Thermal Tolerance Studies on the Heterofermentative Lactobacilli That Cause Greening of Cured Meat Products[1]

C. F. Niven, Jr., L. G. Buettner, and James B. Evans

Division of Bacteriology, American Meat Institute Foundation, University of Chicago, Chicago, Illinois

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Niven, Castellani, and Allanson (1949) described a new species of heterofermentative *Lactobacillus* that frequently is the cause of surface greening on cured meat items. This type of discoloration results from contamination of the meat surface after heat processing followed by holding conditions that allow extensive growth of these bacteria. The green color is the result of hydrogen peroxide produced by these bacteria reacting with the cured meat pigment (Niven, 1951). Lactobacilli with identical serological, physiological, and nutritional characteristics also were isolated from sausages with green cores (Niven and Evans, 1950; Evans and Niven, 1951). This latter type of discoloration is caused by bacteria that survive the smoking and cooking process and multiply to a large population during subsequent storage of the product. The green core does not appear until the cut surface has been exposed to air for several hours, allowing the microorganisms in the core area to produce hydrogen peroxide (Niven, 1951).

Thermal tolerance studies on some of these greening lactobacilli showed that strains isolated from green cores survived 6 to 10 times as long at 145 to 155 F as did strains isolated from cases of surface discoloration. These unexpected results suggested the possibility that conditions of manufacture and handling of these products in some plants are such that progressively more heat-resistant strains are "selected" or "trained".

The heat resistance of lactobacilli from cheese has been studied by Slatter and Halvorson (1947) and Bassett and Slatter (1953) without reporting any similar wide variation in resistance among closely related strains. White (1953), studying the variations in heat resistance of a culture of *Streptococcus faecalis* in different stages of its life cycle, found that survivor curves of young cultures often show a change in slope in the middle of such curves. However, this could have been due to a residue of physiologically old cells in the young culture.

An investigation of some of the factors concerned with variation in heat tolerance of strains of greening lactobacilli is described in the present report.

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Experimental Methods and Results

Thermal tolerance determinations. Cultures were grown in a tomato juice broth containing 1.0 per cent tryptone, 0.5 per cent yeast extract, 0.5 per cent NaCl, 0.5 per cent glucose, 0.2 per cent K_2HPO_4, 0.014 per cent $MnCl_2 \cdot 4H_2O$ and 25 per cent filtered tomato juice, pH 7.0. Five ml of tomato juice broth in a 16-mm tube was inoculated with 1 drop of a 24-hour culture, stoppered with a sterile rubber stopper, and placed in a water bath at the desired temperature. The level of the water in the bath was even with the bottom of the rubber stoppers. The time that the temperature of a control tube reached the temperature of the bath was taken as zero time. At specified intervals tubes were removed to an ice water bath and after cooling were incubated at 30 C for 7 days.

Results with a typical strain from surface greening (M1A) and a typical strain from a green core (S41A) are shown in table 1. Approximately a 6- to 10-fold difference in survival time was found to exist between these otherwise identical organisms. Three other strains from surface greening and three additional strains from green cores gave essentially similar results.

Selection of heat-resistant survivors. An attempt was made to select more heat-resistant strains from two thermolabile cultures. The thermal tolerance of these strains was determined at 150 F (65.5 C) using the method described in the previous section. The tubes that survived the longest heat treatment were used, after growing to a heavy turbidity, as the inocula for another determination of heat resistance. This procedure was repeated 14 times which resulted in about a 2-fold increase in thermal tolerance, as shown in table 2. However, these strains exhibited much less thermal tolerance than did naturally occurring strains isolated from the interior of sausages that had been cooked to an internal temperature of 140–150 F.

Survivor curves. Since attempts to select heat-resistant survivors by the above technique were relatively unsuccessful, it seemed desirable to determine survivor curves on one of these cultures and on a naturally occurring, heat-resistant strain. A flask containing 50 ml of tomato juice broth was inoculated with 5 ml of a 24-hour culture of the respective strains

TABLE 1. *Thermal tolerance of two representative greening lactobacilli*

TEMPERATURE	STRAIN M1A*		STRAIN S41A†	
	Survived	Killed	Survived	Killed
F	*min*	*min*	*min*	*min*
145	40	45	240	270
150	10	12	120	130
155	—	5	45	50
160	—	—	8	10

* Isolated from the green surface of a meat loaf.
† Isolated from the green core of a bologna.

TABLE 2. *Selection of heat-resistant survivors by heat shocking*

STRAIN	TOLERANCE OF PARENT TO 145 F		TOLERANCE AFTER 14 SUCCESSIVE HEAT SHOCKS	
	Survived	Killed	Survived	Killed
	min	*min*	*min*	*min*
M1A	10	12	16	18
S11A	12	14	26	29

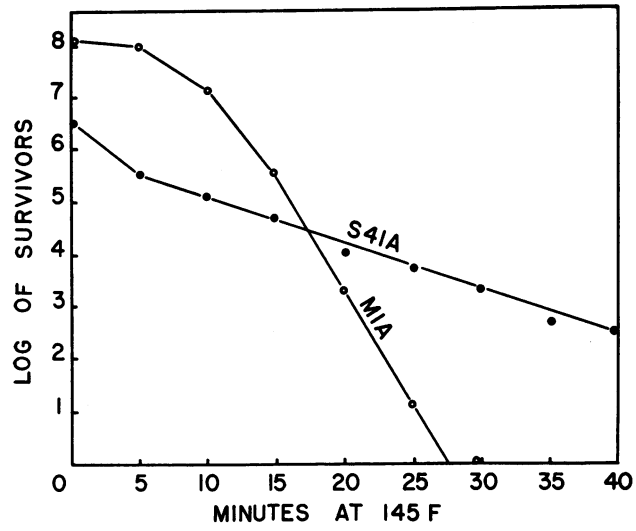

FIG. 1. Survivor curves of typical lactobacilli from surface greening (M1A) and from sausages with green cores (S41A).

and, after mixing, 5-ml aliquots were withdrawn and placed in sterile 16-mm test tubes. The tubes were stoppered and placed in the constant temperature water bath at 145 F (62.8 C). The tubes were withdrawn at specified intervals, chilled in ice water, and plated quantitatively on APT agar (Evans and Niven, 1951). The results are shown in figure 1. It would appear that culture S41A contained two types of cells, a large percentage of thermolabile cells and a small percentage of resistant cells.

In view of the above results, 8 colonies were picked from the plating of an unheated tube of S41A and 8 colonies were picked from the plating of a tube that had been heated for 30 minutes at 145 F. These 16 isolates were tested for thermal tolerance by the usual

TABLE 3. *Thermal tolerance of isolates obtained from a culture of greening* Lactobacillus *before and after heat shocking**

ISOLATES FROM UNHEATED CULTURE		ISOLATES AFTER HEATING 30 MIN AT 145 F	
Survived	Killed	Survived	Killed
min	*min*	*min*	*min*
10	20	120	140
10	20	120	140
80	100	160	180
100	120	180	200
100	120	180	200
120	150	200	220
120	150	200	220
120	150	240	260

* Parent strain survived 120 minutes but not 140 minutes at 145 F.

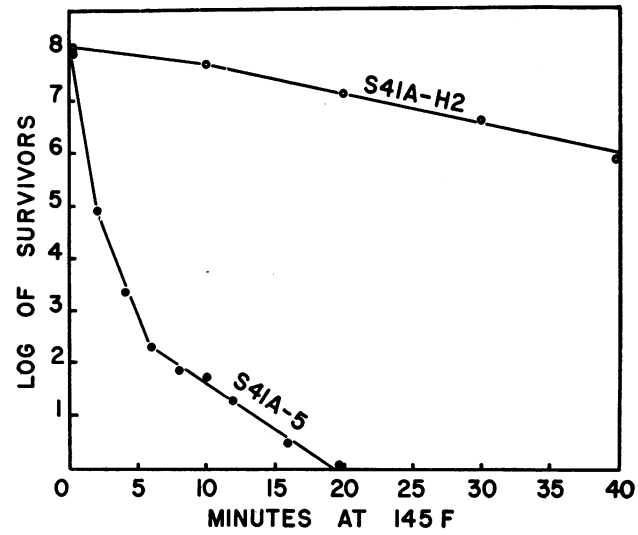

FIG. 2. Survivor curves of a heat-resistant variant (S41A-H2) and a heat-labile variant (S41A-5) isolated from the same parent culture.

method, and the results are shown in table 3. These results indicated that those isolates from the heated parent culture are in general more heat-resistant than the isolates from the unheated parent culture. However, the parent strain was only about half as resistant as when tested several months previously (see table 1), and a year later this strain survived only 40 minutes at 145 F.

Survivor curves next were run on one of the heat-resistant isolates (S41A-H2, that survived 240 minutes at 145 F) and on one of the heat-labile isolates (S41A-5, that survived less than 20 minutes at 145 F). These are shown in figure 2. These results dramatically demonstrate the wide difference in heat tolerance of two isolates from the same parent culture. However, strain S41A-5 appeared to have a few hundred cells that were fairly heat-resistant. Re-isolates from this culture gave some strains that survived 9 but not 12 minutes at 145 F. Survivor curves on these strains still showed the sharp break as did S41A-5, indicating a few cells of greater heat resistance. We

were also able to isolate thermal-tolerant strains from heat-labile cultures that had been heated long enough to kill off most of the labile cells (see table 3). Starting with a parent strain that survived 30 but not 40 minutes, five isolates from heated tubes survived from 90 to more than 120 minutes and three isolates from the parent culture survived 30 or 40 minutes.

Thus it has been possible to isolate variants that will withstand only 9 to 12 minutes at 145 F from a parent strain that was able to withstand 240 minutes at the same temperature. We also have been able to isolate variants that will withstand as much as 120 minutes at 145 F from a parent strain that survived only 30 to 40 minutes at this temperature. All of these variants are identical with the parent strain in their physiological and serological characteristics obviating the possibility of mixed or contaminated cultures.

In the course of this work it was observed that when an unheated culture was plated on APT agar and incubated at 30 C for 48 hours all of the colonies were large and easy to count. However, when a culture was heated sufficiently to kill a majority of the cells and plated in a similar manner many colonies were extremely small or even invisible after 48 hours and the plates had to be incubated for 5 days before an accurate count could be made. When some of these minute colonies were picked into APT broth, incubated for 24 hours, and replated, the colonies produced were all large and readily counted. These results are somewhat similar to those of Curran and Evans (1937), Nelson (1943), and others, that heat-treated bacteria are often more fastidious than the parent culture. It was also noted that when both large and minute colonies from a heat-treated culture were cultured and their thermal tolerance determined in the usual manner, the cultures from large colonies were in most instances more heat-tolerant than those from minute colonies.

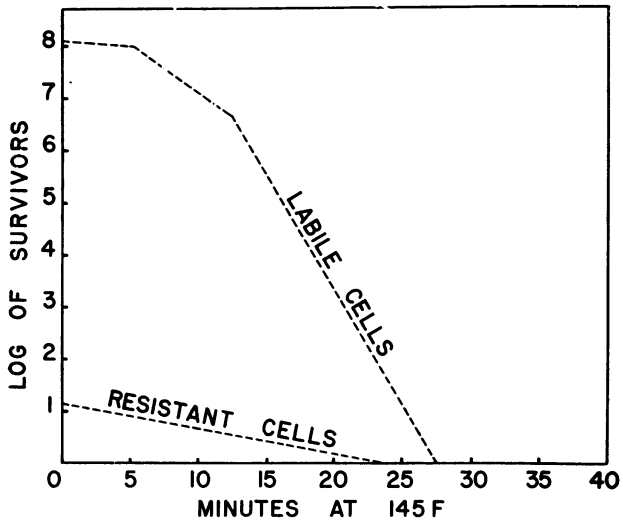

FIG. 3. Hypothetical survivor curves for a large number of thermolabile cells and a small number of resistant cells such as might be found in a fully grown culture of a heat-labile greening *Lactobacillus*.

DISCUSSION

The interpretation of these results in terms of the classical theories of adaptation or mutation and selection is very difficult. However, the recently postulated mechanism of "clonal variation with selection" (Yudkin, 1953) may offer a better framework for explaining our findings.

The heat-resistant strains of greening lactobacilli isolated from naturally occurring outbreaks of green-cored sausage would represent stabilized, resistant strains. These might develop as a result of repeated heat shocking with only a limited amount of multiplication between heatings. In our attempts at training (see table 2), extensive growth in the absence of any selective agent was permitted between heatings. Furthermore, an examination of the hypothetical survivor curves shown in figure 3 indicates that when a mixture of large numbers of labile cells and a small number of resistant cells are heated together the last survivors might well be labile cells. Repeated mild heat treatments separated by limited periods of growth might serve to build up the proportion of heat-resistant cells until they were dominant.

There is some indication that when cultures are maintained without any heat shocking, either in the laboratory or in a natural environment, the heat-labile cells may have slightly faster growth rate than do the resistant cells. Thus, strains isolated from naturally occurring cases of surface greening are not heat-resistant and stock strains of resistant variants gradually lose their high degree of resistance.

Regardless of the theoretical considerations, the occurrence of these heat-resistant strains may necessitate higher processing temperatures for cured meat products than would otherwise be necessary. These organisms that are capable of withstanding as much as 45 minutes at 155 F are among the most heat-resistant spoilage organisms known outside of the spore-formers and the genus *Microbacterium*.

SUMMARY

Heterofermentative lactobacilli isolated from various types of greening of cured meat products have widely different degrees of heat resistance even though they are identical in their physiological, serological, and nutritional characteristics. Those strains isolated from cases of surface greening, caused by contamination of the product after heat processing, are killed by about 10 to 12 minutes at 150 F. Those strains isolated from cases of green cores, caused by organisms that survive the heat processing in the center of the sausage, withstand about 120 minutes at 150 F.

After 14 successive heat shockings two thermolabile strains had approximately doubled in thermal tolerance but still exhibited far less tolerance than did the strains isolated from green cores. On the other hand, after being maintained in the laboratory for an extended length of time, one of the heat resistant strains was about as thermolabile as the strains from surface greening. A survivor curve of this strain appeared to show the presence of two types of cells with respect to thermal tolerance. Re-isolates from the plating of the original culture gave strains that were killed by as little as 12 minutes at 145 F. Re-isolates from the plating of the same culture after heat shocking gave strains that survived as much as 240 minutes at 145 F. All of these re-isolates were serologically and physiologically identical with the parent strain.

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Tetrazolium Bioautography[1]

EARL USDIN, GERALD D. SHOCKMAN, AND GERRIT TOENNIES[2]

Institute for Cancer Research and Lankenau Hospital Research Institute, Philadelphia, Pennsylvania

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Bioautography, the process in which growth factors are identified by the appearance of zones of growing microorganisms wherever such factors are placed on an otherwise nutritionally complete, inoculated solid medium, was introduced as a tool for the identification of paper chromatogram spots by Winsten and Eigen (1948).

Earlier, Kuhn and Jerchel (1941) showed that tetrazolium compounds are reduced to deeply colored formazan derivatives by growing bacteria. We have found[3] that this property can be put to advantageous use for the detection of zones of growth or inhibition on agar plates and the preparation of photographic records thereof. Under optimal conditions the use of tetrazolium extends the sensitivity of the agar method by several decimal powers so that growth factor concentrations can be estimated which previously were detectable only by growth measurements in liquid media. The present communication is concerned with a study of the conditions which govern intensity and visibility of responses in tetrazolium bioautography. The experiments deal with factors of the folic acid group but the general principles have been found applicable to other substances.

METHODS

The medium is that of Toennies, Frank and Gallant (1952) except that the enzymatic casein hydrolysate is replaced by a 5 per cent solution of Hycase[4] (a dry product from acid hydrolysis of casein), 40 mg DL-tryptophane per 100 ml are added, and the cystine supplement is halved.

One and seventy-five hundreth grams of agar (Difco Bacto-agar) in 100 ml of medium (0.625 per cent casein) as well as 1.75 grams agar in 100 ml of water,

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[3] Data were presented on January 29, 1953 (Abstract submitted November 25, 1952) (Usdin, Shockman and Toennies, 1953). The same principle has been proposed and presented (December 19, 1952) independently by Ford and Holdsworth (1953).

[4] Sheffield Farms Co., Inc., New York, N. Y.