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Adaptation to Growth at Low pH by *Clostridium sporogenes*

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To the Graduate Council:

I am submitting herewith a thesis written by Claudia Dee Crosthwait entitled "Adaptation to Growth at Low pH by *Clostridium sporogenes*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

J. Orvin Mundt, Major Professor

We have read this thesis and recommend its acceptance:

John Woodward, David Bemis

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice Chancellor
Graduate Studies and Research

ADAPTATION TO GROWTH AT LOW pH BY *CLOSTRIDIUM SPOROGENES*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee

Claudia Dee Crosthwait

June 1979

DEDICATION

This manuscript is dedicated to my family, especially my parents, Kenneth Jackson and Betsy Shelton Crosthwait, for their unending support and encouragement.

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ABSTRACT

The ability of Clostridium sporogenes to adapt to growth at low pH was studied in tomato serum prepared by enzymatic hydrolysis of canned tomatoes and tomato juice. The lowest pH at which C. sporogenes could grow before adaptation in tomato serum, Trypticase Peptone Glucose Yeast Extract (TPGY) and fluid thioglycollate broths was pH 5.4.

Tomato serum and TPGY or fluid thioglycollate broth adjusted to varying pH levels were inoculated with C. sporogenes from tubes with growth at the lower pH values. Subsequent transfers of C. sporogenes grown in tomato serum were made to tomato serum of equal and lower pH values. Strains emerged which were capable of initiating growth in tomato serum having a pH as low as 4.85. The adaptation to growth at low pH could be maintained only by continuous subculture at pH 5.0. The ability to grow in tomato serum having pH of 5.0 was lost after one passage through neutral media at pH 6.9-7.0.

After storage at 4.4°C, cultures grown in tomato serum at pH 5.0 grew in tomato serum at pH 5.0 if subcultured within 7 days, but not after 21 days.

The addition of mineral salts ($FeSO_4$, $CuSO_4$, $ZnSO_4$, $MnSO_4$, and $MgCl_2$) and components of TPGY medium (sodium thioglycollate, trypticase, peptone, glucose and yeast extract) to tomato serum did not result in growth at pH values below that obtained in tomato serum alone.

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CHAPTER I

INTRODUCTION

Botulism is a food intoxication caused by the ingestion of a highly toxic exotoxin produced by Clostridium botulinum, a gram positive, anaerobic sporeforming rod. Although botulinal intoxication is low in frequency as compared to other food borne intoxications, it is still of great concern since the fatality rate is high, varying from 23% (Odlaug and Pflug, 1978) to 30 to 60% (Jay, 1978).

C. botulinum is divided into seven types according to the distinct toxin produced. Toxins produced by C. botulinum types A and B are incriminated in botulism of home-canned foods.

Botulism occurs primarily in low acid canned foods, but home-canned acid foods were implicated in 4.7% of reported outbreaks from 1899 through 1975 (Odlaug and Pflug, 1978). These outbreaks are of interest because C. botulinum reportedly will not grow at pH <4.7. Odlaug and Pflug (1978) speculate that for an acid food to become a botulinal hazard, there must be several contributing co-existing factors: (1) contamination of the product with a large number of spores of C. botulinum, (2) contamination of the product with other microorganisms, (3) favorable composition of the food and storage conditions conducive to growth and toxin production of C. botulinum, and (4) metabiosis, a condition that exists when one organism makes conditions favorable for growth of another organism. Odlaug and Pflug (1978) consider botulism to be a hazard in acid foods only when contamination by a second microorganism has occurred. However, of the

34 outbreaks of botulism attributed to acid foods from 1899 - 1975 (Odlaug and Pflug, 1978), microorganisms other than C. botulinum were isolated from the food in only three instances (Odlaug and Pflug, 1978). Therefore, the question is still unanswered as to how botulism occurred in the 31 outbreaks involving acid foods.

It is possible that a strain exists in nature that is capable of germination and growth at low pH. Such a strain would be exceedingly rare, however, or it would have been previously isolated. The possibility which this study investigated was that through selection and adaptation to grow at a lower pH by sequential passing from higher pH into progressively lower pH, strains of C. botulinum arise which are capable of initiating growth at low pH.

Clostridium sporogenes was used in this study since it is very closely related to C. botulinum in cultural, biochemical, nutritional and serological properties (Kindler, 1956). This bacterium was used by Savani and Harris (1978) in studies of acidification of tomatoes. C. sporogenes can be differentiated from C. botulinum types A, B, and F only by toxin production (CRC Handbook of Microbiology, 1973).

Since some type of tomato product was incriminated in 50% of the home-canned acid food outbreaks (Odlaug and Pflug, 1978), probably due in part to the popularity of canning tomato products throughout the entire country, serum from home-canned tomatoes and tomato juice were used as a test medium in an attempt to adapt C. sporogenes to grow at a lower pH.

CHAPTER II

SURVEY OF THE LITERATURE

Determining the minimum pH at which C. botulinum will germinate and grow has been the purpose of several studies. Dozier (1924), working with vegetative cells of 37 strains of C. botulinum in phosphate buffered double strength veal infusion - 2% Difco peptone, found the minimum pH for growth to be 4.87.

Later, Townsend et al. (1954) did a study of the pH levels at which spores of C. botulinum would germinate, grow and produce toxin in a variety of foods. The foods included green chili peppers, pimientos, eggplant, pineapple-rice pudding, pork and beans, spaghetti and sauce, prune pudding, strained pears and plums, vegetable juice, zucchini and banana puree. They found that the lowest pH at which growth and toxin production occurred was at pH 4.80 in pineapple rice pudding. Ohye et al. (1966) concluded that the maximum of pH 4.6 in foodstuff where acidification was relied on to prevent growth was an ample safety margin after all strains of C. botulinum which he studied failed to grow at pH 5.0.

Dozier (1924) and Townsend et al. (1954) also showed that some products seemed to be less favorable for the germination of spores of C. botulinum than others.

Ito et al. (1976) working with 5 Type A strains and 6 Type B strains of C. botulinum, observed toxin production in cucumber puree at pH 5.0 but not at pH 4.8 with a spore concentration of 1×10^6 per tube. Ito et al. (1978) found the minimum pH for growth of

C. botulinum in canned figs was 5.45. In a tomato substrate, pH 4.96 was the lowest pH at which growth and toxin formation was observed (Townsend, 1954). Huhtanen et al. (1976) inoculated 10 different strains of C. botulinum, 4 strains of Type A and 6 strains of Type B, into tomato juice at different pH values and found the minimum pH for outgrowth of spores to vary greatly for different strains. The lowest pH at which they observed growth in tomato juice was 5.24. Odlaug and Pflug (1979) found the minimum pH in tomato juice for growth of Type A C. botulinum to be 4.9 and 5.1 for Type B.

Powers (1976), studying the effect of acidification on canned tomatoes, speculated as to why botulism in canned tomatoes was a rare occurrence. He noted several discrepancies between the conditions in laboratory trials and actual canning in the home or commercially. He stated that the spores in nature are generally not as heat resistant as those used in laboratory experiments. Furthermore, he pointed out that the spore load was larger in laboratory trials than in commercial or home canning experiences. He stated that the inocula used in laboratory trials had been thousands or millions of spores, while C. botulinum spore loads in actual canning would probably be considerably lower than this. According to Powers (1976), factors such as these, in addition to the probability of the commercial varieties of tomatoes having a pH as high as 4.8 being 0.000031, account for the low incidence of botulism in canned tomatoes.

Magoon (1926), working with Bacillus mycoides, found that heat resistance of the spores within a given bacterial strain may be increased by a process of selection. He presented evidence that

bacterial spores derived from heat resistant survivors in thermal death time studies possessed greater heat resistance than the original spores and by that process, a strain was obtained whose spores were at least 25 times more resistant than those of the original strain.

Dallinger (1887) acclimated a flagellate unable to grow at 23°C to growth at 70°C over a 7 year period, but Casman et al. (1933) were not successful in acclimating Bacillus subtilis to growth at a higher temperature.

Literature on "training" microorganisms to grow at lower pH is very limited; however, Chung and Goepfert (1970) did attempt it with Salmonella. They were unable to "train" any of the serotypes to grow at lower pH by repeated subculture in an acid environment. There are no reported studies of attempts to "train" either C. botulinum or C. sporogenes to grow at a lower pH.

Powers (1976) mentioned that in laboratory studies dealing with C. botulinum in acid foods, the inoculum had been large - thousands or millions of spores. The National Canners Association (1964) used 10^6 spores as an inoculum in their studies with C. botulinum. Townsend et al. (1954) determined the minimum pH for growth of C. botulinum using 2.0 - 2.5 x 10^6 spores. Ito et al. (1976) was able to get outgrowth in cucumber puree at pH 5.0 using 10^6 spores/ml, but not 10^2 spores/ml. Likewise, Chung and Goepfert (1970) found that in order to obtain growth of Salmonella at minimum pH, an inoculum of 10^4 to 10^6 cells must be used.

CHAPTER III

MATERIALS AND METHODS

Cultures

Two cultures of Clostridium sporogenes were used in this study. C. sporogenes ATCC #7955 was obtained from the American Type Culture Collection. The second culture was obtained from the teaching stocks at the University of Tennessee and will be designated as C. sporogenes (UT).

Maintenance of cultures

Upon receipt, C. sporogenes ATCC #7955 was subcultured in Yesair's Pork Infusion Broth (Speck, 1976), and thereafter was maintained in Trypticase Peptone Glucose Yeast Extract (TPGY) Broth (Speck, 1976) in screw cap tubes.

C. sporogenes (UT) exhibited better growth in standard fluid thioglycollate broth (Rohde, 1967) and was maintained in this medium.

All incubations were at 37°C for seven days. Purity of the cultures was assured by routinely preparing gram stains.

Tubes with growth at pH 5.2 were divided to provide 10 cultures for future experiments.

Preparation of tomato serum

Tomato serum was prepared from home-canned or frozen tomatoes and tomato juice. The tomatoes were homogenized in a laboratory blender. One ml of 2% Cleerzyme, obtained from Dr. J. L. Collins, was added to each 100 ml of tomato juice or homogenate. These were stored

at 5°C for three days. The serum was recovered by centrifuging for 10 minutes at 4500 rpm.

Adjustment of pH

The pH was adjusted as desired with 1N NaOH using an Orion Research Ionalyzer/model 399A. The serum was then tubed in 10 ml quantities in 16 x 125 mm screw cap tubes.

Method of obtaining reduced conditions

Rapid growth of C. botulinum occurs if the Eh is between -6 mv and -436 mv (Odlaug and Pflug, 1978). Canned tomato juice has an Eh of -309.3 mv (Odlaug and Pflug, 1978). To establish and maintain low Eh in the tomato serum, sodium thioglycollate was added to a final concentration of 0.1%.

All media were freshly prepared immediately before use and sterilized for 15 minutes at 15 pounds pressure at 121°C and maintained in a water bath at 45°C until inoculated.

Inoculum

Inocula were grown in TPGY or fluid thioglycollate broth for seven days at 37°C. Inocula were standardized to 10^6 - 10^7 cells as determined by the three tube most probable numbers procedure. Erratic results were obtained when fewer cells were used to inoculate media below pH 5.6. Workers who used C. botulinum were able to use precise inocula because they were able to produce and store pools of very large numbers of spores and could accurately determine their numbers. In this study the apparent rapid death of vegetative cells precluded standardization of inocula prior to experimental work. The

tip of the pipette was placed one to two cm below the surface of the serum to prevent the introduction of oxygen. In all instances, inoculations were made in duplicate.

Incubation of the inoculated media

All media were incubated at 37°C in an air incubator. Observations were made at 72 hours and at 7 days.

Determination of growth of C. sporogenes

Growth was determined visually by observing for turbidity of inoculated media as compared with uninoculated tubes of media. Gram stains were performed routinely to assure that turbidity represented the growth of C. sporogenes.

Experimental plan

Tomato serum and TPGY or fluid thioglycollate broths adjusted to varying pH levels were inoculated with C. sporogenes to determine the minimum pH for growth. Tubes with growth at lower pH values were then used as inocula and transferred to tomato sera and TPGY or fluid thioglycollate broth adjusted to equal and lower pH values. Subsequent transfers were made in this step-wise fashion in an attempt to adapt C. sporogenes to growth at lower pH values.

Insignificant or no sporulation of C. sporogenes occurred at pH below 5.7. Thus, inocula from tubes of tomato serum below pH 5.7 were unheated.

Tomato serum and thioglycollate broth, both with pH 6.9 were inoculated from tubes of media with growth at low pH values and these in turn were used to inoculate serum at the same low pH values to

determine whether the adaptation was permanent or was lost by subculture at neutral pH (6.8 - 7.0).

Effect of additives to tomato serum

To determine whether or not growth of C. sporogenes at lower pH was inhibited due to a chelating effect by citric acid, mineral salts were added to serum to obtain a final concentration of 0.1% $FeSO_4$, 0.01% $CuSO_4$, 0.001% $MnSO_4$, 0.001% $ZnSO_4$ and 0.01% $MgCl_2$.

To determine if the components of TPGY were responsible for the better growth of C. sporogenes in TPGY as compared to growth in tomato serum at low pH, components of TPGY were individually and collectively added to tomato serum. The components were added to serum to obtain a final concentration of 0.1% sodium thioglycollate, 5% trypticase, 0.5% peptone, and 2% yeast extract.

pH of tomato serum after growth of C. sporogenes

The pH of tomato serum in which growth of C. sporogenes had occurred was measured to determine if acid had been produced as a result of growth.

Effect of yeasts upon the growth of C. sporogenes

Tomato serum inoculated with approximately 10^6-10^7 cells of C. sporogenes at 60°C was cooled to 37°C. Tubes were inoculated with 0.01 ml of a 24 hour old mycophil broth culture of Kluyveromyces fragii or Saccharomyces cerevisiae.

Replications

All critical experiments were replicated at least once and some as frequently as five times.

CHAPTER IV

RESULTS

Limiting pH for growth of cultures

The lowest pH at which C. sporogenes could grow in tomato serum, TPGY and in thioglycollate broths before adaptation was 5.4.

Growth at lower pH

Inocula produced in serum at pH 5.4 were maintained in serum at 5.4 and introduced into serum at pH 5.2 and 5.0. When transferred immediately from the first growth in serum at pH 5.4, growth was sporadic. After C. sporogenes was subcultured four times at pH 5.4, each of the 10 strains grew in serum adjusted to pH 5.2, but not in serum adjusted to lower pH values.

After three serial transfers in serum at pH 5.2, tubes of serum at pH 5.0 and 4.8 were inoculated with 1 ml of each of the 10 adapted cultures. Growth ensued in 37 of 100 tubes at pH 5.0, and in none of the 100 tubes at pH 4.8.

Growth in serum at pH 5.0 was erratic. Four of 10 strains stabilized to growth at pH 5.0 after three serial passages. These were used to inoculate serum at pH 4.85. Growth occurred at 7 days, but not at 3 days, in 25 of 32 tubes (Table 1) in one instance. This result could not be obtained in 5 attempted replications with 10 cultures, and transfers from growth at pH 4.85 to serum at pH 4.85 and 5.0 failed to grow. The inoculum for the last replication of this experiment was 3 ml of cultures grown at pH 5.0, with an estimated

TABLE 1

GROWTH OF C. SPOROGENES IN TOMATO SERUM AT pH 4.85 AND 5.00 IN 72 HOURS AND 7 DAYS, FROM TUBES WITH GROWTH AT pH 5.0

Strain	Growth at 72 hours pH 4.85	pH 5.00	Growth at 7 days pH 4.85	pH 5.00
1	-	-	-	+
2	-	-	+	+/-
3	-	-	+	+
4	-	-	+/-	+
5	-	-	+	+
6	-	-	+/-	+/-
7	-	-	-	-
8	-	-	+	+
9	-	-	+/-	-
10	-	-	+	+
11	-	-	+	+
12	-	-	+	+
13	-	-	+	+
14	-	-	+	+
15	-	-	+	+
16	-	-	+	+

\+ : both tubes positive for growth

\- : both tubes negative for growth

+/- : one tube positive for growth; one tube negative for growth

1 x 10^7 cells/ml of inoculum as determined by serial dilutions using triplicate tubes of neutral thioglycollate broth.

Stability after adaptation

To determine the stability after adaptation, C. sporogenes grown in 29 tubes of tomato serum pH 5.0 was transferred to neutral fluid thioglycollate broth and incubated for 72 hours. One ml aliquots of 10^8-10^9 cells/ml were used to immediately inoculate serum and thioglycollate broth adjusted to pH 4.8, 5.0, and 5.2. The results are shown in Table 2. At 72 hours in tomato serum, 17 of 29 cultures grew at pH 5.2. At 7 days all tubes of tomato serum at pH 4.8 and 5.0 were negative, but 54 of the 58 tubes at pH 5.2 contained growth. In thioglycollate broth all cultures grew at pH 5.0 and 5.2 in 72 hours, but no growth occurred in broth at pH 4.8 in 7 days.

Effect of additives to tomato serum

To determine the effect of minerals on the growth of C. sporogenes in tomato serum, 5 cultures of C. sporogenes grown in tomato serum pH 5.0 and transferred for one passage through neutral thioglycollate broth were used to inoculate tomato serum at pH 4.8, 5.0, 5.2, 5.4, and 5.6 with and without mineral salts. The results are shown in Table 3. Mineral salts hindered, rather than encouraged, growth of C. sporogenes in tomato serum. Growth occurred at 72 hours in all tubes of tomato serum without mineral salts at pH 5.2, 5.4 and 5.6. In tomato serum with mineral salts, growth occurred in all tubes at pH $\geq$ 5.4 but only in one set of duplicate tubes at pH 5.2. At 7 days, growth was observed in 4 sets of duplicate tubes of tomato serum

TABLE 2

GROWTH OF C. SPOROGENES ADAPTED TO GROWTH AT pH 5.0 AFTER A SINGLE PASSAGE THROUGH NEUTRAL MEDIUM

Strain	Growth in Tomato Serum						Growth in Thioglycollate Broth					
	72 hours			7 days			72 hours			7 days		
pH	4.8	5.0	5.2	4.8	5.0	5.2	4.8	5.0	5.2	4.8	5.0	5.2
1	-	-	-	-	-	-	-	+	+	-	+	+
2	-	-	+	-	-	+	-	+	+	-	+	+
3	-	-	-	-	-	-	-	+	+	-	+	+
4	-	-	+	-	-	+	-	+	+	-	+	+
5	-	-	+	-	-	+	-	+	+	-	+	+
6	-	-	-	-	-	+	-	+	+	-	+	+
7	-	-	-	-	-	+	-	+	+	-	+	+
8	-	-	+	-	-	+	-	+	+	-	+	+
9	-	-	+	-	-	+	-	+	+	-	+	+
10	-	-	-	-	-	+	-	+	+	-	+	+
11	-	-	+	-	-	+	-	+	+	-	+	+
12	-	-	+	-	-	+	-	+	+	-	+	+
13	-	-	-	-	-	+	-	+	+	-	+	+
14	-	-	+	-	-	+	-	+	+	-	+	+
15	-	-	+	-	-	+	-	+	+	-	+	+
16	-	-	+	-	-	+	-	+	+	-	+	+
17	-	-	+	-	-	+	-	+	+	-	+	+
18	-	-	+	-	-	+	-	+	+	-	+	+
19	-	-	+	-	-	+	-	+	+	-	+	+
20	-	-	+	-	-	+	-	+	+	-	+	+
21	-	-	+	-	-	+	-	+	+	-	+	+
22	-	-	-	-	-	+	-	+	+	-	+	+
23	-	-	-	-	-	+	-	+	+	-	+	+
24	-	-	+	-	-	+	-	+	+	-	+	+
25	-	-	+	-	-	+	-	+	+	-	+	+
26	-	-	-	-	-	+	-	+	+	-	+	+
27	-	-	-	-	-	+	-	+	+	-	+	+
28	-	-	-	-	-	+	-	+	+	-	+	+
29	-	-	-	-	-	+	-	+	+	-	+	+

+ : Both tubes positive for growth

- : Both tubes negative for growth

TABLE 3

THE EFFECT OF MINERAL SALTS ON THE GROWTH OF C. SPOROGENES

Strain	Tomato Serum					Tomato Serum + Mineral Salts				
	4.8	5.0	5.2	5.4	5.6	4.8	5.0	5.2	5.4	5.6
	Growth at 72 hours									
1	-	-	+	+	+	-	-	-	+	+
2	-	-	+	+	+	-	-	+	+	+
3	-	-	+	+	+	-	-	-	+	+
4	-	-	+	+	+	-	-	-	+	+
5	-	-	+	+	+	-	-	-	-	-
	Growth at 7 days									
1	-	+	+	+	+	-	-	+	+	+
2	-	+	+	+	+	-	-	+	+	+
3	-	+	+	+	+	-	-	+	+	+
4	-	+	+	+	+	-	-	+	+	+
5	-	-	+	+	+	-	-	+	+	+

+ : Both tubes positive for growth

- : Both tubes negative for growth

without the mineral salts at pH 5.0, while the lowest pH at which growth occurred in the tomato serum containing mineral salts was 5.2. C. sporogenes grown in the tomato serum pH 5.0 in this experiment was immediately transferred to tomato serum at pH 4.8, 5.0, 5.2, and 5.4. Growth occurred only in serum having a pH $\geq$ 5.2.

None of the components of TPGY - sodium thioglycollate, trypticase, peptone, or yeast extract - added individually or collectively enabled C. sporogenes to grow at a lower pH.

Growth after storage

When cultures grown in tomato serum at pH 5.0 were refrigerated at 4.4°C, growth was obtained at pH 5.0 if subcultured within 7 days, but growth was obtained in 21 days only in neutral thioglycollate broth and no growth occurred in tomato serum at pH 5.0.

pH change after addition of inoculum

Introduction of C. sporogenes grown in neutral thioglycollate broth into tomato serum pH 5.0 caused only a slight (0.05 - 0.10) increase in the pH of the serum.

The pH of the tomato serum did not change after growth of C. sporogenes indicating that either the organism did not produce acid as a result of growth, or that the tomato serum was highly buffered. The buffer capacity was determined to be 6.25 ml of 0.100 N HCl at pH 5.40. This was calculated to be 0.920 milliequivalents.

Effect of yeasts upon the growth of C. sporogenes

Associated yeast growth did not result in growth at pH levels not obtained in the control tubes.

CHAPTER V

DISCUSSION

Few spores were observed upon microscopic examination of cultures grown in tomato serum at pH 5.7. These few spores may have been those from the inoculum that had been unable to germinate at a low pH, or the low pH markedly retarded sporulation. Pederson and Becker (1949) observed that high acid was not conducive to sporulation of Bacillus coagulans. When inoculum grown in tomato serum pH 5.4 and lower was heated, growth was never obtained, but growth did occur if the inoculum was not heated. This suggests that at pH 5.4 and less, spores were not produced and growth from inocula of pH 5.4 and below was from vegetative cells. Pederson and Becker (1949) showed that vegetative cells of Bacillus coagulans grew at a lower pH than that at which spores could germinate. Failure of C. sporogenes to sporulate undoubtedly accounts for the results obtained in further experimentation.

When the cultures of C. sporogenes adapted to growth at low pH were transferred to tomato serum at that low pH, growth could be maintained at that pH. Cultures adapted to growth at pH 5.0 could be maintained when cultured in tomato serum at pH 5.0, but when the pattern was interrupted by only one passage through neutral thioglycollate, none of the cultures grew in serum at pH 5.0 and 54 of 58 grew at pH 5.2. These results indicate that adaptation to growth at low pH was not permanent.

When cultures which had grown for 7 days at pH 5.0 were refrigerated for 7 days, growth was obtained in serum at pH 5.0, but

no growth occurred except in neutral medium when refrigeration was for 21 days. These results are probably due to rapid death of the vegetative cells in the acid environment. The numbers of surviving cells were too low to initiate growth at this pH although they were able to initiate growth at neutral pH. Pederson and Becker (1949) found that vegetative cells of Bacillus coagulans died rapidly in an acid medium.

The necessity of using large inocula in determining the minimum pH for growth was in accordance with procedures of earlier workers. It may be that only a fraction of the spore population is capable of germination and growth at lower pH values and that by increasing the inoculum, the probability of growth is also increased (Odlaug and Pflug, 1979). The same may also be true for vegetative cells. Mundt et al. (1978) found that growth of Klebsiella pneumoniae at minimum pH was determined by the number of cells in the inoculum.

Tomato serum at low pH was not deficient in nutritional components needed by C. sporogenes. This was shown by supplementation with ingredients of the TPGY medium and by the addition of mineral salts in excess of that which might have been chelated. Neither procedure supported growth of C. sporogenes in tomato serum at pH below that supporting growth in unenriched serum. The addition of the mineral salts appeared to hinder growth.

If C. sporogenes is representative of C. botulinum in its ability to adapt to growth at low pH, the possibility of such an occurrence actually happening is exceedingly remote. Two alternative explanations for the occurrence of botulism in acid foods may be considered.

First, preformed toxin may be introduced with tomatoes on which molds have grown before canning. Alkalinizing effect of molds in tomato juice resulting in an elevation of pH, sometimes well above 7.0, has been observed by Huhtanen (1976), Odlaug and Pflug (1979), and Mundt (1978) and in molded fresh tomatoes in this laboratory (current studies). Because of the nature and volume of home-canning, some home-canned tomatoes and tomato juice undoubtedly do not receive the specified heat treatment. Mundt et al. (1977) and Powers and Godwin (1978) with reference to tomatoes, and Thompson et al. (1979) with reference to home-canned vegetables in general, suggest that the prevention of botulism becomes a matter of education of a segment of home-canners who fail to follow the recommended canning procedures.

It has been shown by Woolford and Schantz (1978) that foods with low pH (specifically tomato soup, pH 4.1) provide the most protection to the toxin during heating. They found a 50% inactivation of the toxin in tomato soup after 7 minutes of heating at 68°C, whereas in cream of mushroom soup (pH 6.2) and beef pie blend (pH 5.9) greater than 99% of the toxin was inactivated. After 30 minutes of heating tomato soup at 68°C, the initial toxin level of 60,000 mouse LD_{50}/ml had dropped to about 6,000 mouse LD_{50}/ml. Powers (1976) stated that in some instances, the center temperature in jars of tomatoes probably would not get above 71°C in the home-canning process. One can project from the data presented by Woolford and Schantz (1978) that, after heating for 30 minutes at 71°C, 2,000 mouse LD_{50}/ml would remain from the initial level of 60,000 mouse LD_{50}/ml. Thus, if the toxin were already present in the tomatoes

at the time of canning, and the tomatoes do not receive the proper heat treatment, some toxin would be present to cause botulism if the food were consumed.

Another possibility for the occurrence of botulism in acid foods is based on the work of Odlaug and Pflug (1979). They found that when Aspergillus gracilis and C. botulinum were inoculated and incubated in a hermetic unit, a very thin mycelial mat was formed, and growth of C. botulinum with toxin production was observed immediately beneath the mycelial mat. They suggested that the growth of Aspergillus on the surface of the tomato juice created a microenvironment within or adjacent to the mycelial mat where the pH was probably greater than 4.6 and therefore spores of C. botulinum were able to germinate, reproduce and produce toxin (Odlaug and Pflug, 1979). In the home, probably very few people would consume tomato juice if abundant mold growth were present, but a very thin mycelial mat might be unnoticed or presumed harmless and the tomato juice might be consumed. This could be a hazard if C. botulinum spores were present and had been able to germinate, grow, and produce toxin near the mycelial mat where growth could occur.

CHAPTER VI

CONCLUSIONS

Stock cultures of C. sporogenes were unable to initiate growth in tomato serum below pH 5.4 when large inocula were used.

By sequential transfers from tomato serum at higher pH into tomato serum having progressively lower pH, strains of C. sporogenes were developed which were capable of initiating growth in tomato serum at pH as low as 4.85. The adaptation to growth at low pH is not permanent and can be maintained only by continuous subculture at pH 5.0. The adaptation was lost after one passage through neutral medium.

After storage at 4.4°C, cultures grown in tomato serum at pH 5.0 grew in tomato serum at pH 5.0 if subcultured within 7 days, but not after 21 days of storage.

Spores were not produced at any time in tomato serum at pH 5.4 or below.

Large amounts of inocula must be used in tomato serum adjusted to pH values below initial minimum pH for growth.

Tomato serum at low pH is not deficient in nutritional components needed by C. sporogenes.

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VITA

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