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Pathogenicity of *Salmonella gallinarum* After Metabolic Injury by Freezing[1]

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Freezing (−75 C) and storage (−20 C) of a cell suspension of *Salmonella gallinarum* resulted in a heterogeneous population of dead, metabolically injured, and unharmed cells. Injured cells constituted as much as 40% of those surviving freezing and storage for 1 day. Replica plating of frozen and thawed cells indicated metabolic injury was repairable and not a stable mutation. Penicillin was used to increase the ratio of injured to uninjured cells from a frozen and thawed cell suspension. Pathogenicity was evaluated by observing per cent mortality after injecting injured or uninjured cells into separate sets of chicks. Mortality differences between wholly uninjured and predominantly injured populations were small and consistent (5% level) with a hypothesis of no difference.

Sublethal damage or metabolic injury of microorganisms, due to freezing, has been increasingly demonstrated (1, 13-15, 16, 19). Hartsell (9) and Nakamura and Dawson (15) suggested that *Salmonella typhosa* and *Shigella sonnei* underwent metabolic alteration during freezing and frozen storage. However, there appears to be limited information pertaining to alteration of pathogenicity of salmonellae due to metabolic injury. Simon, et al. (18) reported that the virulence for mice of three strains of *Salmonella typhimurum* did not change appreciably when samples were freeze-dried and stored for 1 or 2 years at 5 C. The present study was undertaken to determine (i) whether *Salmonella gallinarum* undergoes metabolic injury after freezing, and (ii) whether freeze-injured cells demonstrated an alteration in pathogenicity.

MATERIALS AND METHODS

Source and maintenance of test culture. A highly virulent culture for chicks of *S. gallinarum* no.FM1 (isolated from an outbreak of fowl typhoid) was obtained from C. H. Hill, Department of Poultry Science, North Carolina State University. This culture was used throughout this study. The stock culture was maintained by monthly subculture on Trypticase Soy Agar (TSA) slants, incubated 18 hr at 37 C, and subsequently stored at 4 C. Virulence of the culture was tested monthly by using a subculture prepared in Trypticase Soy Broth (TSB) incubated at 37 C for 18 hr. The cells were centrifuged and resuspended in distilled water to contain 10^8 cells/ml. One milliliter of the cell suspension was injected intraperitoneally into each of five chicks. Death usually occurred in 5 to 7 days.

Growth media. TSA served as a complete medium for enumeration of total viable cells. A minimal medium (MA) supplemented with leucine and thiamine (10) was used for the detection of metabolically injured cells. Its composition was K_2HPO_4, 7.0 g; KH_2PO_4, 3.0 g; trisodium citrate·$2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1.0 g; glucose (autoclaved separately), 2.0 g; and agar (autoclaved separately), 15 g; distilled H_2O, 1,000 ml. The *p*H was adjusted to 7.0. Omission of agar provided minimal broth (MB). Prior to use, 1 ml each of filter-sterilized leucine (3 mg/liter) and thiamine (2 mg/liter) was added.

Preparation of uninjured test culture. An initial transfer from the stock culture slant was made into TSB and incubated at 37 C for 18 hr (static). From this subculture a 0.5% inoculation was made into fresh TSB. The freshly inoculated medium was shaken by using a constant-temperature gyrotory shaker at 37 C, until the culture reached the mid-log phase of growth (approximately 4 hr). After incubation the cells were chilled to 2 C and harvested from the medium by centrifugation. The cell pellet was resuspended into a volume of distilled water equivalent to the original culture volume, and this procedure was repeated two additional times. The cells were thoroughly shaken and divided into 5-ml portions for freezing. A 1-ml portion of unfrozen suspension was plated on TSA and MA and served as a control.

Injury of cells by freezing. The 5-ml portions of cells were frozen in a dry ice-acetone bath at −75 C. The rate of freezing was approximately 20 C/min. After freezing, the suspensions were stored at −20 C for 1

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day. After removal from storage, samples were thawed at 37 C for 4 min. Immediately after thawing, the cells were plated on TSA and MA. Death and injury of the cells, after freezing and thawing, were determined as described by Straka and Stokes (19), i.e., (i) unharmed cells—those that grew on MA after freezing; (ii) dead cells—the difference between the plate count on TSA before and after freezing; (iii) injured cells—the difference in plate count, after freezing, on TSA and MA.

Test for mutation. The replica-plating procedure of Lederberg and Lederberg (12) was used to test for the presence of stable auxotrophic mutants. Thawed cell suspension (1 ml) was inoculated into TSB and incubated at 37 C for 4 hr. After incubation, serial dilutions were made and streaked on previously poured TSA plates. The plates were incubated at 37 C until the first visual appearance of colonies and then replica plated onto MA. Both plates were then incubated at 37 C for 24 hr.

Penicillin treatment of cells. A modification of the Davis penicillin method (6) was used to increase the ratio of injured to uninjured cells. Thawed cell suspension (2 ml) was transferred into 20 ml of MB and shaken 13 hr at 37 C by using a rotary shaker. Penicillin (penicillin G potassium U.S.P., 1585 units/mg, General Biochemical) was then added to the culture (final concentration, 475 units/ml) and incubated for 12 hr longer. Penicillinase (10 mg/ml, Nutritional Biochemicals Corp.) was added to inactivate any excess penicillin, and the culture was incubated at 37 C for 15 min. These were used in the chick injections. Unfrozen cells, treated similarly, served as a control for penicillin-treated cells and were also used in chick injections.

Test for pathogenicity of injured cells. Chicks (180 White Plymouth Rock, 6 weeks old, weighing between 300 and 1,000 g) were randomly divided into 18 groups of 10 birds each. Each group of chicks was injected intraperitoneally with 0.5 ml of a different treatment of *S. gallinarum*. All treatments were performed in triplicate. Cages were selected at random and 10 chicks that received the same treatment were assigned to each. The percentage of death of the chicks in each cage was obtained after 7, 9, 11, and 15 days. So that all responses could be considered as having common variance, percentage of death for each cage was transformed into arcsin values.

Analysis of variance was used in summarizing the data. Least significant differences (LSD) were calculated for each day and used as confidence limits for differences observed between the treatments.

RESULTS

When unfrozen cells of *S. gallinarum* were plated on MA, they failed to form visible colonies. When MA was supplemented with leucine and thiamine (11) counts then were equal to those obtained with TSA. Freezing (at −75 C) with no storage caused 86% death of the cells. Of the survivors, 29% were injured (Table 1). After storage for 1 day at −20 C, 88% death was observed, and, of the survivors, 42% exhibited metabolic injury.

TABLE 1. *Effect of freezing and storage on viability of cell suspensions of Salmonella gallinarum as determined by plating on Trypticase Soy Agar (TSA) and minimal medium (MA)*[a]

Treatment	Plating medium	Count	Death	Injury[b]
			%	%
Unfrozen	TSA	180×10^6		
	MA	180×10^6		
Frozen (−75 C)	TSA	25×10^6	86	
	MA	18×10^6		29
Frozen (−75 C), stored 1 day at −20 C	TSA	23×10^6	88	
	MA	13×10^6		42

[a] Average of four trials. Least significant differences: death, 1.3%; injury, 11.5%.
[b] Percentage of survivors injured.

Since injured cells showed an increased nutritional requirement(s), the possible development of mutants was evaluated. After freezing and thawing, cell suspensions of *S. gallinarum* were inoculated into TSB and incubated at 37 C for 4 hr. From this culture, TSA plates were streaked and incubated at 37 C until the first visible appearance of colonies. These were replica plated (12) onto MA. After additional incubation at 37 C for 24 hr, the colonies were compared as to positions. All colonies matched on both media. Approximately 800 colonies were tested, and none appeared to be stable auxotrophic mutants.

To evaluate the pathogenicity of injured cells for chicks, it was desirable to increase the ratio of injured to uninjured cells. Penicillin was used for this purpose since it is known to affect growing cells only. The inability of most injured cells to grow in MB was used to reduce uninjured cells in a mixed population. When penicillin was added to a 13-hr shake culture of unfrozen cells in MB, a rapid decrease in cell numbers occurred in 6 hr as observed by colony counts on TSA and MA (Fig. 1A). During the next 6 hr, the cell numbers continued to decrease, but at a slower rate. Counts on TSA and MA were equal, indicating the absence of metabolically injured cells in the population. When frozen cells were placed in MB, growth of the uninjured cells occurred; however, on addition of penicillin, a rapid decrease in count occurred, and counts on MA were lower than those on TSA (Fig. 1B). After further incubation for 6 hr, the counts on MA continued to decrease, whereas the counts on TSA decreased very little. Thus the penicillin was able to reduce the number of uninjured cells present, leaving the injured cells in predominance. The effect of the penicillin was further analyzed by calculating the rate of

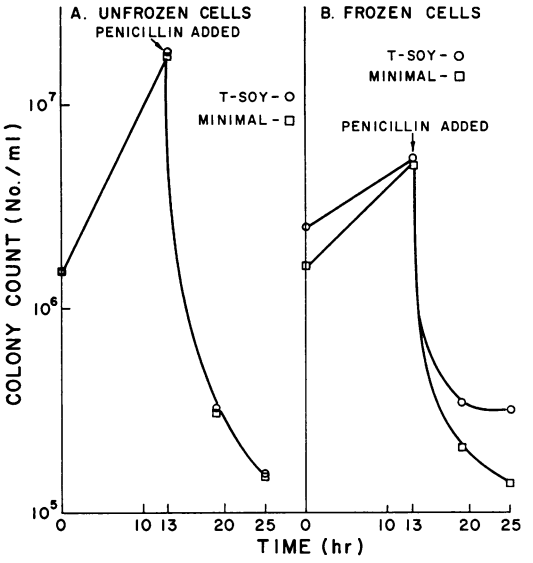

FIG. 1. *Populations of Salmonella gallinarum after penicillin treatment of frozen and unfrozen cells in MB incubated at 37 C.*

change in the ratio of injured to uninjured cells (Fig. 2), using the slope of a straight line approximating the relation between time and per cent of injury. An analysis of variance and LSD was then computed for the calculated rates. The rate for penicillin-treated frozen cells was 3.7% increase/hr and −0.58%/hr for unfrozen cells. This indicated that the penicillin treatment was indeed effective in increasing the ratio of injured to uninjured cells.

In evaluating the effect of metabolically injured *S. gallinarum* on pathogenicity, the control group of chicks consisted of those inoculated with uninjured cells (T_1). The primary test group was inoculated with a suspension containing approximately 60% metabolically injured cells (T_2), as obtained by reducing the uninjured population with penicillin treatment. Both suspensions contained about the same total number of viable cells; this was accomplished by diluting the uninjured cell suspension with the required volume of sterile, distilled water. Other groups of chicks were injected with different treatments (Table 2) of *S. gallinarum* and permitted additional comparisons, namely, (T_3) unfrozen cells treated with penicillin were included so that a comparison could be made of mortalities where the number of uninjured cells was about the same as that in frozen penicillin treated cells (Fig. 1A and B) which remained after treatment; (T_4) frozen cells with approximately 30% injured were included so that a comparison could be made where cell numbers were about the same, but per cent of injured (or per cent of uninjured) differed from T_2; (T_5) a high number of uninjured cells was used so that a comparison could be made where number of uninjured cells was double that of T_1; (T_6) minimal broth served as a control with no pathogens present. The lethality of the different treatments of *S. gallinarum* has been summarized by using analysis of variance. LSD were calculated from the analysis of variance data. Statistical analyses

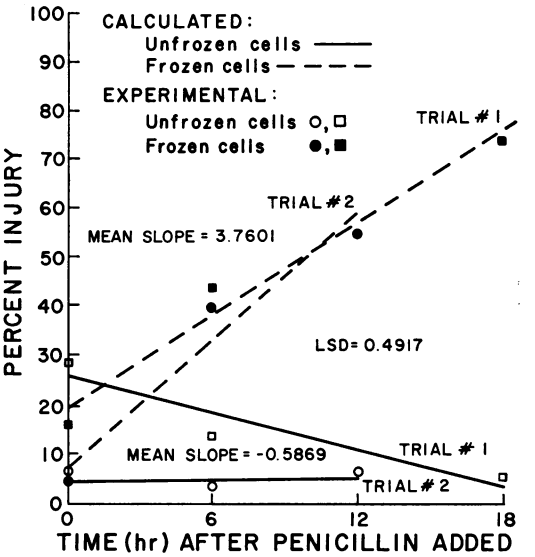

FIG. 2. *Effect of penicillin on the ratio of injured to uninjured cells of Salmonella gallinarum.*

TABLE 2. *Effect of different treatments of Salmonella gallinarum on death of young chicks*

Treatments	No. injected	Day 15[a]
T_1 Unfrozen cells (uninjured)[b]	1.2×10^5	46.92
T_2 Frozen cells (approximately 60% injured)[b]	1.4×10^5	39.06
T_3 Unfrozen cells (penicillin treated)	7.0×10^4	43.07
T_4 Frozen cells (approximately 30% injured)	1.3×10^5	34.92
T_5 Unfrozen cells	2.8×10^5	37.14
T_6 Minimal broth	0	0
LSD (0.05)[c]		13.08

[a] Values under this expressed as average arcsin values of per cent of death.

[b] Main treatment.

[c] If limits for the differences between treatments ± least significant differences (LSD) includes zero, then the difference is not significant at the 5% level.

of the data revealed no significant difference (5%) level in the lethality between uninjured cells and those of which approximately 60% were injured. The results of all the chick injections are presented in Table 2. A split-plot analysis of variance was also conducted, including a complete partition of the treatment × time period sum of squares, for the number of birds dead per day. It failed to indicate (5% level) any detectable relation between time of recording and differences between treatments. Accordingly, only results for number of birds which had succumbed after 15 days are presented.

DISCUSSION

Metabolic injury of *S. gallinarum* in this study was manifested by an increase in nutritional requirements. This was based on a greater number of colonies appearing on the complete medium (TSA) as compared to colony formation on the minimal medium (MA). Metabolic injury was obtained by freezing and thawing the cells without storage. Storage of the cells at −20 C resulted in additional injury. Postgate and Hunter (16) also obtained freeze-injury without storage with *Aerobacter aerogenes*. Straka and Stokes (19), however, observed injury in *Escherichia coli* and *Pseudomonas* species only after frozen storage.

Replica plating indicated injured cells were able to regain their synthetic capabilities when grown on a nutritionally complete medium. MacLeod, et al. (13) reported no stable auxotrophic mutants from frozen and thawed *E. coli* and *A. aerogenes*. Ashwood-Smith (2) concluded that freezing per se was not mutagenic. Postgate and Hunter (16), however, obtained three auxotrophic mutants of *A. aerogenes* from 2,135 survivors after freezing and thawing.

The absence of a detectable difference between the effects of uninjured and injured cells on chick mortality suggests that metabolic injury due to freezing did not alter pathogenicity. Repair of the injury could have occurred while the cells were within the intraperitoneal cavity of the chick. This might be expected since replica plating indicated that injury was repairable in a nutritionally adequate environment. Simon, et al. (18) found *S. typhimurum* remained virulent for mice after being freeze-dried and stored 1 to 2 years at 5 C. Szturm-Rubinsten, et al. (20) reported that *Shigella sonnei*, frozen in ice used in a patients food, was responsible for dysentery.

During repair by injured cells it was possible that the chick could produce antibodies against the organism before establishment in the host. Wolfe and Dilks (21) reported serological "maturity" was reached at 5 weeks in chickens. Six-week-old chicks, presumably unexposed to *Salmonella gallinarum*, were used in this study. However, antibodies are not found circulating in the blood stream until 5 to 7 days after injection with the antigen (5). In this study, death began to occur in 7 days.

The public health significance of these findings should be evident. Certain pathogens can survive freezing for many months in food products (7, 8, 11, 17, 20, 22). These pathogens could go undetected in frozen foods (3, 9, 15) and still be potential hazards. It would be desirable to ascertain whether pathogenicity of other pathogenic microorganisms remains similarly unaffected by freezing.

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