Psychrophiles: a challenge for life

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(Dia 1: Title). Etymologically the word psychrophiles means, “loving cold”, and this translation is not at all usurped since these organisms have been found in all cold environments on earth. Cold environments are in fact the most abundant at the surface of our planet since, next to polar, mountains, glaciers, and permafrost zones, they also include the oceans in which, below 1000m, the temperature is below 5°C, independently of the latitude. The Antarctic occupies a privileged position since it is the coldest habitat on Earth. To fully appreciate the importance of the challenge faced by psychrophiles one has to remember that the rate of a chemical reaction is strongly dependent on temperature.

Indeed, the Arrhenius law, proposed as early as 1889 by the Swedish chemist Svante Arrhenius, (Dia 2) tells that the rate of a chemical reaction is exponentially dependent on temperature. This relation can also be expressed by the Q10, which is the ratio between the rates of a chemical reaction measured at an interval temperature of 10°C. This Q10 is, usually, close to 3 so a drop of 30°C of the environmental temperature should induce an average decrease of the rate of the chemical reactions occurring in the organism by a factor close to 30. This will immediately lead to death or at best to a dormant state of the organism. To prevent this and to become independent of the daily or seasonally fluctuations of the temperature some organisms, like mammals and birds, have succeeded, in the course of evolution, to keep constant their internal temperature.

One can ask the question why not all organisms have developed such a temperature regulation system. In fact all living organisms have the tools to do so but the problem is that the heating system relies on an active metabolism. That means that part of the energy absorbed by the organism under the form of nutriments is used to keep the temperature constant, and this is a very large part since, in the case of mammals, there is a consensus for saying that at least 70 % of the absorbed energy is used to maintain their temperature around 37°C. That is the reason why, in winter, some mammals cannot keep constant their temperature due to the lack of food; so they adopt a dormant state, hibernation, like in the case of some bears and other mammals in which the body temperature can fall well below 37°C in winter. So most of the living organisms on earth leave their temperature fluctuate as a function of the environmental temperature and so are the psychrophiles that cover a wide range of living creatures from microorganisms to fish via invertebrates and insects. They have colonized all cold environments on earth and rather successfully since for example the density of bacterial cell in the Antarctic oceans is similar, around 10^5 cell /ml, to the cell density found in temperate oceans. So one can ask the question of “ How these organisms can cope with the negative effect of low temperatures on reaction rates?” (Dia 3).

We have tried to answer this fundamental question in the course of the last 20 years working successively with fish and then with microorganisms originating from the Arctic and the Antarctic. For the sake of simplicity, I will essentially focus on bacteria, which represents the most important biomass on Earth and in particular in polar regions like the Antarctic. First of all, “Where can we find them? In fact everywhere: on the ground, below the ground, in glacial ice, sea ice and free polar sea waters where the average temperature is typically slightly below 0°C. This moderately low temperature is not at all a limit of survival for psychrophiles. This limit seems on the contrary to go further down with the acuteness of the observations. Recent investigations carried out on bacteria isolated from Antarctic sea ice and from Alaskan soils frozen down to –35°C have indicated that microbial growth and metabolic activities are still significant at temperatures as low as –20°C. This has forced the researchers to distinguish between populations of cold habitats in which water is essentially in liquid phase from organisms living in environments in which water is mainly in a solid state. These special environments are known as the cryosphere and include glaciers, sea and fresh water ice, permanent snow and permafrost zones. In these habitats, the microbial life is mainly located in liquid veins and in the liquid films surrounding the surface of mineral particles trapped into the ice. The extremely low temperatures, largely below the freezing point of water, that prevail in these special environments raise an other question (Dia 4): "How do psychrophiles experimenting temperatures as low as –20°C prevent freezing?"

Indeed the formation of ice into cells is, in general, fatal for the organism. Internal ice causes first mechanical damages to supra-molecular structures such as membranes, induces a severe concentration of solutes, because only pure water freezes, and subsequent irreversible precipitation of various components into the cell. On the other hand, the formation of extracellular ice can also be lethal for the cell since the concentration of the extracellular components induces the loss of water from the intracellular space leading to cell dehydration and death (Dia 5). Psychrophiles, exposed to these extreme conditions, have successfully developed specific molecular adaptations by producing first cryoprotectants and second so-called ice structuring proteins and antifreezes. I will come back to that later.

What about the biodiversity of psychrophiles? In fact, contrarily to what was initially believed, the biodiversity of cold-adapted microorganisms is high and close to that found in temperate environments, exception being in ice, were the availability of water is severely restricted as well as the space. Sea ice is, in this context, an interesting biotope specific to the Arctic and Antarctic waters since even at the lowest temperatures which can drop to as low as –40°C in winter, the ice is channelized with liquid water (Dia 6) forming highly salted brine veins, in which bacteria are found to be metabolically active down to –20°C at brine salinity of 21% as detected by fluorescence techniques.

So we have seen that psychrophilic microorganisms are particularly successful in various cold-environments even in those characterized by the most extreme conditions. The next question is: What are their secrets? To be well adapted to an environment characterized by extreme temperatures, two main components need adaptation: the membrane and the enzymes. Indeed, life cannot be sustained if the exchanges with the environment are not severely controlled. This control is necessary to prevent the diffusion of toxic compounds, to regulate the income of nutriments and to allow the expulsion of waste products resulting from cell activity. To achieve that, a selective membrane permeability is required. For those who are not familiar with membrane structure the next slide shows a schematic representation of the typical double layer structure of a membrane made of two layers of lipids (Dia 7). Each lipid is made of a glycerol polar head oriented towards the outside and inside of the cell and connected to fatty acid chains of varying structure occupying the central part of the membrane. Various proteins are present and play notably a role in the transport of various components in and out the cell. This structure has to present an appropriate fluidity at the temperature of the environment and as low temperature has a freezing effect on membrane structure, in psychrophiles, the fluidity of the membrane has to be notably adjusted by shortening the length of fatty acids and by the introduction of unsaturated and branched fatty acid chains.

Now the other adjustment necessary to cope with low temperatures is related to the rate of the chemical reactions occurring in the organism. In all living organisms most of the chemical reactions, necessary for life, are catalyzed by enzymes which are proteins. That means that in the absence of enzymes or with inappropriate enzymes the rate of the reactions would be too slow to sustain life. And, as in the case of membrane, low temperatures have a freezing effect on enzyme's structure that can preclude the interaction of the enzyme with the chemicals to be transformed. Adjustments of enzyme's structure are therefore necessary and we have shown that this has been achieved by an increase of the flexibility of the enzyme's structure itself resulting from a decrease of the thermal stability of the enzymes. This has led to two very important and general properties of psychrophilic enzymes (Dia 8):

1 - They have a much higher specific activity at low and moderate temperatures than the temperate, the scientific term is mesophilic, counterparts

2 - They have a higher thermosensitivity, meaning that they are less stable than their mesophilic counterparts

These points are illustrated in Dia 9. It is a comparison between the specific activity of a cold-adapted alpha amylase, from an Antarctic bacterial strain, that catalyzes the hydrolysis of starch, and that of a mesophilic counterpart. It can be seen that the specific activity of the cold-adapted enzyme, that means its activity per mass unit, is much higher than that of the mesophilic homologue at low and moderate temperatures. The second point is illustrated by the fact that the activity rapidly decreases at temperature above 30°C meaning that the enzyme's structure is altered by heat and this leads to a complete inactivation of the enzyme around 50°C contrarily to what happens in the case of the mesophilic counterpart. The cold-adapted enzyme is therefore more heat sensitive that its mesophilic homologue.

This has forced the microorganisms living in the cold to shift their growing curves towards low temperatures (Dia 10). From this study, one can see that microorganisms living in Antarctica and in other permanently cold environments, although they are able to survive to moderate temperatures, have their metabolism severely altered at these temperatures. Indeed, at a temperature of about 20°C, they are unable to produce the appropriate enzymes necessary for their survival on a long term. The two properties of cold-adapted enzymes, mentioned above, appear to be extremely valuable for many applications in Biotechnology. I will come back to that later.

Now what happens when the temperature drops well below O°C? It has been mentioned that there is a risk of freezing of the physiological fluids and that can lead to death. In these conditions the organisms can produce antifreeze molecules. These antifreezes are often proteins and the first to be described in the sixties was isolated from an Antarctic fish by Art Devries and co-workers. It is a glycoprotein (Dia 11) made of a repetitive sequence of 3 amino acids Ala-Ala-Thr, bound to a disaccharide.

Five other AFP, not glycoproteins, were also discovered in other fish species adapted to low temperatures (Dia 12). They protect the fish down to the freezing point of sea ice which is around –1.9°C. The particularity of these proteins is that they have no common structural characteristics but they have a common effect, most of them depress the freezing point of water and this creates a difference between the freezing point of water and the melting point of ice, known as a thermal hysteresis. All bind to micro-crystals of ice at specific sites and prevent the growth of these ice crystals preserving in this way a certain fluidity of physiological fluids. The effect is not only colligative, meaning depending on the concentration, since they can be 500 times more efficient in depressing the freezing point of water than sodium chloride. Very efficient antifreeze molecules have been also discovered in insects, they are between 10-100 times more efficient than fish antifreezes and this is compatible with the much colder temperature an insect must survive.

Other antifreezes (known as IR, for ice recrystallization) do not depress the freezing point but prevent the recrystallization, meaning the growth of ice crystals. This is illustrated in the next slide (Dia 13), a courtesy of Prof Peter Davis from Queens University. Antifreeze proteins offer a high potential in biotechnology. Intuitively they appear highly suitable as additives for the preservation of tissue and organs at sub-zero temperatures, and indeed a lot of investigations are oriented towards this very important goal. Some are already valuable products such as Antarticine-NF3, a glycoprotein produced by the bacterium *Pseudoalteromonas antarctica,* and patented by Spanish researchers. It was found that Antarticine is effective for scar treatment and re-epithelization of wounds. This glycoprotein is now included in some cosmetic regeneration creams. In another context, the antifreeze protein from an eel-like fish from the Arctic ocean is now included in several ice cream brands produced by Unilever. It efficiently prevents ice recrystallisation that can lead to a reduction of taste and texture quality.

Now, at lower temperatures than a few degrees below zero the antifreezes cannot alone, in most cases, protect the organism therefore irrevocably exposed to the lethal process of freezing. Cold-adapted organisms such as microorganisms, fungi, insects, plants and even some vertebrates such as certain species of frogs, have however developed additional tools that enable them either to avoid freezing or even to tolerate freezing. Cryoprotectants are produced by a wide diversity of organisms. These products are small molecules such as glycerol, glucose, sorbitol, trehalose, amino acids and various derivatives that act at various levels (Dia 14): they depress the freezing point of water by the well known colligative effect, they prevent the dehydration of cells when extracellular ice is present by their rapid redistribution across the membrane and they protect proteins against cold-denaturation (Dia 15).

Another tool to fight against freezing, when the process is unavoidable, is to produce special proteins that favour the production of extracellular ice; they are named **INP,** for ice nucleating proteins (Dia 16). It could seem paradoxical for an organism to favour the production of ice at temperatures above the normal freezing point. One has however to keep in mind that extracellular ice is not necessarily harmful for the organisms, the idea is to preserve from freezing the intracellular space. It is the reason why some organisms and in particular some bacteria have developed the capacity to produce ice-nucleating proteins that favour the formation of ice a few degrees or fraction of degree above the normal freezing point of extracellular fluids. The idea is to allow the organism to cope with ice by controlling its temperature of formation, its time scale of formation and the size of the crystals.

As, in this way, the ice is progressively formed the organism has the possibility and the time to produce cryoprotectants and to tolerate a more or less limited dehydration in order to keep liquid its intracellular space (Dia 17). The INPs act in adequately positioning the water molecules enabling in this way their association to form a solid phase. Bacteria such as *Pseudomonas syringae* strains, very common on plants, produce ice-nucleating proteins that are responsible for severe damages to plants by inducing the production of ice at a temperature which would normally never cause freezing. For example, in Florida, *Pseudomonas syringae* hosts of citrus blossoms have been shown to cause the lost of buds and of the citrus crop. Some remedy was found by spraying the whole crop with a suspension of the same strain with however the ice-nucleating protein gene deleted. On the positive side, the ice-nucleating protein from *Pseudomonas syringae* is nowadays heavily used for the production of snow on ski tracks. It favours the production of snow by dramatically improving the yield of conversion of water droplets into snow at temperatures slightly above the normal freezing point. The product is known as “Snomax”.

To complete the arsenal of molecules used to control ice formation, one has also to mention (Dia 18) the **ANP,** for anti-nucleating proteins that are proteins acting as inhibitors of ice nucleation. A few years ago, a protein of 55kd was discovered in a bacterial strain. This protein was used in the non-freezing process preservation of livers and it was shown that the number of dead cells was drastically reduced at a temperature of –3°C.

Now, psychrophilic microorganisms such as bacteria start to be used in the field of Bioremediation. Bioremediation of polluted sites is increasingly considered as a potent tool to clean and detoxify soils and waters contaminated by unwanted residues mainly generated by human activities. Two approaches are used: **Biostimulation** that consists in the supply of nutriments to the indigenous microorganisms to stimulate their metabolic activities and **Bioaugmentation** that is carried out by the addition of exogenous microorganisms specialized in a specific degradation capacity not covered by the indigenous microbial population. These processes have nowadays a well-demonstrated efficiency but seasonal temperature variations represent a major drawback since growth and metabolic activities of indigenous microorganisms are depressed at low temperatures. To counteract these effects, psychrophilic microorganisms appear to be an appropriate alternative using the process of Bioaugmentation, thanks to their ability to efficiently thrive at low and moderate temperatures, and to their capacity to produce exo-enzymes that display a much higher specific activity than their temperate counterparts. Several works in this direction have already been quite successful not only for the decontamination of soils and waters contaminated by fats, proteins, and sugar polymers but also contaminated by more nasty components such as petroleum hydrocarbons. I like just to mention one of the works that was carried out in our laboratory in collaboration with the laboratory of Rosi Margesin from Innsbruck. From more than one thousand isolates, collected from different cold environments, one Gram positive strain, *Arthrobacter psychrolactophilus*, collected by us near the Arctic station of Ny-Alesund in Spitzberg, was found to gather all the required properties to be used in a bioaugmentation process. It is a non-pathogenic strain, resistant to lyophilization and storage at room temperature as a dry powder. This strain induces the clarification and degradation of polluted waters at 10°C (Dia 19).

Cold-adapted enzymes produced by psychrophilic microorganisms constitute an extraordinary potential as new tools in biotechnology thanks to their main properties: a high specific activity at low and moderate temperatures and a relatively elevated thermal instability, two properties that can be useful in biotechnological applications. Of course the interest of a high specific activity is obvious whereas that of a higher thermal instability resides in the fact that in many processes, making use of enzymes, the enzymes have to be inactivated quickly after their action. This can be easily achieved with cold-adapted enzymes by mild heat treatment, preserving in this way product quality and other components present in the reactor. The first cold-adapted enzyme that has been commercialized was an alkaline phosphatase from an Antarctic bacterium collected by us near the Antarctic station Dumont d'Urville in Terre Adelie. The product was developed by a Greek colleague, V. Bouriotis, who was our partner in a European research project on these organisms. It is sold nowadays by New England Biolabs, and is used in molecular biology experiments for the radio labelling of nucleotides.

These products have a high added value and are sold in relatively small quantities but cold-adapted enzymes also cover much larger markets. The main one is certainly the detergent field that nowadays requires enzymes capable of working at low temperatures. Enzymes such as lipases, proteases and amylases are systematically introduced in detergents to improve their efficiency in reducing the amount of chemicals used. This is more friendly for the environment, and the temperature can be decreased to some extent and this is of course very interesting in the context of energy saving. This market is very large and represents 30-40% of all enzymes produced worldwide. Actually, the yeast *Candida antarctica* is used to produce two cold-adapted lipases commercially available from Novo. Cold-adapted proteases and sugar hydrolases from cold-adapted organisms are actively sought after and there is no doubt that quite efficient cold-adapted enzymes will emerge on this market in the near future. The next slide summarizes some of the main applications of cold-adapted enzymes; as you can see activities are quite diversified (Dia 20).

Two applications that have been developed in our laboratory and patented are worth mentioning. A lot of people all over the world and especially in Asia and Africa are intolerant to milk. This is due to the fact that these people do not produce any more an enzyme, named lactase or more scientifically beta-galactosidase, able to split, in the digestive tract, the common sugar present in milk, the lactose, into its two well digestible components: glucose and galactose (Dia 21).

So, for the intolerant people, it is necessary to remove the lactose from any milk-based product. This has been, up to now, achieved with a pretreatment of milk using a mesophilic enzyme. We were lucky enough to discover in an Antarctic strain an efficient beta-galactosidase very active in removing the lactose from milk (Dia 22). The cold-adapted enzyme is indeed nearly 3 times more active than the commercial enzyme at 5°C and can be advantageously used during the storage and transport of milk at low temperatures.

Actually, on this basis, we are also developing in collaboration with a Belgian company a new product that consists in the transformation of the liberated galactose into D-tagatose, a high added value sweetener, which is also a natural monosaccharide with low caloric and glycemic index (Dia 23).

The last example of a commercially available cold-adapted enzyme has also been developed in our laboratory, and is a good example of the successful biotechnological transfer from academic research to industry. Nowadays, a few enzymes are used as conditioners of industrial dough in order to improve bread quality. Xylanases are one of the key ingredients. They are glycosyl hydrolases that degrade the ubiquitous polysaccharide, xylan, present in all flours rendering more accessible for fermentation the starch present in the flour and improving also the mechanical properties of the dough. We have isolated, from a bacterium found in the Antarctic a new class of xylanases (Dia 24), which was tested by our industrial partner Beldem-Puratos, a well-known company producing ingredients for the baking industry.

Baking trials carried out with various bread types have revealed that the psychrophilic xylanase was very efficient in improving bread quality. The data are shown in Dia 25, one can see that the cold-adapted xylanase is much more efficient than a currently used mesophilic xylanase. The efficiency is expressed in terms of loaf volume and cut width on so-called Argentinian breads. Indeed, similar loaf volume and even larger cut width are obtained with the cold-adapted xylanase using an amount of enzyme about a 100 times lower than that of the mesophilic counterpart. The product is now on the market and sold by Puratos Belgium. It is nowadays the psychrophilic enzyme produced at the highest amounts to date and in ton units.

So, in conclusion, psychrophilic microorganisms, known as psychrophiles, constitute for the scientist and from the fundamental point of view a nearly unlimited field of investigation in trying to define and to understand the various molecular adaptations which sustain the successful colonization of extremely cold environments by these microorganisms. In addition they also constitute, in various industrial domains, extremely sought-after alternatives to products originating from their temperate counterparts. These investigations are only in their infancy and the field is widely opened to our curiosity. Most of the work carried out in our laboratory on psychrophilic organisms has been rendered possible thanks to our participation in summer campaigns organized by the French Institute for Polar Research at the Antarctic station Dumont d'Urville in Terre Adélie (Dia 26), I like to acknowledge their support for over 20 years as well as that of collaborators from Belgium and from abroad who were not only partners in European contracts but who also became good friends of mine (Dia 27).