PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Cold Adaptation of Microorganisms [and Discussion]

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Cold adaptation of microorganisms

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Psychrophilic and psychrotrophic microorganisms are important in global ecology as a large proportion of our planet is cold (below 5 °C); they are responsible for the spoilage of chilled food and they also have potential uses in low-temperature biotechnological processes.

Psychrophiles and psychrotrophs are both capable of growing at or close to zero, but the optimum and upper temperature limits for growth are lower for psychrophiles compared with psychrotrophs. Psychrophiles are more often isolated from permanently cold habitats, whereas psychrotrophs tend to dominate those environments that undergo thermal fluctuations.

The molecular basis of psychrophily is reviewed in terms of biochemical mechanisms. The lower growth temperature limit is fixed by the freezing properties of dilute aqueous solutions inside and outside the cell. In contrast, the ability of psychrophiles and psychrotrophs to grow at low, but not moderate, temperatures depends on adaptive changes in cellular proteins and lipids. Changes in proteins are genotypic, and are related to the properties of enzymes and translation systems, whereas changes in lipids are genotypic or phenotypic and are important in regulating membrane fluidity and permeability. The ability to adapt their solute uptake systems through membrane lipid modulation may distinguish psychrophiles from psychrotrophs. The upper growth temperature limit can result from the inactivation of a single enzyme type or system, including protein synthesis or energy generation.

Introduction

Man is a mesophilic animal, unable to withstand wide variations of temperature except by taking special measures that do not involve physiological or biochemical adaptations. Not surprisingly, therefore, we regard cold environments as being 'extreme' – particularly those in which the temperature does not rise much above zero. Although we might claim to have colonized all corners of our planet, most of it is permanently cold (that is, normally below 5 °C) and uninhabited by humans. The reason for this is that more than 70% of Earth is covered by seawater, mostly deep ocean of which two-thirds has a remarkably constant temperature of approximately 2 °C. If one includes the polar ice-caps, more than 80% of Earth's biosphere is permanently cold. So we may truly be said to inhabit a cold planet, and should perhaps regard those organisms that are best able to cope with low temperatures as being its most successful colonizers.

If one considers an environmental parameter such as temperature, the species diversity decreases at the extremes and, moreover, it is microorganisms that become the dominant flora (Brock 1985). Representatives of all microbial groups (that is, bacteria, yeasts, algae and fungi) are found in both aquatic and terrestrial cold environments. Besides soils and waters, cold-adapted microorganisms are also found in snow, ice and even beneath the surface of some rocks. They play key roles in the ecology of permanently cold places, which cover such a significant proportion of our planet, and are also important in the spoilage of foods stored at chill

temperatures – a practice that continues to increase worldwide. Therefore, microorganisms capable of growing at low temperatures merit concerted investigation because of their overall contribution to global ecology and Man's well-being.

Psychrophiles and psychrotrophs: definitions

Microorganisms are similar to higher organisms in that each species can usually grow only over a temperature span of some 30–40 °C. As a group, microorganisms can grow at temperatures ranging from sub-zero to boiling point and, although they represent a thermal continuum, it is useful to classify individual species as psychrophiles, mesophiles or thermophiles according to their growth temperature range. The cardinal (lower, optimum and upper) growth temperatures have been used by many workers to define two groups of microorganisms, namely psychrophiles and psychrotrophs, that are both capable of growing at or close to zero (Ingram 1965; Inniss 1975; Morita 1975). Sometimes the terms obligate and facultative psychrophiles are used instead, but as Baross & Morita (1978) have pointed out these should be avoided because they imply that some organisms may be psychrophilic only under certain circumstances. The definitions of psychrophile and psychrotroph that have received most widespread acceptance are those of Morita (1975). He proposed that psychrophiles should include organisms having optimum growth temperatures < 15 °C and upper limits < 20 °C, whereas psychrotrophs (which are still capable of growing at or close to zero) have optimum growth temperatures > 15 °C and upper limits as high as 40 °C in a few cases (Baross & Morita 1978). Compared with psychrotrophs, psychrophiles tend to have narrower growth temperature ranges and some have remarkably low optima of < 10 °C. The most extreme examples of psychrophiles are the species of 'snow algae' found in snow and ice, some of which have optimum growth temperatures as low as 1 °C (Hoham 1975).

Together, psychrophilic microorganisms do appear to represent a distinct thermal group. Whether they possess distinctive biochemical features is considered later in this review. In comparison, the psychrotrophs are a more diverse group of microorganisms whose thermal properties overlap those of some mesophiles; indeed, some so-called mesophilic soil bacteria can grow at 0 °C and should properly be regarded as psychrotrophs (Baross & Morita 1978).

Psychrophiles and psychrotrophs: ecology

There have been many different proposals and much debate about the definitions of and the distinctions between psychrophile and psychrotroph (Morita 1975; Inniss 1975). None the less, they are useful terms because they can be related to particular ecological features. Psychrotrophs are characteristic of cold habitats where temperature fluctuates diurnally and seasonally, because psychrotrophs are regarded as being more 'adaptable' in that they can grow over a wider temperature range. Psychrophiles are generally less frequently isolated but are more likely to be found in those habitats (often aquatic) having stable thermal regimes. However, even in permanently cold freshwater lakes (maximum temperature 5 °C) or some polar seawater (maximum temperature -1 °C) the predominant microorganisms may be psychrotrophic rather than psychrophilic (Herbert & Bhakoo 1979; Leduc & Ferroni 1979).

Of course, there is a danger of oversimplification: besides temperature there are other factors, including the availability of moisture and nutrients, which influence the numbers and

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

distribution of psychrophilic and psychrotrophic microorganisms in an environmental niche. The numerical preponderance of a particular thermal type in a habitat may not match the growth rate at the environmental temperature when tested in the laboratory (Ferroni & Kaminski 1980), even when great care is taken always to maintain cultures at temperatures close to zero (W. J. Wiebe, personal communication); even so, the thermal characteristics of psychrophilic isolates may alter after serial subculturing in the laboratory (R. A. Herbert, personal communication). The relation between nutrient levels and temperature is reflected in the general observation that higher proportions of psychrophiles occur in Antarctic, compared with Arctic, marine ecosystems because the latter are more influenced by terrestrial nutrient inputs (Atlas & Morita 1986). Indigenous psychrophilic yeasts in Antarctic deserts have simple growth requirements that enable them to colonize a severely nutrient-limited environment (Vishniac & Klinger 1986).

Psychrophilic and psychrotrophic microorganisms are particularly important in the ecology of permanently cold environments, such as Antarctica, where they assume key roles in primary biomass production and nutrient cycling (Ellis-Evans 1985; Tearle 1987; Reichardt 1988). The large size of the food web in Antarctica makes it attractive to exploitation by man. Phytoplankton are primary producers of biomass at the bottom of the food chains, their activity being particularly high in places such as the ice edge (Hempel 1985). The recent application of satellite remote sensing techniques in combination with conventional *in situ* observations has given a better appreciation of the scale of phytoplankton blooms in the Antarctic ice edge and has shown that the retreat of pack ice with its attendant microbial flora exerts a substantial control on the marine ecosystem (Sullivan *et al.* 1988). During austral winters, when phytoplankton levels decline, sea-ice algae probably take over at the bottom of the food chain (Stretch *et al.* 1988).

The interplay between factors such as temperature and nutrient levels is difficult to analyse *in situ*. Simpler laboratory-based studies with chemostat-grown cultures can be used to model certain aspects of the natural ecosystem, and have shown that the effects of temperature depend on nutrient levels. Harder & Veldkamp (1971), by using mixed bacterial populations of a psychrophile and a psychrotroph in a chemostat, showed that at −2 °C the former outgrew the latter, irrespective of dilution rate (that is, nutrient supply), whereas at 16 °C the reverse situation occurred; at intermediate tempertures of 4 °C and 10 °C, the dilution rate determined which bacterium became dominant. Ellis-Evans & Wynn-Williams (1985) also found that temperature influenced the outcome of competition experiments involving two species, with different temperature characteristics, grown under glucose limitation in a chemostat. These experiments suggested that at stable low temperatures it is likely to be organisms with more psychrophilic traits that are important in nutrient cycling in natural environments.

A vital consideration in relation to the importance of psychrophiles and psychrotrophs to the ecology of cold environments is how active they are *in situ*. Snow algae and associated bacteria appear to represent one extreme in that some are active *in situ* at temperatures as low as −2 °C (Baross & Morita 1978) and have biochemical characteristics that match closely their thermal habitat (Priscu *et al.* 1987). Others display overlapping maximum rates of autotrophic and heterotrophic growth in sea-ice microbial communities (SIMCO) at 4–7 °C (Kottmeier & Sullivan 1988). These authors' direct observation that primary production (that is, photosynthetic carbon fixation) and biomass production are not uncoupled in SIMCO by the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

differential growth of microalgae and bacteria is contrary to the proposal by Pomeroy & Deibel (1986), who observed that photosynthetic activity of phytoplankton in Newfoundland coastal waters continued at sub-zero temperatures when bacterial growth (based on laboratory measurements) ceased; on the basis of this comparison Pomeroy & Deibel argued that this uncoupling prevented unwanted microbial utilization of the primary products of photosynthesis. There are other examples of the coincidence of growth temperature optima of cold-adapted microorganisms with the *in situ* temperature of their habitat (Reichardt 1987), but this is not generally true of either growth rates or individual metabolic activities (Morita 1975; Vincent 1988). However, it is not necessary for a microbe to function at optimal rates as long as it can compete effectively in its particular environment. In fact, it may be quite advantageous for microorganisms to metabolize sub-maximally and have long generation times in nutrient-poor environments, for example, some Antarctic freshwater lakes, so as to avoid exhausting the food supply (Ellis-Evans 1985). Herbert & Bell (1977) showed that some psychrophiles (for example, *Vibrio* AF-1) with low optimum growth temperatures (10 °C) still grow slowly at zero (generation time, 23 h).

Limits to growth: thermal effects

Temperature influences growth rates by affecting the conformation of cellular macromolecules and other constituents, which determine the rates of enzyme reactions. The relation between temperature and reaction rate (k) is described by the Arrhenius equation:

$$k = Ae^{-E_a/RT},$$

where E_a, activation energy; A, a constant (related to steric factors and collision frequency); R, universal gas constant; T, absolute temperature (K).

The activation energies for most enzymes are usually of the order of 420 kJ mol^{-1}, so a drop in temperature from 20 °C to zero will produce an approximate fourfold decrease in enzyme rate constant. Thus, although reaction rates may fall considerably, there is no thermodynamic restriction on growth at low temperatures: thermodynamics fails to explain why psychrophiles but not mesophiles can grow at zero, and why different psychrophiles have very different growth rates at zero yet none can grow above 20 °C. Identification of the nonlinear events responsible for these discontinuous thermal phenomena should provide an insight into the basis of psychrophily. The following discussion considers the reasons for: (*a*) the lower growth temperature limit of psychrophiles; (*b*) the ability of psychrophiles and psychrotrophs to grow at low temperatures and (*c*) the upper growth temperature limits of psychrophiles and psychrotrophs.

(*a*) *Lower growth temperature limit of psychrophiles*

There are no substantiated reports of microbial growth at temperatures below −12 °C, which is consistent with the known physical state of aqueous solutions at sub-zero temperatures (Mazur 1980). Dilute aqueous solutions will generally supercool to −10 °C, occasionally −20 °C, and most cells remain unfrozen at −10 to 15 °C even though these temperatures are 9–14° C below the freezing point of their cytoplasm and there is extracellular ice in the growth medium. Nucleation of the supercooled cytoplasmic water does not occur above this temperature because small ice-nuclei are barred from entering the cell by the plasma membrane. Supercooled water has a higher vapour pressure than that of the extracellular ice,

so water will move out of the cell thereby concentrating the intracellular milieu (Mazur 1980). At temperatures below −10 to −15 °C, the cell water begins to freeze, further concentrating intracellular salts up to as much as 3 molal; the resulting ionic imbalance, altered pH and lowering of water activity (a_w) have a toxic effect on the microorganism, which will either prevent it from functioning or possibly kill it. Thus the lower growth temperature limit of psychrophiles is fixed, not by the chemical properties of cellular macromolecules, but instead by the physical properties of aqueous solvent systems inside and outside the cell.

(*b*) *Ability of psychrophiles and psychrotrophs to grow at low temperatures*

(i) *Genotypic versus phenotypic adaptation*

In contrast to the lower growth temperature limit, the ability of psychrophiles and psychrotrophs to grow at low, but not moderate, temperatures depends on adaptive changes in cellular proteins and lipids. These adaptations may be genotypic or phenotypic. I shall refer to genotypic changes as those that have occurred over an evolutionary timescale, and which are observed as interspecies differences, whereas phenotypic adaptations are those that occur within the lifetime of an organism and can have timescales ranging from minutes to diurnal, seasonal or longer. Other workers use the terms acclimation and acclimatization for phenotypic adaptation: acclimation refers to laboratory-induced adaptation in response to a single variable and acclimatization refers to multivariables in the natural environment (Hochachka & Somero 1984). An organism's genotype is the result of Darwinian selection and survival in the face of continued stress. Thus genotypic adaptation for an extremely cold environment represents the sum of many end-points of phenotypic adaptation to low temperature of cell structure/metabolism over a long period. Different microorganisms achieve this end-point in different ways and to different extents: hence the variation in thermal growth ranges of psychrophiles and psychrotrophs, and between members of these two groups.

There may be no difference in the outcome of genotypic and phenotypic adaptation in terms of cell composition. However, it is more difficult to evaluate the thermal significance of genotypic change because there are usually additional interspecies differences that are unrelated to effects of temperature. Studies of phenotypic adaptation have the advantages that they provide information about the mechanisms involved (Russell 1988) and, of course, are relevant to temperature variations in natural environments. Hochachka & Somero (1984) have proposed that there are three main 'targets' for biochemical adaptation of an individual organism.

1. Preservation of the structural integrity of macromolecules and assemblies such as membranes.
2. Provision of the necessary energy, metabolites and intracellular environment for metabolism.
3. Regulation of metabolism in response to the changing needs of the organism.

Although it is useful to consider the adaptation of individual cellular systems, for example, those responsible for generating energy, it is the maintenance of functional enzymes and structural molecules upon which all adaptive changes depend. Therefore, it is qualitative and quantitative changes in proteins and lipids, which enable psychrophiles and psychrotrophs to grow at low temperatures.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(ii) *Thermal adaptations in proteins*

The stability of proteins depends on the sum of many weak non-covalent interactions between the polypeptide backbone and between side-chains of amino acids. These interactions include hydrogen bonds, salt bridges, van der Waals interactions and hydrophobic bonds. The conformation of the folded (native) protein is a balance between these stabilizing forces and destabilization due to loss of conformational entropy (Matthews 1987). Studies of the thermal stability of proteins have concentrated almost exclusively on the effects of high temperatures; comparisons have been made of specific enzymes isolated from mesophilic and thermophilic microorganisms (usually bacteria), and of naturally occurring and site-directed mutant forms of enzymes with altered thermal stability (Singleton 1976; Zuber 1979).

Although there is no simple pattern in the type and location of amino acid substitutions that alter protein stability, it is possible to identify some general features (Matthews 1987). Hydrophobic bonds are particularly important in providing thermostability, which is not surprising because their strength increases at temperatures up to 85 °C (Nemethy & Scheraga 1962). It is not the overall hydrophobicity index of the protein that is altered (this is often the same for mesophilic and thermophilic forms of an enzyme), but the hydrophobicity of the interior, often due to changes in very few amino acids. The increase in hydrophobicity may be in α-helical regions in the interior, which gives them greater stability; there may also be an increase in the hydrophilicity of the exterior of the protein (Argos *et al.* 1979). For enzymes containing more than 1 polypeptide chain (for example, lactate dehydrogenase), site-directed mutagenesis has been used (Wigley *et al.* 1987) to confirm the proposal that decreasing the size of water-accessible hydrophobic surfaces increases subunit interactions and gives greater thermostability (Stellwagen & Wilgus 1978; Zuber 1979). Evidence from the structure of α-amylases from mesophilic and thermophilic *Bacilli* shows that additional salt bridges involving lysine residues in the surface may also reduce the extent of protein unfolding at high temperatures (Tomazic & Klibanov 1988). Disulphide bonds are not generally involved in modifying thermal stability, which is achieved through non-covalent interactions. The overall result is to give a protein that is more rigid and retains its native conformation at higher temperatures; this is at the expense of catalytic activity at lower temperatures, when the protein becomes too inflexible to function, which is why thermophiles cannot grow at mesophilic temperatures.

In comparison with thermophilic proteins, there is a paucity of information about psychrophilic proteins. By analogy with mesophilic–thermophilic changes, psychrophilic proteins should have weaker hydrophobic bonding in the interior and stronger hydrophilic interactions on the exterior. Whether this is true in practice remains to be established. It is only recently that the first amino acid sequence of a psychrophilic enzyme has been reported, namely lactate dehydrogenase from *Bacillus psychrosaccharolyticus* (Schlatter *et al.* 1987). This bacterium is a psychrotroph, capable of growing between 0 and 30 °C, and the isolated lactate dehydrogenase has an optimum temperature for activity of 35–40 °C; this is much lower than that of mesophilic and thermophilic lactate dehydrogenases from related *Bacilli*. It remains to be determined if the psychrophilic enzyme really is more flexible that its mesophilic–thermophilic counterparts, and comparative studies (including mutants) are badly needed in order to identify the specific substitutions responsible for the psychrophilic property. The search for the key amino acid substitution(s) is not made easier by the fact that the net

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

difference between the free energy of the native and unfolded states of an enzyme is small, that is, only 21–84 kJ mol^{-1} (Matthews 1987) so only a few amino acid residues may be involved. It is possible that psychrophilic enzymes may not need to be as catalytically efficient as mesophilic and thermophilic counterparts, in view of the often extended generation times of psychrophiles and psychrotrophs at low temperatures, although studies with poikilothermic higher organisms show that enzymes of cold-adapted species have higher catalytic efficiencies than those of warm-adapted species (Hochachka & Somero 1984).

The psychrophilic *Vibrio* spp. strain ABE-1 contains two isocitrate dehydrogenase isoenzymes with different thermal stabilities (Ochiai *et al.* 1984). Isoenzyme I is a dimer and is relatively more thermostable than isoenzyme II, which is a monomer and denatures above 15 °C but is reactivated at 0 °C. This is the only report of the presence of isoenzymes with different thermal stabilities in bacteria or other microorganisms, in comparison with higher organisms in which they contribute to adaptive mechanisms. Therefore, it is not possible to draw comparisons with other microorganisms, but the isocitrate dehydrogenase isoenzymes in *Vibrio* ABE-1 conform to the principles of thermostability outlined above in relation to thermophiles, that is, the monomeric isoenzyme is more thermolabile than the dimeric isoenzyme, because it has greater exposure of its (?hydrophobic) surface to aqueous solvent. Proof of this and further explanation of those structural features that make these and other psychrophilic enzymes active at zero awaits elucidation of their amino acid sequences and three-dimensional structures. Studies of *Vibrio* ABE-1 should provide particularly useful data in this respect because it contains two thermally distinctive isoenzymes in the same genetic background.

Clearly, all the differences in protein structure discussed above in relation to thermal stability are genotypic adaptations. There are no examples of phenotypic adaptation of protein structure, for example, by the temperature-regulated expression of genes coding for isoenzymes having different thermal stabilities. Studies of the heat-shock response show that temperature can regulate gene expression in microorganisms and higher organisms (Schlesinger *et al.* 1982; Neidhardt *et al.* 1984). The synthesis of heat-shock proteins is triggered by a transient rise in temperature and many other stresses, but curiously not by a fall in temperature (which suggests that there is something unique about low temperature stress). There is recent evidence for the synthesis of analogous heat-shock proteins in the psychrotrophic bacterium *Arthrobacter globiformis* grown close to its upper temperature limit (A. R. Hipkiss, personal communication).

In *Escherichia coli* another set of proteins is synthesized in response to cold shock (Jones *et al.* 1987). When cultures are shifted-down from 37 to 10 °C growth stops for several hours until the new rate is established. During this lag period the number of types of proteins made is greatly reduced and they are the only macromolecules made; of these proteins, 13 are made at 3–300 times the rate at 37 °C and the one with the highest rate of synthesis at 10 °C is not made at 37 °C (Jones *et al.* 1987). Several of these cold-shock proteins have been identified; they function mostly in transcription and translation and seem to be concerned with ensuring that the initiation of protein synthesis is blocked. Presumably, by analogy with heat-shock proteins, they prepare the cell for growth at low temperature, but how this is achieved is not clear.

The apparent involvement of cold-shock proteins in the initiation of protein synthesis is reflected in the adaptation of ribosomes from psychrophiles and psychrotrophs. As they are able to grow well at 0 °C, they must be able to make proteins at low temperatures. Krajewska & Szer (1967) showed that a cell-free protein-synthesizing system prepared from the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

psychrophilic *Pseudomonas* species 412 had a very low miscoding rate compared with cell-free systems from mesophiles and thermophiles at the same low incubation temperatures. The ability of the system to function at 0 °C was a property of the ribosomes *per se* rather than the soluble fraction of the cell extract, because a system of psychrophilic ribosomes and *E. coli* supernatant, but not of *E. coli* ribosomes and psychrophilic supernatant, was active at 0 °C. Bobier *et al.* (1972) showed that differences in thermal sensitivity of protein synthesis in two psychrophilic *Bacilli* was also a property of the ribosomes rather than associated factors. Szer (1970) isolated a protein(s) by washing ribosomes of *Pseudomonas* 412; the washed ribosomes, while retaining activity at 25–37 °C, largely lost their capacity to function at 0 °C but this could be restored by addition of the protein washings. These washings probably contain initiation factors for protein synthesis, because protein synthesis in *E. coli* ceases at 0 °C because of a block in initiation (Das & Goldstein 1968; Friedman *et al.* 1969). Broeze *et al.* (1978) found that the initiation of protein synthesis was much more resistant to a sudden decrease in temperature in the psychrotroph *Pseudomonas fluorescens* compared with the mesophile *E. coli*, showing that this early step in translation might be the one that is adapted in cold-loving microorganisms. Although it would be a marathon task, it would be informative to compare the structures of ribosomes and associated factors in psychrophilic and mesophilic bacteria or, better still, those from a psychrophile and its mesophilic mutant. Such an isogenic pair of strains would be invaluable in all studies of psychrophilic proteins.

There have been many attempts to isolate mutants of one thermal group (for example, mesophiles) that are able to grow at temperatures characteristic of another thermal group (psychrophiles), as a way of spotlighting the molecular determinants of psychrophily. Not surprisingly, such mutants are extremely difficult to obtain, because it is presumed that so many mutations are required that the chances of them occurring together in one cell are extremely small. A cold-sensitive mutant of the psychrotroph *Bacillus psychrophilus*, which grew poorly at 5 °C, was isolated by Murray & Inniss (1980). The defect appeared to be due to cold inactivation of ATPase which would lead to an inability to energize membrane transport systems. Farrell & Rose (1967) have reviewed studies of the regulation of a number of enzyme systems in psychrophiles and mesophiles. These do not always reveal differences in, for example, enzyme activation energies, and the enzymes from psychrophiles often have optimum temperatures for activity or stability above the upper growth temperature limit for the microorganism. Optimum growth temperature probably results from some aspect of cellular organization rather than specific enzymes.

(iii) *Thermal adaptations in lipid composition*

In marked contrast to protein structure, there is abundant information about the lipid composition of psychrophiles and psychrotrophs. Most data is on fatty acid composition, which is relatively easy to measure and, moreover, is relevant to thermal studies because it is the acyl (rather than head-group) composition of lipids that has more effect on membrane properties such as fluidity (Russell 1989). Some microorganisms increase the total amount or composition of phospholipid in their membranes at low temperatures (Bhakoo & Herbert 1979), but this is not a general effect and does not seem to be related specifically to psychrophily.

When comparing fatty acid compositions of psychrophiles, mesophiles and thermophiles, it is important to use closely related microorganisms (usually this means the same genus), in order

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

to eliminate as much thermally independent genetic variation as possible. Adopting this approach, Chan *et al.* (1971) compared two thermophilic, a mesophilic and a psychrophilic species of *Clostridia*. The proportion of unsaturated fatty acid increased in the following order: thermophiles (average, 10%), mesophile (37%), psychrophile (52%) and the psychrophile also had a higher proportion of short-chain fatty acids. Both these changes would lower the gel–liquid-crystalline phase transition temperature (T_m) of the lipids. However, no direct measurements of membrane fluidity were reported. Based on experiments with unsaturated fatty acid auxotrophic mutants of *E. coli* and with model membrane systems (McElhaney 1982; Russell 1989), one can predict that the T_m of the *Clostridia* lipids would have occurred at temperatures spanning the lower half of its growth temperature range; thus there would be a sufficient proportion of fluid lipid. In comparison, the T_m of the extracted lipids of the psychrotrophic bacterium *Micrococcus cryophilus* (this bacterium was previously called a psychrophile, but it is a psychrotroph according to the criteria used in this review), in which more than 97% of the lipid fatty acids are unsaturated, is from −30 to −40 °C (McGibbon *et al.* 1985), well below the lower growth temperature limit. These two examples illustrate the fact that there is no uniformity between different psychrophiles/psychrotrophs.

The study by Chan *et al.* (1971) is also complicated by the fact that the two thermophilic species contain branched-chain fatty acids; these are absent from the mesophilic and psychrophilic species, which instead contain unsaturated cyclopropane fatty acids. The authors took care to extract lipids from cultures at the same stage of growth, but had to use a different culture medium for the psychrophile. The growth phase in batch culture, growth rate and composition of the culture medium all influence lipid composition, which makes it very difficult to draw meaningful conclusions about lipid composition in relation to psychrophily by comparing genotypic differences between species. For example, although psychrophilic and psychrotrophic bacteria (Bhakoo & Herbert 1979, 1980) and yeasts (Watson *et al.* 1976) tend to have higher proportions of unsaturated fatty acids in their membrane lipids, there does not seem to be any particular correlation with growth temperature range, as mesophiles may have very similar fatty acid compositions to psychrophiles and psychrotrophs. The minimum amount of unsaturated fatty acid required for the growth of the mesophile *E. coli* is 23% (Cronan & Gelman 1973), so we can assume that psychrophiles and psychrotrophs must have either more than 23% unsaturated fatty acid or a suitable content of some other kind of fluidizing fatty acid.

More meaningful data that is easier to interpret is obtained by investigating temperature-dependent phenotypic changes in lipid composition of cold-adapted microorganisms. Such studies have shown that the observed changes in fatty acid composition are the same as some of those in mesophiles and thermophiles, that is, a decrease in growth temperature results in an increase in fatty acid unsaturation or chain shortening (Russell 1984). Alterations in the amount or type of methyl branching are reported less frequently for psychrophiles and psychrotrophs because they are more often Gram-negative and so do not usually contain branched-chain fatty acids. We have found in a psychrotrophic bacillus (obtained from A. Upton & D. B. Nedwell, University of Essex, England), which has an optimum growth temperature of 10 °C, that the major effect of a decrease in growth temperature is an increase in *anteiso* 15:0 relative to *anteiso* 17:0 with only minor changes in the pattern of *anteiso*/*iso*-branching (N. Fukunaga & N. J. Russell, unpublished results). As in other bacteria, changes

in cyclopropane fatty acid content in psychrophiles and psychrotrophs are not regarded as being related to temperature regulation of membrane fluidity (Russell 1989). There are no psychrophilic archaebacteria, so changes in phytanyl chain cyclization are not observed at low temperatures.

In a definitive study, Bhakoo & Herbert investigated the effect of temperature on fatty acid composition of four psychrophilic *Vibrio* species (Bhakoo & Herbert 1979) and five psychrotrophic *Pseudomonas* species (Bhakoo & Herbert 1980) grown in chemostats to avoid artefacts due to growth rate differences. In two of the psychrophilic *Vibrio* species grown at lower growth temperatures there was a greater proportion of unsaturated fatty acid, whereas in one there was a relative decrease in fatty acid average chain length and in another there was no change in fatty acid composition (Bhakoo & Herbert 1979). None of the psychrotrophic *Pseudomonas* species altered their fatty acid composition when the growth temperature was lowered from 20 to 0 °C (Bhakoo & Herbert 1980). This diversity of thermal response is typical of that reported in the literature for a wide range of psychrophilic and psychrotrophic microorganisms. It probably reflects the fact that a large variety of fatty acid compositions can give the same thermal properties, which are also regulated by other factors such as lipid–protein interactions (McElhaney 1982; Russell 1989). This reasoning can be extended to all microorganisms, which as a group are able to tolerate quite wide variations in their membrane fluidity and may be able to grow with significant proportions of gel-phase lipid in their membranes (McElhaney 1982).

It was shown above that the kinds of temperature-dependent fatty acid modifications in psychrophiles and psychrotrophs are essentially the same as those seen in mesophiles. However, there may be an important difference in the timescale of the adaptive changes after a sudden decrease in temperature. This would be particularly relevant to growth in thermally unstable cold habitats, where thermal fluctuations can span almost the entire growth temperature range of psychrophiles and psychrotrophs (McKay & Friedman 1985). A study of phospholipid and fatty acid turnover in the psychrotroph *M. cryophilus* after temperature shifts showed that the response to a shift-down was faster and cultures appeared to be less stressed compared with the response to a shift-up (McGibbon & Russell 1985). In contrast, the mesophile *Bacillus megaterium* copes better with a shift-up compared with a shift-down, based on the extent and speed of adaptive changes in fatty acid composition (A. Thomas & N. J. Russell, unpublished results). Although their fatty acid compositions are very different, in both these bacteria the fatty acid changes are brought about by enzyme activation and *de novo* fatty acid biosynthesis (Russell 1984). It would be worthwhile comparing this dynamic aspect of lipid compositional change in psychrophiles and psychrotrophs from thermally stable and unstable habitats, as this should reveal information that is relevant to their ability to compete effectively in the natural environment.

One of the most important consequences of membrane lipid changes in microorganisms is to modulate the activity of intrinsic proteins that perform functions such as electron transport, ion pumping and nutrient uptake (McElhaney 1982; Russell 1989). The ability to take up nutrients efficiently at low temperatures has often been mooted as a determinant of psychrophily. Ellis-Evans & Wynn-Williams (1985) found that glucose uptake/utilization (transport was not measured separately) was highest at 0 °C in a psychrotroph from a permanently cold lake, whereas the reverse was true of a psychrotroph from a terrestrial habitat undergoing regular large thermal fluctuations. Most of the psychrophiles but none of the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

psychrotrophs studied by Bhakoo & Herbert (1979, 1980) altered their fatty acid composition and it may well be significant that in one of the psychrophiles glucose uptake/utilization was maximal at 0 °C and decreased up to 15–20 °C, whereas the converse was true of the psychrotrophs. Thus, psychrophiles may adapt their lipid composition so as to increase the efficiency of solute uptake at low temperatures. Although it has been shown that several psychrophilic bacteria and yeasts transport solutes as well or better at 0 °C than higher temperatures (reviewed in Herbert & Bhakoo 1979), several studies did not distinguish transport from subsequent metabolism. In addition, some psychrotrophs (for example, *M. cryophilus*) take up nutrients at the same rate at high or low temperatures (Russell 1971). Moreover, some psychrophiles do not modify their lipid composition in response to temperature changes (Herbert & Bhakoo 1979). Thus the hypothesis that solute uptake efficiency linked to membrane lipid changes is the key to psychrophily remains to be proven.

The above discussions have considered the cold adaptations of proteins and lipids, and some cellular systems including protein synthesis and solute uptake. No single component has been identified as the molecular determinant of psychrophily. All of the cell components of a psychrophile or psychrotroph must be functional for the microorganism to grow at low temperatures. In this sense, there can be no specific determinant of psychrophily and cold adaptation must be an overall cellular phenomenon. In contrast, the upper growth temperature limit of psychrophiles and psychrotrophs can result from the collapse in activity of a single enzyme.

(*c*) *Upper growth temperature limit of psychrophiles and psychrotrophs*

One of the best characterized microorganisms in terms of its upper growth temperature limit is *M. cryophilus*. This psychrotrophic bacterium contains temperature-sensitive amino-acyl-tRNA synthetases for proline, histidine and glutamate that dissociate into inactive subunits at non-permissive temperatures just above the upper limit for growth (Malcolm 1968, 1969*a*). A temperature-resistant mutant was isolated that could grow at 30 °C and contained a glutamyl-tRNA synthetase that did not dissociate into its four subunits at 30 °C (Malcolm 1969*b*, *c*). Thermolabile amino-acyl-tRNA synthetases, as well as several other soluble enzymes associated with protein synthesis, have also been demonstrated in the psychrophilic yeast *Candida gelida* (Nash *et al.* 1969). In several bacteria and yeasts one or more enzymes of energy generation have been identified as being thermolabile (reviewed in Inniss 1975). Not surprisingly, the identity of key thermolabile enzymes varies from one cold-adapted microorganism to another and no unitary hypothesis is possible.

It has often been demonstrated that the cell membranes of psychrophiles and psychrotrophs become indiscriminantly leaky to small and large molecules at elevated temperatures. As membrane permeability depends on lipid composition, it is possible that temperature-dependent changes in fatty acid composition could alter the upper growth temperature limit. This has been demonstrated using fatty acid auxotrophs of *E. coli* and with the wall-less bacterium *Acholeplasma laidlawii*, in which large fatty acid compositional changes can be achieved (McElhaney 1982). Both these bacteria are mesophiles, but there is no evidence to suggest that loss of permeability is the primary determinant of the upper growth temperature limit in psychrophiles and psychrotrophs, although this has not been tested systematically with mutants.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Conclusion

It was more than 100 years ago that Certes (1884) reported the existence of a population of bacteria growing at low temperatures in sediments, and Forster (1887) first isolated bacteria capable of growing at zero. Yet it is only in the past 30 years that the thermal characteristics of psychrophiles and psychrotrophs have been established properly, and we are still a very long way from understanding why this group of microorganisms are capable of growing at such low temperatures. In view of the importance of cold habitats to global ecology it is surprising that psychrophiles and psychrotrophs have not been studied more intensively. Thermophiles have attracted attention because of their presumed potential in biotechnology. Hopefully the current wave of interest in the environment and desire to conserve energy will contribute to a much needed resurgence of research interest in psychrophiles and psychrotrophs. Besides their ecological significance, they could be exploited in low-energy (that is, more economical) biotechnological processes (Sharp & Munster 1986).

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Discussion

P. Harrisson (*British Antarctic Survey, Cambridge, U.K.*). Dr Russell showed a slide of a cryptoendolithic community from the Antarctic and also said that intracellular ice formation hasn't been shown to occur in microorganisms. A paper should be appearing in *Polarforschung* this year, reporting intracellular ice formation in unicellular algae from the algal layer of cryptoendolithic communities. This is part of a study organized by the Friedman group in America and presented at a meeting at Kiel University, F.R.G., run by L. Kappen of the Institüt fur Polaroekolozie in September 1987.

I. A. Johnston (*Gatty Marine Laboratory, U.K.*). Dr Russell described the different temperature optima and maxima of so called psychrophilic and psychrotrophic bacteria. However, comparing bacteria of similar ecologies: to what extent are the growth rates and generation times of psychrophilic bacteria living at or below 0 °C, similar to that of psychrophilic species living at much higher temperatures?

N. J. Russell. Despite the observation that psychrophiles are more likely to be isolated from permanently cold environments that have a stable thermal regime, psychrotrophs may well be the dominant species, in terms of total numbers. The reported growth rates of psychrophilic

bacteria at 0 °C vary widely from only a few hours up to several days, and those species having lower thermal optima tend to grow better at zero. However, I am not aware of any systematic study that has compared all the different bacterial (or other microbial) species in a particular habitat, in terms of growth rates at zero and cardinal growth temperatures (that is, minimum, optimum and maximum). That would be quite an undertaking and would always suffer from the criticism that such parameters measured in the laboratory do not necessarily reflect conditions in the field, for which determinations of the viable cell numbers of each species may be a better indicator.

R. Jaenicke (*Institute for Biophysics, University of Regensburg, F.R.G.*) There are three observations that seemed to me intriguing:

1. How is the desaturase switched on in psychrotrophs when the temperature is reduced; is there cold activation of all enzymes?

2. In the case of the amino-acyl-tRNA synthetases mentioned, one has to postulate endothermic disassembly; how does this occur?

3. How is 'chain elongation' of fatty acids regulated? Is the acyl carrier protein involved or direct acetylCoA addition; does substitution involve phospholipases?

N. J. Russell. These are all interesting questions which I can deal with only briefly here.

1. Nearly all desaturase enzymes are membrane-bound, but not all are regulated by membrane fluidity which provides a 'self-balancing' system thus: when temperature falls suddenly, membrane fluidity is decreased and the desaturase enzymes are activated (presumably via lipid-induced protein conformation changes); intact acyl lipids in the membrane are commonly the substrate and their desaturation *in situ* restores membrane fluidity and switches off the desaturase (Russell 1989). In some other microbial systems, notably the bacilli studied by Fulco, there is a temperature-sensitive repressor molecule (which is protein or RNA), which regulates the transcription of the desaturase gene, so that new enzyme synthesis is triggered by a fall in temperature. However, the molecular details of this system have not yet been elucidated.

2. The temperature-resistant mutants of *Micrococcus cryophilus* that contained the thermostable amino-acyl-tRNA synthetases, to which Professor Jaenicke refers, were generated by UV mutagenesis and were unstable. Unfortunately, they are no longer available and so it is not possible to investigate why the mutant enzymes remained as active tetramers above 25 °C. They would provide, of course, a most interesting system in which to study molecular mechanisms of thermostability.

3. As Professor Jaenicke correctly infers, fatty acid elongation requires removal of acyl chains from acyl lipids, before addition of C2 units as malonyl-CoA or malonyl-ACP. Which substrate is used in bacteria is not known, but in *Micrococcus cryophilus* the interconversion of C16 and C18 acyl chains occurs via a C14 intermediate and all the reactants remain within the membrane where the elongase, which is probably regulated by membrane fluidity is located (Russell 1984).

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

M. Zuber (*Institut für Molekularbiologie und Biophysik, Zürich, Switzerland*). We have been working in recent years on the temperature adaptation of proteins, particularly enzymes. For example, we compared the primary structures of thermophilic, mesophilic and psychrophilic lactate dehydrogenases (LDH) of phylogenetically related bacilli (*B. stearothermophilus*, *B. megaterium* and *B. psychrosuccharolyticus*, respectively). We found, on the basis of a specific matrix analysis, a preference for hydrophobic and typical ion pair-forming residues (for example, Arg) in the thermophilic LDH but polar residues in the mesophilic and psychrophilic LDH. The psychrophilic LDH has even more polar residues than the mesophilic LDH, which has more hydrophobic residues, but also an increased number of charged residues. This shows that in the psychrophilic LDH certain regions (for example, active site) may have increased flexibility (important for activity at low temperatures) and others may have increased numbers of stabilizing (hydrogen bond forming) residues (stability at low temperatures).

N. J. Russell. I thank Professor Zuber for these comments, which are most interesting. It does appear that comparison of thermophilic with mesophilic proteins can be used as a basis for making some assumptions about psychrophilic proteins, if one can be permitted to draw conclusions from a single example. Clearly, we should bear in mind that psychrophiles may have evolved some novel ways of stabilizing their proteins at low temperatures that we have not guessed.

M. Zuber. I find Dr Russell's remarks on the variable temperature-sensitive amino-acyl-tRNAs of *M. cryophilus* (wildtype and mutant) determining the upper growth temperature very interesting. It reminds me of our data on the genetic basis of the temperature adaption of LDH in bacilli. These data show that in evolution the thermophilic, mesophilic or psychrophilic LDH may have been adapted to high, medium or low temperatures on the basis of a positive selection mechanism at the level of the tRNA anticodon–codon system. In translation, thermophilic or mesophilic tRNA types prefer amino acid residues found in thermophilic or mesophilic LDH, respectively. These preferences correspond to a specific structure of the genetic code (Zuber 1988 *Biophys. Chem.* **29**, 171–179).

F. Franks (*Pafra Ltd., Cambridge Science Park, Cambridge, U.K.*). What, if anything, is known about phenotypic or other changes in proteins, e.g. stability/activity changes, which might be induced by sugars and sugar alcohols?

N. J. Russell. That is a most difficult question to answer, because the general features of those compounds that are accumulated as compatible solutes in response to freezing or dehydration stress are not understood. Moreover, there is no evidence to suggest that microorganisms that are capable of growing at low temperatures can do so because they stabilize their intracellular proteins with such small molecules. My guess is that cold stability, like heat stability, will prove to be an intrinsic property of the protein. However, that is not to deny that thermal stability is affected by the binding of such small molecules as the co-factors and substrates of enzymes and (possibly importantly) by metal ions.

D. Wynn-Williams (*British Antarctic Survey, Cambridge, U.K.*). Following Dr Russell's remarks about the lack of information on compatible solutes in microorganisms, it is interesting to note

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

that in the maritime Antarctic (at Signy Island) Dr Paul Tearle has reported small amounts of polyols in snow algae and substantial amounts of arabitol and ribitol in lichens which are essentially assemblages of microorganisms. They seem better able to retain these potential cryoprotectants, despite the frequency of their exposure to freeze–thaw cycles in their exposed habitats. Mosses from adjacent, but more sheltered habitats, readily lose their potential cryoprotectants when exposed to the same freeze–thaw process.

N. J. Russell. Much is known about compatible solutes in microorganisms: the lack of information concerns whether their accumulation is in any way specifically related to the ability to live at low temperatures, which might be the case as some cryoprotectants can also act as compatible solutes (for example, trehalose). In relation to the better retention of cryoprotectants during freeze–thaw cycles, I suspect that this is related to the membrane lipid composition that has a dominating influence on the passive permeability of membranes (Russell 1989).

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES