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Psychrophilic microorganisms and their cold-active enzymes

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The earth's vast and varied cold environments could be rich sources of psychrophilic microorganisms growing at 5°C or below. Unfortunately, the diversity, physiology and potential of these organisms have largely been overlooked. This article focuses on psychrophiles and their cold-active enzymes and emphasizes how future studies could give basic insight into protein structure and could yield industrially useful enzymes. It presents an overview of the characterization of psychrophiles and their growth properties, a summary of biochemical work with cold-active enzymes, a description of comparisons of enzymes with different temperature optima, and a preview of uses for cold-active enzymes in biotechnology.

Keywords: psychrophiles; extremophiles; cold-active enzymes; biotechnology

Introduction

In the past, many researchers avoided studying physiologically diverse microorganisms because their growth required uncommon techniques or specialized equipment. Even if an isolate made potentially useful metabolites or enzymes, it was often set aside because devising special production conditions or obtaining overproducing mutants in an uncharacterized strain was too expensive. Recombinant DNA methods help overcome many of these barriers and make possible the study and use of metabolites and enzymes from extremophiles. Despite this opportunity, the potential held by extremophiles might have smoldered quietly if the exciting work with thermophilic enzymes had not ignited interest. Thermophilic enzymes, especially the success of *Taq* polymerase for the polymerase chain reaction, have indeed paved the way for other enzymes from extremophiles, often nicknamed extremozymes.

Of all the factors affecting a microbial environment, temperature is one of the most important and variable. Temperature is an uncontrollable force in the life of a microbe. Although thermophiles and high temperatures have received more attention, the psychrophilic (cold-loving) microorganisms living at the other end of the temperature scale also hold great promise. In this brief review, I hope to highlight the potential that psychrophiles and their cold-active enzymes hold. This is not a comprehensive discourse on psychrophilic microorganisms, rather it presents an overview emphasizing how little is known about these interesting organisms and their enzymes. It focuses on the following: 1) an introduction and background on the initial isolation and characterization of psychrophiles and their growth properties; 2) a summary of some biochemical work with enzymes that have their highest catalytic activities at low temperatures, which I refer to as cold-active enzymes; 3) information on comparisons of enzymes with different temperature optima to determine structural features responsible for the temperature range; and 4) a glimpse at some ways in which cold-active enzymes can be important in biotechnology. A common thread of all sections is showing how little we know about psychrophiles and posing questions that need to be addressed. A goal of the review is to raise the awareness that psychrophiles hold great potential and that their characterization will enhance our basic knowledge of microbial physiology and enzyme structures and help develop industrial applications.

Background on psychrophiles

Much of our knowledge of psychrophiles accumulated in the late 1960s and early 1970s. There are several excellent reviews of the early isolation and characterization of psychrophiles [10,11,16–18,29,32,37] and I will not repeat their insight here. There has been considerable debate about the definitions of the optimum growth temperatures and ranges for psychrophiles. In the strictest definition, psychrophiles do not grow above 20°C [32]. Organisms growing well at low temperatures, but growing above 20°C are designated as psychrotolerant (or psychrotrophs) [32,37,39]. One reason definitions are difficult is that many microorganisms have evolved to withstand fluctuating temperatures and a continuum of temperature ranges and there is no magic environmental cut-off. As Neidhardt *et al* [35] pointed out, the definitions reflect the interest of bacterial physiologists rather than any fundamental principle relevant to the growth of diverse microorganisms. We create groups; nature does not necessarily select groups. Thus, to avoid the difficulty of dealing with the varying maximum growth temperature, Neidhardt *et al* [35] defined psychrophiles as organisms that grow at 5°C or below to distinguish them from mesophiles which grow best at 37°C.

This definition is especially useful for this review because it is difficult to restrict the discussion of cold-active enzymes to ones arising only from strictly defined psychrophiles. We have observed that organisms capable of growth above 20°C may produce enzymes with substantial activities below 20°C. In fact, some more strictly defined psychrotolerant organisms may have isozymes with temperature optima below those found in more-psychrophilic strains. Furthermore, early workers isolating cold-loving strains

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often did not determine the minimum and maximum temperatures for growth, most likely because they lacked the refrigerated incubators necessary to do the experiments. Since little work has been done with these strains, they have not been separated into psychrophilic or psychrotolerant groups and are usually referred to as psychrophiles. Thus, this review will use the term psychrophilic in the general context of its derived meaning, cold-loving, and will include pertinent work on organisms growing at 5°C and below the mesophilic optima of 37°C. Only when it is especially relevant will the distinguishing terms psychrophilic and psychrotolerant be used.

The potential for physiological diversity among psychrophiles is huge. Cold environments are so vast and varied that psychrophilic organisms with all conceivable combinations of metabolic activities could have evolved. The majority of the ocean and deep lake waters are at temperatures below 5°C. Ground water, springs, and caves generally maintain temperatures below 20°C. Large portions of the earth are in the polar regions or have Northern climates with low winter temperatures. Humans have even increased the potential environments by using air conditioning, refrigerators and freezers. These cold environments house a range of pH values, salinities, oxygen and nutrients. Given these distinct environments, it is reasonable to expect that psychrophiles in physiologically diverse genera would have evolved. This appears to be substantiated by reports that members of distinct genera such as *Pseudomonas, Vibrio, Chromobacterium, Arthrobacter, Cellulomonas, Bacillus, Clostridium, Cytophaga,* as well as cyanobacteria, yeast, diatoms, and fungi have been isolated [10,38]. Specific examples include three alkalophilic-psychrophilic strains able to grow at 0°C and pH 10 [21], gliding bacteria isolated from diseased trout [4], obligate anaerobes [42] and gas-vacuolated bacteria [43]. Highly active and diverse communities were found in the slush layers of lakes [6]. Based on these reports, it is likely that microorganisms with any unique physiology could have adapted to life in some cold, specialized environment.

Researchers not working with psychrophiles often comment on how these organisms must take weeks to form colonies at low temperatures. This is true for some, but not all psychrophiles. The lack of information on psychrophiles and misinformation based on studies using slower growing psychrotolerant organisms, however, may account for the common belief that *all* psychrophiles grow slowly. Interestingly, there are no studies dedicated to enriching for and isolating fast growing psychrophiles nor to optimizing and comparing growth rates of existing strains. Thus, the question of whether any psychrophiles exist with rapid growth rates at low temperatures, for example at 5°C, equivalent to those of *Escherichia coli* at 37°C has not been rigorously addressed. Much of the early work centered on strain identification and did not optimize the nutrients and incubation conditions needed for growth. It also may be that the fastest growing psychrophiles have never been isolated since many early samples could not be kept cold during collection and storage and thermolabile organisms best adapted for rapid growth would have been killed. A few studies do report growth rates. During their characterization of a marine psychrophile, *Vibrio marinus*, Morita and Albright found the generation times to be 80.7 and 226 min at 15 and 3°C respectively [33]. In other work, a *Bacillus* species had a generation time of 8.5 h at 5°C [23] and we are characterizing isolates with generation times of 4–5 h at 5°C (Coombs and Brenchley, unpublished). Although these growth rates do not match those of *E. coli* at 37°C in rich medium (about 30 min), they do show that some strains are capable of reasonable growth rates at 5°C. Because so few psychrophiles have been examined, it is likely that ones with more rapid growth await our isolation attempts.

Furthermore, we should remember that the quest for rapid growth may be more important to the researcher than to the microorganism for several reasons. First, not all mesophiles grow as rapidly as *E. coli*. In fact, researchers deliberately selected faster growing bacteria as their experimental models, leaving many other interesting and useful mesophiles unstudied. It seems reasonable, therefore, that psychrophiles also exist with a range of growth rates. Second, organisms with slower growth rates play crucial roles in the environment and hold great value because of their diversity (weeds may outgrow corn, but corn is still important). Rapid growth rates may be convenient in research, but diversity may be more interesting and useful.

Third, growth rate is only one of many selective factors for survival in nature. Rapid growth may be a competitive advantage in some niches, but withstanding desiccation, adapting to rapid changes in temperature and nutrients, forming biofilms and using and storing limiting nutrients may be more useful in others. Evidence for this comes from chemostat studies [40] comparing the ability of two different strains to adapt to temperature shifts and to use nutrients at different temperatures [34]. It was observed that the response to change was important to survival and that such temporal heterogeneity in environments could increase the microbial diversity. A fourth point is that we may miss other important physiological processes when we examine cells grown only at temperatures giving faster generation times. For example, some *Moraxella* strains produce more lipase at temperatures lower than those for optimal growth [8]. Feller *et al* [7] reported that even though *Alteromonas halopanctis* strain A23 from Antarctic sea water can grow between 0 and 25°C, temperatures higher than those normally experienced in the organism's environment (−2 to 4°C) decreased secretion of amylase. Cultures grown at 25°C had only 6% of the activity of cultures grown at 4°C. Gugi *et al* [13] found the activity of extracellular proteases from *P. fluorescens* MFO was maximal at 17.5°C even though 30°C was optimal for growth. We have observed that proteases with different temperature optima are produced in cells grown at different temperatures (Goldthorp and Brenchley, unpublished). Thus, maximum production of the enzyme does not always correlate with the optimal growth temperature determined in the laboratory.

Characterization of cold-active enzymes

An often-asked question is whether any enzymes exist that have their highest catalytic activities at low temperatures, i.e do cold-active enzymes really exist. The idea of cold-active enzymes strikes some as contradicting the biochemical Q_{10} guide that enzyme activity generally decreases

approximately one-half with each decrease of 10°C until it ceases at low temperature. Cold-active enzymes, however, do not violate this concept; rather they simply shift their peak activities to temperature ranges lower than those generally observed for enzymes from mesophilic organisms; just as enzymes from thermophiles often have optimal activity at temperatures higher than found for mesophilic enzymes. One indication that cold-active enzymes exist rests on the reasoning that if psychrophiles can grow with generation times of even 5 h at 5°C, then the enzymes in this organism must have evolved sufficient activities and efficiencies to support growth at these temperatures.

Another approach to asking if cold-active enzymes exist is to examine the thermodependence of enzymes from psychrophilic isolates. Unfortunately, most studies examine the stability of enzymes at high temperature and only a few report activities over a range of temperatures. It would be useful for future reports to include information on the enzyme activities at different temperatures so that comparative data on ranges for activity could be collected. Some of the earliest attempts to determine the temperature profiles of enzymes from psychrophiles was by Morita and coworkers [32]. They reported that a partially purified malic dehydrogenase from *V. marinus* MP-1 was active between 15°C and 20°C and was inactivated at temperatures above 20°C. Fructose-1,6-bisphosphate aldolase [20] lost activity within 30 min at 35°C and glucose-6-phosphate dehydrogenase lost activity at 36°C. Glyceraldehyde-3-phosphate dehydrogenase, however, was quite thermostable [32]. In other work, a lactate dehydrogenase from *V. marinus* had an optimum between 10 and 15°C [31] and a variety of proteases had their highest activities below 50°C [15,26–28] (Goldthorp and Brenchley, unpublished). Heat-labile phosphatases have been found from an Antarctic bacterium [22] and other soil isolates [24] (de Prada and Brenchley, unpublished). Feller *et al* [8] found lipases of strains of *Moraxella* from Antarctic seawater with optimal temperature at 40°C. In our work with cold-active glycosidases, we found *Arthrobacter* strains producing β-galactosidases with optimal activities between 25 and 30°C, which is about 25°C below the *E. coli lacZ* enzyme [25]. In addition, the enzymes are active at temperatures below 15°C where the *E. coli* enzyme has less than 5% activity. One *Arthrobacter* β-galactosidase had an apparent K_m of 0.4 and a minimum V_{max} value of 1182 U mg^{-1} protein using ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as substrate. This is comparable to that found for the *lacZ* β-galactosidase from *E. coli* with an apparent K_m of 0.1 and V_{max} of 390 U mg^{-1} [44]. Devail *et al* [5] examined a subtilisin from a psychrophilic *Bacillus* that had a temperature optimum 20°C lower, and a specific activity four times higher, than subtilisin Carlsberg.

In order to illustrate the differences in temperature optima, the relative thermodependence of different β-galactosidases from representative psychrophilic (*Arthrobacter* strain D2), mesophilic (*E. coli*) and thermophilic (*Bacillus* isolate) organisms are compared in Figure 1. It is clear that the thermodependence of the three enzymes differ from each other and have specific activity ranges that correspond to, but do not necessarily coincide with, the temperature optimum for growth of the originating organism. Thus, a thermophilic organism would not be a good source for a cold-active enzyme even though a thermophilic enzyme might have fractional activity at a temperature where a psychrophilic enzyme also maintained some fractional activity, for example at 45°C. Since enzymes only need to have sufficient activity to facilitate cell growth, the temperatures for their maximum and minimum activities do not have to match precisely those found for the organism. In addition, we observed that β-galactosidase isozymes within the same organism have different temperature optima. We obtained three different genes, each encoding a β-galactosidase activity when we transformed DNA from *Arthrobacter* isolate B7 into an *E. coli* recipient. The enzyme encoded by the *lacZ*-type gene was not active in the *E. coli* transformant growing at 37°C and had an optimum *in vitro* around 35°C. The other two glycosidases were active in the *E. coli* transformants and had optima between 40°C and 45°C [14,44]. Thus, as has been seen with mesophiles and thermophiles, not all enzymes in a psychrophile need to evolve to the same temperature optimum. The enzymes from psychrophiles have, however, adapted to have their highest catalytic activities at temperatures lower than their mesophilic and thermophilic counterparts.

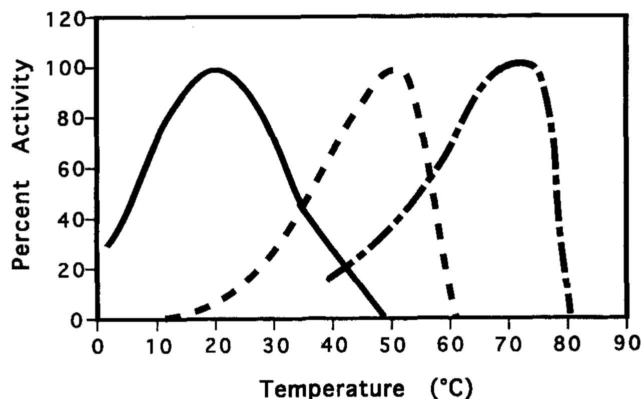

Figure 1 A schematic comparison of the thermodependence of β-galactosidase activities measured in a psychrophile (solid line), mesophile (dashed line) and thermophile (long dashed line). Data for the representation of the activity from the psychrophilic *Arthrobacter* D2 strain and mesophilic *E. coli* were selected from Loveland *et al* [25]. Data for the thermophilic *Bacillus* strain are based on results from Griffiths and Muir [12].

Although work with different psychrophilic microorganisms and their enzymes is in its infancy, some general conclusions can be reached. First, psychrophilic microorganisms are potentially a rich source of cold-active enzymes. In addition, a consequence of the shift in an enzyme's activity to a lower temperature range is that inactivation is likely to occur at lower temperatures than found for its mesophilic counterpart. Thus, heat-lability may be a structural consequence associated with an enzyme's optimal activity at lower temperatures. Second, enzyme activities correspond to, but do not necessarily coincide with, the growth range of the organism. And third, not all enzymes within the same organism necessarily have the same temperature optimum or range.

Comparison of cold-active enzyme structures with their higher temperature counterparts

An interesting biochemical observation, and one illustrated in Figure 1, is that most enzymes have a limited activity range of about 30 and 40°C. This raises questions of which structural parameters set this range and which components dictate the temperature optimum. Most previous investigations of protein structure compared thermophilic and mesophilic enzymes [1,2,30]. The addition of data from psychrophilic enzymes to these studies is helping to clarify the observations. The comparisons of nucleotide sequences of genes from psychrophiles, mesophiles and thermophiles support the view that changes in overall polypeptide structure, rather than changes in the catalytic sites, generally alter the temperature ranges of enzymes. Rentier-Delrue *et al* [36] compared sequences of triosephosphate isomerase (TIM) genes cloned from psychrophilic and thermophilic bacteria. The active site regions were conserved even though the enzymes shared only 34% identity. A cold-active *Arthrobacter* β-galactosidase had only 19% similarity with the *E. coli lacZ* enzyme, but contained highly conserved nucleophilic and acid-base regions and contained the proposed active-site residues Glu-461 and Glu-537 [44]. Two genes encoding lactate dehydrogenase isozymes from *B. psychrosaccharolyticus* were compared to their mesophilic and thermophilic counterparts [45]. The regions of high amino acid replacements were located in the alpha-helices. Interpreting these comparisons is complicated because the enzymes originate from phylogenetically diverse organisms. This makes it difficult to know which changes contribute to the temperature properties of the enzyme and which ones are due to evolutionary changes and genetic drift of the organism. Thus, sequence comparisons remain useful for detecting highly conserved or divergent regions and giving clues to important regions, but they alone do not identify the elements responsible for an enzyme's temperature characteristics.

The limited knowledge of protein structures is not yet sufficient to provide the rules for setting an enzyme's temperature range. However, a general model proposed to explain higher activity at low temperatures is that the enzymes have a more flexible conformation, metaphorically like an open hand, than their thermophilic counterparts. A consequence of this increased flexibility would be the thermal-lability often observed with cold-active enzymes. In contrast, heat-stabile proteins from thermophiles would have more rigid conformations, more like a fist, protecting them against destabilizing forces occurring at higher temperatures. A goal in comparing proteins from extremophiles is to test this, and other models, to determine if and how such changes in flexibility might occur.

Although the precise rules relating a protein's structure to its thermodependence are unknown, there are a growing number of comparisons that give some insight. Jaenicke [19] suggests that changing the number of ion pairs and hydrophobic interactions adjusts the flexibility of proteins so that full catalytic function is maintained at different temperatures. Menendez-Arias and Argos [30] compared thermophilic and mesophilic proteins and suggested that many small changes over the entire polypeptide cause thermostabilization, but that residues in the α-helical regions and domain interfaces displayed decreased flexibility and increased hydrophobicity for enzymes adapted to higher temperatures [30]. Davail *et al* [5] examined a subtilisin with a temperature optimum at about 40°C from the psychrophile *Bacillus* TA41. This comparison took advantage of the detailed knowledge of 50 other subtilisin proteases and the three-dimensional structures analyzed for these industrially important enzymes. The results indicate that the catalytic cavity of the *Bacillus* TA41 (S41) subtilisin is conserved and that the difference in temperature optimum is not due to changes directly affecting the active site. Rather, their analysis pointed to overall changes resulting in increased structural flexibility. Several salt bridges and aromatic interactions conserved in other subtilisins were missing in the *Bacillus* TA41 enzyme. In addition, several polar residues, primarily Asp, arranged on the external shell of the protein provided a more hydrophilic surface. The combination of these features could make the enzyme more flexible, less compact and more thermolabile.

In other work, Feller *et al* [9] analyzed the sequence of a lipase from *Moraxella* TA144, a strain from the Antarctic. They concluded that the structural features responsible for cold-activity could not be deduced simply from the amino acid sequence. Subtle changes may be sufficient to alter the folded state. Investigations [3] with a lipase from *Psychrobacter immobilis* B10 suggested that the presence of glycines close to the consensus peptides might be energetically favorable. Also the molar ratio of basic residues appears lower than for mesophilic and thermophilic proteins. Schlatter *et al* [41] reported the complete amino acid sequence of lactate dehydrogenase from the psychrophile *Bacillus psychrosaccharolyticus*. Zuber [47] compared the primary structures of several lactate dehydrogenases, noting specific amino acid changes, and concluded that the thermophilic lactate dehydrogenase has more hydrophobic interactions and ion pairs which increase the free energy of the folded protein. The lower temperature enzymes appear to have more polar residues.

Although these results are not yet sufficient to predict changes we could make to create enzymes to do our bidding, they do highlight features for analysis in future studies. It is possible that there are many ways in which the thermodependence of enzymes could have evolved and that our search will yield a menu of rules that could be used to alter an enzyme's temperature optimum. Discovering these fundamental rules will require pushing our knowledge beyond analyzing the amino acid sequence into characterizing enzyme structure and eventually towards examining enzyme reaction dynamics. Thus, it may take some time and effort to understand these subtleties and allow us to engineer an enzyme's thermostat at will. However, the combination of the more rapid analysis of crystal structures and the additional insight gained by comparisons that include cold-active proteins should sharpen the view.

Biotechnological potential of cold-active enzymes

Thermophilic enzymes usually star in discussions of industrial uses because their heat-stability makes them ideal bio-

catalysts for many reactions. In contrast, discussions of psychrophilic organisms and cold-active enzymes have often centered on the problems they cause in the food and dairy industry. Success stories with thermophilic enzymes should continue. There are, however, other cases where low temperature is an advantage. The purpose of this section is to illustrate that cold-active/heat-labile enzymes also have great potential in industry.

Cold-active enzymes could be used in chemical manufacturing for organic compounds that are highly volatile and can only be modified at low temperature. In other cases, low temperature may make separations of the product easier and less expensive. Other uses include using enzymes with high activity below 20°C in food processing to limit the growth of other contaminating microorganisms, shorten the process times, and avoid designing expensive heating steps. Treating foods and beverages with enzymes at refrigerator temperatures could prevent spoilage by mesophilic organisms. Cold-active enzymes could remove pectins and other polysaccharides from fruit juices or lactose from milk and whey while they are being refrigerated or accelerate the ripening of cheese. Cold-active amylases and proteases could be used in the brewing industry to speed the mashing phase at low temperatures. Cold-active enzymes could be added to detergents for low-temperature washes or to other solutions for cleaners. Some enzymes might replace chemical preservatives in foods [46] by depleting metabolites required by other organisms, disrupting microbial cells, or degrading other enzymes. The growing number of commercial refrigerated and frozen foods creates new needs for enzymes active at low temperatures for food processing and preservation.

Psychrophilic microorganisms and their enzymes are already crucial to nutrient cycling and biomass degradation and production. We can take advantage of the natural role of psychrophiles and use ones producing useful enzymes in waste-water treatment, biopulping and bioremediation in cold climates. Psychrophilic methanogens would be useful in anaerobic digestors to increase methane production in Northern regions. The development of a host/vector genetic system for psychrophiles would make possible the genetic engineering of better strains for bioremediation and the increased production of a variety of cold-active enzymes.

In other applications we could exploit the heat-lability of cold-active enzymes to stop enzymatic reactions. For example, an enzyme could be added to flour, allowed to react and then inactivated during baking. In research, reactions could be performed at low temperature and then the mixture heated to readily inactivate the enzyme before proceeding to the next step. Other cold-active enzymes could substitute for currently used enzymes that require higher temperatures than the cells or substrates require. For example, many of the enzymes used in molecular biology research were developed for work with *E. coli* or tissue culture cells grown at 37°C. The expansion of experimental models to include plants, nematodes, some cold-blooded animals such as fish and frogs, and other microorganisms may create the need for enzymes with higher activities at lower temperatures. In addition, reporter genes making cold-active enzymes would be valuable additions to the arsenal of molecular tools. These are only a few examples of ways in which we might become creative in finding uses for cold-active/heat-labile enzymes. We are likely to see a long list of applications for metabolites and enzymes unfold as more psychrophiles are isolated and genetic methods developed for their exploration and exploitation.

Concluding comments

I remember participating in a panel meeting where we struggled for a term describing understudied microorganisms requiring special culture conditions, such as high pH, salt or temperature. The need for a convenient space-saving, easy to use word pushed us to adopt 'extremophile.' Although we liked its utility, we realized that it reflects our 'human' mesophilic bias because to a psychrophile, 37°C is extreme. Despite this limitation, however, the word has drawn attention to these wonderfully diverse microorganisms. In this overview on psychrophiles, I spoke of them as extremophiles, but we must not let the emphasis on 'extreme' suggest that they are rare, impossible to isolate or not significant. They can be quite abundant in their specialized niches and, as we are rapidly learning, they are very important. One recurring theme of this review has been the emphasis on how few psychrophiles have been studied and how little we know about them. I hope this article presents a new awareness about the diversity of psychrophiles and their interesting and useful cold-active enzymes. As we learn more about their physiology and usefulness, perhaps our views of psychrophiles as 'extreme' will fade and our quest to learn and use their secrets awaken.

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