meats and revised as additional information is obtained. Finally, each prediction must be tested in the actual food product. One advantage of such a systematic study of the responses of selected pathogens to environmental changes is that previously unsuspected effects may be noted.

Genigeorgis et al. (3) used such an approach to develop equations allowing the percentage of inoculated salmonella cells capable of initiating growth to be predicted when the percentage of brine and the pH of the food or culture medium were known. The most probable numbers of cells initiating growth of *Salmonella saint-paul*, *S. typhimurium*, *S. infantis*, and *S. give* exposed to various pHs and NaCl concentrations in culture media and meat were determined. Response surface methodology was used in developing the predictive equations. For example, the response surface predicted that 79% of the inoculated cells would survive and multiply when inoculated into meat at pH 6.0 containing 3% brine.

Roberts and co-workers investigated the effects of NaCl, pH, various food additives, and processing temperatures on growth and toxin production of *Clostridium botulinum* (10). Multiple linear regression analysis was used to develop equations predicting the probability of toxin production. Nonlinear effects of storage temperatures with salt concentrations were observed (11).

As part of a series of studies to establish quantitatively the effects of environmental factors on the survival and growth of *Salmonella* spp. in foods, the objectives of the present study were to establish the interactive response of *S. typhimurium* ATCC 14028 to temperature, pH, NaCl, and atmosphere in chemically defined medium and to use these data to develop predictive equations that effectively describe the growth of the microorganism in this model system. This work represents our first step toward achieving our long-range goals of developing an effective computer-based model that can be used to predict how changes in formulations and storage conditions will affect the survival and growth of *Salmonella* spp. in foods.

MATERIALS AND METHODS

Organism. *S. typhimurium* ATCC 14028 was obtained from the American Type Culture Collection.

Culture media. The basal medium for all experiments was the E medium of Vogel and Bonner (16), containing 0.20 g of $MgSO_4 \cdot 7H_2O$, 2.00 g of citric acid $\cdot H_2O$, 10.0 g of K_2HPO_4, and 3.50 g of $NaNH_4HPO_4 \cdot 4H_2O$ per liter of water. To this basal medium was added sufficient NaCl to

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TABLE 1. Response surface equations for the effects of pH, temperature, NaCl concentration [NaCl], and atmosphere on the growth of *S. typhimurium* in filter-sterilized glucose[a]

Parameter	Equation
Aerobic	
MLVC	$2.516 + (0.091 \times [NaCl]) + (2.326 \times IPH) - (0.057 \times TEMP) - (0.024 \times [NaCl] \times IPH) + (0.023 \times [NaCl] \times TEMP) - (0.014 \times IPH \times TEMP) - (0.398 \times [NaCl]^2) - (0.141 \times IPH^2) + (0.002 \times TEMP^2)$; $100R^2 = 91.9$
μ	$-0.797 + (0.009 \times [NaCl]) + (0.183 \times IPH) + (0.021 \times TEMP) - (0.001 \times [NaCl] \times IPH) - (0.002 \times [NaCl] \times TEMP) + (0.00001125 \times IPH \times TEMP) + (0.002 \times [NaCl]^2) - (0.014 \times IPH^2) - (0.0002 \times TEMP^2)$; $100R^2 = 89.7$
HMLVC	$624.681 - (39.859 \times [NaCl]) - (24.698 \times IPH) - (25.823 \times TEMP) + (6.857 \times [NaCl] \times IPH) + (1.511 - [NaCl] \times TEMP) - (0.944 \times IPH \times TEMP) - (9.595 \times [NaCl]^2) + (3.333 \times IPH^2) + (0.412 \times TEMP^2)$; $100R^2 = 59.8$
FPH	$0.859 - (0.805 \times [NaCl]) + (0.866 \times IPH) + (0.048 \times TEMP) + (0.155 \times [NaCl] \times IPH) + (0.002 \times [NaCl] \times TEMP) - (0.004 \times IPH \times TEMP) + (0.031 \times [NaCl] - (0.042 \times IPH^2) - (0.0009 \times TEMP^2)$; $100R^2 = 86.9$
Anaerobic	
MLVC	$3.058 - (0.51 \times [NaCl]) - (0.294 \times IPH) + (0.303 \times TEMP) - (0.069 \times [NaCl] \times IPH) + (0.032 \times [NaCl] \times TEMP) + (0.010 \times IPH \times TEMP) - (0.185 \times [NaCl]^2) + (0.063 \times IPH^2) - (0.006 \times TEMP^2)$; $100R^2 = 91.6$
μ	$-0.579 + (0.011 \times [NaCl]) + (0.163 \times IPH) + (0.010 \times TEMP) - (0.007 \times [NaCl] \times IPH) - (0.0006 \times [NaCl] \times TEMP) + (0.001 \times IPH \times TEMP) + (0.003 \times [NaCl]^2) - (0.014 \times IPH^2) - (0.0002 \times TEMP^2)$; $100R^2 = 92.3$
HMLVC	$1094.156 - (44.244 \times [NaCl]) - (161.968 \times IPH) - (28.794 \times TEMP) + (2.743 \times [NaCl] \times IPH) + (1.911 \times [NaCl] \times TEMP) - (0.722 \times IPH \times TEMP) - (6.333 \times [NaCl]^2) + (14.667 \times IPH^2) + (0.416 \times TEMP^2)$; $100R^2 = 66.0$
FPH	$-3.898 - (0.615 \times [NaCl]) + (2.404 \times IPH) + (0.093 \times TEMP) + (0.121 \times [NaCl] \times IPH) + (0.0003 \times [NaCl] \times TEMP) - (0.017 \times IPH \times TEMP) + (0.015 \times [NaCl]^2) - (0.139 \times IPH^2) - (0.0001 \times TEMP^2)$; $100R^2 = 88.2$

[a] Limits for applicable values are pH 5 to 7; sodium chloride concentration, 0 to 5.0%; and temperature, 19 to 37°C. IPH, Initial pH of culture medium.

bring the final concentration to 0, 1.0, 2.0, 3.0, 4.0, or 5.0% (wt/vol) as appropriate. The pH values of the various media were adjusted to pH 5.0, 6.0, or 7.0 with 1 N HCl or NH_4OH. Filter-sterilized glucose was added to bring the final concentration to 2.0%. Baffled 250-ml Erlenmeyer flasks and 250-ml trypsinizing flasks, each containing 150 ml of medium, were used with aerobic and anaerobic cultures, respectively. Studies were conducted initially with autoclaved glucose as the principal carbon source. Although the glucose solution was autoclaved separately and apparent excellent growth occurred under aerobic conditions, the results obtained under anaerobic conditions were extremely erratic and growth was obviously inhibited. All subsequent experiments were made with filter-sterilized glucose, and results obtained with autoclaved glucose were not included in this report.

Growth response in the presence of low concentrations of NaCl was determined in a glucose (0.5%)-mineral salts medium by viable cell count. This medium contained KH_2PO_4, 1.0 g; K_2HPO_4, 2.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4$, 0.05 g; and $CaCl_2$, 0.05 g/liter of distilled water. The final pH was adjusted to 7.0. Phosphates were sterilized separately from the other salts, and glucose was sterilized by filtration.

Inoculum. Each flask was inoculated with approximately 200 cells per ml from a 24-h nutrient broth culture.

Culture conditions. After inoculation, one-half of the flasks were purged with a gas mixture of 4.0% H_2, 10.0% CO_2, and 86.0% N_2. The cultures were incubated at 19, 28, or 37°C on a gyratory shaker at 150 rpm. The cultures were sampled periodically for up to 9 days, and pH values were measured. Appropriate dilutions were made of samples in 0.1% peptone water, and the numbers of CFU were determined with a spiral plater onto tryptic soy agar. Plates were incubated at 37°C for 24 h. All studies were duplicated. The filter-sterilized-glucose studies were duplicated and replicated in their entirety at different times.

Statistical analyses. Responses were expressed as the logarithm of the number of viable bacterial cells at the point of maximum growth (maximum log of viable cells [MLVC]), the number of hours required for each culture to obtain maximum growth (HMLVC), the growth rate (μ) or increase in the logarithm of the viable-cell numbers per hour during the period of logarithmic growth, and the final pH of the culture (FPH). The effects of the experimental factors (initial pH, salt level, temperature, and atmosphere) on MLVC, HMLVC, μ, and FPH were investigated by an analysis of variance with a factorial model. In addition, regression techniques were used to fit second-order response surface models (1). Statistical calculations were performed by the General Linear Models (GLM) procedure of the SAS statistical package (12). The resultant equations are listed in Table 1 with the associated coefficients of determination ($100R^2$).

RESULTS

The aerobic growth response of *S. typhimurium* to various concentrations of glucose in Vogel-Bonner E medium was determined as an aid to the interpretation of the results. At concentrations from 0 to 2.5% (wt/vol), the presence or absence of glucose did not make a significant difference in the number of viable cells obtained in an 18-h incubation period, indicating that the citrate present in the medium could be used by *S. typhimurium* as readily as glucose as a carbon source.

The aerobic growth response of *S. typhimurium* to very low (<0.5%) concentrations of sodium chloride added to a mineral salts medium containing glucose (0.5%) was determined. No significant differences were observed among cultures containing 0, 0.1, 0.2, 0.3, or 0.4% NaCl at a pH of 7.0 and an incubation temperature of 37°C.

Response surface equations for the effects of pH, temperature, NaCl concentration, and atmosphere on growth of *S.*

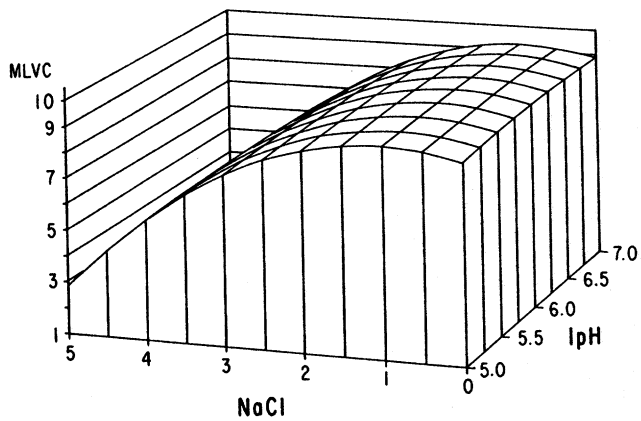

FIG. 1. Predicted MLVC of *S. typhimurium* cultured under aerobic conditions on filter-sterilized glucose, initial pH 5 to 7, temperature 37°C, and NaCl concentrations of 0 to 5% (wt/vol).

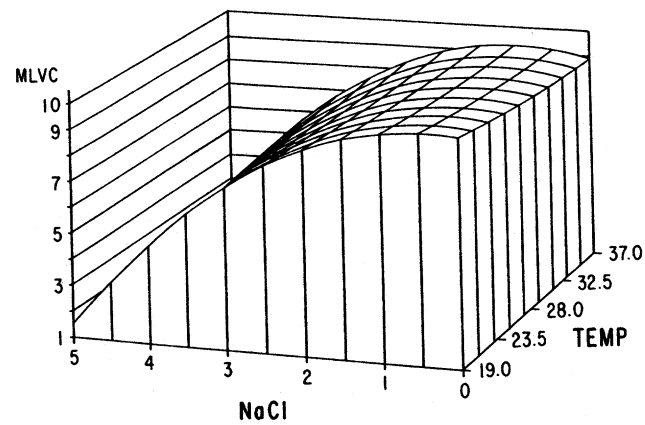

FIG. 3. Predicted MLVC of *S. typhimurium* cultured under aerobic conditions on filter-sterilized glucose, initial pH 7.0, temperature 19 to 37°C, and NaCl concentrations of 0 to 5% (wt/vol).

typhimurium are presented in Table 1, with the associated coefficients of determination ($100R^2$) (3). Response surface curves developed from these equations are presented in Fig. 1 to 4.

Comparisons of the response surface models for aerobic and anaerobic atmospheres (Fig. 1 to 4) and application of the response surface equations indicated that cultivation under an anaerobic atmosphere provided protection to *S. typhimurium* against the inhibitory effects of sodium chloride within the pH range of the study. Comparison of Fig. 1 with Fig. 2 and application of the equations revealed that more growth (MLVC) occurred at NaCl concentrations of 3.0% or greater at pH values of 5, 6, or 7 under anaerobic conditions than under aerobic conditions. The anaerobic cultures produced MLVC values of 5.03, 5.45, and 6.00 versus values of 2.82, 2.96, and 2.81 for aerobic cultures at pH values of 5.0, 6.0, and 7.0, respectively, in the presence of an NaCl concentration of 5.0%. When good growth occurred, final pH values of the cultures tended to be lower under aerobic than under anaerobic atmospheres. Examination of Fig. 3 and 4 indicated enhancement of the protective effect of anaerobiosis against salt as the optimum growth temperature was approached. However, the response surface equation predicted that the effect of anaerobic incubation would produce almost twice as much growth of *S. typhimurium* ATCC 14028 even at an incubation temperature of 19°C in the presence of 5.0% NaCl. Analysis of variance for these data revealed that not only were highly significant ($P < 0.0001$) effects produced by each of the four variables of NaCl, temperature, initial pH, and atmosphere on the maximum number of viable cells as expected, but highly significant ($P < 0.0001$) effects on the MLVC were produced by the interactions between any two, any three, and all four variables.

The MLVC was predicted to occur at an NaCl concentration of 0.5%, a temperature of 19°C, and an initial pH of 7.0 under aerobic conditions. The relative amounts of aerobic growth at 19°C, pH 7.0, and NaCl concentrations of 0, 0.5, 1, 2, 3, 4, and 5% were predicted to be 99.2, 100.0, 98.8, 90.2, 73.5, 48.6, and 15.6%, respectively.

The effects noted for the MLVC numbers of *S. typhimurium* in the presence or absence of air were not apparent when μ was the measured parameter. The predicted growth rates under anaerobic conditions were considerably lower than those predicted for aerobic conditions, indicating a negative correlation between growth rate and ability to produce greater numbers of viable cells under

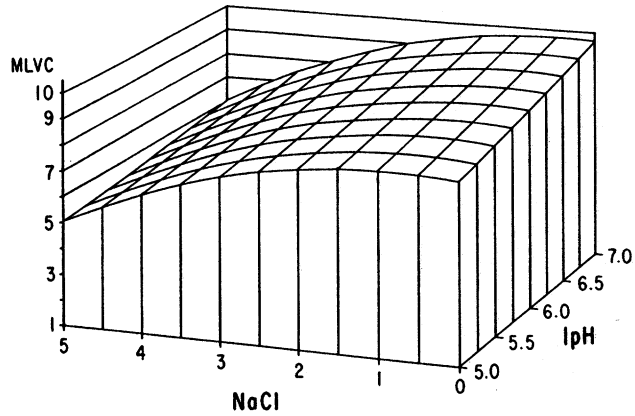

FIG. 2. Predicted MLVC of *S. typhimurium* cultured under anaerobic conditions on filter-sterilized glucose, initial pH 5 to 7, temperature 37°C, and NaCl concentrations of 0 to 5% (wt/vol).

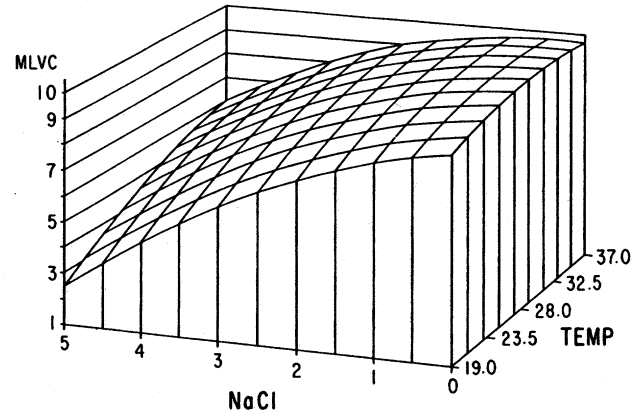

FIG. 4. Predicted MLVC of *S. typhimurium* cultured under anaerobic conditions on filter-sterilized glucose, initial pH 7.0, temperature 19 to 37°C, and NaCl concentrations of 0 to 5% (wt/vol).

adverse conditions. At a given temperature and pH value under aerobic but not anaerobic conditions, the response surface equations predict a nearly linear response of growth rate to NaCl concentration. Under anaerobic growth conditions, the response to decreased NaCl in the culture medium became steeper below 2.0%. Under either aerobic or anaerobic growth conditions the predicted growth rates always increased as the temperature increased ($P < 0.0001$) (Table 1), but those values predicted by linear extrapolation of the rate at 19°C versus that at 37°C were always slightly lower, resulting in a convex response surface. Examination of response surfaces for either aerobic or anaerobic growth of *S. typhimurium* indicated the existence of significant interaction between growth rate and any of the three variables of NaCl, temperature, and initial pH of the culture medium. Analysis of variance revealed that not only were there highly significant ($P < 0.0001$) interactive effects between growth rate and NaCl, temperature, and initial pH, but there were also highly significant ($P < 0.0001$) interactive effects on μ between any two of the four variables of NaCl, temperature, initial pH, and atmosphere except for initial pH and atmosphere. In addition, highly significant ($P < 0.0001$) interactions occurred among any three and among all four of the experimental variables affecting the μ of *S. typhimurium*.

Response surface equations (Table 1) were generated for the hours required to obtain HMLVC to determine the effect of the possible interactions on the length of time which would be required for the cultures to obtain HMLVC. A response surface of this type should be responsive to the μ under a given set of conditions. The extent of the interaction between μ and HMLVC which could be found in a given culture was unexpected. As a result, response surfaces generated by the equations in Table 1 for HMLVC were highly convex with respect to NaCl concentration and highly concave with respect to temperature. Because HMLVC is the result of both μ and MLVC, the associated coefficients of determination are lower than those for the other equations.

The final set of response surface equations that were generated predicts the FPH of the cultures when initiated at a given pH. Under either aerobic or anaerobic conditions in filter-sterilized culture medium at an initial pH of 7.0, response surfaces were nearly linear in their response to either NaCl or temperature. A higher incubation temperature or a lower NaCl concentration resulted in a lower FPH of the culture medium. An aerobic atmosphere resulted in lower FPH values than did anaerobic culture conditions at the same NaCl concentration and temperature. The aerobic response surface at an initial pH of 6.0 was slightly convex in response to temperature. At an initial pH of 5.0, however, markedly different responses to changes in either NaCl concentration or incubation temperature occurred when the cultures were incubated under anaerobic rather than aerobic conditions. Under an anaerobic atmosphere at an initial pH of 5.0, a higher incubation temperature resulted in a higher, not lower, FPH value in the culture medium. Since the culture pH was not maintained at a constant value during growth, it is unknown whether the altered pH values in turn altered μ or MLVC values.

DISCUSSION

The ability of *S. heidelberg* ATCC 8325, *S. typhimurium* ATCC 6994, and *S. derby* ATCC 6966 to grow in nutrient broth containing 0 to 8% added NaCl at temperatures of 8, 12, 22, and 37°C was investigated by Matches and Liston (8). These investigators discovered that in shake cultures at 8°C only *S. heidelberg* and *S. typhimurium* were able to increase in numbers in the presence of 1% NaCl. All three serotypes were able to grow in the presence of up to 4% NaCl at 12°C, and at 37°C growth occurred at 7 to 8% NaCl. Stimulation of growth was observed for all three strains at lower salt concentrations (0 to 2.5%) and was more evident at lower incubation temperatures. If the initial pH of the nutrient broth were assumed to be 7.0, their results could be compared with those of the present study. The MLVC was obtained in cultures with less than 2% NaCl (Fig. 3 and 4) in both studies at all temperatures tested. In the study reported here, pronounced stimulation of growth by low concentrations of NaCl was not observed even though possible stimulation of growth rate was investigated with 0.1% NaCl increments. The present studies were conducted with defined mineral salts media, whereas Matches and Liston (8) used nutrient broth, which may account for the observed differences. They also observed that the largest amounts of growth occurred at suboptimal temperatures, e.g., 19 or 22°C.

Genigeorgis et al. (3) predicted that, when either the brine concentration or the pH was changed to an extreme value, greater contamination levels of salmonellae would be required to initiate growth, and lower brine concentrations would inhibit growth at pH values remote from the optimum. The present study extends these predictions to include the responses of *S. typhimurium* to temperature and atmosphere under conditions in which growth does take place. All four factors were shown to interact and influence μ and the total amount of growth taking place in the medium. The growth response differed when μ rather than MLVC was measured. In terms of MLVC, anaerobiosis provided protection against the effects of NaCl. This prediction of our studies with a pure culture and in a defined medium must be tested under controlled conditions in a meat product before its actual significance can be predicted.

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