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I am submitting herewith a dissertation written by Krishnaswamy Atmaram entitled "The Role of Bacillus cereus in the Sweet-Curdling Defect of Fluid Milk." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

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142

THE ROLE OF *BACILLUS CEREUS* IN THE SWEET-CURDLING DEFECT OF FLUID MILK

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Krishnaswamy Atmaram

March 1973

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1082340

DEDICATED TO

MY MOTHER

ABSTRACT

The recent increase in number of isolations of psychrotrophic bacilli and the sweet curd formation in fluid milk have created the need for more information as to the role of Bacillus cereus. The purpose of this study was to determine the influence of the current trends in the dairy industry towards higher pasteurization temperatures and extended storage times on activation and germination of psychrotrophic Bacillus cereus, as well as the determination of stimulatory influence of the growth of the common Pseudomonas organisms in raw milk on these activated spores.

Twenty-eight percent of commercially pasteurized milk samples procured from various plants throughout Tennessee exhibited the sweet-curdling defect on refrigerated storage within 10 days. This defect was observed many times at the bottom but sometimes throughout the carton of milk. Psychrotrophic Bacillus cereus was isolated as a causative organism from these sweet-curdled milk samples. The isolates differed from the type culture organism only with regard to their ability to grow and bring about sweet curd formation in skimmilk at refrigeration temperature. These isolates of psychrotrophic bacilli even though possessing the same cultural and biochemical characteristics, exhibited marked difference in their response to various heat-activation temperatures as well as the initial excessive growth of Pseudomonas species in raw skimmilk.

Spores of the three isolates exhibited greater activity after activation at 80 C for 15 seconds, which happens to be the higher

pasteurization temperature currently used by many dairy plants, than the standard pasteurization at 71.5 C for 15 seconds. The sweet curd formation was invariably noticed within 10 hours of incubation at 35 C and was always associated with exponential growth of the bacilli with counts ranging from 40 million to more than 60 million.

The two common Pseudomonas species, Pseudomonas fluorescens and Pseudomonas fragi, were isolated from the stored samples of fluid milk for this study. Excessive growth of these Pseudomonas species in the raw skimmilk prior to processing had a stimulatory effect on psychrotrophic Bacillus cereus isolates in combination with heat-activation. Two of three isolates of Bacillus cereus provided statistical evidence of the stimulatory effect due to growth of these Pseudomonas organisms especially when the spores of psychrotrophic bacilli were activated at 80 C for 15 seconds.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	REVIEW OF LITERATURE	4
	Occurrence	4
	Structure and Chemical Nature of Spores	7
	Activation of Spores	16
	The Nature of Activation	17
	Heat Activation of Spores	20
	Activation by Other Agents	24
	Changes in the Activated Spores	28
	Germination Systems Not Requiring Activation	31
	Germination of Spores	33
	Changes in the Germinating Spores	34
	Initiation of Germination	36
	Outgrowth of Spores	39
	Bacilli in Milk	41
III.	MATERIALS AND METHODS	50
	Bacterial Media	50
	Centrifugation	51
	Psychrotrophic *Bacillus cereus* Strains	51
	Source	51
	Isolation and purification	51
	Identification and testing	52

CHAPTER PAGE

Pseudomonas Species 52
Source 52
Isolation 52
Purification 54
Identification and testing 54
Preparation of Biochemically Clean Spore Crops 54
Preparation of *Pseudomonas* Species 57
Sterilization of Skimmilk Prior to Inoculation with the Spores 57
Heat-Activation of Psychrotrophic *Bacillus cereus* Isolates 58
Inoculation of Heat-Activated Spores into Various Sterile Skimmilks 58
Plate Counts of the Skimmilks Inoculated with Heat-Activated Spores 58
Statistical Analyses 59
IV. RESULTS 60
Sweet-Curdling of Commercially Pasteurized Milk 60
Distribution of *Bacillus cereus* in Various Portions of Milk 60
Characteristics of Psychrotrophic *Bacillus* Isolates . . . 61
Biochemical Properties of Psychrotropic *Bacillus* Isolates 62
Cultural and Biochemical Properties of *Pseudomonas* Species 62

CHAPTER PAGE

Initial and Final Counts of *Pseudomonas* Species in Skimmilk 62

The Turbidemetric Standardization of Spore Suspensions . . 66

Results of Experiments with Isolate H-3 66

Influence of Initial Growth of *Pseudomonas* Species on H-3 66

Results of Experiments with Isolate CH-1 72

Influence of Initial Growth of *Pseudomonas* Species on CH-1 72

Results of Experiments with Isolate BC-5 79

Influence of Initial Growth of *Pseudomonas* Species on Isolate BC-5 79

V. DISCUSSION 87

VI. SUMMARY AND CONCLUSIONS 93

REFERENCES 95

VITA 117

LIST OF TABLES

TABLE PAGE

I. Materials and Methods Used in Identification of Bacillus cereus Isolated from Sweet-Curdled Market Milk Samples . . 53

II. Materials and Methods Used in Identifying Pseudomonas fluorescens Isolated from Market Milk Samples 55

III. Materials and Methods Used in Identifying Pseudomonas fragi Isolated from Market Milk Samples 56

IV. Average Plate Counts of ATCC Bacillus cereus 14579 at the Top, Middle and Bottom Portions at Different Intervals of Incubation 61

V. The Biochemical Properties of Psychrotrophic Bacillus cereus Isolates H-3, CH-1 and BC-5 and Bacillus cereus ATCC 14579 63

VI. The Cultural and Biochemical Characteristics of Pseudomonas fluorescens Isolated from Market Milk Samples 64

VII. The Cultural and Biochemical Characteristics of Pseudomonas fragi Isolated from Market Milk Samples 65

VIII. Average Plate Counts of Isolate H-3 at Different Intervals of Incubation and Activation at the Three Temperature-Time Combinations 67

IX. Average Plate Counts of Isolate H-3 Activated at 80 C for 15 Seconds at Various Intervals of Incubation in Sterile Milk and in Milks in which Pseudomonas Species Grew Prior to Sterilization 68

TABLE PAGE

X. Average Plate Counts of Isolate H-3 Activated at 85 C for 10 Minutes at Various Intervals of Incubation in Sterile Milk and Milks in Which Pseudomonas Species Were Grown Prior to Sterilization 69

XI. Average Plate Counts of Isolate of H-3 Activated at 71.5 C for 15 Seconds at Various Intervals of Incubation in Sterile Milk and Milks in Which Pseudomonas Species Were Grown Prior to Sterilization 70

XII. Analysis of Variance of Heat Activation and Influence of Initial Growth of Pseudomonas Species on the Germination and Outgrowth of H-3 71

XIII. Average Plate Counts of Isolate CH-1 at Different Intervals of Incubation and Activation at the Three Temperature-Time Combinations 73

XIV. Average Plate Counts of Isolate CH-1 Activated at 80 C for 15 Seconds at Various Intervals of Incubation in Sterile Milk and Milks in Which Pseudomonas Species Were Grown Prior to Sterilization 74

XV. Average Plate Counts of Isolate CH-1 Activated at 85 C for 10 Minutes at Various Intervals of Incubation in Sterile Milk and Milks in Which Pseudomonas Species Were Grown Prior to Sterilization 75

XVI. Average Plate Counts of Isolate CH-1 Activated at 71.5 C for 15 Seconds at Various Intervals of Incubation in Sterile Milk and Milks in Which Pseudomonas Species Were Grown Prior to Sterilization 76

TABLE PAGE

XVII. Analysis of Variance for Heat-Activation and Influence of Initial Growth of Pseudomonas Species on the Germination and Outgrowth of CH-1 77

XVIII. Treatments and the Treatment Means of Isolate CH-1 78

XIX. Average Plate Counts of Isolate BC-5 at Different Intervals of Incubation and Activation at Three Temperature-Time Combinations 80

XX. Average Plate Counts of Isolate BC-5 Activated at 80 C for 15 Seconds at Various Intervals of Incubation in Sterile Milk and Milks in Which Pseudomonas Species Were Grown Prior to Sterilization 81

XXI. Average Plate Counts of Isolate BC-5 Activated at 85 C for 10 Minutes at Various Intervals of Incubation in Sterile Milk and Milks in Which Pseudomonas Species Were Grown Prior to Sterilization 82

XXII. Average Plate Counts of Isolate BC-5 Activated at 71.5 C for 15 Seconds at Various Intervals of Incubation in Sterile Milk and Milks in Which Pseudomonas Species Were Grown Prior to Sterilization 83

XXIII. Analysis of Variance for Heat-Activation and Influence of Initial Growth of Pseudomonas Species on the Germination and Outgrowth of BC-5 84

XXIV. Treatments and the Treatment Means of Isolate BC-5 85

CHAPTER I

INTRODUCTION

A generation ago milk was delivered to the processing plant, pasteurized, cooled, bottled and delivered to the consumer's doorstep before it was 24 hours old and generally the milk was consumed before it was 48 hours old. Today farm bulk tanks, every-other-day pickup at farms, five-day-a-week plant operation, discontinuing of home delivery and the housewife purchasing milk only on shopping days have increased the age of milk before consumption. In many localities, this age has increased to 7, 10 or even 14 days resulting in a whole set of different quality problems.

In recent years, pasteurization temperatures have been raised above the required standards to keep survival numbers low. Many plants currently heat milk to 80 C or more with a hold of 16 seconds or more. Although psychrophilic Bacillus species were isolated from soil and water (139) it was not until 1969 that the first report of isolation of psychrotrophic sporeforming bacteria from pasteurized milk appeared (87). Following this, many isolations were made from milk (26, 30, 208 210) and the percent of raw milk samples containing psychrotrophic sporeformers ranged from 25 to 84 (30, 87, 210). Bacillus cereus is prevalent in raw milk (159) and heat treatments have been found to activate these spores (32, 47, 163). Milk serves as a good medium for activation and outgrowth of Bacillus cereus, especially the psychrotrophic strains,

when pasteurized milk is held for extended periods of time as a result of twice per week delivery, supermarket sales, larger home refrigerators and bulk packaging. Purchase of a week's supply of milk at one time by the housewife is not unusual. Under such circumstances these psychrotrophic Bacillus cereus produce the exoenzyme which has rennin-like action on the milk casein leading to the sweet curd formation in pasteurized milk. With periods between production and consumption steadily lengthening, psychrotrophic Bacillus cereus has posed a new microbiological problem to the dairy industry.

Psychrophilic bacteria like Pseudomonas fluorescens and Pseudomonas fragi are a part of the normal flora of all raw milk supplies. These psychrophiles are of little significance in raw milk if the milk is pasteurized within 48 hours after milking. But under the present processing procedures, especially during the weekends, raw milk may not be pasteurized for several days. The electrophoretic patterns of caseins from the milk with growth of Pseudomonas species have exhibited some distinct changes (215) and the growth of these organisms in raw milk had stimulatory effect on certain bacteria (176). Undoubtedly, the proteolytic activity of these organisms could liberate various amino acids from milk proteins. Amino acids either separately or in various combinations (103, 148), have been found to cause over 90 percent germination (121) of several strains of bacilli. Germination requirements of bacterial spores are frequently satisfied by amino acids (102, 103, 158) although marked species variations in germination responses to specific amino acids have been reported.

Numerous attempts have been made to obtain information on the nature of changes in milk associated with sweet curd formation by Bacillus cereus (163). This study was conducted in order to determine the influence, of the current trends in the dairy industry, towards higher pasteurization temperatures and extended storage times, on activation and germination of Bacillus cereus and also to determine whether the growth of the common Pseudomonas species in raw milk has any stimulatory influence on this sporeformer.

CHAPTER II

REVIEW OF LITERATURE

Bacterial spores have claimed the attention of technologists, research workers and students of the biological sciences due to their ability to resist not only heat but also radiation, other physical agents, chemicals and enzymes (80, 194). According to Halvorson et al. (96) dormant spores represent both an end point and a beginning in biology and have specialized and complex structures formed as a result of morphogenic change during the vegetative growth. Under appropriate conditions the spores germinated and synthesized the structures and macromolecules characteristic of vegetative cells, in order to begin its life cycle anew (96).

I. OCCURRENCE

Most sporeforming bacteria have been found to reside or originate in the soil and have been considered part of the zymogenous flora (216). In a study of the frequency of isolation of different strains, Bacillus megaterium, Bacillus subtilis and Bacillus cereus made up 350 of 500 isolations, whereas a wide variety of species occurred less frequently (245).

Aerobic sporeformers were among the most common bacterial types isolated from lakes (200). These aerobic sporeformers have also been found in extreme conditions such as acid mine water where the pH was

very low (35, 43, 237). Noble et al. (172) were able to isolate both Bacillus and Clostridium species from hospital air and they assumed that these existed in the air as spores. Fulton and Mitchell (64) found bacteria in the upper atmosphere (altitudes greater than 690 meters). Later Fulton (62) found Bacillus and Micrococcus species comprised 75 percent of all isolates. Studies on a land air mass as it traversed an ocean also indicated Bacillus species (63).

An interesting unusual habitat for Bacillus species was jet fuel. On one study Edmonds and Cooney (45) found that 37 percent of bacterial isolates were Bacillus species. In another study to determine the distribution of microorganisms on the external surfaces of jet aircraft Pfaender and Swatek (181) also found Bacillus species as most common. From the study it was apparent that the jet aircrafts carried certain organisms to new environments in a viable condition on external surfaces and in more protected areas such as wheel wells and door jambs. Slepecky (216) has indicated that there are hopes of finding life in outer space since some sort of life has been suspected on Mars (201), even though there were no spores on the moon.

Extensive consideration of sporeformers as food-spoilage organisms has been presented recently. A number of other reviews and specific papers have appeared on the occurrence of sporeformers in various food environments (5, 8, 25, 44, 58, 74, 75, 88, 204, 224, 240). Food poisoning, definitely attributable to Bacillus cereus has been described in a number of cases (100, 216). Bacillus cereus has also been implicated in cases of septicemia (29), bacteremia (34) and other

infections. Under certain conditions this organism has been found to produce hemolysin, a lethal toxin and phospholipase (117). Slepecky (216) reported that the relationship between these products and the organism's potential as a pathogen was not clear. Bacillus cereus strains, closely related to Bacillus thuringiensis were implicated as the cause of disease in insects. Norris (173) believed this to involve an active phospholipase.

Keynan (120) stated that sporologists of bacteria have always been fascinated with the completely "arrested life" of the dormant bacterial spore. According to him this stage in which metabolism was either slowed down or absent altogether for prolonged periods was called cryptobiosis by Keilin in 1958. In this state organisms have been found to show a minimum of activity and have been presumed to be in the deepest state of dormancy (231). Sussman (230) defined dormancy as any rest period of reversible interruption of the phenotypic development of an organism. Others (153, 244) have indicated that the words dormant and dormancy express the state of microorganisms in which metabolic and developmental processes were arrested. Knaysi (129) indicated that endospores appeared in "healthy cells facing starvation". Others (91, 166, 243) have described that in these sporogenic bacilli facing the limitation of some nutrients, a specialized synthetic mechanism is triggered resulting in formation of resistant dormant cellular form, the spore. Hence, Lewis (153) described dormancy as the central function, the essence of sporulation. He identified the formation of the bacterial endospore as a specialized response to the population explosions so common in the microbial world.

Slepecky (216) recognized the majority of sporeformers as either Bacillus or Clostridium species and most of the knowledge on all aspects of the bacterial spores come from studies of these two genera. According to him there were more species of Clostridium than Bacillus, 93 versus 25 of the over 1,500 species listed in Bergey's Manual (18).

II. STRUCTURE AND CHEMICAL NATURE OF SPORES

Fitz-James and Young (53) summarized that normally, in the growth cycle, sporulation occurred after an extended period of logarithmic growth and division, although it was possible to circumvent this period of increase in cell numbers and proceed directly from a spore to spore or to "microcycle". In either case, normal or microcycle, they (53) found that the time elapsed between germination and the appearance of the crop of spores was the same for any one species. These workers (53) diagrammatically described bacterial spore formation, in the first instance, as a process of cellular division by which protoplast was formed within the cytoplasm of a cell; secondly, as an ordered developmental process in which the two main layers, cortex and coats, were laid down about the internal or spore protoplast. Bayne-Jones and Petrilli (6) were the first to present photographic evidence as to the correct sequence of morphological changes in the living cell in the conversion of vegetative cells into dormant spores. Their evidence indicated these essentials: one cell gave rise to one spore; the spore arose within the cytoplasm at one end of the cell; the developing spore was a dense area of protoplasm incapable of penetration by granules elsewhere in the cell;

the spore area increased in size; and the development of refractility was a rapid terminal process in the formation of the mature spore only to be followed by its liberation from the disintegrating remains of the cell. The use of phase contrast microscopy by Young and Fitz-James (260) confirmed all these features.

Young and Fitz-James (260) found that the vegetative cell of the sporeformers contained two chromatin bodies in stages of division, whereas the spore contained only one in a state of rest. From this finding Fitz-James and Young (52) concluded the average vegetative cell contained approximately twice the quantity of deoxyribonucleic acid as does the spore of the same species. Doi (38) noted that in the vegetative cell of bacilli a particulate flavin-cytochrome electron transport system was responsible for respiration, while in the bacterial endospore, respiration was maintained after germination by a soluble flavoprotein, reduced nicotinamide adenine dinucleotide oxidase, employing riboflavin 5-phosphate as cofactor. In a recent study (48) membrane fragments from isolated forespores and spores were checked to see if they too contained components of the electron transport system. Preliminary evidence indicated that developing structures (forespores) leading to a mature spore did have an electron transport system. When membrane fragments were examined from forespores and spores, similar cytochrome spectra were noted.

Murrell (167) noted that spores differed from vegetative cells in the composition of the various new layers, in having unique substances such as dipicolinic acid, in various new antigens, and in having marked

quantitative differences in calcium. From the electron microscopic studies of thin sections (166, 168, 246), the following sequence of components surrounding the spore cytoplasm has been described by Tipper and Gauthier (234).

(1) Cytoplasmic membrane: Engulfment of the spore cytoplasm by the membrane of the mother cell produced a forespore which was surrounded by two membranes; an inner forespore membrane and an outer forespore membrane. The inner forespore membrane developed into the cytoplasmic membrane of the mature spore.

(2) Germ cell wall: A layer which developed into vegetative cell wall on germination.

(3) Cortex: A unique lysozyme-sensitive peptidoglycan that was solubilized during germination, to give rise to "germination exudate", the material in which muramic acid was first identified according to Strange (227). Cortex has been found to be of low electron density and was so named by Robinow (195).

(4) A second membrane: This membrane was visible in certain sections of Bacillus cereus spores at all stages of development and was derived from the outer forespore membrane (168).

(5) Spore coats: These spore coats were a series of polypeptide-rich layers which may entrap sporangial cytoplasmic components. Thin sections of Bacillus cereus spores showed a series of three distinct layers exterior to the outer forespore membrane and inside the exosporium. The middle layer consisted of two or three juxtaposed lamellae. Recent use of the freeze-etching technique to dissect these multiple layers in

surface view (107, 141) revealed more details. Distinct array of parallel fibres, made up of 50-nm particles, have been seen in spores of all species studied. In Bacillus cereus, the fibrils were short, arranged in small packets and overlaid a pitted layer. The discontinuities in the fibrillar layer of this organism corresponded to the formation of the lamallae from a number of discreet "islands" during sporulation (168).

(6) Exosporium: This outer membranous layer may be loosely or tightly fitted about the spore. According to Tipper and Gauthier (234) in case of Bacillus cereus the exosporium was loosely fitting and it could be selectively removed by mechanical shear. Matz et al. (160) found the chemical composition of the isolated exosporium to be 52 percent protein, 20 percent polysaccharide (glucose: rhamnose: glucosamine: Ribose, 13:9:5:1)), 12.5 percent neutral lipid, 5.5 percent phospholipid (apparently all cardiolipin), and 3.8 percent ash. The protein had a low content of methionine and cysteine which clearly distinguished it from spore coat protein, which had a high cysteine content (234). Gerhardt and Ribi (71) observed a nap or pile of hairlike projections on the exosporium. In Bacillus cereus, the exosporium first appeared after total engulfment of the forespore, as a vesicle on the exterior of the outer forespore membrane at the interior pole of the forespore (234). The exosporium gradually engulfed the forespore as cortex accumulated. Murrell et al. (168) noted the formation of the coat layers of Bacillus cereus from inside the developing exosporium layer.

Fukuda and Gilvarg (65) stated that the presence of dipicolinic acid in spores but not in vegetative cells suggested that this compound

played an important role in spore physiology. The existence of a mutant that neither synthesized dipicolinic acid nor formed spores in its absence supported this view. Spores have been found to contain equimolar dipicolinic acid and calcium ions. Several studies have suggested that heat resistance was related to divalent metal ion or dipicolinic acid content, or both (167). Halvorson and Swanson (95) have indicated that dipicolinic acid and metals might be essential for the maintenance of thermoresistance. Measurement of the dielectric properties of dormant spores of Bacillus cereus revealed that they contain low concentrations of metal ions which suggested that the small molecules were electrostatically bound (22). Nelson and Kornberg (170) found 3-phosphoglyceric acid in high levels in Bacillus cereus spores, firmly bound and not readily exchangeable with external phosphoglyceric acid. Shortly before refractility occurred, 3-phosphoglyceric acid became a major component of the phosphate pool. They (171) also identified a restricted number of free amino acids in the spores, in contrast to the wide variety of amino acids found in vegetative cells. L-Glutamate was the predominant free amino acid in spores of several Bacillus species in addition to significant levels of L-lysine and L-arginine. The glutamate was firmly bound and did not exchange with exogenous glutamate although it could be extracted from the spores by boiling in water.

Pitel and Gilvarg (182) observed membrane proliferation, during sporulation, which according to Tipper and Gauthier (234) was required for the synthesis and expansion of the inner and outer forespore membranes. The latter concluded that the integrity of the spores might be

maintained by high calcium ion concentrations; and hence the internal forespore membrane must be semipermeable to calcium ions, dipicolinic acid and other small molecules which have been found in high concentrations in spores. The outer forespore membrane has been found to be synthesized during engulfment of the forespore. It contained the enzymes that were required for cortex synthesis. This forespore membrane allowed calcium ions, dipicolinic acid and other small molecules to pass through in an external direction and hence functioned only during sporulation (234). Strange and Hunter (228) observed that germ cell wall formed the basis for subsequent vegetative cell wall synthesis. Tipper and Gauthier (234) presumed that the germ cell wall might protect the germinating spore from osmotic lysis. The cortex played a structural role in the maintenance and perhaps in the achievement of dormancy and the dehydrated state of the spore cytoplasm (234). The coat has been found to protect the cortex from physical and enzymatic attack and probably even surfactants, and further, aid dispersal of spores by their hydrophobicity. Exosporium might serve as a protective layer and may have a specific role in directing coat assembly during sporulation (234).

Highton (101) observed changes in the structure of mesosomes and cell membrane of Bacillus cereus during sporulation. Between exponential and stationary phase the cell membrane and mesosomes became more dense and the mesosome structure changed from lamellar to vesicular. He also noted the increase in density produced as a result of increased binding of heavy atoms.

Aronson and Horn (3) attempted to characterize the proteins extracted from the spore coat layers, and found that the proteins in

the spore coat layers were identical. According to these workers there might be only a single major species of polypeptide in the spore coat. As already indicated in this review, dipicolinic acid (pyridine-2, 6-dicarboxylic acid) has been found to be a major constituent of the bacterial spores. According to many reports (2, 15, 28, 151), the calcium salt of this acid was a major contributing factor to heat resistance. Greater evidence in support of this has been provided through isolation of dipicolinic acid-minus mutants by Zytkovicz and Halvorson (263). The biosynthesis of dipicolinic acid has been found to occur from an intermediate in the lysine pathway (4, 24, 262). This very unstable intermediate has been identified as dihydrodipicolinic acid which was later oxidized both enzymatically and nonenzymatically to dipicolinic acid (263). According to Carstensen et al. (22) the low electrical conductivity of bacterial spores indicated the binding of the abundant spore electrolytes in vivo and their nonavailability for conduction of electrical current. Grecz et al. (82) considered the binding of calcium-dipicolinic acid resulted in a hydrophobic condition, whereas the release of calcium-dipicolinic acid increased the hygroscopicity of both the residual spore and the released material. Furthermore, the rate of loss of dipicolinic acid on heating appeared to be related to the rate of thermal death of spores, since heat-resistant strains lost dipicolinic acid less quickly than heat-sensitive strains (83). Hence, heat resistance might be a function of the stability of postulated calcium-dipicolinic acid-spore complexes (184, 259) which determined the amount of heat energy required to dissolve and remove dipicolinic acid from the spore structure. Grecz et al. (84) obtained

data on the dissociation of the 1:1 and 1:2 calcium-dipicolinic acid chelates which showed correlation with the release of calcium ion and dipicolinic acid and with thermal inactivation of spores at pH 2, 7, and 10.5. Although cortex and the core were suggested by Pearce and Fitz-James (179) as the most likely sites of calcium-dipicolinic acid location in the spore, a core location was established by ultraviolet-photoproduct study (40) by the examination of dipicolinic acid content of the cortexless mutant spores of Bacillus cereus (178) and by location of tritium-labeled spore components using beta-attenuation analysis (142).

Wyatt (257) found that the spore coat was of lower optical density than the cortex, contrary to traditional expectations. Further analyses by him revealed that the spore coats had significant variations in thickness and refractive index relative to the more uniform cortex region. Neither the cortex nor the coat contained any free water. The permeability of bacterial spores was assessed by Gerhardt and Black (70) with a discussion by Murrell and the experimental results with free space measurements were detailed in companion articles (12, 13, 14, 16, 69). A new method was described by Scherrer and Gerhardt (206) for determining the quasimonodisperse exclusion threshold in porosity of an intact cell or isolated integument by analyzing the changes in molecular weight distribution of a polydisperse polymer sample before and after uptake. This uptake-distribution method was then applied to dormant spores (205). Intact dormant spores of Bacillus cereus T took up probing solutes passively as a function of molecular size, lipid solubility and charge (69). The passive uptake of otherwise similar solutes was greatly

affected by the net electrostatic molecular charge, even though the potentially ionic chemical groups in dormant spores were electrostatically bound in nonionized forms to an extreme degree (22). Gerhardt et al. (72) have concluded that cytostructural interpretations from direct and indirect evidence suggested that exosporium was either functionless because of an opening or functionally indistinguishable from the coat as the limiting permeation barrier. Smaller lipid-soluble solutes like glycols and water permeated the dormant spore protoplast but smaller lipid-insoluble molecules like sugars and organic acids did not, as evidenced by direct measurements of uptake and by indirect measurements of radioactive exchange.

In recent years there has been increased interest in the understanding of the processes leading to the termination of the cryptobiotic state in the bacterial endospore and the transformation of the dormant spore into a vegetative cell. A good deal of this interest had naturally concerned the mechanisms underlying these transformations. Although, the exact mechanisms have not been fully understood, a generalized picture of the events occurring in the spore during its transformation into a vegetative cell has begun to emerge. On the basis of the present knowledge, at least three different kinds of sequential events occurred in the spore during its transformation into a vegetative cell. These processes have been referred to as activation, germination and outgrowth. These have been caused by different treatments and they differed biochemically and in the kind of changes brought about in the spore (124).

III. ACTIVATION OF SPORES

Activation has been described as a process which conditioned the spore to germinate under appropriate circumstances. All of the evidence indicated that this process was reversible in most bacterial spores based on the change of the macromolecular structure of the spore and this change was not dependent upon metabolic reactions of the spore. Activation resulted in a spore poised for germination, but still retaining most of the spore properties. Activation did not occur in all spores, and the need for this process depended upon the spore itself, its history, and on the circumstances which induced its transformation into a vegetative cell.

Many freshly prepared spore suspensions have been found not to germinate under a given germination condition unless either heated or aged. The activated spore germinated if exposed to the right conditions, even if growth was inhibited by irradiation or by addition of growth inhibiting substances (121). Activation of mold spores was discovered even before the discovery of activation of bacterial spores. Keynan and Evenchik (121) mentioned the name of Weizmann as the first to use heat as a method to activate bacterial spores while developing the technology of acetone fermentation by Clostridium acetobutylicum for which he obtained a U.S. Patent. Curran and Evans (32) noted an increase in colony count of milk after heat treatment. Powell and Hunter (186) observed that the heat activated spores reverted to their dormant state after storage at room temperature. Hence, heat activation has been considered a reversible process, although these spores could be reactivated

by a second treatment (27). The simplest method of activation of spores has been found to be the exposure to sublethal heat, but other treatments such as exposure to low pH (1 to 1.5), thiol compounds and to strong oxidizing agents were effective (121). Because of the potential usefulness in the processing of foods, including milk and milk products this phenomenon of activation of bacterial spores, especially the nature of activation and agents influencing this reaction have been considered important.

IV. THE NATURE OF ACTIVATION

The reluctance to germinate was not confined to bacterial spores alone, but was known to occur in spores of molds, seeds of plants and other cryptobiotic system (124). Activation has been considered as a change in spore permeability, although the dormant spore was to a large extent permeable to water (12, 69). The evidence of altered permeability was based on increased uptake of oxygen by the activated spores when glucose was added (27, 145, 146), need for less L-alanine (175), excretion of calcium dipicolinate and some amino acids during activation (143). More direct proof has been provided by Gould and Hitchins (79) who found that the exposure of spores to mercaptoethanol, a treatment which induced activation, enabled a molecule as large as lysozyme to penetrate the spore coat and reach its substrate in the cortex. Activation has also been considered as change at a definite spore structure or as molecular change occurring in different proteins located in various spore structures. Gould and Hitchins (79) suggested that a disulphide-bond

opens in the coat fraction of the spore as a result of activation (79, 106, 122). The spore coat was the first target to get altered during activation with reducing agents (79) because of a high content of disulphide bonds in the spore coat (241). Others (122) have correlated heat activation with denaturations of the proteins located in the outer parts of the spore coat rather than in the cortex, and hence suggested the spore coat as the site of heat activation. The acid-activation treatment involved cation exchange and the cation load might control the state of activation (154).

The various treatments which induced activation in spores, like heat shock, exposure to reducing agents or high concentration of urea have indicated that the change from dormant state to an active one was accompanied by change in the tertiary structure of some macromolecules of the spores, and activation did not involve metabolic reactions (122). Activation as a possible release of a factor stimulating germination has also been considered. Hyatt and Levinson (110) hypothesized that the fundamental stimulant for germination was a substance which was formed, within the spore and was not an added excitant as suggested by Pulvertaft and Haynes (188). Hyatt and Levinson (110) considered that heating of spores increased production of this germination requirement, which while inadequate to support germination by itself (perhaps because of low concentration), would react with added glucose, or potassium nitrate, to increase the initial germination rate. The identity of this hypothesized internal substance was not known. The increase in alanine concentration on incubation of spore homogenates (144); the

presence of dipicolinic acid complexed with amino acids in the spore (259) and excretion of this dipicolinic acid on heating of the spores (147) lend some credence to the theory that the heat-released internal germination stimulant may be L-alanine or a related substance.

As already stated, calcium and dipicolinic acid have been known to exist in equimolar concentration in the spore in a bound form and were released in a soluble form during activation (98, 126, 252). Lee and Ordal (143) found that when activation was induced by exposure of the spores to calcium dipicolinate, small amounts of amino acids, especially glutamic acid, were released in addition to calcium dipicolinate. Others (61), working with Bacilus subtilis, also demonstrated that under certain conditions calcium dipicolinate did not induce germination, but nevertheless activated the spores for germination with L-alanine.

Mechanism of activation in view of the possibility of inactivation of an inhibitor has to be considered. The hypothesis of the existence of an inhibitor was proposed by several investigators (93). Spontaneous germination of activated Bacillus megaterium spores was inhibited by copper, iron, silver, or mercury salts, saturated o-phenanthroline or solutions having low pH value (126, 143).

On the molecular level, activation has been explained as the result of a reversible change in the tertiary structure of some of the spore macromolecules (122). The thermodynamic and the kinetic data of heat activation were very similar to the data obtained for heat denaturation of proteins or the so-called melting curves for double-stranded nucleic acids (126). The thermodynamic relation was the existence of a critical

temperature range indicating that the energy of activation for the process was very high. Such data suggested the disruption of the tertiary structures in macromolecules (121). Activation at low pH occurred in the range at which proteins were denatured (126). Hence all chemicals which brought about activation were known to cause structural changes of macromolecules. Vinter (241) found spores to contain five times more cystine than vegetative cells and cystine was concentrated mostly in the spore coat (214). Disulphide bonds were typical of the cryptobiotic state and during germination they were reduced to sulphydryl groups (121). Keynan and Evenchik (121) assumed that the macromolecules responsible for the maintenance of the dormant state were the coat proteins rich in cystine, stabilized at certain configuration by S-S linkages. Reduction of these bonds according to them (121) would change the tertiary structure to result in partial unfolding or denaturation of these proteins. Warth et al. (246) reported that the spore coats were mostly composed of protein. The increased permeability brought about in the spore coat as a result of activation would be responsible for the uncovering of the active sites of enzymes or increased accessibility of the substrate to enzymes.

V. HEAT ACTIVATION OF SPORES

Evans and Curran (47) showed that sublethal preincubation heating of aerobic spores helped to accelerate their germination. This process known as heat activation was responsible for breaking dormancy in spores. These workers (47) observed greatest acceleration with a preheating

treatment of 85 C for 8 to 10 minutes although temperatures in the 65 to 95 C range were effective accelerators of spore germination. In addition, the effect of preheating upon spore germination was influenced by the nature of the medium in which the spores were heated and incubated. Curran and Evans (32) found that heat activation was most favorable in heating media like glucose or lactose, peptone, skimmilk and others. Keynan and Evenchik (121) have stated that different species vary in their temperature requirements for activation. They have also indicated the existence of variation among different strains and even among different batches of the same strains. Some strains of Bacillus megaterium required only a few minutes at 60 C for optimal germination rates whereas Bacillus stearothermophilus and other thermophilic and thermotolerant bacteria required from 105 to 115 C for optimal activation (50).

Keynan et al. (126) induced sporulation in the presence of different amounts of calcium, and phenylalanine. The presence of these has been demonstrated by Church and Halvorson (28) to affect the spore composition. The time necessary for optimal activation of the spores correlated well with the content of calcium-dipicolinic acid, but this finding does not necessarily mean that heat activation requirements were dependent only on calcium and dipicolinic acid concentration in spores. Similar correlation between heat resistance and dipicolinic acid content was reported earlier by Halvorson and Howitt (94). Busta and Ordal (20) studied heat activation kinetics of Bacillus subtilis. In this study the response was measured by the plate count and recorded as the heat-activated decimal fraction of the total viable count. They observed

that the exposure time required to achieve the response was extended as temperature decreased. The logarithm of the rate of heat activation was related to the test temperature. In other words, at a lower temperature a longer period of time was required for the response. The kinetics study (70) showed that activation energy was as high as about 28,000 calories. In addition to the stimulation of spores for germination, heat also activated certain enzymes, which were not active in the dormant spore (27). The role of water for activation was clear from the report of Powell and Hunter (186) since they showed that activation must be carried out in the presence of water. Further support was obtained from the work of Beers (7) who demonstrated that it was impossible to activate dry lyophilized spores or spores resuspended in a high concentration of glycerol.

Normally spores are heat activated and germinated at a neutral pH. Keynan and Evenchik (121) have indicated that the degree of activation was pH dependent and exposure to low pH could by itself induce activation in spores which may be subsequently washed and resuspended in a suitable germination medium. Keynan et al. (122) reported that spores of Bacillus cereus T germinated spontaneously after prolonged incubation in a buffer at low pH. At pH above 8.5 these spores were not activated when heated for an hour. When the same spores were washed and resuspended at neutral pH and then heated for an hour at 60 C they behaved like activated spores. This indicated that activation of Bacillus cereus spores was inhibited at a high pH, without injury to the spores. This did not appear to be true with other organisms, since Gibbs (73)

demonstrated optimal activation of Clostridium bifermentans at high pH, and inhibition at low pH.

Heat activation has been observed to be lost during storage and reactivated by a second treatment (27, 186). Hence the process has been considered a reversible process. Powell (183) was the first to note the reversibility of heat activation in Bacillus subtilis when stored for 48 hours after heat activation. She observed the rapid decrease in their germination rates. Church and Halvorson (27) have demonstrated the respiration of the heat-activated ungerminated spores as well as the utilization of oxygen in the presence of glucose.

Heat activation was inhibited to some extent by sodium fluoride (186). Results of heat activation tests using both the complex and synthetic media in case of Bacillus subtilis indicated that 63 percent of the spores did not require heat activation on the complex medium whereas on the synthetic medium from 84 to 96 percent of the spores required heat activation. From this investigation (207) it was apparent that chemical activation was taking place in the complex medium. Some surface active agents in low concentration and certain cations were found to interfere with activation. Heavy metals such as iron and copper inhibited the activation of the spores of Bacillus cereus T (125) and Bacillus megaterium (143). Inhibition of the activation of Bacillus popilliae spores heated for 10 minutes at 80 C in presence of potassium ions has been reported (219). This inhibitory effect of potassium diminished with aging of spores which suggested slow activation during aging of systems blocked by potassium. The influence of several salts on the rate of activation of bacterial spores at 65 C in distilled water

was investigated by Keynan et al. (125). These salts were found to interfere with germination when present in the spore suspension during heating (33, 89, 125).

Deactivation occurred in Clostridium bifermentans, as was shown by Gibbs (73). Under certain conditions such as activation of Bacillus cereus at low pH (125) or Clostridium bifermentans at high pH (73) deactivation did not occur. Hence deactivation depends on environmental conditions (122). Deactivation was also found to be temperature dependent. Gould and Hitchins (79) demonstrated that exposure to disulphide-rupturing agents sensitized spores to lysozyme. Such sensitized spores returned to the normal lysozyme insensitive condition after being dialyzed against water for two weeks. This finding was interpreted as a reoxidation of reduced disulphide bonds. The same reducing agents also cause activation and hence it could be inferred that activation involved rupturing disulphide bonds and deactivation involved reoxidation of reduced disulphide bonds. Hyatt et al. (108) reported another chemically induced deactivation involving Bacillus megaterium where the activated spores were deactivated by absolute alcohol at 60 C for 18 hours. Lee and Ordal (143) were able to deactivate spores activated by calcium-dipicolinic acid for spontaneous germination by exposure to acetic acid for a day. The deactivated spores were found to be less heat resistant than spores before activation.

VI. ACTIVATION BY OTHER AGENTS

Exposure to low pH has been found to induce activation in spores. Keynan et al. (122) observed spontaneous germination of spores of

Bacillus cereus T after a prolonged incubation in a buffer at low pH. They also indicated that another method of activation of bacterial spores was by the use of reducing agents, which imitate or spare the heat treatment (122). Compounds which reduced disulphide bonds did not act as germinating agents but activated spores after prolonged incubation. Keynan and Evenchik (121) have described that freshly harvested spores of Bacillus cereus T when incubated in the presence of thioglycolate or mercaptoethanol at 28 C exhibited partial germination after washing and addition of L-alanine and adenosine. A prolonged time was required for activation with reducing agents since the effect appeared only after exposure of 12 hours. On the other hand, spores of the anaerobe Clostridium bifermentans could not be activated with thioglycolate and this treatment instead inhibited activation (73) as already indicated. Others found that the activation by reducing agents was pH dependent and correlated well with that of spontaneous activation at low pH (122, 12, 145). The optimal activation in presence of reducing agents was around pH 6.

Spores of Bacillus subtilis and Bacillus cereus T germinated without heat activation in solutions of equimolar concentrations of calcium and dipicolinic acid (145, 193). On the other hand spores of Bacillus subtilis-168 did not germinate but were only activated (61). Activation of Bacillus megaterium by calcium and dipicolinic acid occurred at 7 to 10 C (143). The germination of these activated spores was inhibited by cupric ferric, mercuric or silver cations, chelating agents, o-phenanthroline or solutions of a low pH. Lee and Ordal (143)

reported that these inhibitors could be partially or completely reversed by the addition of sodium dipicolinate, which suggested a competitive action of the sodium ion.

Levinson and Hyatt (147) were the first to report the activation of spores of Bacillus megaterium by ionizing radiation. Irradiated spores were able to germinate even when radiation resulted in the inability of the germinated spores to divide. Irradiated spores showed a rise in oxygen consumption rate when exposed to appropriate germinating agents. These results suggested certain similarities in the action of heat and radiation especially at the higher doses of exposure. Gould and Ordal (81) reported activation of Bacillus cereus PX by exposure to gamma radiation. Irradiated spores germinated at a high rate in the presence of germinating agents like L-alanine, inosine, n-dodecylamine and calcium dipicolinate. Similar treatments in the same range of radiation doses had no activation effect on spores of Bacillus cereus T or Bacillus subtilis. Spores activated by irradiation were deactivated on storage similar to the heat activated spores. In contrast to heat, irradiated spores germinated rapidly in n-dodecylamine.

There are various reports about the activation of spores by chemicals. Gould (77) reported activation of Bacillus cereus spores germinating in L-alanine with D-cycloserine (oxamycin) and with o-carbamyl-D-serine. He explained that these antibiotics being analogous to D-alanine inhibited the enzymatic activity of alanine-racemase and thus prevented the formation of D-alanine which consequently allowed more efficient utilization of L-alanine during germination. Widdowson

(249) activated spores of Bacillus pentothenticus by pretreatment with a very polar solvent like dimethylformamide. Lewis et al. (154) used concentrated solutions of chloral hydrate in water or in ethylene glycol as a substitute for heat activation of spores of Bacillus coagulans. This treatment released dipicolinic acid and other spore components irreversibly and the changes resembled germination rather than activation. Levinson and Hyatt (145) observed germination in the presence of manganese salts. Manganese activated proteolytic enzymes in the dormant spores leading to germination and an increase in respiration. The fact that this activation was partially inhibited by D-alanine suggested that the manganese salts were involved in general metabolic activities of the spore. Hyatt et al. (108) were able to activate lyophilized spores of Bacillus megaterium with glucose or with L-alanine after prior exposure to water vapour. Exposure of lyophilized spores to water vapour increased both the percentage of germinating spores and the rate of germination. Suitable time and temperature exposure to water activity as low as 0.6 gave maximal germination. Activation was also achieved by treatment with 60 percent aqueous ethanol at 30 C for five minutes but not with absolute alcohol. The authors suggested that activation by aqueous ethanol was similar to heat shock in permitting water to reach specific sites, as hydration was essential for the functioning of germination. On the other hand, removal of firmly bound water by means of absolute alcohol deactivated already activated spores.

The aging phenomenon was first described by Powell (183) since he observed the slow germination of fresh spores and acceleration in the

rate of germination in spores after 20 days of storage in water at 20 C. Similar changes resulting from storage were observed by Krishnamurthy and Halvorson (136). Aging and heat activation appear to be similar phenomena. Long exposure of spores to low but stable temperatures caused activation. Busta and Ordal (20) noted the activation of spores of Bacillus subtilis exposed to 5 C for 525 days. Thermodynamically there appeared to be no difference between aging and activation (121). Both activation and aging terminated the state of dormancy. The only difference appeared to be the temporary loss of dormancy during activation whereas an irreversible loss of dormancy during aging.

VII. CHANGES IN THE ACTIVATED SPORES

Activated spores have been found to retain most of the important properties of the dormant state such as resistance to heat and radiation, nonstainability and refractility (121) in addition to several measurable changes, in germination rate, germination requirements, morphology, permeability, spore composition and metabolic activity. Keynan and Evenchik (121) have indicated that spores from freshly harvested cultures usually did not germinate or only very slowly. Very long lag period preceded germination, which was slow and unsynchronized. Germination in such unactivated spores was usually incomplete. In many cases highly dormant unactivated spores did not germinate at all and therefore did not form colonies when plated even on a very rich medium. Activation increased the germination rates dramatically. Powell and Hunter (186) observed that the extent of germination depended on the degree of

activation. O'Connor and Halvorson (175) observed that heat shock decreased the lag period prior to germination and increased the rate of germination.

Activation also influenced the qualitative and quantitative requirements of spores for agents which caused germination. Keynan and Evenchik (121) have mentioned Hills as the first to demonstrate the need for specific nutrients for spore germination. Glucose, adenosine, inosine, L-alanine and certain other amino acids, either separately or in various combinations, have been found to cause germination in over 90 percent of spores of several strains of bacilli (121). Powell and Hunter (186) observed that activation might modify this germination requirement. They demonstrated that dormant spores of Bacillus cereus required either inosine or a mixture of alanine, tyrosine and adenosine for germination. The same spores after prolonged aging or heat-treatment germinated easily in the presence of adenosine alone. An even more pronounced effect of heat activation was observed in spores of Bacillus megaterium, which germinated spontaneously after activation. The rate and extent of germination was dependent on the temperature and time of the heat treatment. Hyatt and Levinson (111) found that heat activated spores of Bacillus megaterium germinated when glucose was the sole germinating agent. With the rise in the preheating temperature the requirement for glucose to attain maximum germination rate decreased. O'Connor and Halvorson (175) observed in spores of Bacillus cereus, the heat-treatment decreased the quantitative requirement for L-alanine-induced germination. They suggested that heat treatment might make the

enzyme more accessible to the amino acid thus triggering germination. Some changes in the morphology of the spores were noted when heat activation was carried out at low pH. Acid-activated spores tended to aggregate during heating and their optical density decreased. Additional evidence of morphological and structural changes in activated spores have been reported by Moberly et al. (164), who observed spores after heating for 15 minutes at 65 C under an electron microscope. The heated spores appeared mottled, and the cytoplasm was less granular and more opaque than in the dormant spores.

Activation also influenced the permeability of the spore as was observed in Bacillus subtilis, which germinated in the presence of pyruvate after acid treatment. The chemical composition of spores has been reported to change after activation. Harrell and Mantini (98) demonstrated in spores of Bacillus cereus T the release of dipicolinic acid as a function of time and temperature and suggested a correlation between excretion of dipicolinic acid and degree of activation. The release of dipicolinic acid during heat activation could result from a change in the permeability of the spore coat and the opening of a chemical bond between dipicolinic acid and a spore enzyme. Keynan et al. (125) observed dipicolinic acid release during and after activation of Bacillus cereus T spores which contained varying amounts of endogenous dipicolinic acid.

No metabolic activity was apparent in dormant spores of Bacillus subtilis and Bacillus anthracis as reported by Crook (31), who could not detect any respiration with or without glucose. Activation induced

some measureable metabolism in spores. Church and Halvorson (27) found that aged spores started to oxidize glucose when activated, although no germination was apparent. Krishnamurty and Halvorson (136) demonstrated that different concentrations of alanine or adenosine added during pre-incubation controlled the rate of glucose oxidation. In contrast to Bacillus subtilis the spores of Bacillus cereus T required heating for activation in addition to L-alanine for glucose oxidation. Church and Halvorson (27) noted that no heat treatment was needed when the same spores were incubated in the presence of adenosine and low concentrations of glucose.

In addition to the glucose oxidizing enzymes other enzymatic systems seemed to be activated by heating. Preheated spores of Bacillus cereus T oxidized gluconate as well as 2-ketogluconate and pyruvate (121). Bishop and Doi (11) observed increase in enzymatic activity of protease in spores after heat shock for 15 minutes at 60 C. The activity of enzymes extracted from spores was increased when activation preceded extraction (92).

VIII. GERMINATION SYSTEMS NOT REQUIRING ACTIVATION

Several workers have shown that certain chemicals which were not metabolized could also induce germination. These chemicals were not regarded as nutrients, although they induced germination (145). In this kind of nonphysiological germination, activation was not required. Germination was caused by surface active substances, chelating agents, or by mechanical means. Hence they were referred to as "chemical"

and "mechanical" germination where germination kinetics did not depend on, and were not changed by, heat activation. Keynan and Evenchik (121) have claimed Rode and Foster (198) to be the first to note chemical germination of spores exposed to dodecylamine and several other n-alkyl primary amines. Spores exposed to these agents lost heat resistance and refractility, became stainable, metabolically active and remained viable. This germination seemed to proceed much faster than physiological germination. Rode and Foster (197) found that, when spores were shaken in a Mickle tissue disintegrator for 12 to 14 minutes, they germinated and produced colonies, and the germination kinetics was not dependent on activation. Keynan and Evenchik (121) also reported the investigation of Brown, who demonstrated germination of putrefactive anaerobe (PA 3679) in the presence of the chelating agent, namely ethylene-diamine tetraacetic acid [EDTA]. In contrast, the spores of Bacillus subtilis, Bacillus mycoides, and Bacillus thermoacidurans did not germinate even after five hours of exposure to ethylene-diamine tetraacetic acid (EDTA). Riemann (192) found that although dipicolinic acid alone did not generally induce germination of the spores, it caused rapid germination of many spore suspensions if sufficient calcium chloride was added to the dipicolinic acid solution, such that the number of moles of calcium chloride was at least equal to the number of moles of dipicolinic acid. In some bacterial spores, germination induced by chelates, was dependent on duration of incubation and also on temperature (123, 192). Heat activation did not influence the kinetics of calcium-dipicolinic acid chelate induced germination. Incubation with

calcium-dipicolinic acid chelate at 37 C activated spores for L-alanine germination (61). Germination rates with L-alanine increased by a factor of four after exposure to calcium-dipicolinic acid chelate. Although calcium-dipicolinic acid chelate-induced germination has been classified as "chemical germination", according to Keynan and Evenchik (121) there was some evidence that its effect was in some way metabolically mediated. The mechanism by which chelating agents induced germination was although difficult to understand, could be explained by the chelation of metal ions. Theoretically, chelating agents could chelate an inhibitory ion present in the dormant spore which might have had repressed germination. Chelation has also been found to result in the activation of an enzyme essential for initiation of germination. Calcium and dipicolinic acid were present in spores in a chelated form. Some of the dipicolinic acid was released during heat treatment and aging. This fact, together with the finding of increased metabolic activity of spores during germination, support the assumption that breakage of the enzyme, calcium-dipicolinic acid chelate can be the first signal in a chain of reactions leading to germination (192).

IX. GERMINATION OF SPORES

The second process in the sequential events in the transformation of the spore into a vegetative cell is germination, a process responsible for the definite termination of the dormant state. This process, in contrast to activation, has been found to be irreversible and consequently resulted in a cell which had lost the typical characteristics of a

bacterial spore. Keynan and Halvorson (124) noted that germination did not involve synthesis of macromolecules; instead, it involved degredation of macromolecules. This resulted in the excretion of many typical spore substances into the medium (124). Rapid and complete germination occurred only after activation, and was triggered by specific germination inducing agents such as L-alanine (122). The germinated spore was cytologically distinct and lacked a full complement of typical vegetative macromolecules and enzymatic activities. Germination essentially happened to be the conversion of a resistant and dormant spore into a sensitive and metabolically-active form (78). The germinated spore must in no sense be considered equivalent to a vegetative cell.

X. CHANGES IN THE GERMINATING SPORES

During germination, spores lost their characteristic resistance to environmental stresses such as extremes of temperature, desiccation, pressure, vacuum, radiation, antibiotics, other chemicals and extremes of pH (250, 78). Loss of heat resistance was the criterion first used to measure germination. Germinated spores exhibited increased sensitivity to chemical reagents like phenol (49). This might form the basis of rapid methods for measuring germination rates in place of the more conventional heat challenge (49).

During germination, spores changed from bright to dark when examined by phase-contrast microscopy (185, 188). Phase darkening occurred because of the loss of refractive index of spores during germination. This probably resulted from the combined effects of excretion of dry

matter, slight swelling and possibly the redistribution of water within the spore during germination (78). Lewis et al. (154) indicated that depolymerization of cortex murein could reduce pressure on the core allowing local hydration of that structure or swelling due to the osmotic influence of low molecular weight muropeptides within the spore.

Clean dormant spores are essentially unstainable except at their peripheries unless pretreated, for instance by heat or acids. Germinated spores were readily stained by simple stains and this property of stainability of the germinated spores was used extensively by Powell (183). Rode and Foster (199) explained that germinating spores became stainable by the breach of a permeability barrier. Gould (78) indicated that stainability might accompany the unmasking of reactive groups in the spore. The most likely blocking agent would be dipicolinic acid (for basic groups) and calcium (for acidic groups) or perhaps the 1:1 calcium dipicolinate molecule.

The optical extinction of visible light by a spore suspension was observed to decrease during germination by about 60 percent but its extent was found to differ with different organisms (78). The observed fall in extinction was certainly due to the excretion and solubilization of dry matter from the germinating spores (239) and the consequent fall in refractivity and light absorption by individual spores (185, 183). Powell and Strange (187) found decrease in dry weight of germinating spores. Depolymerization and excretion of spore constituents amounting to 30 percent of their dry weight was observed during germination. The exudate was principally composed of calcium and roughly equimolar amount

of dipicolinic acid and fragments of depolymerized murein (229, 227, 187).

Increase in spore volume was observed in germinating spores (177). The volume increases that accompanied germination were about 20 percent in spores studied including Bacillus cereus. Such increase in size and change in shape suggested that the spore coat was becoming plastic during germination, perhaps by the action of lytic enzymes or disulphide reductases (17, 79, 241).

Enhanced respiratory activity was observed during germination by several workers (37, 155). In addition, the hydration of the spores (197) as well as the increased sensitivity to lysozyme (9) were observed.

XI. INITIATION OF GERMINATION

Hills (102, 103, 104) was the first to discover that germination requirements for bacterial spores could be simple and specific. He found that L-alanine was able to cause germination of spores of a Bacillus subtilis strain, and was inhibited completely by D-alanine. At suitable pH value and ionic strength L-alanine serves in the germination of most strains studied including Bacillus cereus (55, 164, 254, 255). Heat-activated spores germinated in a variety of amino acids when compared to unheated spores as evidenced from germination of heated Bacillus megaterium spores in L-cysteine, L-leucine, DL-isoleucine, DL-methionine, L-proline and L-valine (150). Initiation of germination via oxidative formation of pyruvate from alanine was indicated by enzyme studies of Bacillus cereus T spores (90). Most studies have suggested that L-alanine

was deaminated during germination by enzymes and possible products were pyruvic acid, ammonia, and hydrogen peroxide.

The germinative action of ribosides was first reported by Hills (102). Inosine, which can be derived from adenosine by deamination mediated spore deaminase (186) has generally been found more active. Guanosine and xanthosine also showed some germinative activity for Bacillus cereus T spores particularly when L-alanine was also present (140).

Germination by glucose has been mainly observed with spores of Bacillus subtilis (255) and Bacillus megaterium (111, 112). Hyatt and Levinson (111, 112) found that unheated spores of Bacillus megaterium QMB 1551 could be germinated by hexoses and their derivatives. Heated spores germinated in lower concentrations and also in a wider variety of sugars.

Although spores of some bacterial species were able to germinate substantially in media containing no added ions, a study of 46 strains representing 13 Bacillus species showed that germination of all strains was increased by ions (55) though not all by the same ionic species. Generally organic ions were more effective than inorganic ions. Levinson and Sevag (152) and others (199) observed that manganese and a number of anions stimulated spore germination. Manganese ions have been known to stimulate a number of spore enzymes. A requirement for sodium ions was clearly shown for germination of Bacillus cereus T spores (199). Germination might involve the breaching of a permeability barrier to ions or involve action of ion-dependent enzymes in spores.

Rode and Foster (198) invented the term "chemical germination" to describe germination-like changes induced in spores by surface active agents in order to distinguish from physiological germination and "mechanical germination" (194) as indicated earlier. Cationic surfactants were most active, although some anions caused germination at low pH values. Nonions were not active. Laurylamine (n-dodecylamine), the specially active (cationic) alkyl primary amine, has been extensively studied (198) and found that at concentrations as low as 10 to 100 micromoles induced the following changes: loss of refractility, loss of resistance to staining, chemicals and heat, swelling, fall in optical density of spore suspensions, fall in spore dry weight by about one-third, excretion of calcium, dipicolinic acid and muropeptides (196, 198). The most feasible explanation of germinant activity of surfactants was that the surfactants breached a permeability barrier to solutes within the spores by acting as wetting agents or by solubilizing lipids (198).

The chelate, calcium dipicolinate, a major spore constituent has been found to germinate spores of a number of species of Bacillus (193, 123). Germination was most rapid when equimolar calcium and dipicolinic acid were used. Bacillus species required lower temperatures for successful calcium-dipicolinic acid germination than for amino acid-induced germination (192). Relative nonspecificity of the cation in chelate germination was shown by the germination of Bacillus megaterium spores by strontium and magnesium dipicolinic acid chelates (114). Calcium-dipicolinic acid germination appeared to involve metabolism at some

stage since it was sensitive to general metabolic poisons (123). Various explanations as to the chelate germination of spores, have been given by several workers (54, 192).

XII. OUTGROWTH OF SPORES

The third process in the sequential events in the transformation of the spore into vegetative cell is outgrowth, a process which results in the formation of the first mature vegetative cell. Outgrowth has also been described as the period of development after germination until the beginning of the first cell division (174) or postgerminative development (146). Kobayashi et al. (131) concluded that outgrowth was dependent upon a repair in the protein-synthesizing system and an ordered synthesis of proteins. Hence outgrowth has been considered as a process of synthesis of new macromolecules and a highly ordered sequence of biosynthetic events which result in the differentiation of structures characteristic of vegetative cell.

During outgrowth, progressive changes in structure (51, 118), metabolic activity (76), classes of ribosomal particles (253), patterns of ribonuclei acid synthesis (39), and deoxyribonucleic acid replication (258, 261) have been observed. Appearance of specific vegetative antigens and increase in dry weight and swelling of the cells were also noted (105). Hence, outgrowth can be considered as a process of intercellular differentiation involving a series of distinct structural and functional alterations.

Following dissolution of the spore cortex and rupture of the spore coat, the spore core membrane became the cell wall of the emerging

vegetative cell which then elongated and divided as described by Kawata et al. (118). The conditions for outgrowth were usually different from those supporting germination. Outgrowth and germination had different temperature optima, and most of the spores needed nutrients for outgrowth which were not required for germination (36, 149). Inhibition of the energy supply (236) or the addition of antibiotics which blocked cell wall or macromolecular synthesis (223, 242), inhibited outgrowth.

The spore coat, which was shed intact in some species but absorbed (137) or fragmented (130) in others during outgrowth, contained keratin-like disulphide rich proteins, a phophomuramyl polymer and other proteins (132, 241). Apparently bound sulphur in spore coats was not utilized during outgrowth (109) and the sulphur-amino acids were apparently absent from soluble germination exudates. Hyatt and Levinson (110) observed that an exogenous source of phosphorus was required for outgrowth.

Of importance in context of outgrowth was that the messenger-ribonucleic acid (131) and polysomes (253) appeared to be absent from spores. Protein synthesis during outgrowth was hence dependent upon new messenger ribonucleic acid synthesis. During outgrowth and the first few synchronous division cycles, enzyme synthesis was observed. Synthesis of each of the three enzymes, namely alpha-glucosidase, L-alanine dehydrogenase, and alkaline phosphatase was observed to begin at a specific time and doubled only during a fraction of the generation time (131).

Compared with vegetative cells, activated aerobic spores had low cytochrome content (119) and their respiration was cyanide resistant.

Apparently activated aerobic spores used mainly a soluble flavoprotein system for terminal oxidation rather than a particulate cytochrome system (232). Functional electron transport particles were normally attached to the cytoplasmic membrane of the vegetative cell (156) and the absence of these in the inner membrane of the spore indicated another deficiency to be repaired during outgrowth.

Thus there is sufficient evidence that during outgrowth of spores, changes in structures, the pattern of ribonucleic acid and the relative concentrations of various enzymes and overall metabolic activity occur (228).

XIII. BACILLI IN MILK

Recent developments in the processing and storage of milk have emphasized the importance of sporeforming organisms in the raw milk supply. Considerable attention has been given to the incidence of these organisms in the milk in other countries (21, 66, 67, 68, 86, 191, 226, 238, 251). Franklin et al. (59) conducted a survey of the number and types of aerobic mesophilic spores in milk. The numbers present in raw bulk tanker milk exhibited a seasonal variation, as the counts were higher in winter than in summer. Bacillus cereus spores ranged from <20 to 110 per 100 ml. Stone and Rowlands (226) investigated in detail the problem of "broken" or "bitty" cream in raw and pasteurized milk. The fault was associated with Bacillus cereus and Bacillus mycoides (225, 226). Billing and Cuthbert (9) investigated the significance of Bacillus cereus in raw milk supplies. Rinses of farm dairy equipment

yielded few Bacillus cereus spores but milk cans contained large numbers especially in the summer months, when 10.5 percent of the cans showed more than 5000 per can. In addition, over a period of 12 months they took 50 ml. samples from bottled pasteurized milk from one dairy as delivered to the consumer and incubated at 22 C. The incidence of Bacillus cereus isolations was at its lowest during the spring and early summer months, and highest during the winter months, when it was detectable in almost all samples. When present, Bacillus cereus was not always the dominant organism. Over the year, 95 percent of samples showed some degree of bittiness or a clot after 24 hours at 22 C.

Martin et al. (159) conducted a study to determine the numbers and types of spore-forming microorganisms in the raw milk supplies of Ohio markets. A total of 287 milks were analyzed and 75 percent of the samples had counts of aerobic mesophilic sporeforming organisms exceeding 1000 per 100 ml. of milk, but only 36 percent had counts of sporeforming anaerobic mesophilic organisms at this level. Bacillus cereus represented 37 percent of 10 different Bacillus isolated and identified. Their study did not reveal Bacillus subtilis among the sporeforming organisms isolated and they have attributed this finding in part to the development of more recent methods for differentiating Bacillus subtilis from Bacillus licheniformis.

Cans were obviously not the only source of Bacillus cereus (9). The cow's udder, both the outer surface and the teat canals, was probably an important source of sporeformers. Udder infections resulting in acute mastitis due to Bacillus cereus have been recorded by Brown and

Scherer (19). Other obvious sources of sporeformers were hay, straw, dust, manure and mud. Bacillus cereus content of soil taken from a grazing field at one farm from April to August during 1957 varied from 64,000 to 140,000 per gram wet weight and no significant seasonal variation was observed (9). Samples of manure taken at the same time gave counts varying from 140 to 4,000 per gram. Donovon (42) observed that vegetative cells of Bacillus cereus formed spores readily in thin films of dilute milk, as in a rinsed can. He indicated that diluted milk in thin films provided excellent conditions for the process of spore formation. This appears to be the logical explanation for the appearance of spores in milk cans. Given suitable temperature and sufficient time for the vegetative phase, many spores might be formed in traces of milky water. The metal ions derived from cans might have some importance in case of Bacillus cereus, since trace amounts of certain metals notably manganese and others have been found to help spore formation (248, 57, 23, 33).

Psychrophilic species of Bacillus were isolated from soil and water (139). Grosskopf and Harper (87) first reported isolation of psychrotrophic sporeforming bacteria from pasteurized milk. The pasteurized milk aseptically packaged in standard plastic coated paper containers and stored at 4 C, had a shelf-life of four weeks. Subsequent loss of quality was attributed to the outgrowth of sporeforming microorganisms, which included Bacillus cereus, Bacillus coagulans, Bacillus lentus, and Bacillus licheniformis. These sporeformers were isolated from about 25 percent of producers' milk supplies tested. Their initial results

suggested that all of the psychrophilic sporeformers would survive ultrahigh temperature pasteurization. Similar organisms have since been isolated by others (26, 161, 208). Shehata and Collins (208) believed them to be variant strains that had gained ability to grow at refrigeration temperatures. Subsequently, Shehata et al. (210) reported quantitative data on the influence of temperature on growth of the isolates. Shehata and Collins (209) from their recent study of the sporulation and heat resistance of the psychrotrophic spore-forming bacteria concluded sporulation was best at the optimal growth temperature and on the surface of nutrient agar with magnesium sulfate and manganese sulfate. The D value for Bacillus cereus spores at 85 C (determined in skimmilk) was 18.5 minutes. More than 99 percent of spores of the strain of Bacillus cereus in skimmilk were destroyed by heating at 87.8 C for 20 seconds, storage at 32 C for four hours, and reheating at 76.7 C for 20 seconds, whereas heating at 87.8 C for 20 seconds without the final heat treatment only destroyed 32 percent. Results of the two heat treatments indicated extension of shelf life by about three days at 7.2 C.

Spores of bacilli are essentially found in all milk supplies and consequently they are a major problem in the heat sterilization of dairy foods. Complete elimination of spores requires such drastic heating that the organoleptic and nutritional quality of milk is impaired. The "commercial sterilization" of milk products is increasing in importance as a means of prolonging shelf life, with or without refrigeration. To reduce heat induced flavor changes continuous

sterilization at ultrahigh temperature for few seconds seems to have a promising future. Torres-Anjel and Hedrich (235) have mentioned Simonart and Debeer, who developed a continuous means of removing the vegetative cells and spores from milk by centrifugation. The process eventually became known as bactofugation. The process was applied to market milk (138, 203, 211, 212, 213) and to cheese milk (113, 133, 134, 135, 214). In all these studies two bactofugations effectively reduced bacteria in milk about 99 percent, if the equipment was operated within an established procedure. In general, the keeping quality of pasteurized milk was improved slightly. Research on the reduction of spores in milk by bactofugation has been limited to Clostridium spores (180) in cheese milk. Torres-Anjel and Hedrick (235) studied the practical factors that might affect spore removal from milk by bactofugation and subsequent effects on the bacteriological keeping quality of ultrahigh temperature-processed milk. They obtained removal of Bacillus subtilis spores to the extent of 99.2 to 99.8 percent by one bactofugation, which they considered quite satisfactory. They also noted that the specific gravity of the spores affected the removal in accordance to Stoke's equation. The greater the density, the more easily the spores were removed by centrifugal force. Because of the density similarity of some spores to that of the constituents of the milk solids, such as casein micelles, some spores were impossible to remove economically and practically with a bactofuge. In addition to the cost of several bactofugations the total milk solids loss in the sludges would be high.

Mikolajcik and Koka (163) determined the influence of the heat treatment of the spore and of the milk upon germination, outgrowth, and

subsequent vegetative cell growth of Bacillus cereus 7 (in raw and variously heated skimmilk). In decreasing order of magnitude of germination, the systems with the unheated spores ranked: pasteurized (62.8 C for 30 minutes), high heated (100 C--10 minutes), raw, and autoclaved (121 C--10 minutes), having at the two hour incubation period germinations of 90, 65, 27 and 16 percent respectively. Germination percentages in raw and autoclaved milk did not achieve levels observed in pasteurized or high heated milk. Heat-shocking increased the rate of germination. At two hour incubation, 99.5, 95, 84, and 68 percent of the heat-shocked spores had germinated in pasteurized, high heated, autoclaved and raw skimmilk, respectively. Heat treatment of the spore stimulated germination and subsequent outgrowth, whereas, heat treatment of the milk affected the initial rate of active cell multiplication. This was evident from the comparison of the data for the unheated and the heated spores. Irrespective of heat treatment given to the milk, heat-shocked spores germinated more rapidly and at faster rates than did the unheated spores. The intermediate heat treatments of milk appeared to create an environment favorable for spore germination.

From these investigations it was evident that heat-induced changes in milk created environmental conditions affecting spore germination, outgrowth, and vegetative cell proliferation. Of the heat intensities used, there appeared to be an ideal range above and below which the response of the spore or the vegetative cell was less. It would appear that the factors which restricted the spore response were not necessarily the same for raw and autoclaved milk. There could be more than one

factor responsible for spore and vegetative cell responses at the optimum heat range. Randolph and Gould (189) have shown that the heat-labile immunoglobulins were inhibitory to bacteria. These might be inactivated due to heat-treatment of milk. Mikolajcik and Choudhery (162) indicated that immunoglobulins in normal, mixed raw skimmilk played only minor role as inhibitors of germination of Bacillus cereus spores. Reiter and Dorman (190) identified lactotransferrin as an effective inhibitor of Bacillus subtilis and Bacillus stearothermophilus. The other factors may be the beneficial chemical changes such as the production of sulfhydryl groups and lowering of E_h (162). Green and Jezeski (85), while studying the influence of heating milk on the subsequent response of starter cultures, found that a number of toxic products were formed as a result of autoclaving of milk, which were inhibitory to the organisms. This might be the reason for the restricted spore response, in the investigations of Mikolajcik and Koka (163).

The "sweet curd" defect was commonly associated with moderately or highly heated milk and milk products (67). Jayne-Williams and Franklin (115, 116) found that this defect was related to Bacillus cereus. Hammer and Babel (97) suggested that this defect was due to a rennin-like enzyme produced by sporeforming organisms. Choudhery and Mikolajcik (25) while studying the nature of changes in milk associated with sweet curd formation by Bacillus cereus observed that the sweet curd formation was associated with spore germination and exponential growth phase of this organism. Their findings supported the conclusion that sweet curd formation was associated with exoenzymes released during the

exponential growth phase of organisms rather than with the enzymes involved in spore germination. Webb and Johnson (247) described release of sialic acid (N-acetyl neuraminic acid) from casein during initial stages of rennin action. Choudhery and Mikolajcik (25) also noted the release of the sialic acid moiety of casein to the extent of 4.2 percent during the first eight hours and after 10 hours of incubation, when sweet curd formation was evident, 39.4 percent of sialic acid moiety of casein was released. They concluded that sweet curd formation by Bacillus cereus organisms was at least in part associated with release of sialic acid. The electrophoretic pattern revealed para-k-casein band in all of the systems containing either the organisms or rennin but not in the controls (fortified with k-casein, no added organisms or rennin). Thus, para-k-casein in the systems with organisms or rennin may indicate a similarity of enzyme action on k-casein. The patterns also revealed that the bacterial protease(s) was not selective for k-casein. Both alpha-s and beta-caseins were also degraded with beta-casein disappearing most rapidly (25).

Martin and Blackwood (157) from their investigation of effects of sublethal heat-shock, beta-alanine and L-alanine on germination and subsequent destruction of Bacillus spores by pasteurization concluded that heat-shock alone was more effective as a germination stimulant than was beta-alanine. The combination of the two produced no more germination than either alone. L-alanine alone or combined with heat-shock caused almost complete germination and subsequent destruction of the germinated spores by pasteurization, especially when the treated

product was held for 15 minutes at 37 C. According to these workers the incorporation of germination stimulants and preheating milk at some germination stimulating temperature before pasteurization may be justified in milk processing, especially in ultrahigh temperature pasteurized products where practical sterility is desirable. In their study they used Bacillus cereus and Bacillus licheniformis spores. Heat-shock was at 65, 75, and 85 C with no holding time.

CHAPTER III

MATERIALS AND METHODS

I. BACTERIAL MEDIA

The dehydrated forms of Standard Methods Agar, Trypticase Soy Agar and Trypticase Soy Broth (Baltimore Biological Laboratory, Baltimore, Maryland) were prepared for use in the laboratory according to the instructions on the manufacturer's label. In addition the following ingredients were used in preparation of various media: soluble starch, meat extract, D-mannitol, peptone, Bacto-agar, phenol red, sodium chloride, polymyxin B sulfate, concentrated egg yolk emulsion and manganese sulfate.

Mannitol-Egg Yolk-Phenol Red-Polymyxin Agar used for isolation of Psychrotrophic Bacillus cereus was of the following composition: meat extract, 1 g; peptone, 10 g; D-mannitol, 10 g; NaCl, 10 g; phenol red, 25 mg; agar, 15 g; water, 900 ml; pH, 7.1. After this basal agar was sterilized and cooled to 49 $\pm$ 1 C, to 90 ml. of agar 10 ml of a 20 percent egg yolk emulsion (Oxoid, Ltd., London) was added (10). Polymyxin B sulfate solution sterilized by filtration was added to 100 ml of mannitol-egg yolk-phenol red agar to yield final concentration of 10 µg/ml. (41, 127, 165).

Nutrient Agar (Difco Laboratories, Detroit, Michigan) containing 1.0 percent soluble starch and 0.01 percent manganese sulfate (23, 56, 158) was used as sporulation medium.

Standard Methods Agar (1) containing 0.1 percent soluble starch was used for obtaining plate counts of Bacillus cereus isolates.

II. CENTRIFUGATION

Centrifugations were done in an International 1SB Centrifuge (International Equipment Company, Boston, Massachusetts) operated in a cold room (5 to 10 C).

III. PSYCHROTROPHIC BACILLUS CEREUS STRAINS

Source. More than 10 different isolates were obtained from the periodically procured pasteurized market milk samples from various plants throughout Tennessee which on refrigerated storage for 8 to 10 days had sweet-curdled.

Isolation and purification. Plates of Mannitol-Egg Yolk-Phenol Red-Polymyxin Agar were spread with 0.1 ml. of sweet-curdled market milk samples and incubated at 35 C for 24 to 48 hours. Rough, dry, discrete colonies with a distinct, violet-red background, surrounded by a halo of dense white precipitate were used to inoculate primary cultures in tubes of Trypticase Soy Broth. A plate of Mannitol-Egg Yolk-Phenol Red-Polymyxin Agar was lightly streaked with primary broth culture and incubated at 35 C for 24 to 48 hours. This sequence of cultivations was repeated successively thrice to assure purification of the isolate. Pure culture of each isolate was maintained on slants of Trypticase Soy Agar and stored in the refrigerator (4 to 6 C).

Identification and testing. Gram-stained smears of cells from fresh slant and broth cultures of each strain were examined microscopically for morphological characteristics. Crystal violet stain and Bartholomew and Mittwer's spore stain (218) were used to stain the spores. Each isolate was further tested for various biochemical properties including dissimilation of glucose to acetylmethylcarbinol under anaerobic conditions, hydrolysis of starch and gelatin and reduction of nitrate by the methods summarized in Table I.

IV. PSEUDOMONAS SPECIES

Source. Pseudomonas fluorescens and Pseudomonas fragi were isolated from the market milk samples from various dairy plants in Tennessee.

Isolation. Plates of Trypticase Soy Agar were spread with 0.1 ml. of the homogenized market milk samples and incubated at 21 C for three to four days. Discrete colonies from the Trypticase Soy Agar plates were picked into litmus milk and those showing alkalinity in litmus milk with no coagulation but fluorescence were used to inoculate primary cultures into tubes of Trypticase Soy Broth for purification and identification as possible Pseudomonas fluorescens.

Similarly convex, glistening, smooth colonies from Trypticase Soy Agar showing acidity of litmus milk with acid coagulum at surface with characteristic May-apple odor were used to inoculate primary cultures into tubes of Trypticase Soy Broth for further purification and identification as possible Pseudomonas fragi.

TABLE I

MATERIALS AND METHODS USED IN IDENTIFICATION OF BACILLUS CEREUS ISOLATED FROM SWEET-CURDLED MARKET MILK SAMPLES

Property	Cultural Medium	Reference for Methods
Colonial characteristics	Nutrient Agar	D and B
Morphological characteristics	Glucose Agar	D and B
Liquefaction of gelatin	Gelatin stab	A and D
Hydrolysis of gelatin	Frazier's gelatin medium (modified)	C and D
Hydrolysis of casein	Milk Agar	C and D
Acetylmethylcarbinol production	Glucose broth (modified)	D
Citrate fermentation	Koser Citrate medium	C and D
Nitrate reduction	Nitrate broth	A and D
Anaerobic production of gas from nitrate	Gibson medium (modified)	D
Anaerobic growth in glucose broth	Gibson medium with 1% glucose substituted for the Na NO_3	D
Fermentation of mannitol	Basal medium with 0.5% mannitol	D
Hydrolysis of starch	Starch agar	C and D
Litmus milk reaction	Litmus milk	C
Lecithinase production	Egg-yolk agar	C and B

A = Society of American Bacteriologists (218); B = Breed, Murray and Smith (18); C = Harrigan and McCance (99); D = Smith, Gordon and Clark (217).

Purification. A plate of Trypticase Soy Agar was lightly streaked with primary culture and incubated at 21 C for 3 to 4 days. This sequence of cultivations was repeated successively thrice to assure purification of the isolates. Pure cultures of each isolate were maintained on slants of Trypticase Soy Agar and stored in the refrigerator (4 to 6 C).

Identification and testing. Gram-stained smears of cells from fresh slant cultures of each of the isolates were examined microscopically for morphological characteristics. Each isolate was further characterized by various biochemical tests by methods summarized in Tables II and III for purposes of identification and confirmation.

V. PREPARATION OF BIOCHEMICALLY CLEAN SPORE CROPS

Stock cultures of vegetative cells of the organisms were maintained by semimonthly transfers in Trypticase Soy Broth. Whenever the spores were needed, 1 percent of the stock culture of vegetative cells was inoculated into Trypticase Soy Broth and incubated for 18 hours at 35 C. The 18-hour vegetative cells were then inoculated (2 ml.) onto 125 ml. nutrient agar slants in Roux flask containing 1 percent soluble starch and 0.01 percent manganese sulfate. These flasks were incubated at 35 C for seven days for maximum sporulation.

The spores were washed from the agar slants with 0.01 M phosphate buffer (pH 7.2), and concentrated by centrifugation at 3,000 rpm in an International 1SB centrifuge. The spore pellets were resuspended in

TABLE II

MATERIALS AND METHODS USED IN IDENTIFYING PSEUDOMONAS FLUORESCENS ISOLATED FROM MARKET MILK SAMPLES

Property	Cultural Medium	Reference for Methods
Colonial characteristics	Nutrient gelatin	C and B
Morphological characteristics	Trypticase Soy Broth	A and B
Growth characteristics	Trypticase Soy Agar and Trypticase Soy Broth	A and B
Liquefaction of gelatin	Gelatin stab	A and B
Indole production	Peptone water	C and B
Nitrites from nitrates	Nitrate peptone water	C and B
Acid from glucose	Hugh & Liefson's medium	C and B
Lypolysis	Nile blue sulfate agar	E and B

A = Society of American Bacteriologists (218); B = Breed, Murray and Smith (18); C = Harrigan and McCance (99); E = Knaysi (128).

TABLE III

MATERIALS AND METHODS USED IN IDENTIFYING PSEUDOMONAS FRAGI ISOLATED FROM MARKET MILK SAMPLES

Property	Cultural Medium	Reference for Methods
Colonial characteristics	Trypticase Soy Agar	A and B
Morphological characteristics	Trypticase Soy Broth	A and B
Growth characteristics	Trypticase Soy Agar Trypticase Soy Broth Gelatin Stab	A and B
Growth in Litmus milk	Litmus milk	A and B
Indole production	Peptone water	C and B
Nitrites from nitrates	Nitrate peptone water	C and B
Acid from glucose	Hugh & Liefson's medium	C and B
Lypolysis	Nile blue sulfate agar	E and B

A = Society of American Bacteriologists (218); B = Breed, Murray and Smith (18); C = Harrigan and McCance (99); E = Knaysi (128).

phosphate buffer and the washing and centrifugal steps were repeated sequentially four times.

The spores were incubated for 48 hours at 10 C with papain extract (0.4 mg/ml.) in 0.05 M phosphate buffer (pH 7) containing sodium thioglycolate (0.5 mg/ml.) and a 1:1 toluene and chloroform mixture (0.5 percent). After this treatment with papain (169) three washes were given in glass-distilled water at 1 C. The spores thus treated were considered clean and were then suspended in 0.01 M phosphate buffer (pH 7.2) and stored in the refrigerator (4 to 6 C) until needed.

The spore suspensions in 0.01 M phosphate buffer (pH 7.2) were standardized turbidimetrically in a Bausch and Lomb Spectronic-20 colorimeter at wavelength of 625 nm. (157).

VI. PREPARATION OF PSEUDOMONAS SPECIES

The stock cultures of Pseudomonas fluorescens and Pseudomonas fragi were inoculated into Trypticase Soy Broth and incubated for 16 hours at 21 C. These young cultures were diluted (1 to 100) and inoculated at 0.1 percent into fresh raw skimmilk of high quality and incubated for 72 hours at 10 C. Plate counts were made in duplicate using Standard Methods Agar just after inoculation and again after incubation for 72 hours at 10 C. This milk was subsequently sterilized for inoculation with heat-activated spores.

VII. STERILIZATION OF SKIMMILK PRIOR TO INOCULATION WITH THE SPORES

The raw skimmilk as well as the milk after the growth of Pseudomonas

species were sterilized by autoclaving at 121 C for six minutes (157). The sterilized skimmilk was checked every time for sterility.

VIII. HEAT-ACTIVATION OF PSYCHROTROPHIC BACILLUS CEREUS ISOLATES

The turbidimetrically standardized spore preparations were added to sterile skimmilk at 1 percent level and were heat-activated as required at the following three different temperature and time combinations: (1) 80 C for 15 seconds, (2) 85 C for 10 minutes, and (3) 71.5 C for 15 seconds. The thermostatically controlled waterbaths (Sargent Thermonitor and Precision Scientific Company) and a stopwatch were used for this purpose. After heat-activation for the desired time-temperature combinations the milk with the heat-activated spores was immediately cooled in an icebath to room temperature.

IX. INOCULATION OF HEAT-ACTIVATED SPORES INTO VARIOUS STERILE SKIMMILKS

The heat-activated spores were inoculated 0.6 to 1 percent into the following sterile skimmilks: (1) plain skimmilk-sterilized, (2) skimmilk into which Pseudomonas fluorescens was grown and subsequently sterilized and (3) skimmilk in which Pseudomonas fragi was grown and subsequently sterilized.

X. PLATE COUNTS OF THE SKIMMILKS INOCULATED WITH HEAT-ACTIVATED SPORES

The sterile skimmilks after inoculation with heat-activated spores

were incubated at 35 C up to 11 hours. The plate counts were made in duplicate using Standard Method Agar with 0.1 percent soluble starch (158) beginning with 0 hour (initial count), and at the following intervals of 4 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours and 11 hours of incubation.

Appropriate dilutions were made using dilution blanks (1). The plates were counted after incubation for 24 hours at 35 C. The computing and recording of the counts was done according to the Standard Methods (1).

XI. STATISTICAL ANALYSES

Statistical analyses of the data were based on the methods outlined by Steel and Torrie (222).

CHAPTER IV

RESULTS

I. SWEET-CURDLING OF COMMERCIALLY PASTEURIZED MILK

Out of 54 samples of commercially pasteurized milk obtained from various plants throughout Tennessee over a period of a year 28 percent of the samples developed the sweet-curdled defect on refrigerated storage (5 to 7 C) within 8 to 10 days. In many of the cartons the curd was seen at the bottom but sometimes the entire milk was curdled. The pH of these sweet-curdled milk samples invariably was between 6.2 and 6.6.

II. DISTRIBUTION OF BACILLUS CEREUS IN VARIOUS PORTIONS OF MILK

Sterile tall glass cylinders with sterile skimmilk were inoculated with activated spores of Bacillus cereus ATCC 14579 to obtain an initial count of 650,000 per ml. and incubated at 10 C. The results of the plate counts of four replicates taken on 3, 6 and 9 days at the top, middle and bottom portions are presented in Table IV.

After incubation for three days the counts of the top and the middle portions were the same but the counts of the bottom portion were twice higher. By six days and nine days of incubation the counts of the top portion gradually reduced whereas the plate counts in the middle portion increased from 3.4 to 10 million during the same period. The highest

TABLE IV

AVERAGE PLATE COUNTS OF ATCC BACILLUS CEREUS 14579[a] AT THE TOP, MIDDLE AND BOTTOM PORTIONS AT DIFFERENT INTERVALS OF INCUBATION

Location of the Aliquot in the Cylinder	3 Days	6 Days	9 Days
	----------in millions[b]----------		
Top	1.6	1.4	1.36
Middle	1.6	3.4	10.00
Bottom	3.8	8.0	15.00

[a]Activated at 85 C for 10 minutes.

[b]Average of four replications.

plate counts were from the bottom portion of the milk in the cylinder. The counts at the bottom of the cylinder was greater than the top portion milk over elevenfold by the ninth day of incubation.

III. CHARACTERISTICS OF PSYCHROTROPHIC BACILLUS ISOLATES

The cultural and morphological characteristics of the three isolates, H-3, CH-1, and BC-5 were similar to Bacillus cereus ATCC 14579 in that the colonies were large, rough, flat, irregular, with whiplike outgrowths, whitish with characteristic mottled appearance by transmitted light (resembling galvanized iron or moire silk). Morphologically the spores were 1 to 1.5 micron and ellipsoidal. The vegetative cells were Gram-positive rods 1 to 1.2 micron by 3 to 5 micron with square ends, occurred in tangled chains and were motile. The cells were vacuolated when grown on glucose agar.

IV. BIOCHEMICAL PROPERTIES OF PSYCHROTROPIC BACILLUS ISOLATES

The biochemical properties of the three isolates H-3, CH-1 and BC-5 identified as Bacillus cereus and used in this study are presented in Table V. Bacillus cereus ATCC 14579 was used as a reference culture for purposes of identification and as can be seen in the table did not produce sweet-curdling of milk at refrigeration temperature in 10 days.

V. CULTURAL AND BIOCHEMICAL PROPERTIES OF PSEUDOMONAS SPECIES

Of all the isolates of Pseudomonas species from commercially pasteurized storage samples only one identified as Pseudomonas fluorescens and one identified as Pseudomonas fragi were used in this study to determine the influence of their growth on the germination and outgrowth of the three isolates of psychrotrophic Bacillus cereus, H-3, CH-1, and BC-5. The cultural and biochemical characteristics are presented in Tables VI and VII.

VI. INITIAL AND FINAL COUNTS OF PSEUDOMONAS SPECIES IN SKIMMILK

Raw skimmilk of good bacterial quality was inoculated with 150 to 200 Pseudomonas fluorescens or Pseudomonas fragi to study the influence of their growth on the germination and outgrowth of the three Bacillus cereus isolates. On incubation for 72 hours at 10 C populations of 9.2 to 10.5 million per ml. were attained in the skimmilk prior to sterilization and subsequent inoculation with heat-activated spores.

TABLE V

THE BIOCHEMICAL PROPERTIES OF PSYCHROTROPHIC BACILLUS CEREUS ISOLATES H-3, CH-1 AND BC-5 AND BACILLUS CEREUS ATCC 14579

Characteristics	H-3	CH-1	BC-5	ATCC 14579
Hydrolysis of gelatin	+	+	+	+
Hydrolysis of casein	+	+	+	+
Voges-Proskauer Reaction	+	+	+	+
Citrate Utilization	+	+	+	+
Nitrate reduction to nitrite	+	+	+	+
Anaerobic production of gas from nitrate	+	+	+	+
Anaerobic growth in glucose broth	+	+	+	+
Fermentation of mannitol	-	-	-	-
Hydrolysis of starch	+	+	+	+
Litmus milk--peptonization with coagulation	+	+	+	+
Lecithinase production	+	+	+	+
Sweet curd formation in milk at 5-10 C in 10 days	+	+	+	-

TABLE VI

THE CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF PSEUDOMONAS FLUORESCENS ISOLATED FROM MARKET MILK SAMPLES

Characteristics	Observations
Colonial	Gelatin colonies circular, with greenish center, lobular, liquefying quickly.
Morphological	Gram-negative rods, 0.3 to 0.5 by 1 to 1.8 microns occurring singly and in pairs. Motile, possessing a polar flagellum.
Agar slants	Abundant. The medium showed greenish coloration.
Broth	Turbid, flocculent, with yellowish green pellicle and grayish sediment.
Gelatin stab	Infundibuliform liquefaction.
Litmus milk	Alkaline with no coagulation.
Indole	Not produced.
Nitrites from nitrates	Positive.
Acid from glucose	Positive.
Lypolysis	Positive.
Growth at 37 C	Poor.

TABLE VII

THE CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF PSEUDOMONAS FRAGI ISOLATED FROM MARKET MILK SAMPLES

Characteristics	Observations
Colonial	Convex, glistening butyrous, smooth
Morphological	Gram-negative rods, 0.5 to 1.0 by 0.7 to 4.0 microns, occurred singly, in pairs and in chains. Motile with polar flagellum.
Agar slant	Abundant growth, spreading. Sweet ester-like odor resembling that of the flower of the May apple.
Broth	Turbidity and sediment with a thin pellicle.
Gelatin	Crateriform liquefaction.
Litmus milk	Acid ring and acid coagulum at the surface, Characteristic May-apple or Strawberry odor.
Indole	Not produced.
Nitrites from nitrates	Negative.
Ammonia from peptone	Positive.
Hydrogen sulfide	Not produced.
Acid from glucose	Positive.
Hydrolysis of fat	Positive.
Growth at 25 and 37 C	Grew readily at 25 C but not at 37 C.

VII. THE TURBIDEMETRIC STANDARDIZATION OF SPORE SUSPENSIONS

The spore suspensions of CH-1, H-3 and BC-5 in 0.01 M phosphate buffer (pH 7.2) on standardization had optical densities of 0.5, 0.42 and 0.5 respectively at 625 nm and the respective spore counts were 40, 25 and 32 million per ml.

VIII. RESULTS OF EXPERIMENTS WITH ISOLATE H-3

The average plate counts of six replicates of H-3 isolate at different periods of incubation and activation at the three different temperature-time combinations are presented in Table VIII. The initial spore count (0 hours) varied from 3,300 to 4,700 per ml. The counts from the three temperature-time combinations were not statistically different from each other signifying no apparent difference due to activation at various temperature-time combinations.

IX. INFLUENCE OF INITIAL GROWTH OF PSEUDOMONAS SPECIES ON H-3

The influence of initial growth of Pseudomonas fluorescens or Pseudomonas fragi on the germination and outgrowth of Bacillus cereus isolate H-3 activated at the three temperature-time combinations are presented in Tables IX, X and XI. The analysis of variance is presented in Table XII. Although there are differences in the counts as the result of these treatments these counts were not statistically significant either at 1 percent or 5 percent levels.

Hence in case of Bacillus cereus isolate H-3 the treatments were

TABLE VIII

AVERAGE PLATE COUNTS OF ISOLATE H-3 AT DIFFERENT INTERVALS OF INCUBATION AND ACTIVATION AT THE THREE TEMPERATURE-TIME COMBINATIONS

Intervals of Incubation	Activated at		
	80 C for 15 Seconds	85 C for 10 Minutes	71.5 C for 15 Seconds
	In Millions[a]		
4 hours	0.71	1.45	1.22
6 hours	8.80	9,84	10.08
7 hours	16.26	17.88	16.63
8 hours	23.20	28.25	23.78
9 hours	34.13	35.36	27.45
10 hours	46.38	43.16	36.91
11 hours	53.13	56.06	44.30

[a]Average of six replications.

TABLE IX

AVERAGE PLATE COUNTS OF ISOLATE H-3 ACTIVATED AT 80 C FOR 15 SECONDS AT VARIOUS INTERVALS OF INCUBATION IN STERILE MILK AND IN MILKS IN WHICH PSEUDOMONAS SPECIES GREW PRIOR TO STERILIZATION

Intervals of Incubation	Plate Counts[a]		
	H-3	FrH-3	FlH-3
	In Millions[b]		
4 hours	0.71	0.77	0.79
6 hours	8.80	10.10	9.12
7 hours	16.26	17.13	17.62
8 hours	23.20	23.89	24.55
9 hours	34.13	36.66	34.10
10 hours	46.38	49.56	49.14
11 hours	53.13	57.85	56.56

[a]H-3 = Plain sterile skimmilk in which activated spores of H-3 were inoculated.

FrH-3 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of H-3.

FlH-3 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of H-3.

[b]Average of six replications.

TABLE X

AVERAGE PLATE COUNTS OF ISOLATE H-3 ACTIVATED AT 85 C FOR 10 MINUTES AT VARIOUS INTERVALS OF INCUBATION IN STERILE MILK AND MILKS IN WHICH PSEUDOMONAS SPECIES WERE GROWN PRIOR TO STERILIZATION

Intervals of Incubation	Average Plate Counts[a]		
	H-3	FrH-3	FlH-3
	In Millions[b]		
4 hours	1.45	0.95	0.81
6 hours	9.84	8.87	9.19
7 hours	17.88	16.76	16.68
8 hours	28.25	30.44	30.16
9 hours	35.36	36.86	36.81
10 hours	43.16	43.60	50.21
11 hours	56.06	57.81	61.05

[a]H-3 = Plain sterile skim milk in which activated spores of H-3 were inoculated.

FrH-3 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of H-3.

FlH-3 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of H-3.

[b]Average of six replications.

TABLE XI

AVERAGE PLATE COUNTS OF ISOLATE OF H-3 ACTIVATED AT 71.5 C FOR 15 SECONDS AT VARIOUS INTERVALS OF INCUBATION IN STERILE MILK AND MILKS IN WHICH PSEUDOMONAS SPECIES WERE GROWN PRIOR TO STERILIZATION

Intervals of Incubation	Average Plate Counts[a]		
	H-3	FrH-3	F1H-3
	In Millions[b]		
4 hours	1.22	0.83	0.90
6 hours	10.08	7.65	8.33
7 hours	16.63	11.45	16.05
8 hours	23.78	16.71	28.80
9 hours	27.45	24.75	33.90
10 hours	36.91	34.36	44.36
11 hours	44.30	43.18	52.01

[a] H-3 = Plain sterile skimmilk in which activated spores of H-3 were inoculated.

FrH-3 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of H-3.

F1H-3 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of H-3.

[b] Average of six replications.

TABLE XII

ANALYSIS OF VARIANCE OF HEAT ACTIVATION AND INFLUENCE OF INITIAL GROWTH OF PSEUDOMONAS SPECIES ON THE GERMINATION AND OUTGROWTH OF H-3

Source	Degrees of Freedom	Mean Square	F
Replicates	5	125.3655	0.3058[a]
Treatments	8	320.6841	0.7822[a]
Replicates X treatments	40	50.6728	0.1236[a]
Error	378	409.9843	

[a]Not statistically significant.

not significantly different and the counts obtained as a result of these treatments were not statistically different from each other.

X. RESULTS OF EXPERIMENTS WITH ISOLATE CH-1

The initial count (0 hours) in skimmilk of isolate CH-1 varied from 3,000 to 6,700 per ml. The average plate counts of the six replicates at 4, 6, 7, 8, 9, 10 and 11 hours of incubation at 35 C after activation of CH-1 at the three temperature-time combinations are presented in Table XIII. Although there seems to be an indication of higher plate counts as a result of activation at 80 C for 15 seconds, statistically the counts of these three activations were not significantly different.

XI. INFLUENCE OF INITIAL GROWTH OF PSEUDOMONAS SPECIES ON CH-1

The influence of the growth of Pseudomonas fluorescens and Pseudomonas fragi prior to sterilization on the germination and outgrowth of CH-1 are presented in Tables XIV, XV, and XVI. Analysis of variance for the various treatments of CH-1 is presented in Table XVII. The F value for treatments was significant at 5 percent level as is evident from the table. Hence Duncan's new multiple-range test was used to compare each treatment mean with every other treatment mean, and is presented in Table XVIII.

The treatment mean of activation at 71.5 C for 15 seconds of CH-1 and its growth in sterile skimmilk in which Pseudomonas fluorescens had initially grown was significantly different from the following treatment means: (1) activation of CH-1 at 80 C for 15 seconds and growth in

TABLE XIII

AVERAGE PLATE COUNTS OF ISOLATE CH-1 AT DIFFERENT INTERVALS OF INCUBATION AND ACTIVATION AT THE THREE TEMPERATURE-TIME COMBINATIONS

Interval of Incubation	Activated at		
	80 C for 15 Seconds	85 C for 10 Minutes	71.5 C for 15 Seconds
	In Millions[a]		
4 hours	1.34	1.70	2.02
6 hours	11.31	9.69	11.05
7 hours	18.86	14.41	13.30
8 hours	28.18	22.17	26.96
9 hours	36.05	26.80	29.51
10 hours	49.00	36.50	39.73
11 hours	59.50	45.10	48.60

[a]Average of six replicates.

TABLE XIV

AVERAGE PLATE COUNTS OF ISOLATE CH-1 ACTIVATED AT 80 C FOR 15 SECONDS AT VARIOUS INTERVALS OF INCUBATION IN STERILE MILK AND MILKS IN WHICH PSEUDOMONAS SPECIES WERE GROWN PRIOR TO STERILIZATION

Intervals of Incubation	Plate Counts[a]		
	CH-1	FrCH-1	F1CH-1
	In Millions[b]		
4 hours	1.34	1.22	1.29
6 hours	11.31	12.38	12.26
7 hours	18.86	20.58	20.38
8 hours	28.18	30.05	27.50
9 hours	36.05	38.66	36.51
10 hours	49.00	51.13	49.70
11 hours	59.50	62.08	64.83

[a]CH-1 = Plain sterile skimmilk in which activated spores of CH-1 were inoculated.

FrCH-1 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of CH-1.

F1CH-1 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of CH-1.

[b]Average of six replications.

TABLE XV

AVERAGE PLATE COUNTS OF ISOLATE CH-1 ACTIVATED AT 85 C FOR 10 MINUTES AT VARIOUS INTERVALS OF INCUBATION IN STERILE MILK AND MILKS IN WHICH PSEUDOMONAS SPECIES WERE GROWN PRIOR TO STERILIZATION

Intervals of Incubation	Plate Counts[a]		
	CH-1	FrCH-1	FlCH-1
	In Millions[b]		
4 hours	1.70	1.75	1.61
6 hours	9.69	10.35	9.70
7 hours	14.41	13.05	13.96
8 hours	22.17	22.10	22.71
9 hours	26.80	31.42	30.56
10 hours	36.50	38.88	36.63
11 hours	45.1	50.88	48.28

[a]CH-1 = Plain sterile skimmilk in which activated spores of CH-1 were inoculated.

FrCH-1 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of CH-1.

FlCH-1 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of CH-1.

[b]Average of six replications.

TABLE XVI

AVERAGE PLATE COUNTS OF ISOLATE CH-1 ACTIVATED AT 71.5 C FOR 15 SECONDS AT VARIOUS INTERVALS OF INCUBATION IN STERILE MILK AND MILKS IN WHICH PSEUDOMONAS SPECIES WERE GROWN PRIOR TO STERILIZATION

Intervals of Incubation	Plate Counts[a]		
	CH-1	FrCH-1	F1CH-1
	In Millions[b]		
4 hours	2.02	0.80	0.70
6 hours	11.05	8.15	7.63
7 hours	17.30	12.60	11.25
8 hours	26.96	19.20	17.10
9 hours	29.51	26.15	22.02
10 hours	39.73	34.70	28.68
11 hours	48.60	44.08	37.96

[a]CH-1 = Plain sterile skimmilk in which activated spores of CH-1 were inoculated.

FrCH-1 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of CH-1.

F1CH-1 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of CH-1.

[b]Average of six replications.

TABLE XVII

ANALYSIS OF VARIANCE FOR HEAT-ACTIVATION AND INFLUENCE OF INITIAL GROWTH OF PSEUDOMONAS SPECIES ON THE GERMINATION AND OUTGROWTH OF CH-1

Source	Degrees of Freedom	Mean Square	F
Replicates	5	30.9669	0.0855[a]
Treatments	8	729.3922	2.0140[b]
Replicates X Treatment	40	45.0005	0.1243[a]
Error	378	362.1555	

[a]Not significant.

[b]Significant at 5 percent level.

TABLE XVIII

TREATMENTS AND THE TREATMENT MEANS OF ISOLATE CH-1

Treatment	Treatment Mean
FrCH-1 and activation at 71.5 C for 15 seconds	18.2219[ab]
FlCH-1 and activation at 71.5 C for 15 seconds	15.6722[a]
CH-1 activated at 71.5 C for 15 seconds	21.9202[ab]
FrCH-1 and activation at 85 C for 10 minutes	21.0588[ab]
FlCH-1 and activation at 85 C for 10 minutes	20.4346[ab]
CH-1 activated at 85 C for 10 minutes	19.5686[ab]
FrCH-1 and activation at 80 C for 15 seconds	27.0152[b]
FlCH-1 and activation at 80 C for 15 seconds	26.5623[b]
CH-1 activated at 80 C for 15 seconds	25.5328[b]

a
ab
b Numbers having the same superscript are not significantly different ($P < 0.5$) by Duncan's multiple range test.

FrCH-1 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of CH-1.

FlCH-1 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of CH-1.

CH-1 = Plain sterile skimmilk in which activated spores of CH-1 were inoculated.

sterile skimmilk, (2) activation of CH-1 at 80 C for 15 seconds and growth in sterile skimmilk in which Pseudomonas fluorescens had initially grown and (3) activation of CH-1 at 80 C for 15 seconds and growth in sterile skimmilk in which Pseudomonas fragi had initially grown.

XII. RESULTS OF EXPERIMENTS WITH ISOLATE BC-5

The initial inoculum (0 hours) of activated BC-5 varied from 3,000 to 4,300 per ml. of the skimmilk. The plate counts of BC-5 activated at 80 C for 15 seconds, 85 C for 10 minutes, and 71.5 C for 15 seconds at various intervals of incubation are presented in Table XIX. From these results and the treatment means there is strong indication of greater activation at 80 C for 15 seconds. This was not apparent with isolate H-3.

XIII. INFLUENCE OF INITIAL GROWTH OF PSEUDOMONAS SPECIES ON ISOLATE BC-5

The influence of growth of Pseudomonas fluorescens and Pseudomonas fragi on germination and outgrowth of BC-5 are presented in Tables XX, XXI, and XXII. The results of analysis of variance for various treatments on BC-5 are presented in Table XXIII. The F value for treatments was highly significant at 1 percent level. Hence Duncan's new multiple-range test was used to compare each treatment mean with every other treatment mean as presented in Table XXIV. The treatment mean of activation at 80 C for 15 seconds and inoculation into sterile skimmilk

TABLE XIX

AVERAGE PLATE COUNTS OF ISOLATE BC-5 AT DIFFERENT INTERVALS OF INCUBATION AND ACTIVATION AT THREE TEMPERATURE-TIME COMBINATIONS

Intervals of Incubation	Activated at		
	80 C for 15 Seconds	85 C for 10 Minutes	71.5 C for 15 Seconds
	In Millions[a]		
4 hours	1.35	1.02	1.56
6 hours	12.71	9.47	10.00
7 hours	20.35	17.35	18.91
8 hours	27.40	25.53	24.88
9 hours	38.41	35.01	33.46
10 hours	46.08	44.45	40.26
11 hours	61.88	56.00	49.45

[a]Average of six replications.

TABLE XX

AVERAGE PLATE COUNTS OF ISOLATE BC-5 ACTIVATED AT 80 C FOR 15 SECONDS AT VARIOUS INTERVALS OF INCUBATION IN STERILE MILK AND MILKS IN WHICH PSEUDOMONAS SPECIES WERE GROWN PRIOR TO STERILIZATION

Intervals of Incubation	Plate Counts[a]		
	BC-5	FrBC-5	F1BC-5
	In Millions[b]		
4 hours	1.35	1.02	1.73
6 hours	12.71	13.00	23.58
7 hours	20.35	21.75	46.16
8 hours	27.40	27.25	62.66
9 hours	38.41	36.56	77.66
10 hours	46.08	43.70	98.41
11 hours	61.88	65.50	98.41

[a]BC-5 = Plain sterile skimmilk in which activated spores of BC-5 were inoculated.

FrBC-5 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of BC-5.

F1BC-5 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of BC-5.

[b]Average of six replications.

TABLE XXI

AVERAGE PLATE COUNTS OF ISOLATE BC-5 ACTIVATED AT 85 C FOR 10 MINUTES AT VARIOUS INTERVALS OF INCUBATION IN STERILE MILK AND MILKS IN WHICH PSEUDOMONAS SPECIES WERE GROWN PRIOR TO STERILIZATION

Intervals of Incubation	Average Plate Counts[a]		
	BC-5	FrBC-5	FlBC-5
	----------In Millions[b]----------		
4 hours	1.02	1.00	0.88
6 hours	9.47	9.28	9.78
7 hours	17.35	16.60	17.63
8 hours	25.53	23.97	29.33
9 hours	35.01	34.81	38.48
10 hours	44.45	45.05	45.53
11 hours	56.00	56.41	58.68

[a]BC-5 = Plain sterile skimmilk in which activated spores of BC-5 were inoculated.

FrBC-5 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of BC-5.

FlBC-5 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of BC-5.

[b]Average of six replications.

TABLE XXII

AVERAGE PLATE COUNTS OF ISOLATE BC-5 ACTIVATED AT 71.5 C FOR 15 SECONDS AT VARIOUS INTERVALS OF INCUBATION IN STERILE MILK AND MILKS IN WHICH PSEUDOMONAS SPECIES WERE GROWN PRIOR TO STERILIZATION

Intervals of Incubation	Average Plate Counts[a]		
	BC-5	FrBC-5	F1BC-5
	In Millions[b]		
4 hours	1.56	1.34	1.41
6 hours	10.00	8.16	8.53
7 hours	18.91	13.88	18.01
8 hours	24.88	19.61	29.03
9 hours	33.40	27.70	39.13
10 hours	40.26	37.75	45.36
11 hours	49.45	51.55	55.33

[a]BC-5 = Plain sterile skimmilk in which activated spores of BC-5 were inoculated.

FrBC-5 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of BC-5.

F1BC-5 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of BC-5.

[b]Average of six replications.

TABLE XXIII

ANALYSIS OF VARIANCE FOR HEAT-ACTIVATION AND INFLUENCE OF INITIAL GROWTH OF PSEUDOMONAS SPECIES ON THE GERMINATION AND OUTGROWTH OF BC-5

Source	Degrees of Freedom	Mean Square	F
Replicates	5	113.3649	0.2067[a]
Treatments	8	3763.2210	6.86[b]
Replicates X Treatment	40	42.0114	0.07[a]
Error	378	548.2348	

[a]Not significant.

[b]Highly significant ($P < 0.01$).

TABLE XXIV

TREATMENTS AND THE TREATMENT MEANS OF ISOLATE BC-5

Treatments	Treatment Mean
FrBC-5 and activation at 71.5 C for 15 seconds	20.0014[a]
F1BC-5 and activation at 71.5 C for 15 seconds	24.6047[a]
BC-5, activation at 71.5 C for 15 seconds	22.3108[a]
FrBC-5 and activation at 85 C for 10 minutes	23.3926[a]
F1BC-5 and activation at 85 C for 10 minutes	25.0421[a]
BC-5, activation at 85 C for 10 minutes	23.5224[a]
FrBC-5 and activation at 80 C for 15 seconds	26.0990[a]
F1BC-5 and activation at 80 C for 15 seconds	49.8107[b]
BC-5, activation at 80 C for 15 seconds	26.0251[a]

[a,b]Numbers having the same superscript are not significantly different by Duncan's Multiple Range Test at 1 percent level.

[c]FrBC-5 = Skimmilk which was sterilized after growth of Pseudomonas fragi and subsequently inoculated with activated spores of BC-5.

F1BC-5 = Skimmilk which was sterilized after growth of Pseudomonas fluorescens and subsequently inoculated with activated spores of BC-5.

BC-5 = Plain sterile skimmilk in which activated spores of BC-5 were inoculated.

in which Pseudomonas fluorescens had grown was significantly different from all the other treatment means in the Table.

Thus there is ample statistical evidence to show that the growth of Pseudomonas fluorescens in raw milk has an influence on the activation, germination and outgrowth of BC-5.

CHAPTER V

DISCUSSION

The significance of sweet curd formation in refrigerated storage of commercially pasteurized milk is quite evident. Twenty-eight percent of the samples collected over a period of a year from different plants in the State of Tennessee developed this defect within 8 to 10 days of refrigerated storage (5 to 7 C). Other recent reports have indicated the importance of this defect (87, 26, 208, 210). The organisms isolated and identified from these samples were psychrotrophic Bacillus cereus. The microflora of freshly pasteurized milk has been reported by many to consist of bacilli. The most recent, of Credit and coworkers (30), identified that 84 percent of the isolates from commercially pasteurized milk held at 4.5 C for 30 days, were Bacillus species. The results of this study and the above reports indicate very clearly that psychotrophic bacilli are becoming important as major spoilage organisms in the stored commercially pasteurized milk.

The pH of these sweet-curdled milk samples was invariably between 6.2 and 6.6. Therefore this curd formation must be due to a rennin-like enzyme produced by Bacillus cereus. Hammer and Babel (97) have suggested that this organism has such an ability and in addition, several researchers (220, 221) have isolated an enzyme from Bacillus cereus and have used it as a substitute for rennet extract in Cheddar cheese making.

In most of the milk samples examined during the past year the sweet curd was seen at the bottom of the carton, however in many, the entire

contents of the carton was curdled. Since Bacillus cereus is one of the larger bacilli it seemed plausible that on extended storage these organisms could actually settle to the bottom of the carton. As can be observed in Table IV, page 61, a decided difference in the distribution of the bacilli occurred in the undisturbed milk and the results obtained in this study using Bacillus cereus ATCC 14579 supports the sweet curd formation at the bottom of the carton and subsequent curdling of the entire contents.

In Table V, page 63, the data indicate that there is no difference in the cultural or biochemical properties of the three isolates, H-3, CH-1, and BC-5. All three produced sweet-curdling of milk at refrigeration temperature in 10 days. In contrast to this characteristic, Bacillus cereus ATCC 14579 did not produce sweet-curdling even after 20 days of incubation. Keynan and Evenchik (121) have stated that variation exists among different strains and even among different batches of the same strain. Likewise different isolates vary in their temperature requirements for activation. Evans and Curran (47) have shown that the nature of the medium in which the spores are heated for activation has an influence upon spore germination. The variations that can be seen in the results from these three isolates may be attributed to strain difference and activation temperature requirements even though they were all activated in skimmilk.

The sweet curd defect due to Bacillus cereus is commonly associated with moderately or highly heated milk and milk products (67). Mikolajcik and Koka (163) found that the germination percentage in raw and autoclaved

milk did not achieve levels observed in pasteurized or high heated milk. Also, they found that irrespective of heat treatment given to the milk, heat-shocked spores germinated rapidly and at a faster rate than did the unheated spores and the intermediate heat treatments of milk appeared to create an environment favorable for spore germination. This was also true with the three isolates used in this study. The heat-shocked spores germinated rapidly and the intermediate heat treatment of milk (autoclaving for 6 minutes at 15 pounds pressure) seemed to create a favorable environment for spore germination and outgrowth. With regard to the heat-activation of the spores of H-3, CH-1 and BC-5 there was statistically no significant difference between 80 C for 15 seconds, 85 C for 10 minutes and 71.5 C for 15 seconds although the counts tended to be higher at 80 C for 15 seconds (Table VIII, page 67; Table XIII, page 73; and Table XIX, page 80).

The heat-induced changes in milk have been shown to be destructive to the bacterial inhibitory substances, immune globulins (189). But beneficial changes also occur in the heated milk such as production of sulphydryl groups and a lowered E_h value (163). In this study since the milk used was autoclaved for only six minutes there appeared to be no inhibitory properties due to toxic products which have been demonstrated by Green and Jezeski (85) in milk autoclaved for 15 to 20 minutes at 15 pounds or more of pressure.

Since Bacillus cereus are prevalent in raw milk (159) and pasteurization temperatures have been raised above the required standards to keep survival numbers low it is evident that this may be what is

involved in commercially pasteurized milk which on refrigerated storage shows sweet curd formation.

The sweet curd formation was invariably observed with the three isolates, H-3, CH-1, and BC-5 used in this study by about 10 hours of incubation, which is in complete agreement with the findings of Choudhery and Mikolajcik (25). Sweet curd formation was always associated with exponential growth phase of the three isolates as observed by the same workers.

The sweet curd formation occurred when the counts in the skimmilk were 40 million to more than 60 million. Only in the case of isolate BC-5 in skimmilk in which there was initial growth of Pseudomonas fluorescens sweet curd formation was seen earlier than 10 hours (7 to 8 hours), because the counts by that time were in the above range. By 11 hours proteolysis followed sweet curdling.

The two organisms Pseudomonas fluorescens and Pseudomonas fragi are very common dairy psychrophiles (202). The generation time of Pseudomonas fluorescens and Pseudomonas fragi when grown in skimmilk at 4 C was 7.2 and 5.5 hours respectively according to El-Farekh (46). As these two psychrophiles grow in milk or its products rather subtle changes take place during the early growth period. From a physical standpoint no noticeable changes in milk occur immediately. Skean and Overcast (215) found that the electrophoretic patterns of caseins from the milk with growth of Pseudomonas species exhibited some distinct changes. Glucose, adenosine, inosine, L-alanine and certain other amino acids either separately or in various combinations, have been found to

cause germination (148, 233, 256) in over 90 percent of several strains of bacilli (121). In this investigation an attempt was made to estimate free amino acids in milk after the growth of Pseudomonas species using the ninhydrin technic for free amino acids in milk by Freeman (60). However, no free amino acids were detectable by this technic. The possible role of the various amino acids or other metabolites produced as the result of growth of Pseudomonas species merits further investigation.

Excessive growth of psychrophiles in raw milk had a stimulatory effect on Brevibacterium lipolyticum (176). In this investigation although this type of stimulatory effect of Pseudomonas species tended to be apparent generally from eight hours onwards for H-3 (Tables IX, X, and XI, pages 68 through 70), the difference in counts were not statistically significant. But with isolate CH-1 the treatments were significantly different at 5 percent level (Table XVII, page 77). Thus, there is statistical evidence to show that the initial growth of Pseudomonas fragi and Pseudomonas fluorescens had greater stimulatory effect on the CH-1 spores when activated at 80 C for 15 seconds as against the initial growth of Pseudomonas fluorescens and activation at 71.5 C for 15 seconds (Table XVIII, page 78). From the treatment means (Table XVIII, page 78) it is quite apparent that higher mean counts were seen as a result of activation at 80 C for 15 seconds as against activation at 71.5 C for 15 seconds, although there was not sufficient statistical evidence in this behalf. The stimulatory effect of Pseudomonas fluorescens is very strikingly evident in case of BC-5

when activated at 80 C for 15 seconds (Table XXIV, page 85) and this treatment is different from all the other treatments at 1 percent level (Tables XXIII and XXIV, pages 84 and 85). The trend in terms of counts seems to be higher in case of spores activated at 80 C for 15 seconds. This temperature-time combination happens to be the one used by many plants for pasteurization of milk to keep survival numbers low. In general, the differences in the results of the three isolates can be attributed to strain differences (121).

CRANES CREST

CHAPTER VI

SUMMARY AND CONCLUSIONS

Over a period of a year, 28 percent of commercially pasteurized milk samples obtained from various plants developed a sweet-curdling defect on refrigerated storage within 10 days. The curd formation was observed many times at the bottom but sometimes throughout the carton of milk. Preliminary trials indicated more than a tenfold difference in the counts of causative organisms between the top and the bottom of tall containers of milk. As these organisms grow this defect first appears on the bottom and after extensive growth extends throughout the carton.

Psychrotrophic Bacillus cereus was isolated as a causative organism from sweet-curdled milk samples. These isolates differed from the type culture organism only with regard to their ability to grow and bring about sweet curd formation in skimmilk at refrigeration temperature. The psychrotrophic bacilli even though they had the same cultural and biochemical characteristics exhibited marked difference in their response to various heat-activation temperatures. Spores of two of the three isolates exhibited greater activity after activation at 80 C for 15 seconds, which incidentally happens to be the higher pasteurization temperature currently used by many plants, than the standard pasteurization at 71.5 C for 15 seconds.

The sweet curd formation was invariably noticed within 10 hours of incubation at 35 C. This defect was always associated with exponential

growth of the bacilli with counts ranging from 40 million to more than 60 million.

Pseudomonas fluorescens and Pseudomonas fragi were isolated from the stored samples of commercially pasteurized milk and used for this study. Excessive growth of these Pseudomonas species in the raw milk prior to processing had a stimulatory effect on Bacillus cereus isolates in combination with heat-activation.

Two of three isolates of Bacillus cereus provided statistical evidence of the stimulatory effect due to growth of these Pseudomonas species especially in combination with the activation of the spores at 80 C for 15 seconds.

In conclusion, the results of this work present evidence which contributes to the knowledge of the sweet curd formation in fluid milk. First, the psychrotrophic sporeformer, Bacillus cereus, is a causative organism. Secondly, higher temperatures currently used in the pasteurization of milk are more effective in activating the spores of this organism than the standard pasteurization temperature. Thirdly, the growth of psychrotrophic Pseudomonas species may influence the germination and outgrowth of certain Bacillus cereus strains.

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VITA

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Atmaram was the recipient of financial assistance from the Government of Mysore throughout his higher educational career in India. His study at The University of Tennessee was financed by the United States Agency for International Development. He completed the Doctor of Philosophy degree in March 1973, with a major in Animal Science and minor in Food Technology.

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Atmaram was married to the former Kasturi Padmanabhan in 1964. They are the proud parents of two girls and a boy, Usha, Latha, and Nirmalanand.