Journal of Food Protection, Vol. 66, No. 2, 2003, Pages 200–207

Comparison of Predictive Models for Growth of Parent and Green Fluorescent Protein–Producing Strains of *Salmonella*†

T. P. OSCAR*

U.S. Department of Agriculture, Agricultural Research Service, Microbial Food Safety Research Unit, 1124 Trigg Hall, University of Maryland Eastern Shore, Princess Anne, Maryland 21853, USA

MS 02-149: Received 9 May 2002/Accepted 25 August 2002

ABSTRACT

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* can be expressed in, and used to follow the fate of, *Salmonella* in microbiologically complex ecosystems such as food. As a first step in the evaluation of GFP as a tool for the development of predictive models for naturally contaminated food, the present study was undertaken to compare the growth kinetics of parent and GFP-producing strains of *Salmonella*. A previously established sterile chicken burger model system was used to compare the growth kinetics of stationary-phase cells of parent and GFP strains of *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Dublin. Growth curves for constant temperatures from 10 to 48°C were fit to a two- or three-phase linear model to determine lag time, specific growth rate, and maximum population density. Secondary models for the growth parameters as a function of temperature were generated and compared between the parent and GFP strain pairs. The effects of GFP on the three growth parameters were significant and were affected by serotype and incubation temperature. The expression of GFP reduced specific growth rate and maximum population density while having only a small effect on the lag times of the three serotypes. The results of this study indicate that the growth kinetics of the GFP strains tested were different from those of the parent strains and thus would not be good marker strains for the development of predictive models for naturally contaminated food.

Predictive microbiology is a subdiscipline of food microbiology that quantifies the behavior of microorganisms as a function of food and environmental factors such as temperature, pH, and water activity *(7, 16).* The traditional approach to predictive model development involves collecting kinetic data on the growth, survival, or inactivation of microorganisms in laboratory medium under a matrix of conditions (i.e., combinations of temperature, pH, and water activity) and then using these data to develop primary, secondary, and tertiary models *(7, 16).* Kinetic data on changes in viable cell counts are usually used to develop predictive models. The increase in the number and sophistication of predictive models in recent years can be attributed to the development of automated microbiology equipment, such as plate pourers, spiral platers, and colony counters, and the personal computer, which make the collection, processing, and analysis of quantitative microbiological data fast, accurate, and convenient *(7).* Predictive models developed in this manner have formed the basis for user-friendly computer software applications, such as the United Kingdom's Food Micromodel *(16)* and the U.S. Department of Agriculture's Pathogen Modeling Program *(5, 6),* that can be used to predict the shelf life and safety of food, to evaluate the safety of new food formulations, to plan predictive modeling experiments, and to educate the public about microbial behavior in food *(31).*

The ease of collecting large amounts of kinetic data has made broth cultures the system of choice in predictive modeling studies. However, the effects of important food and microbial factors, such as the chemical composition of food, bacterial attachment, and microbial competition, on pathogen behavior cannot be completely emulated and modeled in a broth culture system. A major obstacle to the development of predictive models with naturally contaminated food is the difficulty in detecting and enumerating the pathogen of interest in a complex mixture of microorganisms in which the pathogen is usually present in very low numbers. To circumvent this problem, the food can be inoculated with a marker strain of the pathogen that possesses a phenotypic characteristic, such as luminescence *(2),* antibiotic resistance *(23),* or fluorescence *(12),* that makes it easy to detect and enumerate the pathogen on a viable cell count plate in the presence of other microorganisms. However, for the development of a successful predictive model, the marker strain of the pathogen should exhibit growth, survival, or inactivation kinetics that are not different from those of the parent strain from which it was derived.

The green fluorescent protein (GFP) is a small polypeptide (27 kDa, 238 amino acids) from the jellyfish *Aequora victoria* that has been cloned *(22)* and expressed in both prokaryotic and eukaryotic cells *(9, 15).* In bacterial applications, the *gfp* gene has been inserted in a low-copy plasmid *(13),* in a high-copy plasmid *(12),* and in the bacterial chromosome *(26).* Colonies of bacterial cells expressing GFP can be easily detected and counted with the illumination of viable cell count plates with ultraviolet light

* Author for correspondence. Tel: 410-651-6062; Fax: 410-651-6568; E-mail: toscar@mail.umes.edu.

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

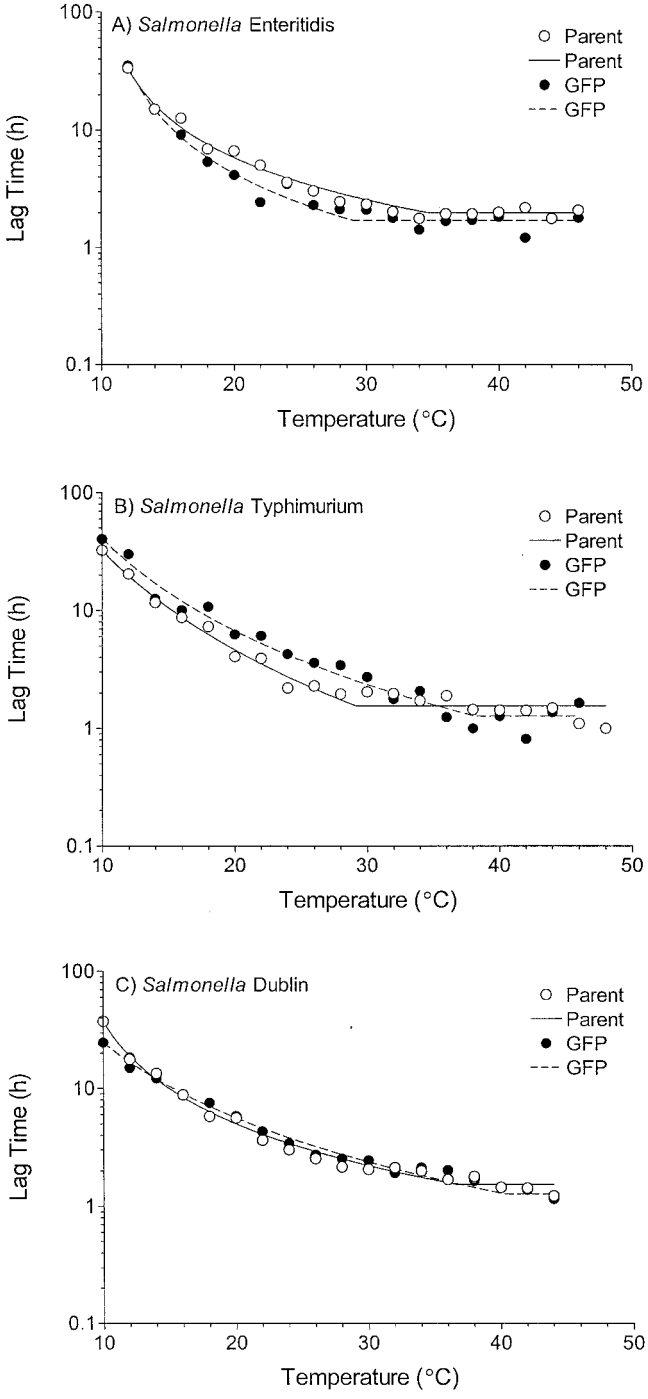

FIGURE 1. *Secondary-model fits for lag time as a function of incubation temperature for parent and GFP-producing strains of (A)* Salmonella *Enteritidis, (B)* Salmonella *Typhimurium, and (C)* Salmonella *Dublin. Symbols represent observed values, whereas lines represent predicted values.*

(365 nm). This is a desirable characteristic for predictive model development because it allows the automated counting of large numbers of plates without the need for the addition of exogenous substrates, which would be required for luminescent marker strains, or lengthy confirmation tests, which would be required for purely antibiotic-resistant marker strains. In fact, automated counting of GFP colonies on agar plates in the presence of competing microorganisms can be accomplished by adding an external charge-coupled-device camera in a darkroom box equipped with an ultraviolet light to an automated colony counting system (unpublished data).

A number of studies *(4, 11, 12, 14, 21, 27, 28)* involving GFP-producing strains of bacteria have indicated that GFP expression does not alter the biochemical, morphological, or growth and survival characteristics of the bacterium. However, only anecdotal or limited (i.e., at one temperature) data regarding the effects of GFP expression on microbial growth are provided in these studies *(4, 11, 12, 21).* Consequently, the objective of the present study was to conduct a systematic comparison of the growth kinetics of parent and GFP-producing strains of *Salmonella* over a broad range of temperatures. The hypothesis tested was that the GFP strains have growth kinetics that are not different from those of the parent strains and thus would be suitable marker strains for the construction of predictive models using naturally contaminated food.

MATERIALS AND METHODS

Strains. Dr. P. M. Fratamico (U.S. Department of Agriculture, Agricultural Research Service, Wyndmoor, Pa.) provided the parent and GFP-producing strains of *Salmonella* Enteritidis S1952, *Salmonella* Typhimurium TML, and *Salmonella* Dublin 15480 used in this study. A high-copy (pUC19) plasmid encoding the wild-type GFP from *A. victoria* and ampicillin resistance was introduced by calcium chloride precipitation into the parent strains to produce the GFP strains *(12).* Stock cultures of the parent and GFP strains were maintained at −70°C in brain heart infusion broth (Difco Laboratories, Sparks, Md.) that contained 15% (vol/vol) glycerol.

Challenge studies. Five microliters of the stock culture was inoculated into 5 ml of brain heart infusion broth for the parent strains and brain heart infusion broth plus 100 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml for the GFP strains. Stationary-phase cells for inoculation were obtained by incubating the cultures at 37°C for 23 h under aerobic conditions *(18).*

Stationary-phase cultures were diluted in buffered peptone water (Difco), and then 100 μl was inoculated into a well (1.2 cm^2) on the surface of sterilized (autoclaved at 121°C for 18 min) chicken breast (*Salmonella* Enteritidis) or thigh (*Salmonella* Typhimurium and *Salmonella* Dublin) burgers, which were prepared as previously described *(19).* The target level of *Salmonella* was 10^6 CFU/cm^2. Burgers were inoculated at 4°C and then incubated at constant temperatures from 12 to 46°C for *Salmonella* Enteritidis, from 10 to 48°C for *Salmonella* Typhimurium, and from 10 to 44°C for *Salmonella* Dublin. Each growth curve involved one strain and eight burgers.

At each sampling time, a chicken burger (6 g after autoclaving) was homogenized in a stomacher blender (model 400, Seward, London, UK) for 1 min in 94 ml of buffered peptone water. Undiluted and diluted homogenate (50 μl) was spiral plated (Whitely Automatic Spiral Plater, Don Whitley Scientific, West Yorkshire, UK) onto brain heart infusion agar for the parent strains and onto brain heart infusion agar plus ampicillin (100 μg/ml) for the GFP strains. Ampicillin was included in the agar medium for the GFP strains so that only inoculated cells that retained the GFP plasmid (pGFP) after passage on chicken burgers without ampicillin were counted. Spiral plates were inverted and incubated at 30°C for 24 h before automated colony counting was carried out (Protocol, Microbiology International, Frederick, Md.).

TABLE 1. *Comparison of secondary-model parameters for lag time between parent and GFP-producing strains of* Salmonella

Serotype	Parameter	Best-fit value (SE) for: Parent strain	Best-fit value (SE) for: GFP strain	*t* test significance[a]
Salmonella Enteritidis	p	44.29 (6.76)	28.14 (2.81)	*
	q	9.38 (0.62)	9.39 (0.70)	NS
	m	1.24 (0.16)	1.50 (0.23)	NS
	$T_{\lambda_{min}}$	34.62 (4.13)	28.95 (1.92)	NS
Salmonella Typhimurium	p	34.93 (1.29)	41.48 (3.43)	NS
	q	−1.02 (2.92)	0.45 (4.78)	NS
	m	3.02 (0.62)	2.53 (0.92)	NS
	$T_{\lambda_{min}}$	29.17 (1.77)	38.17 (11.93)	NS
Salmonella Dublin	p	40.32 (3.48)	44.99 (2.13)	NS
	q	6.59 (0.55)	0.24 (2.66)	*
	m	1.47 (0.14)	2.10 (0.39)	NS
	$T_{\lambda_{min}}$	36.78 (4.32)	40.4 (7.86)	NS

[a] *, $P < 0.05$; NS, $P > 0.05$.

Primary modeling. Viable cell counts (N, log CFU/ml of homogenate) were graphed as a function of sampling time (t, h) and then lag time (λ, h); specific growth rate (μ, log CFU/h) and maximum population density (MPD; log CFU/cm^2) were determined by nonlinear regression (Prism, version 3.0, GraphPad Software Inc., San Diego, Calif.) with a two- or three-phase linear model *(8)*:

$$N = \begin{cases} N_0 & \text{if } t \le \lambda \\ N_0 + \mu(t - \lambda) & \text{if } \lambda < t < t_{max} \\ \text{MPD} & \text{if } t \ge t_{max} \end{cases}$$

where N_0 is the initial viable cell count (log CFU/ml of homogenate) and t_{max} is the time (h) at which MPD was reached.

Secondary modeling. Secondary models predicting lag time as a function of incubation temperature (T, °C) were obtained by nonlinear regression (Prism) with a two-phase hyperbola model:

$$\lambda = \begin{cases} [p/(T - q)]^m & \text{if } T < T_{\lambda_{min}} \\ \lambda_{min} & \text{if } T \ge T_{\lambda_{min}} \end{cases}$$

where p describes the change in lag time from q to $T_{\lambda_{min}}$, q is the temperature (°C) at which lag time was infinite, m is a shape parameter, λ_{min} is the minimum lag time or bottom plateau of the lag time curve, and $T_{\lambda_{min}}$ is the incubation temperature (°C) at which lag time became minimal. The two-phase hyperbola model is a modification of an earlier modification *(20)* of the hyperbola model for lag time *(34)*. In the previous modification *(20)*, the shape parameter m was introduced to eliminate prediction bias at high incubation temperatures, whereas in the present modification a bottom plateau was introduced to better fit the lag time data at high incubation temperatures.

Secondary models predicting specific growth rate as a function of T were obtained by nonlinear regression (Prism) with a cardinal temperature model *(24)*:

$$\mu = \begin{cases} 0 & \text{if } T \le T_{min} \quad \text{or} \quad T \ge T_{max} \\ \mu_{opt}(D/E) & \text{if } T_{min} < T < T_{max} \end{cases}$$

$$D = (T - T_{max})(T - T_{min})$$

$$E = (T - T_{min})(T - T_{max}) - (T - T_{opt})^2$$

where T_{min} is the minimum growth temperature (°C), T_{max} is the maximum growth temperature (°C), T_{opt} is the optimum growth temperature (°C), and μ_{opt} is the optimum specific growth rate.

Secondary models predicting MPD as a function of T were obtained by fitting the data to the model of Wijtzes et al. *(32)*:

$$\text{MPD} = \alpha\left[\frac{(T - T_{min})(T - T_{max})}{(T - T_{sub\,min})(T - T_{sup\,max})}\right]$$

where α is a regression coefficient, $T_{sub\ min}$ is a temperature (°C) just below T_{min}, and $T_{sup\ max}$ is a temperature (°C) just above T_{max}. To achieve convergence of the curve fits, T_{min} and T_{max} were fixed at constant values on the basis of the results of the secondary-model fits for lag time and specific growth rate.

Statistical analysis. The original experimental design involved one growth curve at each 2°C increment in temperature from 8 to 48°C for the three pairs of parent and GFP strains. However, differences in the minimum and maximum growth temperatures were observed among the serotypes. Notably, *Salmonella* Enteritidis grew over a narrower temperature range than did *Salmonella* Typhimurium or *Salmonella* Dublin. Accurate determination of growth parameters was more difficult at temperatures close to the growth and no-growth interfaces. Consequently, the final temperature ranges for secondary-model development were narrower than originally planned and were slightly different among the serotypes. Although six strains were studied, statistical comparisons of secondary-model fits were limited to the parent and GFP strain pairs because the type of chicken meat (i.e., breast or thigh) used and the temperature range of the growth kinetic measurements were the same within but not between the parent and GFP strain pairs.

The secondary model fits for lag time, specific growth rate, and MPD for the parent and GFP strain pairs were compared by an F test *(17)*:

$$F = \frac{(SS_c - SS_s)/(df_c - df_s)}{SS_s/df_s}$$

where SS_c is the sum of squares of the curve fit for the combined parent and GFP strain data, SS_s is the sum of squares of the curve fit for the parent strain plus the sum of squares of the curve fit for the GFP strain, df_c is the degrees of freedom for the curve fit of the combined data, and df_s is the degrees of freedom for the curve fit for the parent strain plus the degrees of freedom for the curve fit for the GFP strain. The curve fits were carried out with

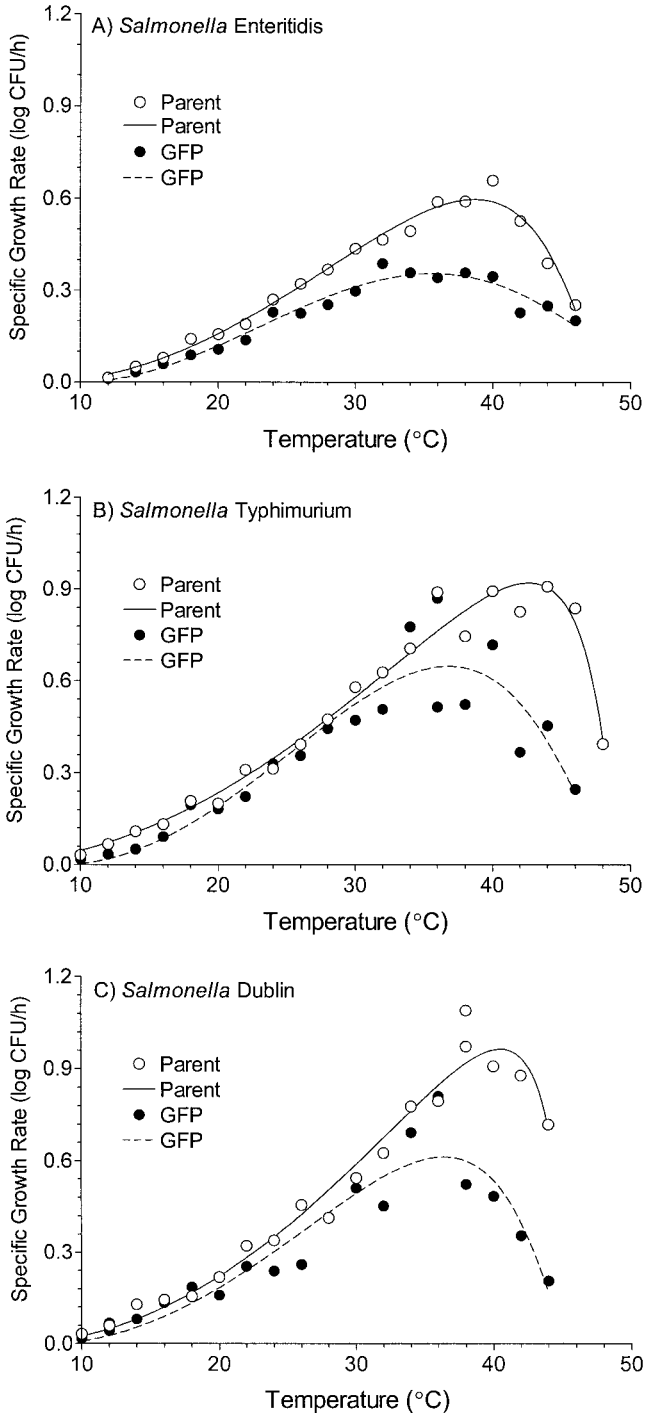

FIGURE 2. *Secondary-model fits for specific growth rate as a function of incubation temperature for parent and GFP-producing strains of (A)* Salmonella *Enteritidis, (B)* Salmonella *Typhimurium, and (C)* Salmonella *Dublin. Symbols represent observed values, whereas lines represent predicted values.*

Prism, whereas the F test was conducted with a Microsoft Excel 2000 spreadsheet (Microsoft Corporation, Redmond, Wash.).

The parameters of the secondary-model fits for the parent and GFP strain pairs were compared by a t test with a Microsoft Excel 2000 spreadsheet *(17)*:

$$t = \frac{B_p - B_{gfp}}{\sqrt{SE_p^2 + SE_{gfp}^2}}$$

where B_p is the best-fit value for the parent strain, B_{gfp} is the best-fit value for the GFP strain, SE_p is the standard error of the best-fit value for the parent strain, and SE_{gfp} is the standard error of the best-fit value for the GFP strain. P values of <0.05 were considered significant for both the F test and the t test.

RESULTS

The MPD of the broth starter cultures was 1 to 1.5 log cycles lower for the GFP strains. To adjust for this difference, a 10^{-3} dilution of the parent strain cultures and a 10^{-2} dilution of the GFP strain cultures were used to inoculate the chicken burgers. The inoculation of these dilutions resulted in the initial density on the burgers being 0.5 log cycles lower (i.e., 5.5 versus 6 log CFU/cm^2) for the GFP strains than for the parent strains of *Salmonella* Enteritidis and *Salmonella* Typhimurium, whereas the initial density of the GFP strain of *Salmonella* Dublin was the same as that of the parent strain. In a separate study, the growth kinetics of the GFP strain of *Salmonella* Typhimurium at 25°C were not different at initial densities of 5 and 6 log CFU per cm^2 (unpublished data). In addition, the effects of GFP on lag time, specific growth rate, and MPD on the chicken burgers in the present study (see below) were generally similar among the serotypes, which suggested that the small difference in initial density between the parent and GFP strains of *Salmonella* Enteritidis and *Salmonella* Typhimurium did not grossly affect the results of this study.

Differences in lag time on the chicken burgers were observed between the parent and GFP strains of *Salmonella.* Predicted lag time (i.e., the secondary-model curve fit) was shorter ($F = 8.95$, $P < 0.05$) for the GFP strain than for the parent strain of *Salmonella* Enteritidis at incubation temperatures of 16 to 46°C (Fig. 1A). Specifically, the p parameter, which describes the rate of change in lag time as a function of temperature from q to $T_{\lambda_{min}}$, was lower for the GFP strain than for the parent strain of *Salmonella* Enteritidis (Table 1). Other parameters of the two-phase hyperbola model for lag time did not differ between the parent and GFP strains of *Salmonella* Enteritidis (Table 1). The λ_{min} value (minimum lag time, or the bottom plateau of the lag time curve as a function of temperature) was 2.01 h for the parent strain and 1.73 h for the GFP strain (Fig. 1A). Whether λ_{min} values were statistically different between the parent and the GFP strains was not determined because the λ_{min} values were predicted (i.e., calculated from the secondary-model fit) rather than best-fit (i.e., obtained by fitting the lag time data to the secondary model) values.

Differences in lag time between the parent and GFP strains of *Salmonella* Typhimurium were also observed. Predicted lag times were longer for the GFP strain at temperatures of 10 to 34°C and shorter at temperatures of 36 to 46°C (Fig. 1B). The F test of the secondary-model fits indicated that the lag time of *Salmonella* Typhimurium was affected ($F = 10.84$, $P < 0.05$) by GFP. Nonetheless, none of the parameters of the two-phase hyperbola model were statistically different between the parent and GFP strains of *Salmonella* Typhimurium (Table 1). The λ_{min} values were 1.55 h for the parent strain and 1.27 h for the GFP strain, and the results for *Salmonella* Enteritidis were similar.

Lag time for the parent and GFP strains of *Salmonella*

TABLE 2. *Comparison of secondary-model parameters for specific growth rate between parent and GFP-producing strains of* Salmonella

Serotype	Parameter	Best-fit value (SE) for:		*t* test significance[a]
		Parent strain	GFP strain	
Salmonella Enteritidis	T_{min}	6.89 (1.26)	9.95 (1.79)	NS
	T_{max}	48.30 (0.51)	53.59 (2.49)	*
	T_{opt}	38.69 (0.39)	35.66 (0.73)	*
	μ_{opt}	0.77 (0.01)	0.60 (0.01)	*
Salmonella Typhimurium	T_{min}	2.15 (1.76)	8.61 (3.73)	NS
	T_{max}	49.24 (0.37)	49.41 (2.33)	NS
	T_{opt}	42.64 (0.49)	36.80 (1.33)	*
	μ_{opt}	0.96 (0.01)	0.80 (0.03)	*
Salmonella Dublin	T_{min}	4.98 (2.15)	7.35 (3.02)	NS
	T_{max}	45.81 (1.04)	45.72 (1.09)	NS
	T_{opt}	40.54 (0.64)	36.39 (1.00)	*
	μ_{opt}	0.98 (0.02)	0.78 (0.02)	*

[a] *, $P < 0.05$; NS, $P > 0.05$.

Dublin appeared similar at most incubation temperatures (Fig. 1C). Nonetheless, the F test indicated that the lag time for *Salmonella* Dublin was affected ($F = 49.6$, $P < 0.05$) by GFP. Predicted lag times for the GFP strain were slightly longer or shorter than those for the parent strain, depending on the incubation temperature (Fig. 1C). Only the q parameters (temperature where lag time was infinite) of the two-phase hyperbola model were different between the parent and GFP strains of *Salmonella* Dublin (Table 1). The λ_{min} values were 1.53 h for the parent strain and 1.27 h for the GFP strain of *Salmonella* Dublin.

In summary, the effects of GFP on lag time were significant and slightly different among the serotypes of *Salmonella*. Whether GFP increased, decreased, or did not alter lag time depended on the incubation temperature and serotype. The most consistent observation was that λ_{min} was 0.26 to 0.28 h shorter for the GFP strains than for the parent strains. Thus, at high incubation temperatures, the GFP strains initiated growth slightly earlier than the parent strains did.

The effects of GFP on specific growth rate were more consistent among the serotypes. The predicted specific growth rate of the GFP strain of *Salmonella* Enteritidis was lower ($F = 63$, $P < 0.05$) at all temperatures (Fig. 2A). The only parameter of the cardinal temperature model for specific growth rate that was not affected by GFP expression was T_{min} (Table 2). It is notable that the expression of GFP lowered T_{opt} by 3°C and reduced μ_{opt} by 0.17 log CFU/h.

As it did for *Salmonella* Enteritidis, GFP decreased ($F = 17.05$, $P < 0.05$) the specific growth rate of *Salmonella* Typhimurium at low (i.e., 10 to 22°C) and high (i.e., 30 to 46°C) incubation temperatures (Fig. 2B). However, specific growth rates were similar at ambient (24 to 28°C) temperatures, which is consistent with the results of another study (unpublished data) in which specific growth rates at 25°C were not found to be different between the parent and GFP strains of *Salmonella* Typhimurium. The T_{min} and T_{max} parameters for specific growth rate were similar for the parent and GFP strains; however, GFP lowered T_{opt} by 6°C, and GFP lowered μ_{opt} by 0.16 log CFU/h (Table 2).

The specific growth rate of *Salmonella* Dublin was also reduced ($F = 25.1$, $P < 0.05$) by GFP, with the extent of reduction increasing with incubation temperature (Fig. 2C). Similar to the case for *Salmonella* Typhimurium, T_{min} and T_{max} for specific growth rate were not affected by GFP expression, whereas T_{opt} was decreased by 4°C and μ_{opt} was reduced by 0.20 log CFU/h in the GFP strain (Table 2).

Thus, GFP reduced specific growth rate for the three serotypes tested. The magnitude of the reduction in specific growth rate was dependent on the incubation temperature and the serotype. The most consistent effects were that GFP reduced T_{opt} for specific growth rate by 3 to 6°C and decreased μ_{opt} by 0.17 to 0.2 log CFU/h.

Not all of the growth curves exhibited three phases of growth. In some instances, sampling was not extended for enough time to detect the stationary phase. Consequently, MPD data were not obtained for all incubation temperatures. Nonetheless, as it did for the MPD of the broth starter cultures, GFP lowered ($F = 8.71$ to 17.33, $P < 0.05$) the MPD on the chicken burgers (Fig. 3). However, the only parameters of the secondary-model fits for MPD that were affected by GFP expression were $T_{sup\ max}$ for *Salmonella* Enteritidis and α and $T_{sup\ max}$ for *Salmonella* Dublin (Table 3). Predicted MPDs were 1 to 2.4 log cycles lower for the GFP strain than for the parent strain of *Salmonella* Enteritidis (Fig. 3A), 1 to 2 log cycles lower for the GFP strain than for the parent strain of *Salmonella* Typhimurium (Fig. 3B), and 0 to 2 log cycles lower for the GFP strain than for the parent strain of *Salmonella* Dublin (Fig. 3C).

DISCUSSION

The hypothesis tested in this study was that GFP expression does not alter the growth kinetics of *Salmonella* and that the GFP strains would therefore be suitable marker strains for the development of predictive models for naturally contaminated food. To test this hypothesis, predictive

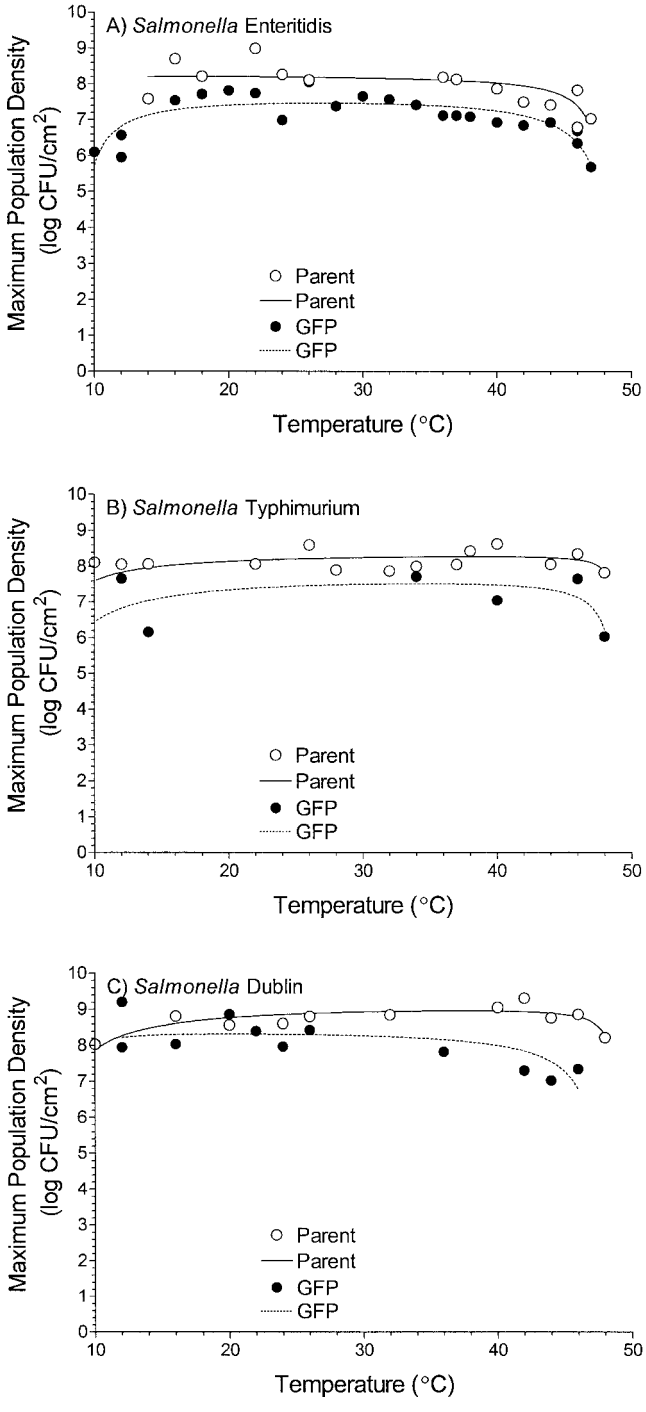

FIGURE 3. *Secondary-model fits for MPD as a function of incubation temperature for parent and GFP-producing strains of (A)* Salmonella *Enteritidis, (B)* Salmonella *Typhimurium, and (C)* Salmonella *Dublin. Symbols represent observed values, whereas lines represent predicted values.*

models for the growth of three parent and three GFP strains of *Salmonella* on sterile chicken burgers over a broad temperature range were developed and compared. The results of this study indicate that GFP expression altered lag time and specific growth rate on sterile chicken burgers and that it altered MPD on sterile chicken burgers and in broth starter cultures. The effects of GFP on the growth of *Salmonella* were affected by serotype and incubation temperature, but in general, GFP expression reduced specific growth rate and MPD while having a small and inconsistent effect on lag time. Thus, the hypothesis was rejected and it was concluded that the GFP strains of *Salmonella* tested would not be good marker strains for the development of predictive models for naturally contaminated food.

The failure of the three GFP strains tested to display growth kinetics similar to those of the parent strains may have resulted from the overexpression of GFP. The plasmid encoding GFP in the present study was a high-copy plasmid in which *gfp* was under the control of the *lac*Z promoter, for which most *Salmonella* do not have a *lac*I gene encoding for the *lac* repressor protein *(25).* In fact, the addition of a *lac*Z inducer, isopropylthio-β-galactoside, to the agar medium in this study did not result in the enhanced fluorescence of the GFP strain colonies (results not shown), which suggests that GFP was being constitutively expressed in the three serotypes tested. Crameri et al. *(10)* reported that GFP accounts for up to 75% of the total cellular protein in bacteria that constitutively express GFP. Such a high level of marker protein expression could slow growth and decrease MPD by creating a competition for and an eventual deficiency of essential nutrients *(30).* Alternatively, hydrogen peroxide produced in the terminal reaction of GFP formation could create a toxic effect that could reduce specific growth rate and MPD *(30).*

Although the results of this study indicate that growth kinetics of the GFP strains tested are different from those of the parent strains and thus would not be good strains for the development of predictive models for naturally contaminated food, it should be possible to construct marker strains of *Salmonella* that do not overexpress GFP and grow in a manner similar to that of the parent strains. For example, if *gfp* were placed under the control of a different promoter, such as P_{BAD}, that requires an inducer (i.e., arabinose) not found in food *(3),* the expression of GFP could be repressed during the growth of the pathogen on the food but could then be induced during the growth of the pathogen on the viable cell count plate through the inclusion of the inducer in the agar medium. Unlike other applications of GFP in which high levels of expression are needed for in situ detection of single cells *(33),* lower levels of expression would be acceptable for the detection of GFP colonies on viable cell count plates. Thus, the use of a low-copy plasmid, such as pCM18 *(13),* or the insertion of a single copy of the *gfp* gene into the pathogen's chromosome *(29),* both of which result in lower expression of GFP than do high-copy plasmids, may be acceptable alternatives for the construction of GFP strains for predictive microbiology applications.

A limited number of studies have reported on the effects of GFP expression on microbial growth. Bae and Knudsen reported *(1)* that the mycelial growth of the fungus *Trichoderma harzianum* was slowed by GFP, consistent with the results of the present study. In contrast, Fratamico et al. *(12),* who developed the GFP strains used in this study, reported that GFP did not alter the growth of *Escherichia coli* O157:H7 strains at 37°C in broth culture. Likewise, Prachaiyo and McLandsborough *(21)* did not observe any difference in the growth kinetics of parent and GFP

TABLE 3. *Comparison of secondary-model parameters for maximum population density between parent and GFP-producing strains of* Salmonella

Serotype	Parameter	Best-fit value (SE) for: Parent strain	Best-fit value (SE) for: GFP strain	*t* test significance[a]
Salmonella Enteritidis	α	8.29 (0.32)	7.86 (0.16)	NS
	T_{min}	8.24 (fixed)	8.72 (fixed)	NA[b]
	T_{max}	48.07 (fixed)	48.71 (fixed)	NA
	$T_{sub\ min}$	8.22 (0.41)	8.27 (0.11)	NS
	$T_{sup\ max}$	48.32 (0.09)	49.31 (0.12)	*
Salmonella Typhimurium	α	8.44 (0.14)	7.89 (0.58)	NS
	T_{min}	5.37 (fixed)	5.65 (fixed)	NA
	T_{max}	48.82 (fixed)	49.00 (fixed)	NA
	$T_{sub\ min}$	4.86 (0.15)	4.71 (0.52)	NS
	$T_{sup\ max}$	48.87 (0.04)	49.24 (0.18)	NS
Salmonella Dublin	α	9.24 (0.10)	8.55 (0.23)	*
	T_{min}	5.88 (fixed)	8.00 (fixed)	NA
	T_{max}	48.98 (fixed)	48.00 (fixed)	NA
	$T_{sub\ min}$	5.18 (0.09)	7.90 (fixed)	NA
	$T_{sup\ max}$	49.08 (0.03)	48.51 (0.20)	*

[a] *, $P < 0.05$; NS, $P > 0.05$.
[b] NA, parameter was fixed during curve fitting, and therefore a *t* test comparison was not applicable.

strains of *E. coli* O157:H7 in a laboratory medium incubated at 37°C. Also, Bloemberg et al. *(4)* reported no effect of GFP on growth rate or MPD for *Pseudomonas* spp., and de Palencia et al. *(11)* reported no effect of GFP on growth rate or MPD for *Lactococcus lactis* in laboratory medium incubated at unspecified temperatures. Differences in microorganism types, plasmid constructs, test matrices, and other experimental conditions may explain the differences in results obtained in these studies and those obtained in the present study, in which specific growth rate and MPD were significantly reduced at 37°C on sterile chicken burgers. Notably, *E. coli* typically possesses *lac*I, and thus GFP expression may be lower than that in transformed *Salmonella* with *gfp* under the control of the *lac*Z promoter, as in the present study. Nonetheless, the aforementioned studies indicate that GFP strains that are suitable for predictive model development in naturally contaminated food may already exist. However, a more detailed (i.e., involving multiple temperatures) comparison of the parent and GFP strains from these studies may be warranted, as the effects of GFP expression on the growth kinetics of *Salmonella* were affected by incubation temperature and serotype in the present study.

In summary, GFP has great potential as a cellular tag to aid in following and modeling the growth kinetics of human pathogens in food. However, the expression of GFP needs to be designed such that overexpression or other unknown effects of GFP in pathogens growing on food does not compromise the rate and extent of growth. In the present study, the constitutive expression of GFP in three serotypes of *Salmonella* is offered as an explanation for the reduced specific growth rates and MPDs of the GFP strains. A strategy is proposed for predictive microbiology applications of GFP in which the expression of GFP is repressed during the growth of the pathogen on food but is induced during the growth of GFP pathogens on viable cell count plates. The use of an inducible promoter or a low-copy plasmid or the insertion of a single copy of the *gfp* gene into the chromosome are proposed as possible solutions to the problem of overexpression of GFP during the growth of the GFP marker strains of *Salmonella* on food.

ACKNOWLEDGMENTS

The author appreciates the excellent technical and administrative assistance of Jaci Wheatley, Pat Shannon, Doane Dariah, and Gabe Hughes, which made this project a success.

REFERENCES

1. Bae, Y. S., and G. R. Knudsen. 2000. Cotransformation of *Trichoderma harzianum* with beta-glucuronidase and green fluorescent protein genes provides a useful tool for monitoring fungal growth and activity in natural soils. *Appl. Environ. Microbiol.* 66:810–815.
2. Bautista, D. A., J. Chen, S. Barbut, and M. W. Griffiths. 1998. Use of an autobioluminescent *Salmonella hadar* to monitor the effects of acid and temperature treatments on cell survival and viability on lactic acid-treated poultry carcasses. *J. Food Prot.* 61:1439–1445.
3. Berry, E. D. 2000. Development and demonstration of RNA isolation and RT-PCR procedures to detect *Escherichia coli* O157:H7 gene expression on beef carcass surfaces. *Lett. Appl. Microbiol.* 31:265–269.
4. Bloemberg, G. V., G. A. O'Toole, B. J. J. Lugtenberg, and R. Kolter. 1997. Green fluorescent protein as a marker for *Pseudomonas* spp. *Appl. Environ. Microbiol.* 63:4543–4551.
5. Buchanan, R. L. 1991. Using spreadsheet software for predictive microbiology applications. *J. Food Saf.* 11:123–134.
6. Buchanan, R. L. 1993. Developing and distributing user-friendly application software. *J. Ind. Microbiol.* 12:251–255.
7. Buchanan, R. L. 1993. Predictive food microbiology. *Trends Food Sci. Technol.* 4:6–11.
8. Buchanan, R. L., R. C. Whiting, and W. C. Damert. 1997. When is simple good enough: a comparison of the Gompertz, Baranyi and three-phase linear models for fitting bacterial growth curves. *Food Microbiol.* 14:313–326.
9. Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher.

1994. Green fluorescent protein as a marker for gene expression. *Science* 263:802–805.
10. Crameri, A., E. A. Whitehorn, E. Tate, and W. P. C. Stemmer. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.* 14:315–319.
11. de Palencia, P. F., C. Nieto, P. Acebo, M. Espinosa, and P. Lopez. 2000. Expression of green fluorescent protein in *Lactococcus lactis*. *FEMS Microbiol. Lett.* 183:229–234.
12. Fratamico, P. M., M. Y. Deng, T. P. Strobaugh, and S. A. Palumbo. 1997. Construction and characterization of *Escherichia coli* O157:H7 strains expressing firefly luciferase and green fluorescent protein and their use in survival studies. *J. Food Prot.* 60:1167–1173.
13. Hansen, M. C., R. J. Palmer, C. Udsen, D. C. White, and S. Molin. 2001. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology* 147:1383–1391.
14. Himathongkham, S., H. Riemann, S. Bhari, S. Nuanualsuwan, P. Kass, and D. O. Cliver. 2000. Survival of *Salmonella typhimurium* and *Escherichia coli* O157:H7 in poultry manure and manure slurry at sublethal temperature. *Avian Dis.* 44:853–860.
15. Inouye, S., and F. I. Tsuji. 1994. *Aequorea* green fluorescent protein—expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett.* 341:277–280.
16. McClure, P. J., C. D. Blackburn, M. B. Cole, P. S. Curtis, J. E. Jones, J. D. Legan, I. D. Ogden, M. W. Peck, T. A. Roberts, J. P. Sutherland, and S. J. Walker. 1994. Modelling the growth, survival and death of microorganisms in foods: the UK Food Micromodel approach. *Int. J. Food Microbiol.* 23:265–275.
17. Motulsky, H. J. 1999. Analyzing data with GraphPad Prism®, p. 211–232. GraphPad Software, Inc., San Diego, Calif.
18. Oscar, T. P. 1998. Growth kinetics of *Salmonella* isolates in a laboratory medium as affected by isolate and holding temperature. *J. Food Prot.* 61:964–968.
19. Oscar, T. P. 1999. Response surface models for effects of temperature and previous temperature on lag time and specific growth rate of *Salmonella* Typhimurium on cooked ground chicken breast. *J. Food Prot.* 62:1111–1114.
20. Oscar, T. P. 2002. Development and validation of a tertiary simulation model for predicting the potential growth of *Salmonella* Typhimurium on cooked chicken. *Int. J. Food Microbiol.* 76:177–190.
21. Prachaiyo, P., and L. A. McLandsborough. 2000. A microscopic method to visualize *Escherichia coli* interaction with beef muscle. *J. Food Prot.* 63:427–433.
22. Prasher, D. C., V. K. Eckenrode, W. W. Ward, F. G. Prendergast, and M. J. Cormier. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* 111:229–233.
23. Rice, K. M., and M. D. Pierson. 1982. Inhibition of *Salmonella* by sodium nitrite and potassium sorbate in frankfurters. *J. Food Sci.* 47:1615–1617.
24. Rosso, L., J. R. Lobry, S. Bajard, and J. P. Flandrois. 1995. Convenient model to describe the combined effects of temperature and pH on microbial growth. *Appl. Environ. Microbiol.* 61:610–616.
25. Sanderson, K. E., and J. A. Hurley. 1987. Linkage map of *Salmonella typhimurium,* p. 877–918. *In* F. C. Neidhart (ed.), *Escherichia coli* and *Salmonella typhimurium.* ASM Press, Washington, D.C.
26. Scott, K. P., D. K. Mercer, A. J. Richardson, C. M. Melville, L. A. Glover, and H. J. Flint. 2000. Chromosomal integration of the green fluorescent protein gene in lactic acid bacteria and the survival of marked strains in human gut simulations. *FEMS Microbiol. Lett.* 182:23–27.
27. Siemering, K. R., R. Golbik, R. Sever, and J. Haseloff. 1996. Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr. Biol.* 6:1653–1663.
28. Tolker-Nielsen, T., U. C. Brinch, P. C. Ragas, J. B. Andersen, C. S. Jacobsen, and S. Molin. 2000. Development and dynamics of *Pseudomonas* sp. biofilms. *J. Bacteriol.* 182:6482–6489.
29. Tombolini, R., A. Unge, M. E. Davey, F. J. Debruijn, and J. K. Jansson. 1997. Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. *FEMS Microbiol. Ecol.* 22:17–28.
30. Tsien, R. Y. 1998. The green fluorescent protein. *Annu. Rev. Biochem.* 67:509–544.
31. Whiting, R. C., and R. L. Buchanan. 1994. Microbial modeling. *Food Technol.* 48:113–120.
32. Wijtzes, T., J. C. de Wit, J. H. J. Huis in't Veld, K. van't Riet, and M. H. Zwietering. 1995. Modeling bacterial growth of *Lactobacillus curvatus* as a function of acidity and temperature. *Appl. Environ. Microbiol.* 57:780–783.
33. Wu, H., Z. Song, M. Hentzer, J. B. Andersen, A. Heydorn, K. Mathee, C. Moser, L. Eberl, S. Molin, N. Hoiby, and M. Givskov. 2000. Detection of *N*-acylhomoserine lactones in lung tissues of mice infected with *Pseudomonas aerugionsa*. *Microbiology* 146:2481–2493.
34. Zwietering, M. H., J. T. de Koos, B. E. Hasenack, J. C. de Wit, and K. van't Riet. 1991. Modeling of bacterial growth as a function of temperature. *Appl. Environ. Microbiol.* 57:1094–1101.