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Transformation of *Escherichia coli* K-12 with a High-Copy Plasmid Encoding the Green Fluorescent Protein Reduces Growth: Implications for Predictive Microbiology†

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ABSTRACT

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been widely used as a biomarker and has potential for use in developing predictive models for growth of pathogens on naturally contaminated food. However, constitutive production of GFP can reduce growth of transformed strains. Consequently, a high-copy plasmid with *gfp* under the control of a tetracycline-inducible promoter (pTGP) was constructed. The plasmid was first introduced into a tetracycline-resistant strain of *Escherichia coli* K-12 to propagate it for subsequent transformation of tetracycline-resistant strains of *Salmonella.* In contrast to transformed *E. coli* K-12, which only fluoresced in response to tetracycline, transformed *Salmonella* fluoresced maximally without tetracycline induction of *gfp.* Although pTGP did not function as intended in *Salmonella,* growth of parent and GFP *E. coli* K-12 was compared to test the hypothesis that induction of GFP production reduced growth. Although GFP production was not induced during growth on sterile chicken in the absence of tetracycline, maximum specific growth rate (μ_{max}) of GFP *E. coli* K-12 was reduced 40 to 50% ($P < 0.05$) at 10, 25, and 40°C compared with the parent strain. When growth of parent and GFP strains of *E. coli* K-12 was compared in sterile broth at 40°C, μ_{max} and maximum population density of the GFP strain were reduced ($P < 0.05$) to the same extent (50 to 60%) in the absence and presence of tetracycline. These results indicated that transformation reduced growth of *E. coli* K-12 independent of *gfp* induction. Thus, use of a low-copy plasmid or insertion of *gfp* into the chromosome may be required to construct valid strains for development of predictive models for growth of pathogens on naturally contaminated food.

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been widely used as a biomarker in eukaryotic and prokaryotic cells *(8, 20, 21, 31, 36).* In prokaryotes, GFP has been used to follow growth, death, and dissemination of bacteria in water *(9, 24),* food *(2, 7, 11, 15, 16),* and environmental systems *(13, 19, 34, 38).* Many reports indicate that GFP production does not alter the biochemical, morphological, or behavioral characteristics of the transformed microbial cells *(4, 12, 14, 15, 19, 28, 37).* Transformation methods include use of high-copy plasmids *(1, 15)* or low-copy plasmids *(17, 37)* and insertion of *gfp* into the chromosome *(16, 18, 22, 35).* Production of GFP has been constitutive *(14, 25, 26)* or inducible *(27, 29, 30),* depending on the application of the marker organisms.

A few reports have recently appeared indicating that GFP reduces growth of transformed bacteria. Rang et al. *(29)* found that doubling time of enteric bacteria increased in proportion to the amount of GFP produced, which was altered with an inducible promoter. Oscar *(26)* reported that constitutive expression of *gfp* reduced growth of *Salmonella* and that the magnitude of the effect depended on the strain and incubation temperature.

In a recent exposure assessment for food pathogens, the lack of models that predict behavior of pathogens in naturally contaminated food was noted *(10).* Pathogens transformed with *gfp* have potential as marker organisms for developing predictive models in food *(26, 37).* However, before a GFP-transformed pathogen can be used to develop a model in food with competitive microflora, the behavior of the GFP pathogen must be confirmed as the same as that of its parent strain *(26, 37).*

In predictive microbiology applications, production of GFP is not required during growth of a pathogen on food. Rather, GFP production is only required during pathogen enumeration. In the current study, a tetracycline-resistant strain of *Escherichia coli* K-12 and four tetracycline-resistant strains of *Salmonella* were transformed with a high-copy plasmid encoding *gfp* under the control of a tetracycline-inducible promoter (pTGP). This promoter was selected because tetracycline is not normally found in food. *E. coli* K-12 was selected to create a strain for propagation of pTGP for transformation of *Salmonella.* The objective was to determine whether induction of GFP production altered microbial growth by comparing growth of parent and GFP strains of *E. coli* K-12. Growth of parent and GFP *Salmonella* was not compared because transformed cells were maximally fluorescent in the absence of tetracycline. Thus, effects of induction of GFP production on growth of *Salmonella* could not be directly assessed.

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MATERIALS AND METHODS

Plasmid construction. Two different high-copy plasmids, pEGFP (3.4 kb) and pPRO*tet*.E (2.2 kb) (Clontech Company, Palo Alto, Calif.), were used to construct pTGP (2.9 kb). pPRO*tet*.E has a chloramphenicol-resistant gene and a tetracycline-inducible promoter, and pEGFP has the gene for enhanced GFP. Plasmids were digested overnight at 37°C with the restriction enzymes *Not*I and *Bam*HI (New England Biolabs, Beverly, Mass.) in a buffer composed of 150 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin (all from Sigma Chemical Co., St. Louis, Mo.). Digested plasmids were separated in a 1% low-melting-point agarose gel, and bands with desired genes, pPRO*tet*.E (2.2 kb) and *gfp* (0.7 kb), were excised from the gel and ligated using T_4DNA ligase (New England Biolabs) to generate pTGP.

Transformation. Competent cells of a tetracycline-resistant strain of *E. coli* K-12 (Carolina Biological Supply Company, Burlington, N.C.) and tetracycline-resistant strains of *Salmonella* Hadar, *Salmonella* Kentucky, and *Salmonella* Typhimurium ($n = 2$) were transformed with pTGP *(32)*. The ligation mixture containing pTGP was mixed with competent cells in a microcentrifuge tube and incubated on ice for 30 min. The tube was then incubated at 37°C for 2 min in a water bath and chilled on ice for 5 min, 950 μl of Luria-Bertani (LB) broth (Becton Dickinson, Sparks, Md.) was added, and the transformant mixture was incubated at 37°C for 60 min and then streaked onto LB agar that contained tetracycline (100 ng/ml; Sigma) and chloramphenicol (25 μg/ml; Sigma). After overnight incubation at 37°C, the streak plate was examined for fluorescence with a hand-held 366-nm UV light.

Stock cultures. A fluorescent colony from the streak plate of the transformant mixture was used to inoculate 5 ml of brain heart infusion (BHI) broth (Becton Dickinson) that contained 25 μg/ml chloramphenicol in a 25-ml Erlenmeyer flask sealed with a foam plug. The cultures were incubated at 30°C and 150 orbits per min (opm) for 23 h. Aliquots (100 μl) of the 23-h culture were transferred to freezer storage vials that contained 900 μl of BHI broth supplemented with 15% (vol/vol) glycerol. Vials of the GFP strain and vials of the parent strain, which were prepared in a similar manner but with BHI broth as the growth medium, were stored at −70°C until they were used in the growth experiments.

Sterile chicken. Ten-gram portions of ground chicken breast meat were formed into uniform patties that were cooked in an autoclave at 121°C for 18 min to kill all microorganisms and to inactivate residual tetracycline that could be present from treatment of live birds with the antibiotic. After cooling, a sterile 1.5-cm-diameter cork borer was used to cut plugs from the center of the patties. The plugs were cut in half and trimmed with a sterile scalpel to yield 1-g samples of sterile chicken, which were transferred to individual wells of a sterile 12-well tissue culture dish for subsequent inoculation.

Inoculum culture. Thawed and resuspended stock culture (5 μl) was added to 5 ml of BHI broth for the parent strain or 5 ml of BHI broth plus chloramphenicol (25 μg/ml) for the GFP strain in a 25-ml Erlenmeyer flask sealed with a foam plug. Inoculum cultures for the parent and GFP strains were incubated in air at 30°C and 150 opm for 23 h before serial dilution in buffered peptone water (BPW; Becton Dickinson).

Growth experiments. Diluted inoculum culture (5 μl) was pipetted onto the surface of sterile chicken portions for an initial density of $10^{3.8}$ CFU/g. Inoculated chicken samples were incubated in air at 10, 25, or 40°C. At selected incubation times, a 1-g portion of sterile chicken was homogenized (model 80 stomacher blender, Seward, London, UK) for 1 min at normal speed in 9 ml of BPW in a Whirl-Pak bag (9.4 by 17.5 cm) containing a filter screen (Nasco, Fort Atkinson, Wis.).

Samples of stomachate (50 μl), either undiluted or serially diluted in BPW, were spiral plated (Whitley Automatic Spiral Plater, Microbiology International, Frederick, Md.) onto BHI agar (Becton Dickinson) for the parent strain and onto BHI agar plus chloramphenicol (25 μg/ml) and tetracycline (100 ng/ml) for the GFP strain. Tetracycline was included in the BHI agar for the GFP strain to induce production of GFP and to simulate how the GFP strain would be used in a predictive microbiology experiment with naturally contaminated food. Spiral plates were incubated in air at 30°C for 24 h, and then colonies were counted with an automated counter (ProtoCol, Microbiology International). Three replicate trials of the growth experiment were conducted per temperature, each with a different batch of chicken portions.

Growth modeling. Colony counts (log CFU per gram) were graphed as a function of time and fit to a three-phase linear model *(6)* using version 4.0 of Prism (GraphPad Software, Inc., San Diego, Calif.):

$$N(t) = \begin{cases} N_0 & \text{if } t \le \lambda \\ N_0 + [\mu_{max} \cdot (t - \lambda)] & \text{if } \lambda < t < t_{max} \\ N_{max} & \text{if } t \ge t_{max} \end{cases}$$

where $N(t)$ is *E. coli* K-12 density (log CFU per gram) at time t (hours), N_0 is initial density (log CFU per gram), λ is lag time (hours), μ_{max} is maximum specific growth rate ($hours^{-1}$), N_{max} is maximum population (log CFU per gram), and t_{max} is the time (hours) when growth reaches N_{max}.

Statistical analysis. The experimental design was a 2×3 factorial arrangement of strain (parent or GFP) and temperature (10, 25, or 40°C). Main effects and interaction of strain and temperature on best-fit values of growth parameters (λ, N_0, μ_{max}, and N_{max}) were evaluated by two-way analysis of variance with GraphPad Prism software. Effects with $P < 0.05$ were considered significant.

RESULTS

Growth curves on sterile chicken were not conducted for sufficient time in all cases to accurately assess N_{max}. Consequently, only results for N_0, λ, and μ_{max} were evaluated (Table 1). Although the same dilution and volume of inoculum culture were used to inoculate sterile chicken portions, N_0 was on average 0.6 log CFU/g lower ($P < 0.05$) for the GFP strain than the parent strain. Temperature did not alter N_0 ($P > 0.05$). Although the final density of the inoculum cultures was not measured, these results suggested that the final density of inoculum cultures for the GFP strain was lower than that of inoculum cultures for the parent strain.

Lag time was affected ($P < 0.05$) by temperature but not by strain (Table 1). As expected, λ decreased as temperature increased, and thus transformation with pTGP did not affect the ability of *E. coli* K-12 to initiate growth at temperatures corresponding to abuse of cooked food during refrigerated storage (10°C) or at normal (25°C) or high (40°C) room temperatures.

In contrast to λ, both strain and temperature affected μ_{max} (Table 1). Maximum specific growth rate was reduced

TABLE 1. *Comparison of the growth of parent and green fluorescent protein (GFP) strains of* Escherichia coli *K-12 on sterile chicken incubated at 10, 25, or 40°C*[a]

Temp (°C)	Parameter	Parent		GFP	
		Mean	SEM	Mean	SEM
10	N_0 (log CFU/g)[b]	3.80	0.08	3.28	0.08
	λ (h)[c]	62.9	9.8	65.2	19.4
	μ_{max} (h^{-1})[d]	0.0089	0.0014	0.0048	0.0001
25	N_0 (log CFU/g)	3.77	0.07	3.03	0.03
	λ (h)	3.66	0.32	2.15	0.15
	μ_{max} (h^{-1})	0.304	0.004	0.160	0.013
40	N_0 (log CFU/g)	3.77	0.08	3.17	0.06
	λ (h)	1.66	0.15	1.78	0.14
	μ_{max} (h^{-1})	0.891	0.027	0.554	0.035

[a] SEM, standard error of the mean; N_0, initial density; λ, lag time; μ_{max}, maximum specific growth rate.
[b] Significant main effect of strain, two-way analysis of variance, $P < 0.05$.
[c] Significant main effect of temperature, two-way analysis of variance, $P < 0.05$.
[d] Significant interaction of strain and temperature, two-way analysis of variance, $P < 0.05$.

($P < 0.05$) by transformation of *E. coli* K-12 with pTGP, and the magnitude of the effect depended on temperature. Transformation decreased μ_{max} to a larger extent at 10°C (46%) and 25°C (47%) than at 40°C (38%).

Fluorescent colonies of *E. coli* K-12 were observed only when tetracycline was included in the medium. Consequently, the observed slower growth of the GFP strain of *E. coli* K-12 on sterile chicken without tetracycline was not expected and did not support the hypothesis that high levels of GFP production are responsible for the reduced growth of transformed *Salmonella (26).* The current results suggested that the slower growth of the GFP strain resulted from transformation with pTGP and not from induction of GFP production. To test this possibility, a second experiment was conducted.

In the second experiment, growth of the parent strain of *E. coli* K-12 in BHI broth was compared with growth of the GFP strain in BHI broth with chloramphenicol (25 μg/ml) and 0 or 100 ng/ml tetracycline. Broth cultures (100 ml) were incubated at 40°C and 150 opm in 250-ml Erlenmeyer flasks sealed with foam plugs. Inoculum preparation, *E. coli* K-12 enumeration, and growth modeling were as described in ''Materials and Methods'' for the sterile chicken experiment. However, different volumes of the diluted inoculum cultures (i.e., 50 μl for the parent strain and 158 μl for the GFP strain) were inoculated into the BHI broth media to achieve the same initial density (3 log CFU/ml) of the parent and GFP strains. Effects of treatments on growth parameters were assessed by one-way analysis of variance with the Prism software. When analysis of variance indicated a significant difference among treatments ($P < 0.05$), treatment means were compared by using Tukey's multiple comparison test.

In contrast to the sterile chicken experiment, N_0 was not different ($P > 0.05$) between parent and GFP strains and was close to the target value of 3 log CFU/ml for all treatments (Table 2). There was also no significant difference in λ among treatments. However, transformation of *E. coli* K-12 with pTGP reduced both μ_{max} and N_{max}, and the extent of reductions were equivalent in the absence and presence of tetracycline induction of GFP production. Thus, reduced growth of the GFP strain did not result from induction of GFP production but rather from transformation with pTGP. These results also suggest that reduced growth of the GFP strain did not result from induction of proteins involved in the resistance response to tetracycline. The μ_{max} was reduced 49%, and N_{max} was reduced 0.42 log CFU/ml (62%) by transformation of *E. coli* K-12 with pTGP (Fig. 1).

In the sterile chicken experiment, the lower μ_{max} of the GFP strain could have resulted from the enumeration method used. A subpopulation of cells that lost pTGP could have progressively developed over time on sterile chicken in the absence of chloramphenicol selection. If this occurred, then enumeration on BHI agar with chloramphenicol would have progressively underestimated the number of *E. coli* K-12 over time and resulted in an underestimation of μ_{max}. However, in the sterile broth experiment, lower μ_{max} and reduced N_{max} were observed in transformed *E. coli* K-12 cells grown in the presence of chloramphenicol selection (Table 2). Thus, progressive loss of pTGP over time was not a logical explanation for the reduced growth of GFP *E. coli* K-12 in this study.

DISCUSSION

In a previous study *(26)*, predictive models for λ, μ_{max}, and N_{max} on sterile chicken as a function of temperature (8 to 48°C) were compared among pairs of parent and GFP *Salmonella* strains. Differences in growth parameters between parent and transformed strains were observed and were dependent on strain and incubation temperature. A general observation was that μ_{max} and N_{max} were reduced in GFP strains of *Salmonella (26).* In that study, *Salmonella* strains were transformed with a high-copy plasmid (pUC19) in which *gfp* was under control of the *lac*Z promoter. However, most strains of *Salmonella* do not carry a gene for *lac*I *(33)*, the repressor protein for the *lac*Z promoter. Consequently, expression of *gfp* was not repressed,

TABLE 2. *Comparison of growth of parent and green fluorescent protein (GFP) strains of* Escherichia coli *K-12 in brain heart infusion broth supplemented with chloramphenicol (Chl) and tetracycline (Tet) and incubated at 40°C*[a]

Strain	Chl (μg/ml)	Tet (ng/ml)	N_0 (log CFU/g)		λ (h)		μ_{max} (h^{-1})		N_{max} (log CFU/g)	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Parent	0	0	2.85	0.16	1.56	0.29	0.860 A	0.026	9.31 A	0.01
GFP	25	0	2.92	0.09	1.66	0.32	0.460 B	0.018	8.85 B	0.05
GFP	25	100	2.94	0.13	2.48	0.41	0.421 B	0.048	8.92 B	0.07

[a] SEM, standard error of the mean; N_0, initial density; λ, lag time; μ_{max}, maximum specific growth rate; N_{max}, maximum population. When a significant effect of treatment (one-way analysis of variance, $P < 0.05$) was found, means were compared by using Tukey's multiple comparison test. Means followed by different letters within a growth parameter are significantly different ($P < 0.05$). Means without letters within a growth parameter are not significantly different ($P > 0.05$).

FIGURE 1. *Growth of a parent and a green fluorescent protein (GFP)–producing strain of* Escherichia coli *K-12 in brain heart infusion broth incubated at 40°C.*

which led to the hypothesis that constitutive production of GFP had created a metabolic burden (e.g., a deficiency of an essential amino acid) that resulted in reduced growth of transformed strains *(26).*

The goal in the present study was to control *gfp* expression using an inducible promoter and thus prevent a metabolic burden that would reduce growth of transformed strains during growth on food. A high-copy plasmid with *gfp* under control of a tetracycline-inducible promoter (pTGP) was successfully created and introduced into a tetracycline-resistant strain of *E. coli* K-12, and fluorescent colonies were observed only when tetracycline was included in the enumeration medium. In contrast, when four strains of tetracycline-resistant *Salmonella* were transformed with pTGP (results not shown), maximally fluorescent colonies were observed in the absence of tetracycline. Thus, similar to the *lac*Z promoter *(26),* the *tet*E promoter was not repressed in *Salmonella.* These findings indicated that pTGP does not provide controlled expression of *gfp* in all transformed bacteria. Another limitation of pTGP is that it will work only in tetracycline-resistant bacteria, such as those used in the current study.

Growth of parent and GFP strains of *E. coli* K-12 were compared on sterile chicken to test the hypothesis that controlled expression of *gfp* results in GFP marker bacteria with growth kinetics indistinguishable from those of the parent strains. Growth was compared at 10, 25, and 40°C because in a previous study *(26)* there were some temperatures (e.g., 25°C for *Salmonella* Typhimurium) at which growth was similar between parent and GFP strains and some temperatures (e.g., 10 and 40°C for *Salmonella* Typhimurium) at which growth was different between parent and GFP strains. Thus, multiple temperatures were investigated to provide a better evaluation of the effect of transformation on microbial growth.

Regardless of the incubation temperature, transformation with pTGP reduced growth of *E. coli* K-12 on sterile chicken. Because GFP was not induced when *E. coli* K-12 grew on sterile chicken, these results indicate that transformation with pTGP created a fitness problem that was in-

dependent of *gfp* expression. This result was confirmed and expanded in a second experiment in sterile broth at 40°C. In that experiment, both μ_{max} and N_{max} were reduced to the same extent when GFP production was not induced and when it was induced by tetracycline. Thus, induction of GFP production did not produce a further reduction in μ_{max} and N_{max}, which would have been expected had synthesis of high levels of GFP created a metabolic burden as previously hypothesized *(26).* These results also suggest that reduced growth of the GFP strain did not result from induction of proteins involved in the resistance response to tetracycline.

Rang et al. *(29)* recently compared GFP production and doubling times for a panel of enteric bacteria transformed with pGEN91, which contains *gfp* under control of a sodium chloride–inducible promoter, and pGEN91$^{\Delta gfp}$, in which *gfp* is deleted. When sodium chloride was increased from 42 to 171 to 300 mM, the ratio of doubling times (pGEN91/pGEN91$^{\Delta gfp}$) for *E. coli* DH5α increased from 1.34 to 1.50 to 1.79. These results indicated that GFP production, which was measured, was positively correlated with increased doubling times *(29).* Significant effects of GFP on increasing doubling time were observed for all strains and levels of sodium chloride induction except for enterohemorrhagic *E. coli* and *Shigella flexneri* at the two lowest sodium chloride concentrations *(29).* Similar to previous results *(26),* the effect of GFP on doubling times of enteric bacteria *(29)* was variable among strains. In part, slower growth of transformed strains of enteric bacteria *(29)* resulted from inhibition of cell division; some cells of *E. coli* and *Salmonella* Typhi (2 to 40% depending on the growth condition) were elongated by 10 to 25 cell lengths. A small population of *Salmonella* Typhi cells (0.5%) transformed with pGEN91$^{\Delta gfp}$ exhibited the elongated phenotype, indicating that the plasmid has a small effect on cell division that is independent of *gfp* expression *(29).* In the current study, transformation with pTGP had a large effect on growth of *E. coli* K-12 cells (about a 40 to 60% reduction of μ_{max} and N_{max}) that was independent of *gfp* expression.

A potential limitation of GFP as a biomarker for modeling studies is that some transformed cells may retain the plasmid but fail to produce GFP, resulting in an underestimation of pathogen growth on naturally contaminated food. Using flow cytometry, Maksimow et al. *(25)* found that 2 to 9% of transformed *E. coli* cells retained the plasmid but failed to produce GFP. Likewise, Kohler et al. *(23)* found that 3 to 4% of transformed *Legionella pneumophila* cells retained the plasmid but failed to produce GFP. In the current study, all colonies of GFP *E. coli* K-12 were fluorescent on enumeration medium with chloramphenicol and tetracycline. Thus, pTGP transformation would not result in an underestimation of *E. coli* K-12 numbers on food with competitive microflora due to a subpopulation of transformants that retained pTGP but did not produce GFP on viable count plates.

Initial cell density can affect bacterial growth kinetics. In the current study, initial density of GFP *E. coli* K-12 on sterile chicken was on average 0.6 log CFU/g lower than that of the parent strain. In a previous comparison of parent and GFP strains of *Salmonella* Dublin *(26),* a 1-log CFU/ml difference in starter culture density was compensated for by using a 1-log lower dilution of the GFP strain culture for inoculation. Despite the same initial density on sterile chicken, the GFP *Salmonella* Dublin cells still exhibited slower growth than did the parent strain over a broad range of temperatures *(26).* In various studies with pathogenic bacteria in pure broth cultures, small differences in initial density (<1 log CFU/ml) did not affect growth from high initial densities (>3 log CFU/ml) *(3, 5, 39).* Thus, a slightly lower initial density (0.6 log CFU/g) is an unlikely explanation for the reduced growth of GFP *E. coli* K-12 on sterile chicken. In the broth culture experiment, initial density was normalized among the parent and GFP strains of *E. coli* K-12 and the reduced growth of the GFP strain was still observed (Table 2).

In conclusion, transformation of *E. coli* K-12 with a high-copy plasmid with *gfp* under the control of a tetracycline-inducible promoter (pTGP) was successful (i.e., resulted in the controlled production of GFP) but resulted in slower growth and reduced N_{max} that was independent of *gfp* expression. In contrast, transformation of *Salmonella* with pTGP resulted in constitutive production of GFP. These results indicate that transformation of bacteria with pTGP is unlikely to produce validated GFP strains for modeling pathogen growth on food with competitive microflora. Nonetheless, other transformation approaches, such as use of a low-copy plasmid or chromosomal insertion of *gfp,* may result in successful validation and use of GFP as a biomarker for predictive microbiology.

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