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COLD TOLERANT MICROBES IN SPOILAGE AND THE ENVIRONMENT

edited by
A. D. Russell & R. Fuller

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Preface

This volume, Number 13 in the Society for Applied Bacteriology Technical Series, is based on the demonstrations made at the Autumn 1977 Demonstration Meeting of the Society.

The subject of the meeting was "Cold Tolerant Microbes in Spoilage and the Environment". The book brings together contributions from experts on psychrotrophic bacteria and contains chapters on various aspects of low temperature microbiology as they affect fundamental metabolism, ecology and food spoilage. Providing as it does an excellent survey of the importance of psychrotrophic bacteria it will be of value generally to bacteriologists and is essential reading for those engaged in this particular field.

We would like to thank those who presented demonstrations at this meeting, members of the staff of the Department of Biology, Brunel University, especially Professor J. D. Gillett, Mr. F. Jones (Senior Lecturer) and Mrs. S. Bannerman (Chief Technician) who helped in the staging of this demonstration, and Mr. D. W. Lovelock of Heinz Co. Ltd., for his help and advice.

February 1979

A. D. Russell
R. Fuller

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Microbial Growth at Low Temperatures

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Introduction

Temperature is undoubtedly one of the most important environmental parameters operating in any ecosystem. The fundamental role of temperature in controlling the rate of all physico-chemical reactions underlies its significance when considering the complexities of biological processes. Micro-organisms, since they can be cultivated with relative ease in the laboratory, provide a convenient experimental tool for elucidating the effects of temperature on living cells. Most micro-organisms can grow only over a relatively narrow temperature range, from about −10°C to +70°C, and within this range, temperature affects the growth rate, nutritional requirements, enzyme activities and chemical composition of the cells. Considering the profound effects of temperature, it is somewhat disturbing that many microbiologists conveniently neglect the fact that most natural environments are at low temperatures. The literature is, however, replete with examples of micro-organisms whose physiology was studied at temperatures in excess of those from which they were obtained.

Traditionally, micro-organisms have been classified on the basis of temperature optima for growth into thermophiles, mesophiles and psychrophiles according to their ability to grow at high, medium and low temperatures, respectively. The first two groups can be readily differentiated by their growth temperature optima. Psychrophiles have also been defined following their discovery by Forster (1887) but it is only during the last decade that some semblance of agreement has been achieved amongst microbiologists in defining these organisms. For detailed discussions on the definition of psychrophiles the reader should consult reviews by Ingraham and Stokes (1959) and Morita (1966, 1975). For the purpose of this review the definition of psychrophiles as proposed by Morita (1975) will be used, i.e. organisms having an optimal growth temperature of about 15°C or lower, a maximal growth temperature of

about 20°C and a minimal growth temperature of 0°C or lower. Bacteria which grow at 0°C and at maximum temperatures exceeding 25°C will be considered to be psychrotrophic.

Distribution of Psychrophilic Bacteria

The ability of micro-organisms, and in particular bacteria, to grow at low temperatures was first demonstrated by Forster (1887) and subsequently they have been shown to have a widespread distribution in a range of natural environments (Ingraham and Stokes, 1959; Morita. 1966). The majority of the isolates obtained by the early investigators were psychrotrophic and the occurrence of truly psychrophilic bacteria, which conform to the present definition, were not reported until 1964. Morita and Haight (1964) isolated the first authentic psychrophilic bacterium, *Vibrio marinus* MP-1, and subsequently they have been isolated from a range of habitats (Harder and Veldkamp 1967; Stanley and Rose, 1967; Moiroud and Gounod, 1969; Herbert and Bell, 1977). The principal reason for the inability of early microbiologists to isolate psychrophiles was the failure to use pre-cooled media and to ensure that the samples were never exposed to lethal temperatures. These organisms are abnormally thermolabile and even exposure to room temperature for a period of time is likely to be lethal. However, if pre-cooled media are used, psychrophiles can be obtained relatively easily from permanently cold environments. The majority of psychrophiles studied have been isolated from the marine environment but they are not confined to this habitat.

Taxonomy of Psychrophilic Bacteria

A notable feature of psychrophilic bacteria is the predominance of Gram negative species isolated and the relative rarity of Gram positive organisms. Most of the psychrophiles studied belong to the genera *Vibrio* and *Pseudomonas* (Morita and Haight, 1964; Harder and Veldkamp, 1967; Herbert and Bell, 1977). Chromogenic isolates have been assigned to the genus *Flavobacterium* (Stanley and Rose, 1967; Sieburth, 1967), although the taxonomic status of the genus is at present uncertain. Gram positive psychrophiles have been isolated. Sieburth (1967) isolated *Arthrobacter* spp. from Narragansett Bay, Rhode Island and a similar organism, *A. glacialis*, has been isolated from sediments below arctic glaciers (Moiroud and Gounod, 1969). Until recently there had been no reports of psychrophilic anaerobes. Sinclair and Stokes (1963) successfully isolated from soil, mud and sewage *Clostridium* spp. which were

capable of sporulating at 0°C, but these were shown to be psychrotrophs. Liston *et al.* (1969) and then Finnes and Matches (1974) were the first to report psychrophilic *Clostridia* in marine sediments from Puget Sound. Sixteen of these isolates had growth temperature maxima below 15°C.

Herbert and Bell (1973, 1974) made an extensive survey of the waters and sediments of the lakes and offshore coastal waters for psychrophilic

TABLE 1. Bacterial groups isolated from freshwater lakes and coastal offshore waters at Signy Island, South Orkney Islands, Antarctica

Bacterial group	Marine	Freshwater
Azotobacter sp.	+	+
Nitrosomonas sp.	−	+
Nitrobacter sp.	−	+
Dentrifier sp.	+	+
Proteolytic sp.	+	+
Purple non-sulphur sp.	+	+
Purple sulphurs	+	+
Green sulphurs	+	+
Thiobacillus sp.	+	+
Filamentous S-oxidizers (*Beggiatoa*)	ND	+
Sulphate reducers	+	+
Sulphur oxidizers	+	ND
Cellulose decomposers	+	+

ND, not determined.

representatives of other bacterial groups. Table 1 shows the diversity of bacterial types that were isolated. The majority of the isolates obtained were psychrotrophic. Although psychrophilic representatives of some groups have been obtained they are present in relatively small numbers. It seems paradoxical that in such permanently cold-environments (lake temperature maximum 5°C, sea temperature maxima −1°C) that a greater proportion of the microflora is not psychrophilic.

Growth of Psychrophiles

The growth characteristics of psychrophilic bacteria have been studied extensively over the last decade. Unfortunately, many of these studies have been performed on batch grown cultures which make interpretation of the data difficult. Our data, like those of Harder and Veldkamp (1967), relate to psychrophilic bacteria grown in a chemostat. In this way, by growing the cells at a constant growth rate, the effect of temperature can be studied as a single environmental factor without the complication of

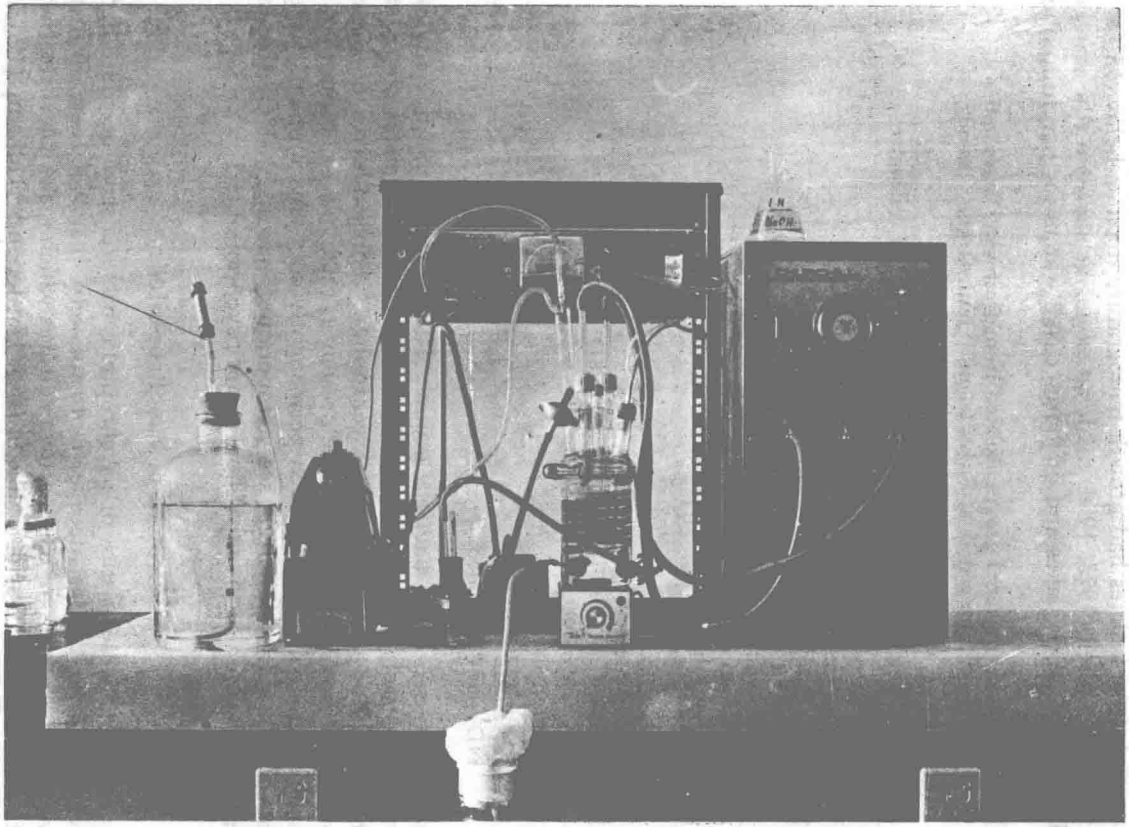

FIG. 1. Chemostat arrangement for growing psychrophilic bacteria

changes in growth rate, nutrient concentration, pH, dissolved gas concentrations which occur during the growth of bacteria in batch culture. In our own work we have used a simple single stage 1 litre chemostat (Fig. 1) based on the design of Baker (1968). Cooling is provided from an external thermocirculator (Churchill Instruments Ltd) circulating coolant via a "cold-finger" or cooling coil in the chemostat. In order to standardize the growth conditions we have used a defined mineral salts medium (Brown and Stanley, 1972) supplemented with glucose as carbon source and either KNO_3 or NH_4Cl as nitrogen source.

Temperature Effects

Effect of temperature on growth rate

It is a widely held belief that psychrophilic bacteria grow slowly at near zero temperatures. A closer examination of the literature shows that this is not necessarily the case. *Vibrio marinus* MP-1, for example, has a mean generation time of 226 min at 3°C (Morita and Albright, 1965) whilst Larkin and Stokes (1969) claim that *Bacillus cryophilus* will double its population every 6 h at −5°C. Other psychrophilic bacteria are much slower growing. *Micrococcus cryophilus* has a doubling time of 28·33 h at 0°C and *Vibrio* AF-1 (Herbert and Bell, 1977) has a mean generation time of 23 h. It may well be that it is not the rate of growth which is important but the efficient conversion of substrate carbon into cell carbon which is significant.

Following the derivation by Arrhenius (1889) of an equation to describe the effects of temperature on chemical reactions,

$$K = Ae^{-E/RT} \quad (1)$$

where K is the reaction rate, R the gas constant, T the absolute temperature, $E(\mu)$ the activation energy and A a constant, many microbiologists have attempted to apply the equation to the growth of bacterial cultures. By substituting bacterial growth rate for K in the equation the temperature characteristic for growth (μ) can be determined. Ingraham (1958, 1962) originally proposed that this could be used to determine whether or not a particular micro-organism was a psychrophile or a mesophile since the former should have a lower μ value. Ingraham's data have been challenged by Janota-Bassalik (1963) and Hanus and Morita (1968) on the grounds that his calculations were erroneous. The latter authors could find no significant difference between the μ values of a psychrophile, a psychrotroph and a mesophile. Similar findings have been reported for

TABLE 2. Optimal growth temperatures and temperature characteristic of growth (μ) for 5 psychrophilic *Vibrio* spp.

Isolate number	Optimal growth temperature (0°C)	Temperature characteristic for growth
AM1	6	27 400
AM10	10	32 000
AF1	15	9900
BM2	4	8200
BM4	8	38 700

yeasts (Shaw, 1967) and Gram positive bacteria (Brownlie, 1966). We determined μ values for 5 psychrophilic *Vibrio* spp. (Table 2) and these similarly show no consistent values. Whilst μ values may be of doubtful validity in describing the growth characteristics of psychrophiles and mesophiles, the Arrhenius plot of log specific growth rate against $^1/_{°K}$ is significant. In the case of psychrophiles, the slope of the Arrhenius plot is linear down to 0°C and below, whilst psychrotrophs deviate from linearity about 4–5°C and mesophiles tend to deviate from a straight line at higher temperatures (Harder and Veldkamp, 1971).

Effect of temperature upon substrate uptake

The theory that permeability and the associated control of solute transport may constitute a major factor in the ability of psychrophilic microorganisms to grow at low temperatures has gained considerable support and evidence in recent years. The minimum temperature for the growth of mesophiles is considered by many authors to be controlled by the low temperature inhibition of substrate uptake (Ingraham and Bailey, 1959; Rose and Evison, 1965; Morita and Buck, 1974). Even in a psychrotrophic *Vibrio*, Paul and Morita (1971) found that uptake of ^{14}C-glutamate was much reduced at low temperatures. However, Baxter and Gibbons (1962) and Cirillo *et al.* (1963) showed that in a psychrophilic *Candida* sp. sugar transport was largely independent of temperature. These data have been subsequently confirmed for Gram positive and negative bacteria (Wilkins, 1973). In *M. cryophilus* uptake of lysine occurred at the same rate when the cells were grown at 0°C as at 20°C (Russell, 1971). Herbert and Bell (1977) showed that in *Vibrio* AF-1 maximum uptake of ^{14}C-glucose and lactose occurred at 0°C and decreased with increasing temperature. Using statistical analysis (analysis of variance) these authors found that the temperature of assay and not the temperature at which the cells were grown was the factor controlling the rate of sugar uptake, i.e. there appear to be no quantitative differences in the number of glucose or

lactose permease molecules present in the cells when grown at different temperatures.

Closely allied to the effects of temperature on substrate uptake are the effects on the composition of the membrane lipids. Micro-organisms, in common with most other poikilothermic and many homeothermic organisms, synthesize increased proportions of unsaturated fatty acids, at the expense of saturated acids, when the growth temperature is lowered (Farrell and Rose, 1967). It is well known that an increase in the degree of unsaturation in lipids causes a decrease in the melting point of the lipids. A number of workers have argued that the physiological effect of the increased synthesis of unsaturated fatty acids at low temperatures is to maintain the membrane lipids in a fluid, and therefore mobile, state. This "lipid solidification" theory has been difficult to demonstrate experimentally. Circumstantial evidence for the theory has been provided by studies on cold shock in mesophilic and psychrophilic pseudomonads (Farrell and Rose, 1968). *Pseudomonas aeruginosa*, a mesophile, when grown at 30°C is susceptible to cold shock and loses viability whereas when grown at 10°C the cells are no longer susceptible. At the lower temperature (10°C) increased levels of unsaturated fatty acids are synthesized and Farrell and Rose (1968) postulate that these prevent lesions forming in the cytoplasmic membrane, thus maintaining their integrity. Additional evidence to support this hypothesis was the demonstration that a psychrotrophic pseudomonad which had increased unsaturated fatty acid levels at 30°C, was less susceptible to cold shock than the mesophile at this temperature. These data provide evidence that solute transport may well be influenced by the degree of unsaturation of the fatty acid side chains of the membrane lipids.

Several studies have been made of the changes in fatty acid composition of membrane lipids with temperature; however, the interpretation of certain of these data is difficult since the organisms were grown in batch culture and it is known that fatty acid composition alters in respect of growth rate as well as temperature. Comparative studies of mesophilic and psychrophilic yeasts have shown that the psychrophiles are generally endowed with a higher proportion of unsaturated fatty acids than the mesophiles (Kates and Baxter, 1962; Brown and Rose, 1969a). Data on psychrophilic bacteria have not provided such clear-cut findings. Kates and Hagen (1964) examined mesophilic and psychrophilic *Serratia* spp. and concluded that the psychrophilic species produced increased unsaturated fatty acids (hexadecenoic and octadecenoic acids) compared with the mesophiles. In *M. cryophilus* the response to low temperature is somewhat different and involves a shortening of the fatty acid chain length rather than increased synthesis of unsaturated fatty acids (Russell,