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Psychrophilic Lifestyles: Mechanisms of Adaptation and Biotechnological Tools

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Abstract

Cold-adapted microorganisms inhabiting permanently low temperature environments were initially just a biological curiosity but have emerged as rich sources of numerous valuable tools for application in a broad spectrum of innovative technologies. To overcome the multiple challenges inherent to life in their cold habitats, these microorganisms have developed a diverse array of highly sophisticated synergistic adaptations at all levels within their cells; from cell envelope and enzyme adaptation, to cryoprotectant and chaperone production, and novel metabolic capabilities. Basic research has provided valuable insights into how these microorganisms can thrive in their challenging habitat conditions and into the mechanisms of action of the various adaptive features employed, and such insights have served as a foundation for the knowledge-based development of numerous novel biotechnological tools. In this review, we describe the current knowledge of the adaptation strategies of cold-adapted organisms and the biotechnological perspectives and commercial tools emerging from this knowledge. Adaptive features and, where possible, applications, in relation to membrane fatty acids, membrane pigments, the cell wall peptidoglycan layer, the lipopolysaccharide component of the outer cell membrane, compatible solutes, anti-freeze and ice-nucleating proteins, extracellular polymeric substances, biosurfactants, chaperones, storage materials such as polyhydroxyalkanoates and cyanophycins, and metabolic adjustments are presented and discussed.

Keywords: Psychrophiles ● Cell Envelope ● Cryoprotection ● Enzymes ● Chaperones ● Metabolic Adjustments

Introduction

Habitats of permanently low temperature dominate the Earths biosphere and have been successfully colonised by a wide variety of organisms collectively termed psychrophiles, or cold-adapted organisms. Microorganisms prevail in these cold habitats, especially bacteria, archaea, yeasts, cyanobacteria and protists, but microalgae and viruses are also common, and the ability of psychrophilic microorganisms to thrive in such environments reveals an adaptation to their habitat (Margesin and Collins 2019). It demonstrates a capacity to surmount the constraints inherent to life at low temperatures, which can only be achieved via a complex range of structural and functional adaptations of all cellular components, from the level of single molecules up to whole cells and even complete ecosystems (D'Amico et al. 2006; De Maayer et al. 2014; Morgan-Kiss et al. 2006; Siddiqui et al. 2013).

Since the first reports of cold dwelling organisms (Forster 1887), various comparative physiology, microbiology, biochemistry, biophysics and molecular-based approaches have been used in identifying the physiological adaptations, biogeographical distribution, diversity and ecological roles of cold adapted organisms. More recently, various new emerging technologies, namely various 'omics' approaches including genomics, transcriptomics, proteomics and metagenomics, have broadened our understanding of these; enabling identification of novel adaptation mechanisms and biotechnological tools, detection of key functions and acquirement of a more global view of the structures and roles of microbial communities in cold ecosystems (Barauna et al. 2017; Bowman 2017; Koh et al. 2017; Raymond-Bouchard et al. 2018; Singh et al. 2014; Tribelli and Lopez 2018).

Life in the cold is characterised by a multitude of stresses in addition to low temperatures. Indeed, besides a reduced thermal energy, low temperatures also provoke further physicochemical constraints such as an increase in solvent viscosity and solubility of gasses, namely an increased solubility of oxygen and reactive oxygen species (ROS), a decrease in the solubility of solutes and nutrients, reduced diffusion, increased osmotic stress, desiccation and ice formation. Furthermore, many cold ecosystems are often also characterised by fluctuating environmental conditions and/or multiple additional ecological limiting factors, including low nutrient levels, high salinity, oxidative stress, freeze-thaw cycles and low water activity. In the deep sea and sub-glacial environments for example, microbial populations are additionally subjected to high pressure stress. Extremes of light exposure are also common in many cold ecosystems, from high light and UV irradiation in high altitude cold environments, to low light exposure in ice-covered lakes and deep within ice layers and permafrost. Thus,

life in the cold biosphere requires a multitude of synergistic adaptations, not only to respond to the low temperature challenge but also to the multitude of other interacting stresses imposed by the particular environmental conditions.

Cold-adapted microorganisms have responded to the low temperature challenge via the development of a cold-adaptation toolkit constituted by a number of elegant physiological and structural adaptations, many of which are only beginning to be understood. Importantly, many of these tools serve overlapping functions and may be used to respond to the various different challenges or combinations of challenges encountered in a specific cold habitat. In fact, a common problem encountered in characterising cold-adapted microorganisms is in unravelling the different interacting parameters and deciphering the precise function of a specific trait, whether it is a specific response to low temperatures or to another (or other) environmental stressor(s) common in the particular habitat. Importantly also, microorganisms do not always make use of all tools in their 'cold-adaptation' toolbox, and in fact, each specific organism will use its own strategy, or combination of strategies, depending on its own specific requirements and on the environmental parameters and microbial community structure.

In the present review, we will discuss various adaptation strategies used by cold-adapted microorganisms to enable life in the harsh environmental conditions of their habitat, and show how an understanding of these different strategies is leading to the development of various novel tools of commercial interest. See Table 1 for an overview of the cold-adaptation tools with commercial potential. In this review, the various stresses to which microorganisms in cold habitats are exposed will be presented, the adaptation strategies developed by psychrophiles to cope with the challenges and their underlying mechanism of action discussed, and examples of the biotechnological tools being developed from these will be given.

Cell Envelope

The cell envelope and its various components serve the critical functions of providing shape, support and protection as well as regulating the movement of substances into and out of cells. It protects cells from the surroundings and against turgor pressure, acts as a semipermeable membrane functioning in nutrient uptake, product export and solute transport, and participates in cell division, sensing, signaling and adhesion. Low temperatures adversely affect cell envelope properties and functions by leading to reduced membrane fluidity, permeability and diffusion rates, in addition to deceasing mobility and function of embedded proteins,

increasing turgor pressure and even leading to physical cell rupture by ice formation and/or freeze-thaw cycles. While the cold adaptation traits of the cell membrane have been known for some time, the adaptation strategies of the other envelope components; the outer membrane, peptidoglycan layer and even exterior cell coatings; are now also beginning to be understood.

Cell Membrane

Homeoviscous adaptation of cell membranes to low temperatures is accomplished in cold-adapted microorganisms via modification of the fatty acid composition of the lipid bilayer (D'Amico et al. 2001; Siddiqui et al. 2013). Cells mainly increase the content of unsaturated fatty acids, but increases in the content of short chain, methyl branched and/or cis-isomeric fatty acids are also common (Chintalapati et al. 2004; Russell 1997; Russell 2008). These fatty acids disrupt the packing order and reduce the packing density of the phospholipid bilayer, leading to a lowering of the liquid phase to gel phase transition temperature and maintenance of functional fluid bilayers even at low temperatures. Indeed, in agreement with this cold adaptation strategy, an overrepresentation and upregulation of genes encoding various proteins involved in membrane biogenesis and fatty acid synthesis as well as in fatty acid desaturation (desaturases, which simultaneously also protect against ROS), production of branched chain fatty acids (KAS-II, KAS-III) and cis-isomerisation (fatty acid cis/trans isomerases) have been reported for numerous cold-adapted organisms (De Maayer et al. 2014; Goordial et al. 2016; He et al. 2015; Medigue et al. 2005; Methé et al. 2005). In addition, an increased genome content of genes for proteins involved in the degradation of membrane rigidifying molecules naturally present in the environment has also been observed (Medigue et al. 2005) and may serve as a further means for reducing membrane rigidity at low temperatures.

Finally, in relation to the other major components of the cell membrane, i.e. the proteins embedded in the lipid bilayer, an upregulation of membrane transport proteins has been observed in some psychrophiles and is believed to act in counteracting the reduced diffusion rates and transport inherent to low temperatures (Bakermans et al. 2007; De Maayer et al. 2014).

Cell Membrane LC-PUFAs

While the role of unsaturated fatty acids with low numbers of double bonds in membrane cold adaptation is well understood, the precise role of long chain polyunsaturated fatty acids (LC-PUFAs) is still under discussion. These have been considered part of the cold adaptation tactic for maintaining membrane fluidity and indeed some LC-PUFAs, such as

eicosapentaenoic (EPA, 20:5ω3), docosahexaenoic (DHA, 22:6ω3) and arachidonic (ARA, 20:4ω6c) acid, are preferentially distributed in marine organisms, with higher levels being often produced at lower temperatures (Feng et al. 2014; Okuyama et al. 2008; Yoshida et al. 2016). In line with this, comparative genomics has indicated that the polyketide synthase gene cluster (*pfaA,B,C,D* and *E*), responsible for the synthesis of these, is largely restricted to marine organisms (Shulse and Allen 2011). Nevertheless, recent studies have indicated that, rather than serving in cold adaptation, LC-PUFAs may primarily serve antioxidative functions, protecting against reactive oxygen species naturally present at high levels in the marine environment and augmented at low temperatures (Nishida et al. 2007; Okuyama et al. 2008). It is believed that LC-PUFAs act as membrane shields, forming more hydrophobic interfaces between the lipid bilayers and thereby preventing entry of ROS such as H_2O_2 into the cells. Therefore, their presence in low temperature environments may not be a direct response to the cold, *per se*, but a response to other stress(es) inherent to low temperatures, in this case, oxidative stress. Interestingly, in addition to their antioxidative function, functions as chaperones for membrane proteins, in efflux processes and in cell division have also been proposed (Okuyama et al. 2008; Yoshida et al. 2016).

PUFAs have considerable nutritional and pharmaceutical value (Yoshida et al. 2016). They are components of neuronal and thrombocytes cells, neutrophils and monocytes and are found at high concentrations in the brain and retina. Importantly also, they are precursors of eicosanoid signaling molecules and endocannabinoid neurotransmitters (Ochsenreither et al. 2016). As such they have a wide array of functions in human health; from regulating the cardiovascular system, immune system and inflammation, to participating in the development and proper functioning of the brain, eyes and central nervous system. Some PUFAs such a linoleic acid (LA) and α-linolenic acid (ALA) are essential fatty acids which have to be obtained from the diet while LC-PUFAs such as DHA and ARA are recommended in infant diets. Indeed, a balanced intake of various ω-3 (ALA, EPA and DHA) and ω-6 PUFAs (LA, ARA) is recommended as part of a normal healthy diet. Fish oils are the main source of PUFAs, especially LC-PUFAs, but problems associated with flavor, allergies, effects on global fish stocks and presence of various environmental pollutants has resulted in the search for alternative sources, including microorganisms. However, membrane levels of phospholipid PUFAs in microorganisms are in general too low for commercial production. On the other hand, oleaginous microorganisms, which produce PUFAs as components of triacylglycerol in stored oils, and which can accumulate lipids at concentrations of up to 80% of dry biomass weight, may prove suitable sources. In this sense, cold-adapted organisms,

with increased levels and/or varied distributions of PUFAs may be of interest, especially for animal, and in particular aquaculture feed. Indeed, marine microalgae are potent producers of various LC-PUFA and the marine dinoflagellate *Crypthecodinium cohnii* is used in commercial production of DHA as an infant formula additive. In addition, filamentous fungi of the genus Mortierella, many of which are cold adapted, and various cold-adapted yeasts are also reported to accumulate high levels of PUFAs (Amaretti et al. 2010; Ochsenreither et al. 2016) and may prove potent commercial sources.

Membrane Pigments

Pigments, and especially carotenoids (pigmented polyisoprenoid hydrocarbons), have also been suggested to play a role in the modulation of cell membrane fluidity. Pigment production is common in psychrophilic microorganisms, being reported in isolates from ice cores and glaciers (Shen et al. 2018), marine surface waters (Dieser et al. 2010) and high altitude soils (Pandey et al. 2018). A few reports have shown an increased production of these at low temperatures (Chattopadhyay et al. 1997; Pandey et al. 2018), with a preference for polar carotenoids (Chattopadhyay et al. 1997; Jagannadham et al. 2000), yet a recent study identified a decrease in pigmentation upon slowly reducing the temperature for a number of Arctic bacteria (Singh et al. 2017). Interestingly, polar carotenoids are believed to enhance membrane rigidity and hence increased concentrations should be counterproductive to cold-adapted organisms at low temperatures. It has been suggested that these may serve in homeoviscous adaptation by counterbalancing the fluidizing effects of the unsaturated fatty acids and stabilizing the membrane (Jagannadham et al. 2000). Furthermore, these pigments have been shown to also play a number of other important roles, including, in photoprotection (acting, in conjunction with other molecules such as scytonemin and mycosporine-like amino acids, as light screeners protecting against high light and UV radiation common to many cold habitats,), as antioxidants (protecting against ROS, also commonly produced in low temperature and/or high light environments), as light harvesters (in photosynthetic microorganisms), and even as antimicrobials (Pandey et al. 2018). Importantly, a potential role as cryoprotectants, imparting resistance to freeze-thaw cycles, has also been demonstrated (Dieser et al. 2010). These various overlapping functions would variably influence pigmentation levels and could lead to the conflicting pigmentation levels observed at low temperatures. Further studies are therefore required to unravel the true role and specific functions of membrane pigments in cold-adapted organisms and their relationship to pigment type and specific structure, as well as the precise effects of the different pigments on membrane properties.

Numerous carotenoids have various beneficial health effects (Kirti et al. 2014). They have been shown to be effective antioxidants and pro-vitamins (vitamin A), and to be important for healthy growth and development, in maintenance of the immune system, and in eye health. In addition they can be used as natural food colorants, in skin care, in sunscreen products and as precursors of chemicals for fragrance products, as well as in animal/aquaculture feed to impact desired colours on products, e.g. egg yolks, salmon. Cold-adapted organisms with enhanced levels of various different carotenoids may prove interesting microbial sources of carotenoids for such commercial applications.

Cell Wall: Peptidoglycan Layer

Cold adaptation of other cell envelope components has been much less investigated than the cell membrane but recent studies have begun to unveil possible strategies. In relation to adaptation of the cell wall; an upregulation of peptidoglycan biosynthesis genes and a thickened peptidoglycan layer at low temperatures has been reported in some cold-adapted bacteria (Mykytczuk et al. 2013; Rodrigues et al. 2008). Similarly, *Planococcus halocryophilus* Or1 also displays a thickened outer cell surface, but this is achieved by a rather unique mechanism involving extracellular cell wall associated hydrophobic encrustations composed of peptidoglycan, calcium carbonate and choline (Mykytczuk et al. 2013). Such strategies for thickening and strengthening the cell outer surfaces would obviously lead to a reinforced physical barrier which could protect psychrophiles against cell disruption by ice formation, freezing-thawing, and/or increased osmotic pressure at low temperatures.

Cell Wall: Outer Membrane

Cold adaptation traits have also been observed in the outer membrane layer of the cell wall of gram negative bacteria, and namely in the lipopolysaccharide (LPS) structure which accounts for ~75% of this layer (Corsaro et al. 2017). Only a few cold adapted LPS structures have been analyzed to date but it appears that most produce LPS solely as rough LPS (i.e. without the specific O-chain component) of shortened length at low temperatures (Carillo et al. 2013; Carillo et al. 2011; Corsaro et al. 2017; Corsaro et al. 2002). Many more cold-adapted LPS structures need to be analyzed to determine the extent of these observations, but it has been suggested that these alterations may increase outer membrane flexibility and stability at low temperatures (Corsaro et al. 2017). As regards to the other LPS components, a similar strategy to that used for the lipid bilayer of cell membranes is observed for the lipid A component of LPS, i.e. increased content of short chain and/or unsaturated fatty acids to increase fluidity, while a high negative charge of the core oligosaccharide component has been suggested to be

important in sequestration of divalent cations common in many cold environments (Casillo et al. 2017b; Corsaro et al. 2017; Sweet et al. 2015). Finally, in common with that observed for other cell envelope components, transcriptome analyses have shown an upregulation of genes involved in biosynthesis of outer membrane components, with LPS biosynthetic genes (mainly glycosyltransferases) and outer membrane proteins being upregulated at low temperatures (De Maayer et al. 2014; Frank et al. 2011; Gao et al. 2006). In agreement with this, a recent study showed how mutation of a core LPS glycosyltransferase gene (*wapH*) impaired growth of an Antarctic bacterium at low temperatures (Benforte et al. 2018).

Cryoprotection

Subzero temperatures provoke ice formation which can lead to cryoinjury, osmotic stress, dehydration and even cell rupture and death. In the natural environment, onset of ice formation is usually delayed in the cell interior as compared to the cell exterior due to the lower volume and densely packed, highly crowded nature of the former. Intracellular ice crystal formation, which is normally lethal to cells, can indeed occur when temperatures decrease at high rates, but in the natural environment cooling is usually relatively slow and ice formation is thus mostly restricted to the extracellular space (Fonseca et al. 2016). Extracellular ice formation, which can lead to physically damaging membrane fracturing, occurs with exclusion of solutes and removal of available liquid water. This leads to elevated extracellular solute concentrations and provokes intra/extracellular osmotic imbalances. In turn, this leads to stresses related to osmotic shrinkage and dehydration of the cell interior, negatively affecting cell function and survival, but also preventing intracellular ice formation and instead leading to a non-crystalline amorphous (colloidal glassy) state with inhibition of cell metabolism (Fonseca et al. 2016). In addition, at relatively high subzero temperatures, in partially frozen environments subjected to freeze-thaw cycles and during temperature fluctuations, cells can also be subjected to harmful ice recrystallization stress, a thermodynamically driven process causing ice crystal coalescence and growth of large, fatally damaging ice crystals at the expense of smaller crystals (Bar Dolev et al. 2016b). Cold-adapted microorganism respond to these multiple freezing related detrimental challenges by production of a variety of novel tools including, compatible solutes, ice-binding proteins (antifreeze and ice-nucleating proteins), extracellular polymeric substances (EPS) and/or biosurfactants.

Compatible Solutes

Compatible solutes are low molecular mass, non-toxic organic osmolytes. Many cold-adapted microorganisms have an increased genome content of compatible solute biosynthesis, uptake and degradation genes and often accumulate up to molar concentrations of various different compatible solutes; with glycine betaine, trehalose, glycerol, sucrose, sarcosine, mannitol and sorbitol being commonly reported (Ghobakhlou et al. 2015; Goordial et al. 2016; Mykytczuk et al. 2013). Accumulation of these organic osmolytes contributes to restoring osmotic balance and thereby counteracts water loss and cell shrinkage during freezing. In addition, they depress the freezing point of solution and, importantly also, the intracellular colloidal glass transition temperature (Tg) (Fonseca et al. 2016). In fact, a recent study showed reductions of as much as 30 ºC in the Tg of the cytoplasm of bacterial cells and improved post thaw viability upon glycerol addition (Fonseca et al. 2016). Furthermore, compatible solutes are also believed to be involved in scavenging free radicals and, due possibly to their preferential exclusion from protein surfaces and/or water entrapment effects, compacting effects on proteins and destabilizing effects on the unfolded state, also play roles in preventing protein aggregation, enhancing protein folding and stabilising proteins and membranes at low temperatures.

In relation to applied aspects, various compatible solutes are commonly used in the stabilisation, preservation and cryopreservation of diverse biological materials, ranging from enzymes to whole cells and tissues. In addition, their use in increasing the freshness and stability of foods, in cosmetics and skin care products, as well as in extending the growth performance of plants in saline, dry and low temperature environments has also been investigated (Wani et al. 2013).

Ice-Binding Proteins: Antifreeze Proteins

Antifreeze proteins (AFPs), also known as ice structuring or thermal-hysteresis proteins, are noncolligative biological antifreezes which can bind to ice and inhibit ice growth and recrystallisation (for recent reviews on AFPs see e.g. Bar Dolev et al. (2016b); Lorv et al. (2014) and Voets (2017)). They were first identified in the blood of Antarctic fish but have since been reported in various bacteria, fungi, diatoms, plants, insects and crustaceans. Various types of AFPs of diverse structure exist in nature, being frequently glycosylated and/or lipidated and varying in size from ~2kDa to ~50 kDa, with a 1.5 MDa multi-domain ice adhesion AFP being reported for the Antarctic bacterium *Marinomonas primoryensis* (Bar Dolev et al. 2016a). AFPs are believed to function by irreversibly binding to specific ice crystal planes, thereby blocking secondary nucleation events and shaping a unique ice morphology. Due to the Kelvin effect, ice surface expansion between the adsorbed AFPs

leads to local curvature of the ice face which is energetically unfavourable for further ice growth. This leads to thermal hysteresis (TH) in which the freezing point is depressed below the equilibrium melting point to create a thermal-hysteresis gap in which ice crystal growth is halted. TH activities from ~0.1 ºC to as high as ~13 ºC have been measured (Duman et al. 2004; Voets 2017) and these activities can be increased in the presence of other AFPs, solutes and ions. AFPs are also effective ice recrystallisation inhibitors (IRI), being frequently more effective in IRI (often requiring only sub-µM concentrations) than in TH (often requiring mM concentrations). Indeed, IRI is believed to be the primary function of many secreted AFPs from cold-adapted organisms and AFPs from Antarctic algae and glacier ice bacteria were shown to effectively stabilise brine pockets and contribute to the preservation of a liquid environment in the cell vicinity (Raymond et al. 2008). Finally, in addition to their TH and IRI activities, AFPs are also believed to help stabilize cell membranes and protect structural integrity, and, in some cases, via their adhesion domains and ice binding function, are thought to play a role in positioning cells on ice so as to enhance access to oxygen and nutrients in the phototrophic zone (Bar Dolev et al. 2016a; Lorv et al. 2014; Voets 2017).

AFPs are already being applied in the food industry for improved food preservation during freezing. They are used to preserve a smooth texture in ice creams and frozen yoghurts and can reduce drip loss in frozen meats and fish, enhance frozen dough bread quality, and improve post-freezing quality of currently difficult to freeze foods such as fruit and vegetables (Muñoz et al. 2017; Regand and Goff 2006; Voets 2017). They also have obvious potential as cryoprotective agents and have been shown to improve cryopreservation efficiency and post thaw viability of various types of biological materials. Furthermore, the ability of certain AFPs at high concentrations to induce formation of needle-like structures which penetrate and destroy cells is being investigated for use in cryosurgery, e.g. in tumor ablation. They are also being developed for use in improving freeze tolerance of crops and aquaculture fish, in gas hydrate inhibition in the petroleum industry and in anti-freeze/de-icing materials via surface coating of materials for prevention/decreasing of ice formation, e.g. on aircrafts, power lines, roads (Bar Dolev et al. 2016b; Lorv et al. 2014; Voets 2017).

Ice-Binding Proteins: Ice-Nucleating Proteins

Ice-nucleating proteins (INPs) are large membrane bound proteins that facilitate ice formation. They initiate heterogeneous ice crystallisation at high subzero temperatures and are proposed to act by providing a template for the ordering and stabilisation of water molecules in an ice-like structure (Lorv et al. 2014; Pandey et al. 2016; Pummer et al. 2015). This lowers the activation energy barrier for freezing and nucleates ice growth at temperatures as high as -

2 ºC (Li et al. 1997). INPs are produced by diverse organisms, most commonly as large, extracellular, repetitive multimeric aggregates, with larger complexes enabling higher activity (Bar Dolev et al. 2016a). In cold-adapted microorganisms, INPs are believed to counteract low temperature damage by directing ice nucleation to the extracellular space (Lorv et al. 2014) which can prevent formation of lethally damaging intracellular ice via removal of the available intracellular liquid water as discussed above. In addition, INPs favour development of small extracellular crystals which are less damaging to the cell than large crystals while the release of latent heat of crystallization during the freezing process could also be beneficial in preventing further temperature decreases (Pummer et al. 2015). In addition to their role in cold adaptation, INPs also play a role in nutrient mining by many plant pathogenic organisms where they are used to induce frost damage in plants and enable access to nutrients.

INPs are presently being commercialised for artificial snow production (Cochet and Widehem 2000) but are believed to have a number of other potential applications. These include, reducing freezing energy costs in the food industry (Li et al. 1997), freeze-concentrating beverages, in freeze–thaw valves of microfluidic devices (Gaiteri et al. 2017), as anchoring motifs for cell surface display applications (Jung et al. 1998), as well as in cloud seeding for climate control (Pummer et al. 2015).

Extracellular Polymeric Substances

Extracellular polymeric substances (EPS) are multifunctional, high molecular weight biopolymer complexes secreted by various organisms into their local environment. They are large, complex, highly diverse architectural structures composed principally of carbohydrates (homo- or hetero-polysaccharides), but also proteins and lower concentrations of nucleic acids, lipids, phenols and humic substances. EPS are produced by a wide variety of organisms and are found either attached to the cells outer surface or released into the surrounding environment. They form hydrated gels that play an important role in the formation of biofilms and in the modification of the physical, chemical and biological characteristics of the cell environment. Indeed they are believed to have multiple functions, including in cell adhesion and nutrient scavenging, but are also thought to be important in protective functions such as osmoprotection, ROS scavenging, extracellular protein protection and even in cryoprotection. For reviews of the subject see e.g. Deming and Young (2017) and Ewert and Deming (2013), and references therein.

In relation to cold-adaptation, metagenomics studies have identified numerous genes for EPS biosynthesis in both Antarctic and Arctic ice shelf ponds (Varin et al. 2012) and cold-adapted bacteria have been found to produce high concentrations of EPS at low and especially

subfreezing temperatures (Caruso et al. 2018; Feng et al. 2014; Marx et al. 2009; Mykytczuk et al. 2013). The hydrated EPS gel matrix is believed to protect against low temperatures by forming a protective shell around cells which acts as a diffusion barrier to solutes and a physical-like barrier to ice formation (Caruso et al. 2018; Deming and Young 2017; Ewert and Deming 2013; Krembs et al. 2011). This diminished solute diffusion limits freezing induced osmotic stress and desiccation damage. Furthermore, the gel like state of EPS reduces the available free energy for ice nucleation while solute exclusion to the surrounding liquid phase impedes ice crystal growth in this, thereby protecting cells from ice damage and increasing the available habitable liquid space. Interesting also, recent studies have indicated ice binding functions and IRI activity similar to AFPs (described above) for EPSs produced by cold adapted organisms (Casillo et al. 2017a). As compared to other cold-adaptation traits, EPS are much less well studied and the important role of these in coping with low temperatures is only beginning to be unraveled. Further studies are warranted to gain a better understanding of the different interrelated cold adaptation roles and physiological and ecological functions of EPS and their relationship to composition and structure.

Microbial EPS and their various components, as biodegradable, biosustainable, non-toxic and biocompatible biopolymers, have been recognized as potential alternatives to chemical polymers in a number of applications, ranging from the pharmaceutical to the cosmetics and food industry and environmental biotechnology. Their capacity to adsorb heavy metals and organic pollutants combined with their flocculation properties for removal of suspended solids, organic matter etc. are advantageous for use as bioflocculants and bioabsorbants in soil and water bioremediation and decontamination as well as in wastewater and sludge treatment (More et al. 2014). Their use, especially the polysaccharide component, as cryoprotectants is also under study and an ability to improve the freeze-thaw survival of various Antarctic bacteria has already been shown (Caruso et al. 2018). The high emulsifying activity of some EPS indicates potential as bioemulsifiers (Caruso et al. 2018) while their role as bioadhesives has also been indicated (Muralidharan and Jayachandran 2003). In the food industry, EPS polysaccharides can be used as thickening agents and emulsifiers, conferring improved texture and stability to foods and beverages while, more recently, beneficial effects of EPS on human health via potential immunomodulatory, anticoagulant, anti-inflammatory, and antioxidant capacities have been suggested (Colliec Jouault et al. 2001; Leroy and De Vuyst 2016). Furthermore, their use in tissue engineering has even been suggested (Kumar et al. 2018). A number of microbial EPS carbohydrates, e.g. xanthan gum, gellan gum and dextran, have already been commercialised for a number of applications, but studies of cold-adapted

EPS components with high potential for identification of unique structures and functions could be expected to reveal novel products with novel applications.

Biosurfactants

Biosurfactants are surface active amphiphilic compounds of microbial origin which reduce surface and interfacial tension between liquids, solids and gases. Generally they are of low molecular weight, in contrast to bioemulsifiers, such as for example EPS, which tend to be of high molecular weight, and can be composed of sugars, amino acids, fatty acids and/or functional groups such as carboxylic acids. They are structurally diverse compounds but the most commonly reported are glycolipids (rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol lipids etc.), lipopeptides (surfactin, iturin, fengycin, viscosin etc.), phospholipids, fatty acids and neutral lipids. In nature, they are believed to play roles in enhancing the bioavailability of poorly soluble hydrophobic substrates, in regulating biofilm structure and surface attachment/detachment, in bacterial pathogenesis and quorum sensing, as well as acting as antibacterial and antifungal agents. In relation to cold adaptation, a glycolipid biosurfactant isolated from an Antarctic yeast was shown to have IRI activity (Kitamoto et al. 2001), while a role for biosurfactants as osmolytes has also been suggested (Perfumo et al. 2018). Interestingly, even though biosurfactant production appears to be widespread among cold adapted organisms (Gesheva et al. 2010; Malavenda et al. 2015; Perfumo et al. 2018; Vollú et al. 2014), studies investigating their potential role in cold adaptation are scant. Further studies are obviously required to clarify whether these compounds constitute part of the cells toolkit for adapting to cold and to elucidate their precise role and mechanism of action in this.

Biosurfactants offer non-toxic, sustainable, biodegradable, and ecofriendly alternatives to the chemical surfactants currently used in a huge variety of applications as diverse as bioremediation, in cleaning agents, cosmetics, pharmaceuticals, agriculture and the petroleum industry. Interest in ‘cold’ biosurfactants has only recently been initiated, but already potential in a number of applications has been demonstrated; they have been shown to have potential for use as antiagglomerants in ice–water slurry technologies (Kitamoto et al. 2001), as low temperature detergents (Perfumo et al. 2018), as fuel additives to improve the flow properties of biodiesel and diesel at low temperatures (Madihalli et al. 2016), and for recovery of natural gas hydrates in gas hydrate technologies (Arora et al. 2016; Perfumo et al. 2018).

Enzymes

One of the principal challenges that cold-adapted microorganisms have to contend with is the negative effect of low temperatures on reaction rates. All reactions, including enzyme catalysed reactions, are influenced by temperature according to the Arrhenius Law wherein any decrease in temperature induces an exponential decrease in the reaction rate (Arrhenius 1889). Indeed, with most non-adapted enzymes, a reduction in temperature from 37 °C to 0 °C results in a 16-80 fold reduction in activity. In contrast, most enzymes produced by psychrophilic organisms are adapted to their environment and maintain high specific activities enabling appropriate metabolic rates in their cold habitats (for recent reviews see e.g. Collins et al. (2002a); Collins and Gerday (2017); Fields et al. (2015); Gerday (2013); Santiago et al. (2016) and Siddiqui (2015)).

With few exceptions, e.g. Oswald et al. (2014); Roulling et al. (2016), most cold-adapted enzymes studied to date are characterised by a higher catalytic activity at low to moderate temperatures, a shift in the optimum temperature for activity towards lower temperatures and a decreased stability as compared to mesophilic and thermophilic homologous enzymes. These 'cold enzymes' successfully reduce the activation energy, ΔG^*, and temperature dependence of reactions through a reduced activation enthalpy, ΔH^*, thereby implying a decreased number and/or strength of enthalpic interactions that are broken during the catalytic cycle (Lonhienne et al. 2000). This is accompanied by a lower, more negative activation entropy value, ΔS^*, suggestive of greater re-ordering during activation, which, in conjunction with a more negative heat capacity change during activation, suggestive of a higher heat capacity and thus increased vibrational modes for the ground state (Arcus et al. 2016), and a lower substrate affinity (higher K_m) (Collins et al. 2002a), as compared to mesophilic and thermophilic homologs, implies a more disordered enzyme-substrate (ES) ground state for psychrophilic enzymes. Such a disordered state could give rise to an ES complex of reduced stability and higher free energy and hence lead to the observed reduced ΔG^* and enhanced catalytic rates for these enzymes.

A number of different studies using a range of techniques have investigated this disorder, or structural flexibility, in various psychrophilic enzymes. It is important to note here that protein flexibility is a complex parameter which is difficult to measure and further in-depth comparative studies characterising the specific amplitudes, time frames, regions involved and temperature dependence of protein motions are called for, with use of powerful techniques such as NMR being key to better understanding this parameter. Nonetheless, notwithstanding current limitations, it is now generally accepted that psychrophilic enzymes do indeed display an increased structural flexibility and that they have overcome the low temperature challenge

by increasing the plasticity or flexibility of specific regions (at or near the catalytic site) or of the whole protein. This flexibility enables the conformational changes necessary for activity at a low energy cost, but also leads to a decreased stability. At the structural level, this increased flexibility is mainly achieved by a reduction in the number and/or strength of stabilising interactions such as H-bonds, salt bridges, aromatic interactions, disulphide bonds, ion binding sites and/or a weakening of the hydrophobic core. In addition, a reduction in the proline content and increase in glycine content, especially in loop regions, and a greater exposure of hydrophobic residues have also been reported. Such modifications would allow for the increased flexibility necessary for increased low temperature activity but would also lead to the reduced stability inherent to most psychrophilic enzymes (Collins and Gerday 2017). Importantly, it has been found that different enzymes can use different structural adaptation strategies, with each enzyme using its own specific strategy, employing any one or combination of these modifications depending on its own specific characteristics, its environment and its requirements.

The inherent characteristics of a high activity at low to moderate temperatures and a reduced stability of psychrophilic enzymes offers many advantages in a variety of applications. They can be used to enhance the efficiency and economics of low to moderate temperature processes, reduce the process temperature and hence improve process economics and environmental impact, and enable more simplified enzyme inactivation. Cold enzymes are already being employed in all three sectors of the industrial enzymes markets; food, feed and technical, and are expanding into new areas in pharmaceutical and fine chemical synthesis. For a recent in-depth review see Barroca et al. (2017a). Some of the better known commercial successes include a cold-adapted lipase used in the organic synthesis of various pharmaceutical, cosmetic and flavor compounds (Kirk and Christensen 2002); a xylanase for enhancing bread quality (Barroca et al. 2017b; Collins et al. 2012; Collins et al. 2006; Collins et al. 2002b; Dutron et al. 2010-2012); various hydrolases in detergents for low temperature cleaning (Sarmiento et al. 2015), and various enzymes (alkaline phosphate, nuclease and uracil-DNA N-glycosylase) used in molecular biology for their high activity and ease of inactivation (Barroca et al. 2017a).

Chaperones

Protein and RNA/DNA chaperones, which facilitate efficient protein and RNA/DNA folding, respectively, play important roles in counteracting low temperature stabilisation of RNA and DNA secondary structures as well as protein misfolding and aggregation. DNA and RNA

chaperones are important in maintaining efficient transcription, translation and DNA replication. They are transiently produced as part of the cold-shock response in mesophilic and thermophilic microorganisms but are often continuously overexpressed as cold-acclimation proteins or up-regulated at low temperatures in psychrophiles (Lim et al. 2000). In relation to protein chaperones in cold-adapted microorganisms, continuous overexpression, upregulation of production, and production of cold adapted variants, but also no overexpression and cold repression, have been reported (Ferrer et al. 2003; Godin-Roulling et al. 2015). Importantly, while protein misfolding and precipitation are believed to be strongly reduced at low temperatures, due mainly to a weakening of hydrophobic interactions, proteins at the low range of the temperature spectrum are faced with another phenomenon; cold denaturation (Collins and Gerday 2017; Romero-Romero et al. 2011). Cold denaturation is thought to be due to a preferential hydration and weakening of hydrophobic and ionic interactions at low temperatures and intriguingly it appears that psychrophilic enzyme may be more susceptible to this than their higher temperature adapted homologs (D'Amico et al. 2003). Nevertheless, the underlying basis for this is still not fully understood and future studies should address this.

Protein and RNA/DNA chaperones have potential for use in recombinant protein production, in the low temperature production of proteins prone to aggregration/misfolding and an *E. coli* host producing protein chaperones from an Antarctic bacterium has already been commercialised for such a use (Ferrer et al. 2003).

Metabolic Adjustments

Recent studies making use of modern ‘omics’ approaches such as genomics, transcriptomics and proteomics have revealed a number of additional traits common to various cold adapted microorganisms (see e.g. Tribelli and Lopez (2018) for a recent review. In particular, studies have indicated various metabolic adjustments at low temperatures, including a down regulation of primary metabolism pathways and substitution with abridged or alternative secondary pathways, as well as accumulation and metabolism of reserve compounds. Oxidative metabolic processes, namely, glycolysis, the pentose phosphate pathway, the TCA cycle, and/or the electron transport chain, but also pathways involving metal ions and molybdopterin metabolism are reported to be downregulated at low temperatures in some psychrophiles (Medigue et al. 2005; Piette et al. 2011; Tribelli et al. 2015). While still poorly understood, studies have indicated their substitution with alternative/shortened pathways such as the glyoxylate, methyglyoxal and 2-methylcitrate cycles, the ethanol oxidation pathway,

589 acetate metabolism and propionyl-CoA catabolism (Ayala-del-Río et al. 2010; Tribelli and
590 Lopez 2018). The actual alternative pathway(s) used being dependent on the organism and its
591 ecological niche. Such a strategy of metabolic reprogramming may alleviate oxidative stress
592 inherent to low temperatures by avoiding ROS producing pathways but may also be important
593 in energy conservation and long term survival.
594 Screening studies have indicated a high content of polyhydroxyalkanoate (PHA) producers in
595 cold habitats (Ciesielski et al. 2014; Goh and Tan 2012; Pärnänen et al. 2015) and many
596 psychrophiles can accumulate and degrade PHA (López et al. 2009; Methé et al. 2005; Ting
597 et al. 2010) and/or cyanophycin-like (Duchaud et al. 2007; Methé et al. 2005; Vollmers et al.
598 2013) compounds. These compounds can act as dynamic reserves of carbon, nitrogen,
599 reducing equivalents and energy in cells and are thought to be important in overcoming low
600 temperature challenges to carbon and nitrogen uptake. Nevertheless, functions in
601 cryoprotection, oxidative stress resistance, maintenance of cellular redox balance and cell
602 motility have also been suggested for PHAs (Methé et al. 2005; Tribelli and Lopez 2018). In
603 relation to applied aspects, these compounds, or derivatives, are suggested as non-toxic,
604 biodegradable, biocompatible biopolymers to replace petrobased polymers. PHAs are a family
605 of microbial polyesters with interesting thermoplastic and elastomeric properties and potential
606 for application in almost all areas of the conventional plastics industry. For reviews on PHA
607 applications see e.g. Chen (2009) and Singh et al. (2019). A specific focus of application has
608 been in the medical (tissue engineering, bio-implants, drug delivery, sutures etc.), and fine
609 chemical synthesis fields (chiral starting materials for the synthesis of antimicrobials,
610 vitamins, fragrances etc.), but also in materials (packaging, smart materials etc.) and biofuels.
611 Recently novel applications such as in enhancing stress tolerance in plants has even been
612 suggested (Stritzler et al. 2018). Cyanophycin-like compounds are polyamides composed
613 mainly of aspartic acid and arginine. Derivatives of this have been suggested for use in
614 nutrition (sources of highly bioavailable amino acids/peptides), biomedicine (as polyaspartic
615 acid hydrogels), laundry detergents (polyaspartic acid), anti-scalants (polyaspartic acid) and in
616 bioflocculation (polyaspartic acid). For a recent review on cyanophycins and their
617 applications see e.g. Frommeyer et al. (2016).
618
619 **Other Applications**
620 In addition to the numerous biotechnological tools related to the cells response to low
621 temperatures presented above, an array of other bioactive metabolites and bioproducts,
622 including novel antimicrobials, anti-fungal agents, anti-cancer drugs, anti-tumor and anti-

inflammatory agents, antioxidants, alkaloids, organic acids etc. have also been identified in psychrophilic microorganisms. For reviews on these see e.g. Avila (2016); Borchert et al. (2017) and Soldatou and Baker (2017). The majority of these bioproducts used today originate from moderate temperature or warm environments whereas cold environments have been much less investigated and hence offer an extraordinary opportunity as an underexplored source of potential novelty. Indeed, the abundance and diversity of psychrophiles, combined with the vastness, enormous diversity and severity of their habitats points to their tremendous potential as rich reservoirs of novel biomolecules and metabolites of applied interest. Recent growing interest in cold environments has led to identification of numerous new products, mainly from microbes, (Soldatou and Baker 2017), and further bioprospection of these environments using modern high throughput techniques such as metagenomics-based approaches will surely lead to discovery of further novel tools with diverse bioactivities and applications (Borchert et al. 2016).

Cold-adapted microorganisms have also been shown to have potential for use in bioremediation, as probiotics and as cell factories. Their potential for degrading a wide range of organic compounds of environmental concern, including mineral oil hydrocarbons, phenolic compounds, polyaromatic hydrocarbons, pesticides and persistent pollutants, but also proteins, carbohydrates and lipids, has found broad application in bioremediation of polluted cold soils and waters, and their use in wastewater and groundwater treatment has also been suggested (reviewed by e.g. Bajaj and Singh (2015) and Margesin (2017)). As probiotics, psychrophiles are believed to have potential for use as dietary supplements in aquaculture to improve health and nutrition of livestock (Makled et al. 2017; Sun et al. 2011; Wanka et al. 2018). While studies in this are scarce, their adaptation to low temperatures is suggested to be beneficial for a more efficient utilisation in the marine habitat as compared to currently available terrestrial and/or moderate temperature adapted probiotic organisms. Finally, in relation to their utilisation as cell factories, cold-adapted microorganisms can be used for the production of heat-sensitive compounds and difficult to express or aggregation-prone proteins at low temperatures with reduced environmental and economic impact due to the absence of heating requirements (Miyake et al. 2007; Parrilli and Tutino 2017).

Conclusions

Cold adapted microorganisms inhabiting permanently low temperature environments have evolved a suite of highly sophisticated adaptations at all levels of their cells to cope with their challenging habitat conditions. Many of these adaptive features and their underlying

mechanism of action are beginning to be understood but questions remain in a number of areas. These include, among others, the structure-function relationships and precise role(s) of EPS, PUFA, LPS, biosurfactants, membrane pigments and accumulated compounds such as PHA in cold adaptation; the effects of cold denaturation on proteins adapted to different temperatures and the protective measures employed by psychrophiles to counteract this; a better understanding of the dynamic motions, amplitudes and time frames of protein structures and their role in temperature adaptation and function; and a better understanding of the various metabolic adjustments employed by psychrophiles. Further studies making use of modern technologies will advance our understanding in these areas and will undoubtedly unveil further adaptive traits and novel metabolic peculiarities employed by these microorganisms, leading to identification of further novel biomolecules with novel properties for use as innovative biotechnological tools. Indeed, while a number of psychrophile derived commercial tools are already being commercialised, the adaptive features, abundance and diversity of psychrophiles and their habitats points to their high potential as rich sources of further novel biomolecules and compounds of applied interest, and further bioprospection of these organisms and their environments should lead to an improved valorisation of their commercial potential.

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Compliance with ethical standards

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

691 **Conflict of interest**

692 The authors declare that they have no conflict of interest.

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Table 1. The toolkit for a psychrophilic lifestyle. Bioproducts believed to play a role in cold adaptation of micro-organisms, their proposed function in cold-adaptation and their potential biotechnological applications are listed. Only those tools with a commercial potential and described potential role in cold adaptation are given. '?' indicates that questions still remain as to the true function in cold adaptation.

Bioproduct	**Proposed Cold-adaptation Functions**	**Potential Applications**
Membrane fatty acids: unsaturated fatty acids, long chain polyunsaturated fatty acids (LC-PUFA)	Unsaturated fatty acids: maintenance of membrane fluidity LC-PUFA: maintenance of membrane fluidity?	Nutrition and health
Membrane pigments: carotenoids	Maintenance of membrane fluidity? Cryoprotection?	Nutrition and health
Compatible solutes	Osmoprotection: against freezing induced osmotic stress Desiccation protection: against freezing induced desiccation Freezing point depression Colloidal glass transition temperature depression Protein and membrane stabilisation	Cryopreservation Stabilisation/preservation of biological materials Food stability and freshness Cosmetics and skin care Plant resistance enhancement
Antifreeze proteins	Ice growth inhibition (thermal hysteresis) Ice recrystallisation inhibition Membrane stabilisation? Ice adhesion?	Frozen foods preservation and quality enhancement Cryopreservation Cryosurgery Freeze tolerance enhancement, e.g. crops, fish Gas hydrate inhibition 'Ice-prevention' materials
Ice-nucleating proteins	Extracellular ice crystal nucleation - prevention/reduction of damaging intracellular ice formation - small ice crystals? - release of latent heat of crystallisation?	Artificial snow production Frozen foods and beverages industries Microfluidic devices: freeze–thaw valves Cell surface display Climate control
Extracellular polymeric substances	Ice growth inhibition Osmoprotection: against freezing induced osmotic stress Desiccation protection: against freezing induced desiccation Ice-recrystallisation inhibition	Biopolymers Bioflocculants Bioabsorbants Bioemulsifiers Bioadhesives Thickening agents Cryopreservation
Biosurfactants	Ice-recrystallisation inhibition?	Biosurfactants

	Osmoprotection?	
Cold-adapted enzymes	Maintenance of adequate metabolic flux	Enzymes markets: low to moderate temperature processes
Chaperones	Promotion of protein folding and stability Destabilisation of RNA/ DNA secondary structures	Low temperature recombinant protein production
Storage compounds: polyhydroxyalkanoates, cyanophycins	Overcoming carbon and nitrogen uptake deficiencies	Biopolymers Fine chemical synthesis Biofuels Stress tolerance enhancement: plants