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Susceptibility of Suspended and Surface-Attached *Salmonella enteritidis* to Biocides and Elevated Temperatures

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The differential resistance of substratum-attached, detached, and planktonic cells of *Salmonella enteritidis* phage type 4 was studied by using several inimical processes and in vivo bioluminescence as a nondestructive, real-time reporter of metabolic activity. Bioluminescence in this strain was mediated by a construction containing the entire *lux* operon from *Photorhabdus luminescens*. An excellent correlation between bioluminescence and classical plate count data was obtained when we compared attachment profiles, biocide concentration exponents, and thermal inactivation D values (D value was the time required for a 10-fold reduction in the number of survivors). Biocide challenge of surface-adherent *S. enteritidis* resulted in concentration exponents that were experimentally indistinguishable from those obtained with Luria-Bertani broth-grown planktonic cells. It appears that cleansing regimes developed by using planktonic cell data are effective against surface-attached cells of this bacterium. Both attached and detached cells exhibited an approximately twofold increase in D values at 52°C compared with values calculated for planktonic cells, strongly indicating that the detached cells exhibited an attached phenotype during the heating process. A model of a physiological adaptive response induced in attached cells and also reflected in detached cells is presented.

Salmonella enteritidis phage type 8 is the most common serotype of the genus *Salmonella* reported in the United States (41, 51), and *S. enteritidis* phage type 4 is the major causative agent of human salmonellosis in Europe (2, 31). Investigations of gastrointestinal outbreaks caused by *S. enteritidis* frequently implicate contaminated eggs as the source of infection (34, 41, 42, 46). Eggshell contamination is common, and a damaged shell permits contamination of the internal portion of an egg. Transovarian transmission, however, poses an additional threat as the shells do not have to be damaged for surface contamination to reach the egg contents (50). Preventing food contamination by *S. enteritidis* is important, and the U. S. Department of Agriculture is promoting improved handling of eggs together with implementation of hazard analysis critical control point programs to identify, monitor, and solve potential contamination points in the food industry (18, 25).

Effective hygiene control measures coupled with the use of appropriate disinfection regimes are important tools for the control of *S. enteritidis* in food production and processing environments. However, control measures must take into account the different susceptibilities of surface-attached and planktonic cells (9). The mechanisms of bacterial attachment and the resulting hygiene and economic problems caused in the food and dairy industries have recently been reviewed (8, 10, 60). Compared with their planktonic counterparts, attached bacteria typically exhibit enhanced resistance to inimical processes such as antimicrobial agents (7), biocides (3), and heat (15). Yet, despite the economic and health significance of *S. enteritidis*, these factors have not been critically evaluated with surface-attached cells of this bacterium.

Recently, novel tools for assessing the physiological status of planktonic, attached, and detached cells have become available, and these tools are proving to be invaluable to our understanding of how cells acquire enhanced resistance to inimical processes (28, 56, 59). In this respect, the use of bacterial bioluminescence for environmental, diagnostic, and analytical applications is preeminent (1, 6, 22, 48, 49). The bacterial bioluminescence reaction is catalyzed by the enzyme luciferase and depends on O_2, reduced flavin mononucleotide, and a long-chain aldehyde substrate to yield blue-green light (39). Gram-negative bacteria can be transformed to a bioluminescent phenotype by introducing appropriate host vectors containing either *luxAB* (the genes encoding the α and β subunits of bacterial luciferase) or the complete *luxCDABE* operon (*luxCDE* encodes the fatty acid reductase complex required to produce the aldehyde substrate) (23). Since the production of light by *lux*-containing bacteria depends on the presence of a functional intracellular biochemistry, stress and adaptive responses are readily monitored through suboptimal bioluminescence production as such responses either directly or indirectly affect the intracellular levels of reduced flavin mononucleotide (26, 39).

A good correlation between bioluminescence and viability has been demonstrated in biocide efficacy studies performed with *Listeria monocytogenes* (53, 55). In particular, the equivalence of concentration exponent (η) data, which describe how rapidly the antimicrobial activity of a biocide is lost upon dilution (24), as well as thermal inactivation data has made workers confident to use bioluminescence in circumstances where direct determinations of viable counts are difficult or impractical (12–14, 54).

In this work we demonstrated that bioluminescence can be used as a noninvasive, real-time reporter of the physiological status of *S. enteritidis* cells attached to glass and stainless steel following heat and biocide challenges. We demonstrated the accuracy, robustness, and sensitivity of in vivo bioluminescence for direct evaluation of the activities of planktonic, attached, and detached cell populations. The significance of our data for the control of *S. enteritidis* in food environments is discussed.

MATERIALS AND METHODS

Microorganism and plasmid. *S. enteritidis* (isolated from fresh chicken) was transformed to a bioluminescent phenotype with the constitutive *luxCDABE*

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expression plasmid pSB311 (the *luxCDABE* genes in pSB311 were derived from *Photorhabdus luminescens*) (11). The organism was maintained at 4°C in Luria-Bertani (LB) medium (10.0 g of tryptone [Difco Laboratories, Detroit, Mich.], 5.0 g of yeast extract [Difco Laboratories], 5.0 g of NaCl, and 0.16 g of NaOH in 1 liter of water purified by reverse osmosis) (47). This medium was supplemented with tetracycline (10 μg ml^{-1}) to maintain plasmid selection, and agar (1.5%, wt/vol) was added to produce a solid medium. Stock plates were subcultured monthly.

Substrata. Glass coverslips (18 by 18 mm; Chance Propper, Ltd., Smethwick, England) were acid washed in a 50% (vol/vol) aqueous H_2SO_4 solution for 24 h and then rinsed six times in sterile water purified by reverse osmosis (Elga, Ltd., Buckinghamshire, England). Stainless steel coupons (type 304 with a 2B finish; Campden Food and Drink Research Association, Chipping Campden, England) were cut into 10- by 10-mm squares, cleaned by scrubbing in a neutral detergent, and then rinsed in sterile water. After rinsing, both substrata were heated in a sterilization oven for 4 h at 140°C.

Measurement of bacterial growth. Overnight cultures grown at 37°C and 70 rpm in LB medium (47) and in minimal medium (MM) (27) [7.0 g of K_2HPO_4, 3.0 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$), 1.0 g of $(NH_4)_2SO_4$, and 2.0 g of glucose in 1 liter of sterile water purified by reverse osmosis; pH 7.0] were diluted 1:10,000 and 1:1,000, respectively, in 300-ml Erlenmeyer flasks containing 50 ml of the same medium so that the initial A_{600} was approximately 0.002. Bacterial growth at 37°C and 70 rpm was recorded for 12 h by measuring the A_{600}, and bioluminescence was measured by determining the number of relative light units per milliliter in scintillation vial inserts (4 ml; FSA Laboratory Supplies, Loughborough, England) with a Turner Designs model 20e luminometer (Steptech, Ltd., Hertfordshire, England).

Viable counts. Viable counts were determined by the method of Miles et al. (40) by spotting onto LB agar duplicate 10-μl samples from serial dilutions prepared in phosphate-buffered saline (PBS) (pH 7.3) (Oxoid, Ltd., Hampshire, England) or an appropriate neutralizer solution during biocide experiments. The plates were incubated inverted at 37°C for 24 to 48 h before counting; this extended incubation period was used to ensure maximal recovery of damaged cells.

Attachment of bacteria to substrata. Sterile petri dishes (diameter, 55 mm; Bibby Sterilin, Ltd., Staffordshire, England) containing LB medium or MM (5 ml) were inoculated (1:1,000 dilution) with overnight cultures of *S. enteritidis*(pSB311) grown in the same medium. The glass or stainless steel substratum was immersed into the broth and incubated for 12 h at 37°C and 70 rpm. Following incubation, the substratum was carefully removed with sterile forceps and washed with 200 ml of PBS at a flow rate of approximately 400 ml min^{-1} to remove the residual cells that were still in suspension or were loosely attached. The resulting prepared substrata were used for subsequent biocide and heat challenge experiments.

Biocide challenge experiments. Fresh stock solutions of phenol (FSA Laboratory Supplies), chlorhexidine diacetate (Sigma Chemical Co., St. Louis, Mo.), and Virkon (Antec International, Ltd., Suffolk, England) in sterile water were prepared before each experiment. All experiments were performed at 22°C, and the data are reported below as the number of surviving bacteria compared with the number of control bacteria, expressed as the percentage of survivors.

(i) Suspension tests. *S. enteritidis*(pSB311) was grown in LB medium or MM under conditions equivalent to those described above for adhered cells. Cells (10 ml) were harvested by centrifugation at 7,000 × *g* for 10 min, washed three times in sterile water, and adjusted to an A_{600} of 0.1 (approximately 10^8 CFU ml^{-1}). Samples (1 ml) of each cell suspension were diluted with 1-ml portions of the appropriate double-strength biocide solution so that the correct final biocide concentration was obtained. The control suspensions contained 1 ml of sterile water instead of biocide. At intervals (5, 15, 30, 45, and 60 min), triplicate 50-μl samples were dispensed into microtiter tray wells (Dynatech Laboratories, Inc., Chantilly, Va.) containing a suitable biocide-neutralizing agent. The biocide-neutralizing agents used were 2% (vol/vol) Tween 80 (Sigma) for phenol (24), 2.5% (wt/vol) letheen broth (Difco Laboratories) for chlorhexidine (24), and 2% (wt/vol) sodium thiosulfate (Sigma)–20% (vol/vol) horse serum (Oxoid) for Virkon. The preparations were then examined for bioluminescence output with an Amerlite luminometer (Amersham International plc, Amersham, England). Parallel viable count experiments were performed as described above.

(ii) Surface tests. Triplicate glass coverslips with cells adherent by growth in LB medium or MM (see above) were exposed to 5 ml of phenol, 5 ml of chlorhexidine, or 5 ml of Virkon at various concentrations for 15 min. For phenol, additional contact times ranging from 0 to 60 min were also investigated. Control coverslips were exposed to 5 ml of sterile water for the same times. The treated coverslips were aseptically removed with sterile forceps and immersed for 5 min in 10 ml of an appropriate neutralizer. Coverslip bioluminescence was then determined with a Turner Designs model 20e luminometer by placing the coverslip into an empty 24-ml scintillation vial (FSA Laboratory Supplies), after which 2% Tween 80 was used to disperse the attached cells by vortexing the preparation at the maximum speed for 2 min; the resulting suspension was used for viable count experiments (see above).

(iii) η determination. The η was calculated from the following equation: $\eta = (\log t_2 - \log t_1)/(\log c_1 - \log c_2)$, where t_1 and t_2 are the times necessary to produce a given reduction in the number of survivors with biocide concentrations c_1 and c_2, respectively (24). Thus, for example, if a biocide had an η of 6, its activity would be reduced, after dilution, by the dilution factor to the power of 6 (i.e., a threefold dilution would reduce the activity 3^6, or 729, times) and the killing time would be extended by the same factor.

Heat treatment experiments. (i) Suspension tests. *S. enteritidis*(pSB311) planktonic cells were prepared as described above for the biocide suspension tests, except that the cells were resuspended in PBS and the A_{600} was adjusted to 0.01 (approximately 10^7 CFU ml^{-1}). Cell suspensions (1 ml) were placed into two sterile loosely capped plastic universal bottles (30 ml; Bibby Sterilin, Ltd.) in plastic tubs in static water baths (FSA Laboratory Supplies); one cell suspension was used as the control and was kept at 30 ± 0.3°C, and the second cell suspension was kept at 52 ± 0.3°C. The sample temperatures were monitored with a Squirrel probe (Grant Instruments, Ltd., Cambridge, England) placed at the center of a universal bottle containing 1 ml of PBS. The measured heating rate for the 52°C sample was 2.7 ± 0.2°C min^{-1} ($n = 10$). After the temperature reached 52°C, the cells were incubated for various lengths of time. After each exposure time, 9 ml of prewarmed (37°C) LB medium was immediately added to the control and treated cells. Levels of bioluminescence were recorded by using 1-ml samples and a Turner Designs model 20e luminometer, and then the samples were returned to their respective universal bottles. Viable counts were also determined as described above. The control and treated cells were then incubated in a static water bath at 37°C, and levels of bioluminescence and viable counts were determined after 30 and 60 min of incubation.

(ii) Surface tests. *S. enteritidis*(pSB311) cells attached to substrata as described above were placed in empty sterile loosely capped universal bottles and exposed to a temperature of 52°C in a humid environment for different times, with the exposure time monitored after the temperature reached 52°C. A control substratum was placed in a 30°C humidity chamber for the same times. The attached cells at 52°C were subjected to heating rates comparable to those in the PBS suspension tests (see above). The humidity chambers were used so that we could avoid undesirable drying effects during the heating period. After heating, the substrata were removed aseptically with sterile forceps and immediately placed into 10 ml of prewarmed (37°C) LB medium in a static water bath to allow the cells to recover. Cell attachment to the substrata and detachment into the LB medium were monitored by measuring bioluminescence after 0, 30, and 60 min of incubation; the viable counts in the LB recovery medium, which reflected the number of detached cells, were determined at the same times. After 60 min of incubation with LB medium, the adhered cells were dispersed with Tween 80 (see above), and the viable counts of the suspensions were determined.

(iii) D value determination. The ratios of the mean numbers of surviving treated bacteria to the mean numbers of control bacteria (expressed as percentages) were determined, and these values were plotted against time of exposure to heat. The time required for a 10-fold reduction in the number of survivors (i.e., D value) was determined from the steepest portion of the resulting profiles. D values at 52°C ($D_{52°C}$ values) were calculated from both bioluminescence and viable count data for planktonic, attached, and detached cells. All of the heat exposure experiments were performed in triplicate.

RESULTS AND DISCUSSION

Biocide challenge of planktonic *S. enteritidis* cells. One of the advantages of using *luxAB* in isolation from *luxCDE* is that in the absence of exogenously added aldehyde there is no endogenous drain on cellular energy through the bioluminescent pathway (39). It has been perceived that under conditions of bacterial stress this provides a real-time reporter with minimal perturbation of the physiology being studied; aldehyde can be added at the time of light measurement when preservation of the energy status of the cells is no longer relevant (12, 14, 23, 53–55). The disadvantage of this approach is that exogenous addition of aldehyde is not suited to continuous bioluminescence monitoring, as would be ideal for biofilm studies. In previous studies workers have successfully employed full *lux* constructions for continual real-time noninvasive monitoring of cell viability and gene expression, but in these studies cell injury and recovery were not evaluated (19, 36, 52). Therefore, we decided to compare the η values for *S. enteritidis* for phenol, chlorhexidine, and Virkon derived by using both classical viable count and bioluminescence measurement methods. We reasoned that equivalent η values would reflect minimal perturbation of the assay mixture by constitutive continuous light output.

The growth curves for *S. enteritidis* with and without the *luxCDABE* plasmid pSB311 were indistinguishable. The growth rates for this organism in LB medium and MM were 2.5 and 1.4 generations h^{-1}, respectively (Fig. 1). We observed a

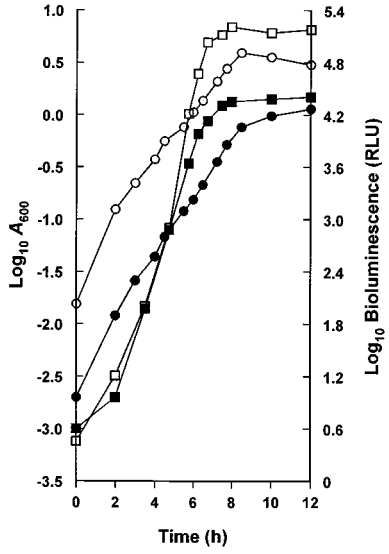

FIG. 1. Growth of *S. enteritidis*(pSB311) as determined by bioluminescence (open symbols) and A_{600} (solid symbols) for LB medium-grown (■ and □) and MM-grown (● and ○) planktonic cells. RLU, relative light units.

linear correlation between bioluminescence and A_{600} throughout the exponential growth curve for *S. enteritidis*(pSB311) in either LB medium or MM ($r_{LB} = 0.988$; $r_{MM} = 0.996$) (Fig. 2). A comparison of the logarithmic relationships between phenol concentration and the D value for planktonic *S. enteritidis*(pSB311) cells derived by using viable count data or bioluminescence measurements revealed a close correlation between the two plots (Fig. 3); this was true for cells grown in LB medium and MM ($r_{LB} = 0.998$; $r_{MM} = 0.990$). The corresponding η values for phenol were calculated from the gradient of the line of best fit (Fig. 3), and the η values for chlorhexidine and Virkon were calculated from equivalent plots (Table 1). Experimentally equivalent η values were obtained from the bioluminescence and viable count data for each of these biocides in both media.

The η values for chlorhexidine and phenol for cells grown in LB medium fall within the generally accepted ranges observed with these biocides and enteric bacteria (24, 26). Nominal variations in η values for LB medium- and MM-grown cells were observed with chlorhexidine and Virkon. However, the fact that the phenol η values for MM-grown cells were less than the lowest expected value may indicate that there was an

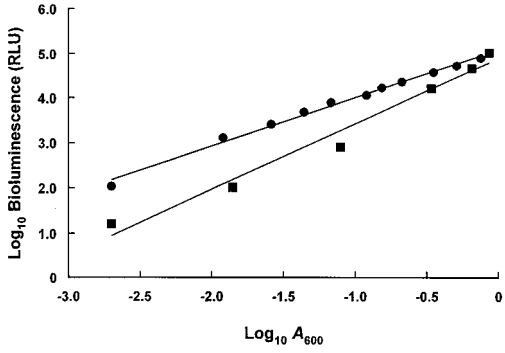

FIG. 2. Relationship between bioluminescence and A_{600} for LB medium-grown (■) and MM-grown (●) *S. enteritidis*(pSB311) cells. RLU, relative light units.

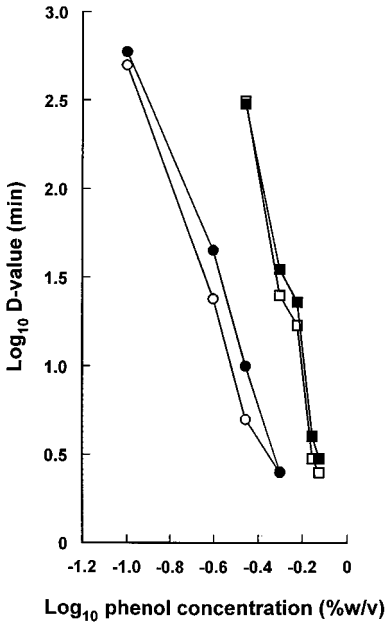

FIG. 3. Relationship between D value and phenol concentration as determined by the bioluminescence (open symbols) and viable count (solid symbols) methods for planktonic *S. enteritidis*(pSB311) cells grown in LB medium (■ and □) and MM (● and ○).

altered target for phenol accessibility, as nutritionally distinct media are known to influence certain surface and cellular characteristics of bacterial cells (16, 17, 29, 30, 43, 45). As Virkon is a relatively new, multipurpose, strongly oxidizing disinfectant, limited published material concerning this biocide is available. Nevertheless, the η values for Virkon calculated for *S. enteritidis*(pSB311) fall within the range for compounds that promote cellular leakage (24), which is consistent with previous data for this biocide with *L. monocytogenes* (53). The well-correlated results obtained from biocide challenge of planktonic cells demonstrate the ability of the bioluminescence phenotype in *S. enteritidis*(pSB311) to accurately respond to a range of biocides with different modes of action and validate the use of a complete *lux* operon as a bioluminescence reporter for inimical processes.

Surface adsorption of *S. enteritidis*. Glass coverslip and stainless steel coupon surfaces were colonized by high numbers of *S. enteritidis*(pSB311) cells, as determined by both the viable count method (Fig. 4A) (after removal of cells from the surface) and the direct real-time bioluminescence method (Fig. 4B). There was a rapid increase in the number of adherent bacteria during the first 6 h, after which saturation of bacterial binding to the two substrata was observed. This saturation coincided with a plateauing of cell numbers in the suspension at approximately 10^9 CFU ml^{-1} (Fig. 4C). In subsequent protocols for preparing attached cells for heat and biocide treatments we used a 12-h attachment time to ensure that there was a stable adherent population with optimal bioluminescence output. For cells grown in LB medium the mean levels of attached *S. enteritidis*(pSB311) cells after 12 h were $1.9 \times 10^6 \pm 0.3 \times 10^6$ CFU ml^{-1} ($n = 3$) for the glass coverslips and $6.5 \times 10^6 \pm 0.5 \times 10^6$ CFU ml^{-1} ($n = 3$) for the stainless steel coupons; for cells grown in MM the corresponding values were $3.6 \times 10^7 \pm 1.8 \times 10^7$ and $2.8 \times 10^8 \pm 1.8 \times 10^8$ CFU ml^{-1}, respectively. The linear correlation coefficients calculated for the comparative viable count and bioluminescence data are as

TABLE 1. Biocide η values for planktonic and surface-attached *S. enteritidis*(pSB311) cells as determined by the viable count and bioluminescence methods

Growth medium	η value for planktonic cells challenged with:						η value for surface-attached cells challenged with phenol	
	Phenol		Chlorhexidine		Virkon			
	VC method[a]	BL method[a]	VC method	BL method	VC method	BL method	VC method	BL method
LB medium	6.00	6.31	1.28	1.41	3.08	2.96	5.92	5.94
MM	3.37	3.40	1.37	1.82	2.71	3.02	5.84	5.87

[a] VC, viable count; BL, bioluminescence.

follows: r_{LB} (glass) = 0.957; r_{LB} (steel) = 0.989; r_{MM} (glass) = 0.973; and r_{MM} (steel) = 0.983. These data demonstrate the effectiveness of bioluminescence for reporting total viable counts in situ and in real time.

Biocide challenge to surface-adherent *S. enteritidis* cells. The η values for phenol were determined with both LB medium- and MM-grown cells attached to glass coverslips. Interestingly, the values obtained experimentally were indistinguishable and were equivalent to the η value obtained for LB medium-grown planktonic cells (Fig. 5 and Table 1). However, the η value obtained for planktonic MM-grown cells was approximately one-half of the η value obtained for the equivalent surface-attached cells. This implies that cells grown in MM exhibit a significant increase in resistance to phenol after surface attachment; however, this increased resistance does not exceed the resistance of LB medium-grown cells in either the attached or planktonic state. It has been demonstrated (24) that the maximum η value for phenol with *S. enteritidis* is approximately 6.0. On the basis of our data this suggests that cleansing regimes developed with planktonic cell data should be effective against surface-attached cells of this bacterium.

To extend this conclusion to biocides known to have alternative mechanisms of action, we examined the concentration-dependent survival curves obtained with two other biocides and a single contact time (15 min). The phenol susceptibility profiles of LB medium-grown suspended and attached *S. enteritidis*(pSB311) cells were similar (Fig. 6A), which supported the finding obtained from the η values (Table 1) that there was no significant difference in the levels of resistance of LB me-

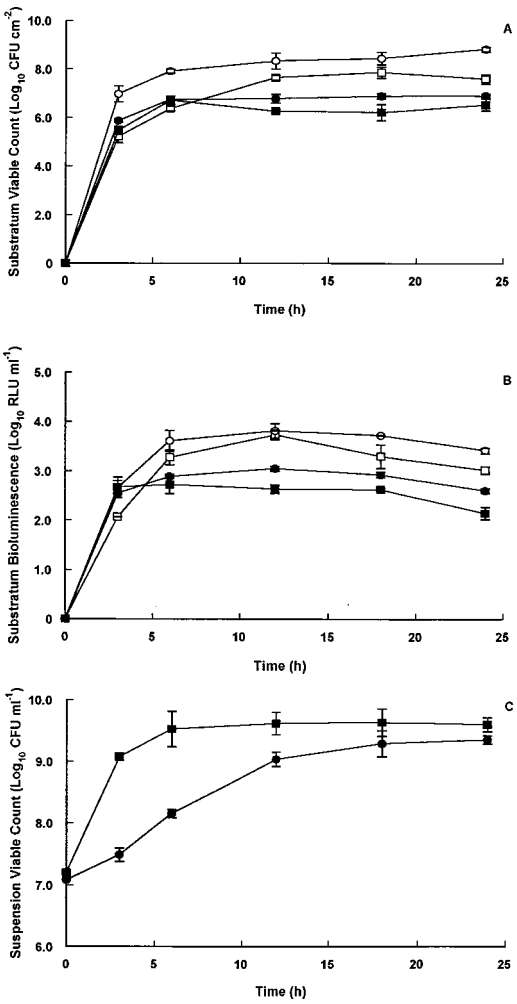

FIG. 4. Adsorption of *S. enteritidis*(pSB311) grown in LB medium (solid symbols) and MM (open symbols) as determined by the viable count method (A) and the bioluminescence method (B). Symbols: ■ and □, glass coverslips; ● and ○, stainless steel coupons. (C) Suspension viable counts for LB medium-grown cells (■) and MM-grown cells (●). The datum points represent means ± standard errors of the means (n = 3). RLU, relative light units.

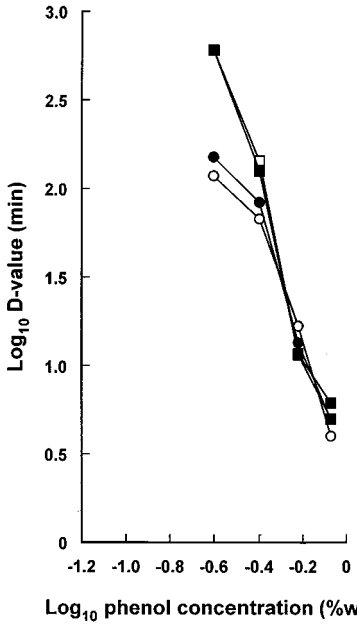

FIG. 5. Relationship between D value and phenol concentration as determined by the bioluminescence method (open symbols) and the viable count method (solid symbols) for LB medium-grown (■ and □) and MM-grown (● and ○) surface-attached *S. enteritidis*(pSB311) cells.

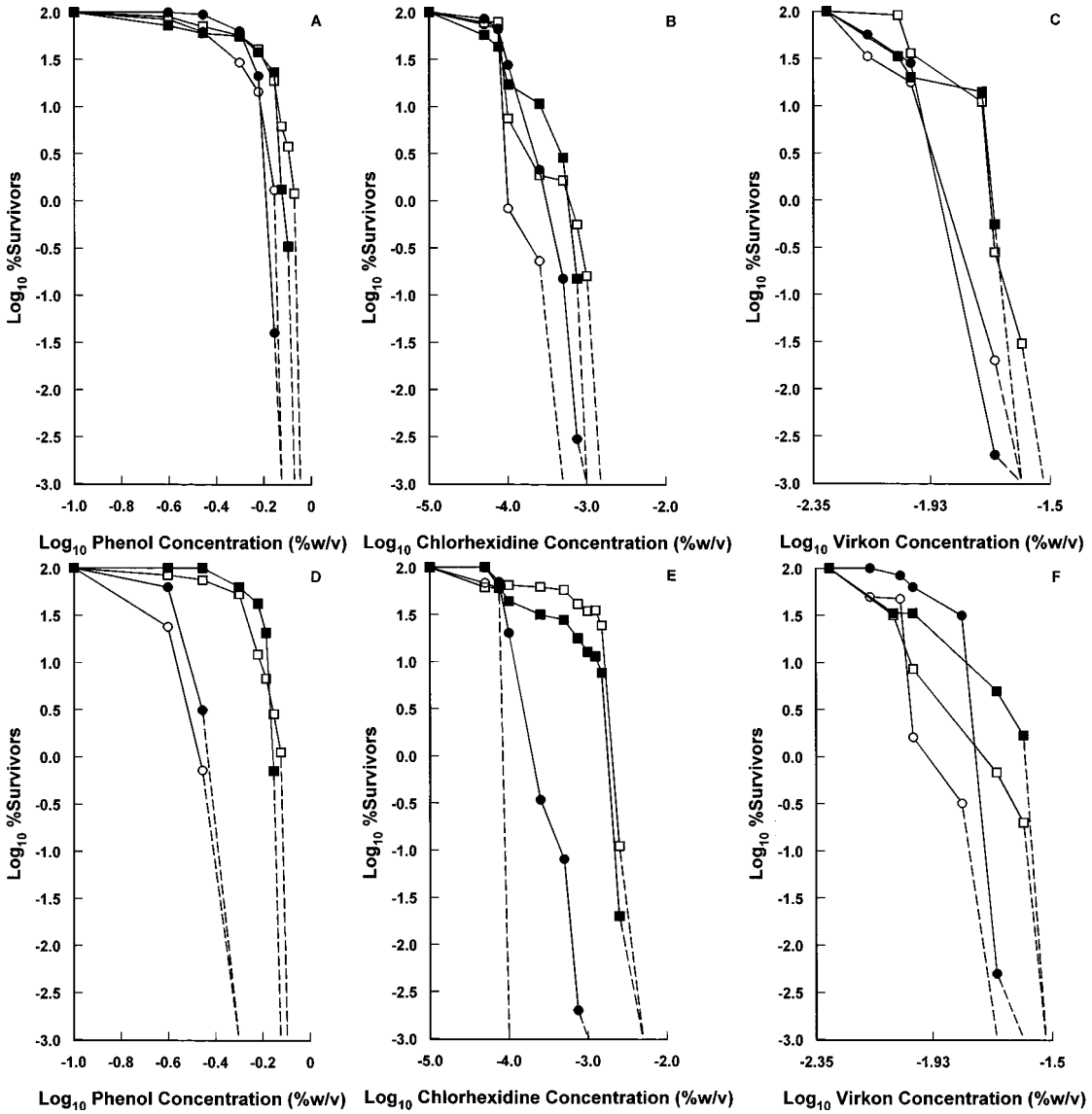

FIG. 6. Biocide concentration-dependent survival curves for planktonic (● and ○) and surface-attached (■ and □) *S. enteritidis*(pSB311) cells grown in LB medium (A to C) and MM (D to F) as determined by the bioluminescence method (open symbols) and the viable count method (solid symbols). The plots show the results for 15-min contact times for phenol (A and D), chlorhexidine (B and E), and Virkon (C and F). The dashed lines indicate the biocide concentrations at which bioluminescence and viable count values were below the limits of detection.

dium-grown planktonic and attached cells to this biocide. Comparative plots for chlorhexidine and Virkon (Fig. 6B and C) also revealed no significant difference in levels of resistance between attached and planktonic cells. In contrast, the MM-grown attached cells displayed well-defined susceptibility shifts when they were compared with their planktonic counterparts (≈1.5-fold for phenol, ≈3.5-fold for chlorhexidine, and ≈1.5-fold for Virkon), clearly illustrating that resistance increased upon surface attachment (Fig. 6D through F). These findings are consistent with the increase in the η values for phenol (and hence increased resistance) observed with MM-grown surface-attached cells (Table 1). Except for chlorhexidine, the increase in resistance did not surpass the increase obtained for LB medium-grown cells either in planktonic or surface-attached environments. Resistance to chlorhexidine was maximal with MM-grown surface-attached cells, and thus this finding provides a caveat to the general conclusion that surface attachment of *S. enteritidis* does not substantially enhance resistance to biocide inactivation compared with LB medium-grown cells. The complete equivalence of the data generated by 24-h classical viable counting methods and real-time bioluminescence measurement methods provides considerable support for the finding that *luxCDABE* constitutive bioluminescence can be used in studies of surface-attached bacteria. The only exception was observed with chlorhexidine-treated MM-grown planktonic cells, in which the bioluminescence measurements indicated a greater level of susceptibility than the level of susceptibility obtained by the viable counts method. No unequivocal explanation can be suggested for this finding, and further work is needed to examine this anomaly.

Extending the inimical processes to heat treatment. Bioluminescence has proved to be a very effective way to monitor injury and recovery from temperature-induced cellular damage in bacteria (12–14). However, in previous studies, workers used

TABLE 2. D values for planktonic, surface-attached, and detached *S. enteritidis*(pSB311) cells as determined by the viable count and bioluminescence methods

Growth medium	$D_{52°C}$ values (min)									
	Planktonic cells		Glass coverslips				Stainless steel coupons			
			Attached cells		Detached cells		Attached cells		Detached cells	
	VC method[a]	BL method[a]	VC method	BL method	VC method	BL method	VC method	BL method	VC method	BL method
LB medium	4.78 (4.95)[b]	5.00	10.80	11.25	10.80	11.20	9.79	10.00	9.79	10.25
MM	3.75 (4.50)[b]	3.75	9.17	9.25	9.38	10.00	8.33	8.50	8.75	8.88

[a] VC, viable count; BL, bioluminescence.
[b] The values in parentheses are *S. enteritidis* parent strain D values.

only recombinant constructions based on *luxAB* technology, which required the addition of aldehyde to initiate bioluminescence at the time of assay. As discussed previously, this was done primarily to eliminate the energy drain of constitutive bioluminescence on stress-induced injury and subsequent recovery, but it is difficult to extend the previous planktonic cell studies to surface-attached and biofilm populations of cells. Given the correlations which we obtained for viable count and bioluminescence data in biocide work with both planktonic and surface-attached *S. enteritidis* cells, we tried to establish the validity of using the constitutive *luxCDABE*-based construction pSB311 for monitoring heat-induced injury of surface-attached *S. enteritidis* cells. The *lux* genes present in pSB311 originated from the naturally bioluminescent terrestrial microorganism *P. luminescens*, which expresses the most heat stable of the available bacterial luciferase enzymes (23, 35).

The protocol for monitoring the recovery of heat-treated *S. enteritidis*(pSB311) cells permitted frequent bioluminescence sampling of the substratum (i.e., attached cells) and the surrounding LB recovery medium (i.e., detached cells). The mean levels of detached cells ($n = 6$) were significant (86 ± 4.3, 85 ± 4.3, 73 ± 5.9, and 55 ± 3.6% for LB medium-grown cells on glass coverslips, LB medium-grown cells on stainless steel coupons, MM-grown cells on glass coverslips, and MM-grown cells on stainless steel coupons, respectively). Such levels of detachment are not unusual, however, as previous workers using various substrata and bacteria have also detected significant levels of detachment or desorption of cells after simple surface attachment (4, 5, 33, 37, 38, 44, 57). Typically, approximately 50 to 85% of the total bacterial population can become detached (4, 5, 57).

The calculated D values ($D_{52°C}$ values) for planktonic, attached, and detached *S. enteritidis*(pSB311) cells revealed good equivalence between bioluminescence and viable count data (Table 2). The planktonic *S. enteritidis* parent strain $D_{52°C}$ values compared well with the recombinant *S. enteritidis* $D_{52°C}$ values, thereby confirming that the *lux* plasmid does not disrupt or interfere with the thermal death kinetics of *S. enteritidis*. The $D_{52°C}$ values calculated in this study (Table 2) compare favorably with the values obtained for *S. enteritidis* ATCC 4931 heated in casein-peptone–soymeal-peptone broth and skim milk ($D_{52°C}$ values, 5.4 and 6.1 min, respectively), as determined by Xavier and Ingham (58). The slightly higher $D_{52°C}$ values calculated by Xavier and Ingham (58) were probably a result of differences in the heating media and strain used in their study. Both attached and detached cells exhibited an approximately twofold increase in $D_{52°C}$ values compared with the values calculated for planktonic cells (Table 2), strongly indicating that attached and detached cells exhibit greater resistance to heat inactivation than planktonic cells. The observation that detached cells exhibited the same thermal resistance phenotype as their attached counterparts indicates that there was no significant heterogeneity in the attached population at the time of heat treatment.

The differences in $D_{52°C}$ values for MM-grown planktonic, attached, and detached *S. enteritidis*(pSB311) cells were only marginally lower than the differences calculated for LB medium-grown cells. Overall, the stainless steel $D_{52°C}$ values for attached and detached LB medium- and MM-cultured cells were less than the glass coverslip $D_{52°C}$ values (Table 2). Lee and Frank (32) observed similar substratum-dependent variations in thermal death kinetics for *L. monocytogenes* and attributed the differences to the lower heat conductivity and insulation properties of glass compared with stainless steel.

Using bioluminescence to probe the physiological status of planktonic and attached cells. The $D_{52°C}$ values from the heat treatment studies described above, revealed that planktonic cells are less thermotolerant than attached cells, which is consistent with previous findings (15). The greater thermotolerance of the detached cells is explained by the fact that the cells were attached at the time of heating. Although the levels of thermotolerance of the attached and detached cells were essentially the same (Fig. 7), the MM-grown planktonic and detached cells exhibited an approximate 10-fold reduction in the level of bioluminescence per cell compared with the attached cells. This finding suggests either that attached cells are metabolically more active than detached or planktonic cells or, more probably, that the geometry of the detector which we used allowed greater efficiency of light accumulation from cells attached to surfaces than from cells not attached to surfaces.

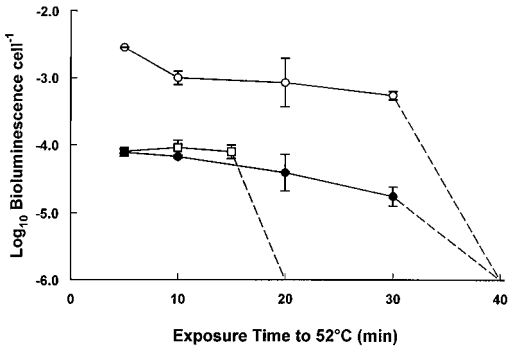

FIG. 7. Relationship between bioluminescence per cell and time of exposure to 52°C for planktonic (□), surface-attached (○), and detached (●) *S. enteritidis*(pSB311) cells grown in MM. The dashed lines indicate the times at which values for bioluminescence per cell were below the limits of detection. The datum points represent means ± standard errors of the means ($n = 3$).

Thermotolerance in *Escherichia coli* is enhanced following the synthesis of the disaccharide trehalose, which preserves membrane function and integrity (21). The genes responsible for trehalose synthesis are known to be controlled by *rpoS*. The observed thermotolerance of attached cells may thus be a consequence of prior gene expression during the period of cellular attachment (for example, expression of starvation-induced or stationary-phase genes regulated by the RNA polymerase sigma factor RpoS [20], which in turn induces expression of trehalose synthesis genes, if analogous systems exist in *S. enteritidis*). A consequence of such a theory is that, as detached cells were previously attached, these cells may well retain the postulated physiological adaptive responses induced in attached cells and could therefore be inherently more thermotolerant than their planktonic counterparts. We are currently studying the nature of thermotolerance in surface-detached cells of *S. enteritidis* and the putative role of *rpoS*.

Conclusions. It has been shown that engineering a full *lux* construction into a food isolate of *S. enteritidis* provides a sensitive and robust method for monitoring the effects of biocide challenge and heat treatment. As it is generally believed that the introduction of full *lux* constructions into bacteria for such physiological studies might result in spurious data because of the increased metabolic burden imposed upon the cells, successful validation of the system, as demonstrated in this study, is timely. When planktonic *S. enteritidis* cells are grown in MM, they are more susceptible to biocide challenge than attached cells and exhibit a lower level of thermotolerance. The general findings obtained in this study provide the foundation for future work involving the use of full *lux* constructions for in situ monitoring of single bacterial components within multixenic consortia or biofilms and also provide a unique opportunity with which to examine the physiological status of detached cells and the reversion of attached cells to the planktonic state.

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