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Growth Kinetics of *Salmonella* Isolates in a Laboratory Medium as Affected by Isolate and Holding Temperature†

ABSTRACT

Salmonella isolates were surveyed for their growth kinetics in a laboratory medium for the purpose of identifying isolates suitable for modeling experiments. In addition, the effect of holding stationary phase *Salmonella* cultures at different temperatures on their subsequent growth kinetics was evaluated for the purpose of developing a protocol to prevent the need for midnight sampling in modeling experiments. In Experiment 1, 16 isolates of *Salmonella,* 2 from the American Type Culture Collection (ATCC) and 14 from broiler operations, were surveyed for their growth kinetics in brain heart infusion (BHI) broth at 40°C. Lag time ($P = 0.005$) and growth rate ($P = 0.022$) were affected by identity of the isolate. Lag time ranged from 0.73 to 1.38 h, whereas growth rate ranged from 0.78 to 0.94 $\log_{10}$ CFU/ml/h. Overall, isolate S1 (*Salmonella infantis* from ATCC) was the fastest growing. In Experiment 2, 4 isolates of *Salmonella,* 1 from ATCC and 3 from broiler operations, were used to determine whether holding temperature influences subsequent growth kinetics. *Salmonella* isolates were grown to stationary phase at 37°C in BHI and then held for 24 h at 5, 22, or 37°C before dilution and reinitiation of growth in BHI at 37°C. Holding temperature did not alter or interact with identity of the isolate to alter subsequent growth kinetics. From the latter finding, a protocol was devised in which a dual-flask system is used to prevent the need for midnight sampling in modeling experiments. Similar to the results obtained in Experiment 1, identity of the isolate had only minor effects on growth kinetics in Experiment 2 indicating that all isolates examined were suitable for modeling experiments.

Development of mathematical models that predict growth of *Salmonella* strains associated with poultry products requires extensive kinetic data on responses of the *Salmonella* strains to combinations of environmental and food formulation factors. Collection of such data in food is labor intensive, thus making it difficult to obtain sufficient data for development of accurate predictive models *(10).* Consequently, like other modelers, we plan on developing predictive models for *Salmonella* strains using extensive kinetic data collected in studies with a laboratory medium and then validating the models by comparison of their predictions to less extensive kinetic data collected in studies with poultry foods.

A problem encountered when developing predictive models for *Salmonella* growth is the problem of which strain to use as there are over 2,000 serotypes and many more strains of *Salmonella* to choose from *(8).* Surprisingly, there is limited published information about the growth kinetics of *Salmonella* strains to help with this decision. Nonetheless, the available data suggest that there are only minor differences in growth rates *(4)* and the minimum *(18, 19)* and maximum *(7)* temperatures at which different strains of *Salmonella* will initiate growth. Although these data suggest that growth kinetics of *Salmonella* strains are not highly variable, a systematic study of the effect of identity of strain on growth kinetics of *Salmonella* strains has not been reported. Consequently, the current study was undertaken to survey isolates of *Salmonella* from broiler operations for their growth kinetics in a laboratory medium for the purpose of identifying strains that would be suitable for modeling experiments.

A second problem encountered when conducting modeling experiments is the need to collect kinetic data in the middle of the night. Stagger-starting of two cultures per growth condition at 12-h intervals has potential for eliminating the need for midnight sampling. However, one must establish that the *Salmonella* culture used to inoculate the second flask has similar growth kinetics to that used to inoculate the first flask. Considering that previous growth conditions have been found to alter subsequent growth kinetics of other foodborne pathogens *(3, 13),* the second objective of this study was to determine the effect of holding temperature on the subsequent growth kinetics of *Salmonella* cultures with the goal of devising a dual-flask system that would eliminate the need for midnight sampling in modeling experiments.

MATERIALS AND METHODS

Isolates. Table 1 lists the sources and serotypes of the isolates of *Salmonella* used in this study. Isolates S1 and S2 were from ATCC (American Type Culture Collection, Rockville, Md.), whereas the other isolates were from broiler operations. The broiler isolates were serotyped by the *Salmonella* Reference Center at the University of Pennsylvania (Kennet Square, Pa.). Stock cultures of all

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† Mention of a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE 1. *Sources and serotypes of* Salmonella *used to measure growth kinetics in laboratory medium*

Isolate	Serotype	Source
S1	*S. infantis*	ATCC 51740
S2	*S. typhimurium*	ATCC 14028
S5	*S. worthington*	Broiler ceca
S7	*S. mbandaka*	Broiler ceca
S10	*S. binza*	Broiler ceca
S14	*S. typhimurium*	Broiler feed
S16	*S. senftenberg*	Broiler ceca
S17	*S. binza*	Broiler feed
S20	*S. brandenburg*	Broiler ceca
S22	*S. senftenberg*	Broiler ceca
S26	*S. senftenberg*	Broiler house litter swab
S30	*S. thompson*	Broiler processing plant sludge
S31	*S. schwarzengrund*	Broiler processing plant sludge
S33	*S. indiana*	Broiler house litter swab
S38	*S. senftenberg*	Broiler feed
S44	*S. simsbury*	Broiler carcass rinse
S52	*S. worthington*	Broiler feed
S62	*S. typhimurium*	Broiler house beetles

isolates were maintained at −70°C in brain heart infusion (BHI) broth (pH 6) that contained 15% glycerol.

Starter cultures. Starter cultures of each isolate were initiated by inoculating 50 ml of BHI (pH 6) with 50 µl of the resuspended stock culture, which had a *Salmonella* viable cell concentration of 9.2 $\log_{10}$ CFU/ml. Starter cultures, with an initial *Salmonella* cell level of 6.2 $\log_{10}$ CFU/ml, were incubated for 24 h at 30°C in Experiment 1 and for 24 h at 37°C in Experiment 2 to obtain stationary phase cells. Starter cultures were incubated in 250-ml Erlenmeyer flasks that were sealed with foam plugs and shaken at 150 orbits per minute (opm). In Experiment 1, starter cultures were used at 24 h of incubation to inoculate growth cultures, whereas in Experiment 2, starter cultures were held an additional 24 h at 5, 22, or 37°C before being used to inoculate growth cultures.

Growth cultures. Growth cultures in both experiments were inoculated to an initial level of 4.2 $\log_{10}$ CFU/ml and were incubated under aerobic conditions in 250-ml Erlenmeyer flasks that contained 50 ml of BHI (pH 6) shaken at 150 opm. In Experiment 1, growth cultures were incubated at 40°C, whereas in Experiment 2 they were incubated at 37°C.

Viable counts. At 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 8 h after inoculation, the viable count of *Salmonella* cells in the growth cultures was determined. A sample of the growth culture was withdrawn, diluted in peptone water, and spiral plated onto BHI agar. The BHI agar plates were incubated at 30°C until the colonies that formed were large enough to count; this usually occurred between 18 and 24 h of incubation. Colonies were counted using a Protos Colony Counter (Synoptics Ltd., Cambridge, UK).

Curve fitting. Growth curves of viable counts (Y, $\log_{10}$ CFU/ml) versus time (X) were iteratively fit using GraphPad PRIZM (GraphPad Software, San Diego, Calif.) to a two-phase linear growth model:

$$Y = \text{Baseline} + \text{Increase}$$

$$\text{Increase} = 0 \qquad \text{if } X \leq (\text{lag time})$$

$$= (\text{growth rate}) \times \Delta X \qquad \text{if } X > (\text{lag time})$$

$$\Delta X = X - (\text{lag time})$$

where the viable count Y was equal to Baseline (initial viable count) plus Increase (increase of viable count). In turn, Increase was equal to zero if the sample time X was less than or equal to the lag time; otherwise Increase was equal to the growth rate times ΔX (the sample time minus the lag time).

Statistical analysis. Effects of identity of the isolate and holding temperature on growth characteristics (i.e., lag time and growth rate) were evaluated by analysis of variance using the Statistical Analysis System *(24)*. The model for Experiment 1 contained a term for identity of the isolate, whereas the model for Experiment 2 contained terms for identity of the isolate, holding temperature, and the interaction of identity of the isolate with holding temperature. When a significant F test was encountered, individual means were compared using Duncan's multiple range test. Three to five replicate growth curves were obtained for each isolate in Experiment 1, whereas two or three replicate growth curves were obtained for each combination of isolate and holding temperature in Experiment 2.

RESULTS

In Experiment 1, 16 isolates of *Salmonella* were surveyed for their growth kinetics in laboratory medium incubated at 40°C. Analysis of variance indicated that identity of the isolate affected lag time ($P = 0.005$) and growth rate ($P = 0.022$). Lag time ranged from 0.73 h for isolate S1 to 1.38 h for isolates S31 and S38 (Figure 1). Growth rate ranged from 0.78 $\log_{10}$ CFU/ml/h for isolate S62 to 0.94 $\log_{10}$ CFU/ml/h per hour for isolate S44 (Figure 2). Overall, the maximum difference between isolates was 90% for lag time and 20% for growth rate with most isolates showing similar lag times and growth rates.

In an effort to identify fast- and slow-growing isolates in Experiment 1, the observed lag time (Figure 1) and growth rate (Figure 2) for each isolate were used to calculate the time needed for an increase of 0, 1, 2, or 3 log cycles in *Salmonella* numbers. The calculated time for a given log increase was plotted against the corresponding log increase, as shown in Figure 3. The most striking revelation from this

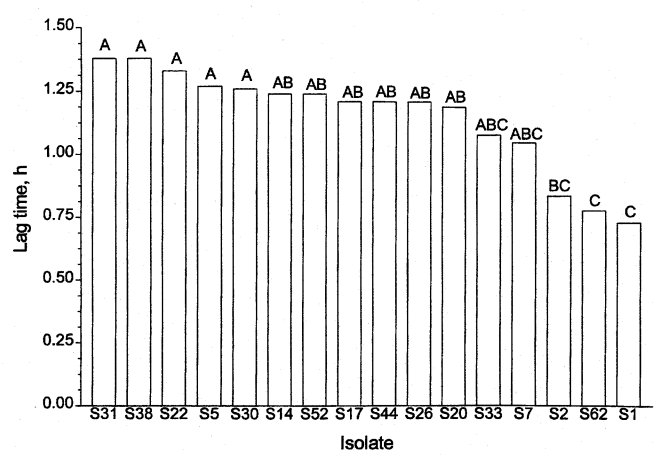

FIGURE 1. *Effect of identity of the isolate on lag time of* Salmonella *isolates in laboratory medium incubated at 40°C (Experiment 1). Each bar is the mean of three to five replicate lag time determinations. Analysis of variance indicated that identity of the isolate affected lag time ($P = 0.005$). Bars having the same letter in common are not significantly different at $P < 0.05$ as determined by Duncan's multiple range test.*

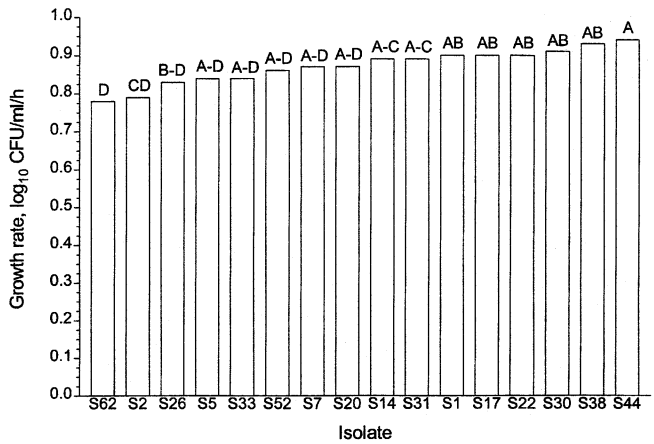

FIGURE 2. *Effect of identity of the isolate on growth rate of* Salmonella *isolates in laboratory medium incubated at 40°C (Experiment 1). Each bar is the mean of three to five replicate growth rate determinations. Analysis of variance indicated that identity of the isolate affected growth rate ($P = 0.022$). Bars having the same letter in common are not significantly different at $P < 0.05$ as determined by Duncan's multiple range test.*

plot was that isolate S1 (*Salmonella infantis* from ATCC) was clearly the fastest growing (i.e., had the shortest time for a given log increase in numbers). In general, most isolates exhibited similar rates of growth, as indicated by the clustering of lines in Figure 3.

In Experiment 2 four isolates of *Salmonella* were used to determine whether previous holding temperature influences subsequent growth kinetics. After growth to stationary phase (24 h at 37°C), *Salmonella* cultures were held for 24 h at 5, 22, or 37°C before dilution and reinitiation of growth in laboratory medium at 37°C. Holding temperature did not alter ($P = 0.90$ for lag time and 0.22 for growth rate) or interact with identity of the isolate to alter ($P = 0.98$ for lag time and 0.62 for growth rate) subsequent growth kinetics (Table 2). Again, minor differences in growth kinetics were seen between isolates. Most notable, the lag time of isolate S10 was 40% longer than the lag time of isolates S2 and S62. A plot of time for a given log increase in numbers versus the

TABLE 2. *Effect of isolate and holding temperature on growth kinetics of* Salmonella *in laboratory medium*[a]

	Lag time, h				Growth rate, log CFU/ml/h			
	Holding temperature				Holding temperature			
Isolate	5°C	22°C	37°C	Main effect	5°C	22°C	37°C	Main effect
S2	1.14	0.98	1.16	1.09B	0.86	0.86	0.90	0.87A
S10	1.57	1.46	1.52	1.52A	0.84	0.82	0.85	0.84B
S16	1.24	1.29	1.32	1.28AB	0.81	0.84	0.84	0.83B
S62	1.11	1.12	1.01	1.08B	0.86	0.85	0.85	0.85AB
Main effect	1.26A	1.21A	1.25A		0.84A	0.85A	0.86A	

[a] *Salmonella* were grown to stationary phase (i.e., 24 h at 37°C) in brain heart infusion (BHI) broth (pH 6), and then held at 5, 22, or 37°C before measurement of growth kinetics in BHI, pH 6, incubated at 37°C. Holding temperature did not alter or interact with isolate to alter growth kinetics. However, isolate had a significant effect on lag time and growth rate. Means having the same letter in common within the same main effect are not significantly different at $P < 0.05$ as determined by Duncan's multiple range test.

log increase calculated from lag time and growth rate revealed that isolates S2 and S62 grew at a similar rate but they grew faster than isolate S16, which grew slightly faster than isolate S10 (Figure 4).

Subsequent to Experiment 2, my colleagues and I adopted a protocol in which the starter culture is diluted by a factor of 10^{-4} in buffered peptone water and the diluted culture is then used to inoculate two flasks, one at 8 a.m. (i.e., flask A) and one at 8 p.m. (i.e., flask B), for each growth condition being modeled. The diluted culture used to inoculate flask A is stored at 4°C for 12 h before it is used to inoculate flask B. Although this protocol differs from that formally tested in Experiment 2, growth curves obtained by sampling flasks A and B usually yielded kinetic data that fell in line with each other. An example is illustrated in Figure 5. Here, the data points enclosed in boxes were collected from flask B, whereas the data points not enclosed in boxes were

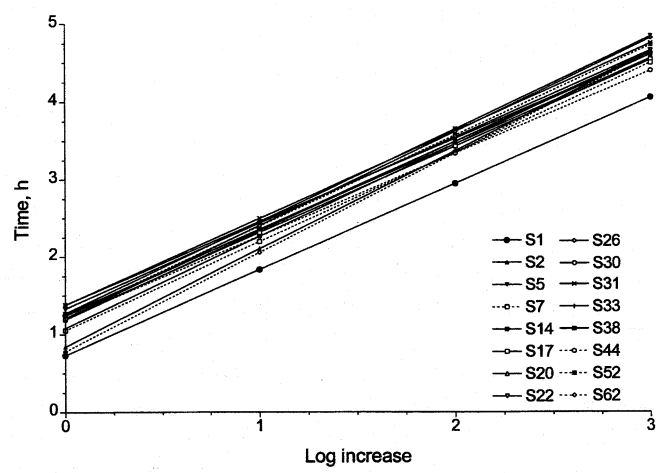

FIGURE 3. *Effect of identity of the isolate on time for a given log increase in* Salmonella *viable cell numbers in laboratory medium incubated at 40°C (Experiment 1).*

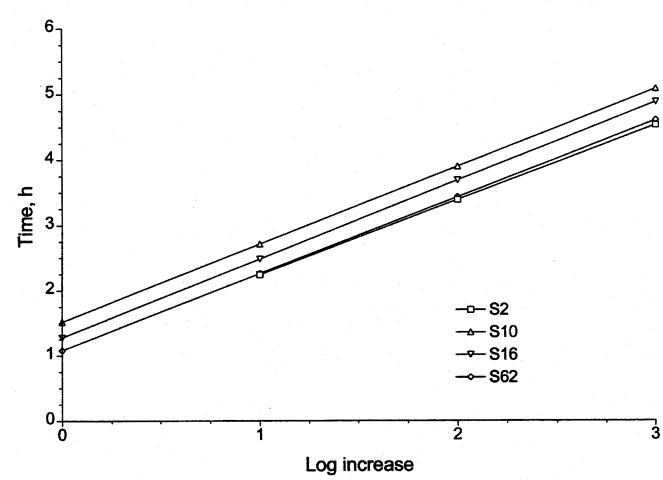

FIGURE 4. *Effect of identity of the isolate on time for a given log increase in* Salmonella *viable cell numbers in laboratory medium incubated at 37°C (Experiment 2).*

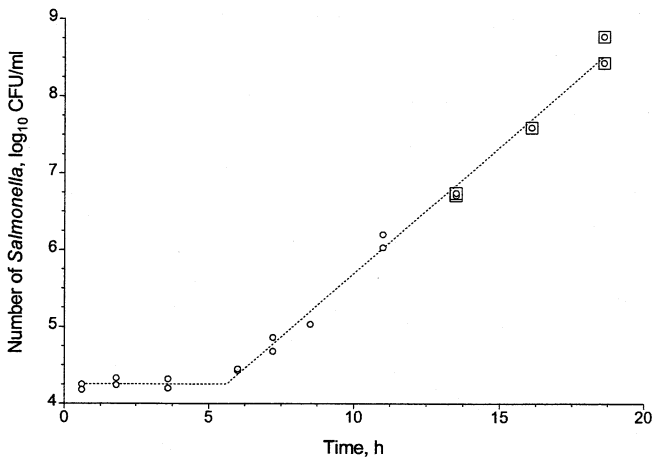

FIGURE 5. *An example of a growth curve obtained using a dual-flask system. Viable count data indicated by circles enclosed in boxes were collected from flask B, which was inoculated with isolate S2,* S. typhimurium, *at 8 p.m., whereas other viable count data (indicated by circles with no boxes) were collected from flask A, which was inoculated at 8 a.m.* S. typhimurium *was grown in brain heart infusion broth adjusted to pH 6.5 and incubated at 22.5°C.*

collected from flask A. Figure 5 also shows how well the two-phase linear equation fits the *Salmonella* growth data. The r^2 values, a measure of goodness of fit, for all 92 curve fits in this study ranged from 0.9364 to 0.9993 with a mean ± standard error of the mean of 0.9902 ± 0.0012.

DISCUSSION

Thermal death time studies indicate that although most *Salmonella* strains exhibit similar rates of inactivation there are a number of heat-tolerant *Salmonella* strains *(1, 21, 25, 26).* Notably, *Salmonella senftenberg* 775W is as much as 20-fold more heat tolerant than other serotypes of *Salmonella (21, 22)* and other strains of *S. senftenberg (5, 14).* However, at low water activity *(12)* or in exposure to dry heat conditions *(11, 23),* heat resistance of *S. senftenberg* 775W is similar to that of other *Salmonella* strains. Thus, strain identity and environmental conditions interact to influence thermal inactivation kinetics of *Salmonella* strains.

The influence of strain and environmental conditions on growth and nonthermal inactivation kinetics of *Salmonella* strains is less well defined. The available data suggest that there are only minor differences in growth kinetics (data from references *4* and *20* and this study) and minimum *(18, 19)* and maximum *(7)* temperatures at which strains of *Salmonella* grow. In addition, environmental factors appear to interact with strain to influence growth kinetics of *Salmonella* strains *(2, 9).* However, unlike heat tolerance, there do not appear to be strains of *Salmonella* with unusual growth characteristics, such as the extreme heat resistance of *S. senftenberg* 775W.

In the present study, a systematic investigation was conducted of the effect of identity of the isolate on growth kinetics of *Salmonella* isolates in a laboratory medium under two sets of environmental conditions. In Experiment 1 two ATCC isolates and 14 broiler isolates of *Salmonella* were surveyed for their growth kinetics in a laboratory medium (pH 6) at 40°C. Although most isolates exhibited similar growth kinetics, identity of the isolate was found to influence growth of *Salmonella* isolates. The maximum difference between isolates was 90% for lag time and 20% for growth rate.

The importance of assessing strain differences under a variety of growth conditions for the predictive microbiologist is to identify strains that can be used to develop predictive models that are accurate and err on the side of food safety. An appropriate strain is one that grows slightly faster than other strains pertinent to the situation being modeled. In the present study, isolate S1 was found to grow slightly faster than other isolates and thus would be a good choice for model development. This of course assumes that isolate S1 grows slightly faster under other conditions not tested but included in development of a predictive model. Again, one may want to verify the latter assumption before a final decision on use of a particular strain for modeling is made. A safe conclusion, based on the observed similarity of growth kinetics in this study, is that all isolates of *Salmonella* examined are suitable for modeling experiments, i.e., an empirical choice is acceptable.

The previous incubation temperature has been shown to influence subsequent thermal inactivation kinetics of *Salmonella* strains. Ng et al. *(21)* found that *Salmonella* strains grown at 44°C were more heat tolerant than *Salmonella* strains grown at lower temperatures (15 to 35°C). Other investigators *(6, 17)* have also observed that increasing the growth temperature enhances subsequent thermal resistance of *Salmonella* strains. Previous temperature also alters subsequent growth kinetics of foodborne pathogens. Mackey and Derrick *(15, 16)* demonstrated that the lag time of *Salmonella* growth increases when strains are previously exposed to low temperatures (−5 to −20°C) or high temperatures (50 to 57°C). Buchanan and Klawitter *(3)* for *Listeria monocytogenes* and Hudson *(13)* for *Aeromonas hydrophila* found that growing these pathogens to stationary phase at different temperatures altered their subsequent lag time but not growth rate. In contrast, I found that holding stationary phase *Salmonella* cultures at nongrowth temperature (5°C) and growth temperatures (22 and 37°C) for 24 h did not alter their subsequent growth kinetics. These results suggest that it is possible to prevent effects of preconditions on subsequent growth of *Salmonella* strains by imposing preconditions that prevent cell division and death.

Based on the latter supposition, my colleagues and I devised a protocol to prevent the need for midnight sampling in modeling experiments. In this protocol, we grow a *Salmonella* culture to stationary phase, dilute it, and then use it to inoculate two flasks; one at 8 a.m. and one at 8 p.m. The diluted *Salmonella* culture used to inoculate the first flask is held at 4°C for 12 h before being used to inoculate the second flask. By inoculating flasks at 12-h intervals, we are able to achieve 24-h coverage of sampling between 8 a.m. and 8 p.m. without the need to come into the laboratory during the middle of the night. We have used this protocol to successfully develop a predictive model for combined effects of previous pH (5.5 to 8.5), temperature (15 to 40°C),

and pH (5 to 7) on growth of isolate S2 in laboratory medium (unpublished data).

In summary, 16 broiler isolates of *Salmonella* and two ATCC isolates were surveyed for their growth kinetics in a laboratory medium under two sets of environmental conditions. In general, growth kinetics were similar between isolates indicating that all of them would be suitable for modeling experiments. However, the limited number of isolates tested and growth conditions investigated justify further research defining the variability of growth kinetics among isolates of *Salmonella.* In addition, an experiment was conducted that led to development of a two-flask system to prevent the need for midnight sampling in modeling experiments. It was demonstrated that one can inoculate two flasks at 12-h intervals and obtain similar growth kinetics in each flask by taking advantage of the inability of *Salmonella* strains to grow at refrigerated temperatures *(18, 19).*

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