Combined Effects of Water Activity, Solute, and Temperature on the Growth of *Vibrio parahaemolyticus*

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*Vibrio parahaemolyticus* was grown at 36 C in tryptic soy broth (pH 7.8) containing added levels of NaCl ranging from 0.5 to 7.9% (wt/wt). The fastest generation time was 16.4 min in tryptic soy broth containing 2.9% NaCl (TSBS) which corresponded to a water activity (a_w) of 0.992 ± 0.005. Tryptic soy broth containing lower or higher levels of NaCl resulted in higher or lower a_w, respectively, and slower generation times. Growth was measured turbidimetrically at 36 C in TSBS containing added amounts of NaCl, KCl, glucose, sucrose, glycerol, or propylene glycol. The solutes used to reduce a_w to comparable levels resulted in extended lag times of varied magnitude, dissimilar growth rates, and different cell numbers. Reduction of a_w with glycerol was less inhibitory to growth than similar a_w reductions with NaCl and KCl. Sucrose, glucose, and propylene glycol generally had the greatest effect on extending the lag times of *V. parahaemolyticus* when the addition of these solutes was made to establish similar a_w levels lower than 0.992. Minimal a_w for growth at 15, 21, 29, and 36 ± 0.2 C for each of four strains of *V. parahaemolyticus* was tested in TSBS containing added solutes. Reduced a_w was generally most tolerable at 29 C, whereas higher minimal a_w for growth was required at 15 C. Solute added to TSBS to achieve reduction in a_w, minimal a_w for growth after 20 days, and incubation temperatures were as follows: glycerol, 0.937, 29 C; KCl, 0.945, 29 C; NaCl, 0.948, 29 C; sucrose, 0.957, 29 and 36 C; glucose, 0.983, 21 C; and propylene glycol, 0.986, 29 C. Each of the four strains tested responded similarly to investigative conditions. It appears that minimal a_w for growth of *V. parahaemolyticus* depends upon the solute used to control a_w.

The relationships between available water content and the potential for spoilage of foods by microorganisms have been of interest for many years. Various minimal, optimal, and maximal moisture levels, usually expressed in terms of water activity (a_w), have been reviewed (17, 20). Much of the available data on a_w requirements for bacteria have resulted from studies on foodborne pathogens such as salmonellae (3, 4, 6), staphylococci (5, 15, 16, 19), and *Clostridium* spp. (1, 7, 10, 18). In light of the recent recognition of *Vibrio parahaemolyticus* as a cause of foodborne disease outbreaks in the United States (2) and the lack of definitive information regarding the organism's tolerance to reduced a_w levels, several experiments were designed to determine the growth response of *V. parahaemolyticus* over a wide range of a_w.

In the present study, the optimal a_w in a medium containing added NaCl was established. Effects from minor changes in optimal a_w levels resulting from the addition of several solutes to growth media on lag times of *V. parahaemolyticus* are discussed. Minimal a_w levels for growth at 15, 21, 29, and 36 C were achieved by the addition of solutes to a basal medium.

**MATERIALS AND METHODS**

*Test cultures.* Four strains of *V. parahaemolyticus* were studied: M5256J-2 (O1:K22), received from W. E. DeWitt, Center for Disease Control, Atlanta; 4750 (O2:K3) and T-3755-1 (O3:K7), obtained from H. Zen-Yoji, Tokyo Metropolitan Research Laboratory of Public Health; and 8700 (O4:K11), acquired from M. Fishbein, Food and Drug Administration, Washington, D.C. Stock cultures were maintained at room temperature in a long-term preservation medium consisting of 0.3% yeast extract, 1.0% peptone (Difco), 2.0% NaCl, and 0.3% agar in distilled water as suggested by M. Fishbein.

*Media.* The basal medium used for all investigations was tryptic soy broth (TSB, Difco), which contains 0.5% NaCl when prepared according to the manufacturer's directions. For studies involving
growth of the organism at elevated NaCl levels, quantities of NaCl were added to known volumes of TSB, and the percentage of NaCl was expressed on the basis of grams of NaCl per grams of final NaCl-TSB mixture, assuming the density of TSB to be 1.0. Each of the remaining solutes studied (KCl, glucose, sucrose, glycerol, and propylene glycol) were added individually to TSB containing 2.93% NaCl (TSBS) in progressively increasing amounts to achieve amounts above and below which V. parahaemolyticus would grow. Percentages of these solutes, which are referred to throughout this report, were calculated as grams of solute per grams of final mixture (solute plus TSBS), again assuming the density of TSBS to be near 1.0. All growth media were adjusted to pH 7.8 by adding 2 N NaOH, dispensed into either 15 by 100 mm or 16 by 150 mm screw-cap tubes and sterilized at 121 C for 15 min. Random measurement of pH after sterilization revealed changes of no greater than ±0.2.

**Growth studies.** Since V. parahaemolyticus is facultatively halophilic, an initial experiment was designed to determine the optimal NaCl concentration at which the organism would grow. Strain M5250J-2 was cultured in TSBS at 29 C on a gyratory shaker (150 rpm) for 16 h. The culture was diluted 100-fold in distilled water containing 0.1% peptone (Ditco) and 3.0% NaCl; 1 ml of the diluted culture was then inoculated into 150-ml portions of fresh TSB containing concentrations of NaCl ranging to 7.87%. The 500-ml Erlenmeyer flasks containing the cultures were returned to the shaker at 29 C. Samples were withdrawn at selected times and appropriate dilutions were made in the peptone-NaCl diluent prior to surface-plating on TSBS containing 1.5% agar (TSBSA) and on thiocyanate citrate bile salts sucrose agar (TCBS). Counts were made after 12 h (TSBSA) and 24 h (TCBS) of incubation at 36 C, and generation times were calculated for the organism cultured in each of the TSB media containing added NaCl.

The above experiment showed strain M5250J-2 to have the fastest generation time in TSB containing 2.93% NaCl (TSBS). Addition of various quantities of NaCl, KCl, glucose, sucrose, glycerol, or propylene glycol to TSBS was made to achieve only slight reductions in amounts. The effects of these reductions on lag times of strain M5250J-2 were then examined by measuring absorbance of the growing cultures at 520 nm after inoculation with a loop of 16-h TSBS culture. Growth temperature for the inoculum and the test cultures was 36 C. Amounts of solutes added, their weight percents, and resulting amounts are summarized in Table 1.

Minimal amounts for growth of each of four strains of V. parahaemolyticus at 15, 21, 29, and 36 C were determined. Each strain was cultured in TSBS for 16 h at 29 C and standard loop inocula were transferred to 10-ml portions of TSBS containing individually added quantities of test solutes. Each test was performed in quintuplicate. In all cases, sufficient solute was added to achieve several amounts above and below that required for growth. Caps were tightened on the tubes (16 by 150 mm), and the inoculated media were incubated for 20 days in walk-in incubators adjusted to 15, 21, 29, and 36 ± 0.2 C. Obvious turbidity during the 20-day period was recorded as positive growth, and tubes were discarded. After 20 days all remaining tubes were examined for number of viable cells by plating on TCBS and for number of total cells by using a Petroff-Hauser counting chamber. Tubes were recorded as negative if viable cell population and direct microscope counts per milliliter were both less than the original number of cells per milliliter of culture at the time of inoculation. Direct counts were necessary to determine whether cell division was followed by death of significant numbers of cells during the 20-day incubation period.

**Determination of amount.** Equilibrium relative humidity measurements were made at 29 C with Hygrosensor elements (no. 4-4822; Hygrodynamics, Inc., Silver Spring, Md.) mounted in lids of 8-oz jars. Hygrometer sensors were attached to a Hygrometer Indicator (model no. 4-4900; Hygrodynamics, Inc.) and an equilibration time of not less than 8 h was allowed before measurements were recorded from 25-ml portions of each test solution. Sensory calibrations were made against saturated KNO₃. Mean values were determined from triplicate readings for several concentrations of each solute and sorption isotherms were plotted (Fig. 1). All amounts reported in this paper were taken from isotherm curves. Accuracy of the measuring system is considered to be no better than ±0.005 amount. However, for purposes of comparison, as will become evident upon examination of results, data are presented at the ±0.001 amount level.

**RESULTS AND DISCUSSION**

Inhibitory effects of amounts levels higher and lower than optimum on the growth and metabolism of several bacterial species have been reported (3, 5, 6, 16, 17). Extreme sensitivity to relatively minor variations in osmotic and ionic conditions in growth media have been most dramatic with V. costicola (8) and V. metchnikovi (13). Reduction in amount from 0.999 to 0.995 resulted in a fivefold increase in the rate of growth of V. metchnikovi. Further reduction in amount generally resulted in decreased rates of growth, depending upon the solute added to control amount and the nutrient composition of the growth medium. Several reports indicate conflicting ranges and optima for percentage of NaCl tolerance for V. parahaemolyticus (11, 21, 22, reviewed by 14; C. R. Lazarus and J. A. Koburger, Abst. South-eastern Branch Amer. Soc. Microbiol., 51st and 52nd Ann. Mtgs., p. 19, 1973). It is difficult to compare these data because methods for preparing percent NaCl levels in media were not detailed and amounts levels were not reported. Initial experiments were therefore designed to determine the optimal NaCl concentration (and corresponding amount) for growth of V. parahaemolyticus by using TSB as a basal medium. Results are shown in Fig. 2. As amount was decreased from 0.998 to 0.992 (0.5 and 2.93% NaCl, respectively), the generation time of V.
parahaemolyticus M5250J-2 decreased from 24.4 to 16.4 min. Increased NaCl levels above 2.93% prolonged generation times. Whether $a_w$ manipulation by the addition of other electrolytes or nonelectrolytes alone or in combination to TSB would have resulted in different $a_w$ optima and faster growth rates was not determined. The 16.4-min generation time was considered to result from nearly optimal culture conditions, and therefore TSBS (TSB containing 2.93% NaCl) was used as a basal medium in subsequent studies involving the effects of added solutes on lag phase extension and minimal $a_w$ tolerance of the four test strains.

Realizing that only slight differences in $a_w$ achieved from the addition of NaCl to the growth medium resulted in substantial differences in generation times of strain M5250J-2, it was decided to measure the effects of low amounts of added KCl, glucose, sucrose, glycerol, and propylene glycol, in addition to NaCl, on lag times of the organism. Data are plotted in Fig. 3. Grams of solute added per 100 ml of TSB or TSBS, calculated solute concentrations, and corresponding $a_w$ levels are summarized in Table 1. Some trends can be observed in these data. NaCl studies show that 0.992 $a_w$ results in the shortest lag time, fastest growth rate, and highest total biomass production. Departure from 0.992 $a_w$ resulted in longer lag times with slower growth rates and depressed cell production. These data tend to confirm those derived from generation time studies. Addition of solutes to TSBS (0.992 $a_w$) reduced $a_w$ in all cases, but the magnitude of change in growth parameters was varied, depending upon the solute added. Glycerol had the least inhibitory effect on V. parahaemolyticus of all solutes tested. Addition of glycerol to achieve a 0.961 $a_w$
extended the lag phase only by 4 h compared to greater lag phase prolongation at even higher $a_w$ levels for the other test solutes. Inhibitory effects of NaCl and KCl were similar at comparable reduction in $a_w$ levels, whereas propylene glycol, glucose, and sucrose, in that general order, were most effective in delaying logarithmic growth and depressing cell production by strain M5250J-2. The glycerol data are in agreement with those reported for Staphylococcus aureus survival (15) and enterotoxin production (19) and growth of Salmonella oranienburg (4), Bacillus cereus (9), and Clostridium perfringens (10), wherein glycerol was found to be less inhibitory than other test solutes. Marshall et al. (12), on the other hand, reported that at $a_w$ levels between 0.96 and 0.90 the inhibition of S. aureus growth rate was about 10% greater in glycerol than in NaCl. Glucose and propylene glycol proved to be most bacteriostatic to V. parahaemolyticus when compared to the other solutes at similar $a_w$ levels. Total inhibition of growth of V. metchnikovi in nutrient broth containing glucose in amounts to achieve $a_w$ less than 0.997 has been noted (13). These authors reported no growth of the organism in brain heart infusion broth containing glucose. Glucose appears, therefore, to exhibit a toxic effect on V. metchnikovi and V. parahaemolyticus. By-products arising from chemical reactions between glucose and medium constituents during sterilization may account for these inhibitors. The reasons for V. parahaemolyticus sensitivity to propylene glycol cannot be explained.

Table 2 shows the minimum $a_w$ for growth of V. parahaemolyticus in TSB and TSBS adjusted to reduced $a_w$ levels by the addition of various solutes. Calculated concentrations of added solutes are also listed. The four test strains responded similarly in their minimal $a_w$ levels at various temperatures. In most cases the $a_w$ levels listed in Table 2 are representative of at least three of the four strains. Therefore, only one minimal $a_w$ is listed for each solute-temperature combination. In general, solutes which were more inhibitory with respect to prolonging lag phase of growth were also more effective in completely inhibiting growth. Glycerol was least effective in controlling growth, followed by NaCl and KCl which were approximately equal, and then sucrose, glucose, and propylene glycol. The 29°C incubation temperature proved to be most satisfactory for the organism's tolerance to low $a_w$, whereas 15°C adversely effected the response to $a_w$ stress. V. metchnikovi was reported to have lower tolerance to glycerol than to NaCl when $a_w$ adjustment was made in quarter-strength brain heart


The relationship between limiting $a_w$ levels for growth of microorganisms and the solute added to achieve those levels is unclear. Several authors (7, 17) have stated that biological response to $a_w$ by some organisms is independent of the types of solutes used to reduce the $a_w$. Other reports have shown that nutrient availability (3), pH (1), oxygen level (6, 16), and moisture content (15), in addition to the test solute, affect a microorganism’s ability to grow at limiting $a_w$. It appears that the limiting $a_w$ for growth of the four *V. parahaemolyticus* strains examined in this study depends upon the solutes used to attain these lower limits. Notwithstanding the possibility of nutrient dilution effects inherent in the methods employed to prepare the test media, the differences in physico-chemical properties of the solutes apparently have a significant influence on the ability of *V. parahaemolyticus* to tolerate suboptimal $a_w$ levels. Further experiments are required to determine the response of *V. parahaemolyticus* to low $a_w$ levels achieved by the addition of other halogen salts to growth media. Studies involving the combined effects of various electrolytes and nonelectrolytes on tolerance of the organism at reduced $a_w$ would also provide valuable information on growth characteristics of this facultative halophile.

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