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Salmonella enteritidis Phage Type 4 Isolates More Tolerant of Heat, Acid, or Hydrogen Peroxide Also Survive Longer on Surfaces

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In a comparative study of different *Salmonella enteritidis* phage type 4 isolates we found that those isolates with enhanced heat tolerance also survived better than isolates that were heat sensitive either at pH 2.6, in 10 mM H_2O_2, or on surfaces. Culture to the stationary phase increased the heat tolerance of all isolates and the acid and H_2O_2 tolerance of heat-tolerant isolates. With heat-sensitive isolates, however, extended culture had no impact on survival in H_2O_2 and only a marginal impact on acid tolerance. The growth phase had no appreciable impact on the surface survival of any of the isolates.

Different serotypes of salmonellas have been found to vary in their heat tolerance (8, 10). It is also possible to increase the heat tolerance of salmonellas by prior exposure to elevated temperatures (15, 20), alkaline conditions (14), or low-nutrient environments (16). In contrast, heat sensitivity can be induced by exposure to low temperatures (13). Acid tolerance can be enhanced by exposure to mildly acidic environments (11, 12, 18) and by growth at or shifts to higher temperatures (15). Conversely, cells exhibit reduced acid tolerance when they are cultured under alkaline conditions (24) or when certain virulence plasmids are introduced (7). Studies have also shown that the challenge conditions can have a marked impact on measured heat or acid tolerance (4, 5, 9).

Salmonella enteritidis phage type 4 (PT4) is an internationally important human pathogen, and *S. enteritidis* PT4 outbreaks have been associated with both cooked and acidified foods (1). In previous work in our laboratory workers have examined the survival of *S. enteritidis* PT4 at high temperatures (13–15) and low pH values (15). We decided to extend these studies as part of a large-scale study of behavior and virulence in *S. enteritidis* PT4 and to investigate possible isolate-to-isolate differences in tolerance of heat, acid, or hydrogen peroxide (H_2O_2) and in the ability to survive on surfaces.

In preliminary experiments, the rates of death of stationary-phase cultures of 40 isolates of *S. enteritidis* PT4 obtained from either chickens, eggs, or patients from unrelated incidents of *S. enteritidis* PT4 infection were determined at 52 ± 0.2°C by using previously described techniques (15), except that the diluent used was the Maximal Recovery Diluent (Oxoid Ltd.) and the cells were enumerated by using a spiral plater (Don Whitley Ltd.).

Ten isolates were examined in more detail, and the D(52°C) values (time to kill 90% of the population at 52°C) of these isolates are shown in Table 1. Our data indicated that the 10 isolates could be separated into two groups on the basis of heat tolerance. The four human isolates, which had D(52°C) values of 26.2 ± 1.0 min (mean ± standard error), were more heat tolerant than the six chicken or egg isolates, which had D(52°C) values of 17.3 ± 0.2 min. Additional work on heat and other tolerances was carried out by using two human isolates (isolates A and E) and two isolates from chickens (isolates C and I).

As the growth phase of an organism can influence its resistance to stressful conditions (6, 17), we were careful to ensure that cultures were in the same phase in comparative studies. The growth rates of static cultures of all four strains in lemco broth at 37°C were determined by monitoring both viable counts and optical density at 600 nm. Our results showed that the mean lag periods (43.3 ± 0.9 min) and the mean generation times (30.5 ± 0.7 min) were essentially the same for all four isolates. Not only did the strains all grow at the same rate, but they also took the same length of time to enter the stationary phase. To obtain log-phase cells, the optical densities of 18-h cultures were adjusted to 0.2, 1 ml of each culture was added to 9 ml of fresh lemco broth, and the preparations were incubated at 37 ± 0.5°C for 3 h. To obtain stationary-phase cells, three or four colonies of each isolate were inoculated into a separate tube containing 9 ml of lemco broth; the optical densities were standardized to 0.2, 1 ml of each standardized suspension was added to 9 ml of lemco broth, and the resulting cultures were incubated at 37 ± 0.5°C. All isolates entered the stationary phase at 11 h and were used at 15 h, and the number of viable cells used was the same for each isolate.

The cultures described above were used on at least five separate occasions to measure death rates in lemco broth at either 52 ± 0.2°C or pH 2.6 ± 0.02, in the presence of 10 mM H_2O_2 in distilled H_2O, or in droplets of sterile, lysed horse blood on Formica squares exposed to air at 20 ± 0.5°C. Heat tolerance was measured as described previously. To measure death rates at pH 2.6 at 37 ± 0.2°C, we used previously described techniques (15). To study survival in the presence of H_2O_2, cultures were inoculated into distilled H_2O (pH 7.0) containing 10 mM H_2O_2 and incubated at 37 ± 0.5°C. Portions (1 ml) were removed at intervals and placed into 9 ml of Maximal Recovery Diluent containing 10% sterile whole blood. Cells were enumerated as described above. To determine death rates on Formica, 0.1-ml samples of cultures were added to 10-ml portions of sterile, hemolyzed whole horse blood. One 0.02-ml drop of blood was then placed on each of up to 80 2-cm^2 squares of Formica. These squares were put in sterile petri dishes and kept at 20 ± 0.5°C for up to 5 weeks in

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TABLE 1. D(52°C) values for some *S. enteritidis* PT4 isolates

Isolate	Source	D(52°C) value (min)[a]
A	Human stool	26.8
B	Human stool	24.2
C	Chicken carcass	18.1
D	Human stool	25.1
E	Human stool	28.6
F	Eggshell	17.3
G	Chicken carcass	16.9
H	Egg contents	17.0
I	Chicken carcass	16.8
J	Egg contents	17.9

[a] 15-h cultures.

air. At intervals ranging from 6 h to 1 week, depending on the *S. enteritidis* isolate and time after contamination of the squares, three squares were removed, and each was placed in 10 ml of Maximal Recovery Diluent in a sterile 25-ml screw-cap plastic container. The blood, which dried within 3 h of being placed on the square, was removed from the square and into Maximal Recovery Diluent by using a sterile plastic loop and was distributed by vigorous shaking. The cells were enumerated as described above. The initial levels of *S. enteritidis* were approximately 10^6 cells per ml in the acid, heat, and H_2O_2 tolerance experiments and approximately 10^5 cells per square in the survival experiments. To minimize experimental variation, death rates were determined by using media, H_2O_2, or blood from the same batch in each experiment. The levels of significance for differences in either tolerance or survival were determined by using paired or unpaired *t* tests.

The results of a more detailed analysis of the survival profiles of isolates A, C, E, and I supported previous observations concerning differences in heat tolerance. There was also apparent linkage between tolerance of one hostile environment and the ability to survive in another, and this linkage was influenced by the growth phase of the salmonella isolates. D values are shown in Table 2, and the levels of survival after defined exposure periods are shown in Table 3. Log-phase (3-h) cells were very sensitive to both heat and acid, and the levels of survival after incubation for 10 min at 52°C or for 5 min at pH 2.6 were low (Table 3).

As expected, culture to the stationary phase increased tolerance of both heat and acid, but the impact of extended culture appeared to differ between the two challenge conditions and between isolates. All four salmonella isolates exhibited markedly increased heat tolerance in the stationary phase (Tables 2 and 3). The differences between log phase and stationary phase were greater with isolates A and E, and a consequence of this was that these salmonellas were significantly ($P < 0.05$) more heat tolerant in the stationary phase than either isolate C or isolate I.

The death curves at pH 2.6 were biphasic. During the first 1 to 2 min after exposure to acid the death rates were relatively low, with the viability of the log-phase cultures reduced by approximately 90% and the viability of the stationary-phase cultures reduced by approximately 10%. After this initial period the death rates were higher, particularly with log-phase cells. The four isolates appeared to be equally acid sensitive in the log phase. Culture to the stationary phase increased acid tolerance in all isolates but had a greater impact on the two isolates obtained from humans. Thus, with stationary-phase cultures (15-h cultures) the D values for the human isolates were significantly higher ($P < 0.05$) than the D values for the isolates from chickens (Table 2). The differences were even more marked when levels of survival after 5 min were compared. With log-phase cultures, the measured values were low, and <0.01% of each isolate survived. Culture to the stationary phase had relatively little impact on the levels of survival of the chicken isolates but increased the levels of survival of the human isolates more than 500-fold (from <0.01 to >5%) (Table 3). The differences between the values obtained for the human and chicken isolates in the stationary phase were highly significant ($P < 0.001$) (Table 3).

The results obtained for tolerance of H_2O_2 and the ability to survive on surfaces differed from the heat and acid tolerance results. In the log phase, the human isolates survived much better ($P < 0.001$) than the chicken strains either in the presence of 10 mM H_2O_2 or on surfaces at 20°C (Tables 2 and 3). Culture to the stationary phase had almost no impact on the H_2O_2 tolerance of the chicken isolates but greatly increased the levels of survival of the human isolates. Thus, for example, after 10 min in the presence of 10 mM H_2O_2, 18.2% ± 1.1% of the log-phase cells of isolate A remained viable. When stationary-phase cells of this isolate were challenged under identical conditions, 75% ± 5.0% of the population survived. With isolate C the corresponding figures for log- and stationary-phase cells were 0.3% ± 0.02% and 0.8% ± 0.1%, respectively (Table 3).

Survival on surfaces was not affected by the growth phase, and the D values for the 3- and 15-h cultures were very similar (Table 2). In all experiments, the human isolates survived sig-

TABLE 2. D values for *S. enteritidis* PT4 isolates

Condition(s)	Growth phase[a]	D value[b]			
		Human stool isolates		Chicken carcass isolates	
		Isolate A	Isolate E	Isolate C	Isolate I
52°C	Log	2.0 ± 0.1	3.1 ± 0.1	1.9 ± 0.1	2.3 ± 0.1
	Stationary	31.9 ± 0.7	32.5 ± 2.5	19.8 ± 1.8	18.9 ± 0.7
pH 2.6	Log	1.7 ± 0.1	1.9 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
	Stationary	4.3 ± 0.1	4.7 ± 0.3	2.9 ± 0.2	3.0 ± 0.2
10 mM H_2O_2	Log	13.3 ± 0.4	12.2 ± 0.7	3.0 ± 0.2	3.5 ± 0.1
	Stationary	30.3 ± 0.9	26.7 ± 2.9	3.9 ± 0.4	4.3 ± 0.3
Blood droplets at 20°C	Log	>35	>35	0.4 ± 0.05	0.3 ± 0.02
	Stationary	>35	>35	0.3 ± 0.01	0.3 ± 0.01

[a] Log-phase cells were cultured at 37°C for 3 h, and stationary-phase cells were cultured for 15 h.

[b] Means ± standard errors, calculated from at least five separate experiments. The data obtained at 52°C, at pH 2.6, and in the presence of 10 mM H_2O_2 are expressed in minutes, and the data obtained with blood droplets at 20°C are expressed in days.

TABLE 3. Survival values for *S. enteritidis* PT4 isolates exposed to different damaging environments

Conditions	Growth phase[a]	% Survivors[b]			
		Human stool isolates		Chicken carcass isolates	
		Isolate A	Isolate E	Isolate C	Isolate I
10 min at 52°C	Log	0.02 ± 0.01	<0.01	0.05 ± 0.02	0.05 ± 0.03
	Stationary	37.0 ± 6.0	35.8 ± 3.0	26.7 ± 3.0	20.3 ± 0.9
5 min at pH 2.6	Log	<0.01	<0.01	<0.01	<0.01
	Stationary	6.1 ± 0.1	4.4 ± 0.3	0.02 ± 0.01	<0.01
10 min in 10 mM H_2O_2	Log	18.2 ± 1.1	12.7 ± 1.8	0.3 ± 0.02	0.2 ± 0.02
	Stationary	75.0 ± 5.0	71.0 ± 4.0	0.8 ± 0.1	1.3 ± 0.3
24 h in blood droplets at 20°C	Log	52.5 ± 4.6	45.0 ± 2.6	2.4 ± 0.3	1.9 ± 0.2
	Stationary	59.0 ± 3.4	46.5 ± 5.0	1.7 ± 0.2	2.2 ± 0.3

[a] The culture conditions are described in Table 2, footnote *a*.
[b] Means ± standard errors, calculated from at least five separate experiments.

nificantly better ($P < 0.001$) than the isolates obtained from chickens (Table 2).

During the initial screening of the various *S. enteritidis* PT4 isolates, some isolates appeared to be more heat tolerant (Table 1). The results of a more detailed analysis of four isolates, two obtained from humans and two obtained from chickens, supported these observations and also demonstrated that tolerance of elevated temperatures was accompanied by better survival in the presence of acid or H_2O_2 or on surfaces. It would be interesting to screen many more isolates to see whether greater tolerance is a common feature of salmonellas recovered from humans.

The observations described in this paper may have practical significance. Thus, isolates which exhibit enhanced tolerance of heat and acid may be better able to survive cooking or conditions in the stomach. Destruction of ingested bacteria by phagocytes involves the liberation of H_2O_2, and enhanced tolerance to this compound may facilitate infection. Cross-contamination during catering is known to be important in food poisoning outbreaks caused by *S. enteritidis* PT4 (1) and other salmonellas (23). The extent to which this occurs is governed, in part, by the ability of the bacteria to survive on surfaces. Thus, acid-, heat-, and H_2O_2-tolerant cells which also exhibit prolonged survival on surfaces may be more likely to come into contact with foodstuffs than cells which die quickly on surfaces.

A variety of studies have demonstrated that stationary-phase cells exhibit greater tolerance than cells in the log phase (6, 17). The results of our study are in general agreement with previous observations. However, differences in growth phase cannot explain the observed differences between the human and chicken isolates. In this study we performed extensive experimental work on the growth curves of the four isolates which we examined. Whether measurements were obtained by determining optical densities or by viable counting, the growth curves appeared to be identical and cultures entered the log and stationary phases at essentially the same times. There was also rigid standardization of inocula and growth conditions in all experiments.

As stated above, our findings are consistent with previous findings showing that cells in the stationary phase survive better under a range of adverse conditions than cells in the log phase. It also appears, however, that the impact of the stationary phase differs not only with the challenge conditions but also with the PT4 strain. For example, the increases in heat and acid tolerance in stationary-phase cells were much greater in strains A and E than in either strain C or strain I. Extended culture, however, had no impact on the ability to survive on surfaces and only increased H_2O_2 tolerance in the human isolates.

Previous studies in our laboratory and elsewhere have shown that it is possible to increase the acid or heat tolerance of salmonellas and related bacteria by exposure to or growth under certain conditions. This study was the first study to examine a range of apparently inherent tolerances in *S. enteritidis* PT4 strains cultured under identical conditions, at pH 7.0 and 37°C, and in the same phase of growth. Our results show that different PT4 isolates can have significantly different survival profiles. More importantly, cells which exhibit enhanced tolerance to high temperatures are also better able to survive in the presence of acid or H_2O_2 or on surfaces. Given the results of previous studies (16, 19, 21, 22, 25), these observations may have been expected. The differing impacts of growth phase and isolate type on the observed tolerances, however, suggest that the cellular attributes which increase tolerance of one damaging environment may not be the same attributes which permit enhanced survival in another environment even when apparent cross-tolerance is observed.

Recently, Ak et al. (2, 3) demonstrated that the survival of *Salmonella typhimurium* is influenced by the nature of the material onto which it is placed. Our results demonstrate that isolates of the same salmonella phage type have very different survival profiles on the same surface. The significance of our observations has yet to be fully assessed, but work is under way to compare isolates obtained from foods and infected persons in the same outbreak.

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