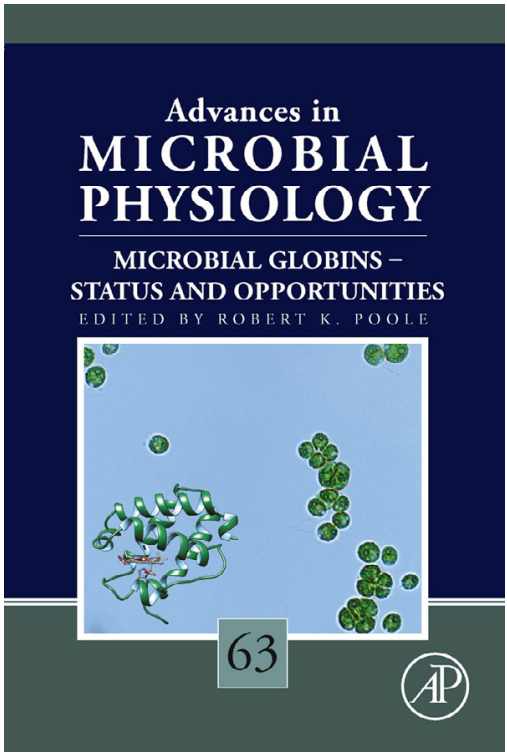

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CHAPTER EIGHT

The Globins of Cold-Adapted *Pseudoalteromonas haloplanktis* TAC125: From the Structure to the Physiological Functions

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Contents

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Abstract

Evolution allowed Antarctic microorganisms to grow successfully under extreme conditions (low temperature and high O_2 content), through a variety of structural and physiological adjustments in their genomes and development of programmed responses to strong oxidative and nitrosative stress. The availability of genomic sequences from an increasing number of cold-adapted species is providing insights to understand the molecular mechanisms underlying crucial physiological processes in polar organisms. The genome of *Pseudoalteromonas haloplanktis* TAC125 contains multiple genes encoding three distinct truncated globins exhibiting the 2/2 α-helical fold. One of these globins has been extensively characterised by spectroscopic analysis, kinetic measurements and computer simulation. The results indicate unique adaptive structural properties that enhance the overall flexibility of the protein, so that the structure appears to be resistant to pressure-induced stress. Recent results on a genomic mutant strain highlight the involvement of the cold-adapted globin in the protection against the stress induced by high O_2 concentration. Moreover, the protein was shown to catalyse peroxynitrite isomerisation *in vitro*. In this review, we first summarise how cold temperatures affect the physiology of microorganisms and focus on the molecular mechanisms of cold adaptation revealed by recent biochemical and genetic studies. Next, since only in a very few cases the physiological role of truncated globins has been demonstrated, we also discuss the structural and functional features of the cold-adapted globin in an attempt to put into perspective what has been learnt about these proteins and their potential role in the biology of cold-adapted microorganisms.

ABBREVIATIONS

CAP cold-adapted protein
CSP cold-shock protein
Cygb cytoglobin
FHb flavohaemoglobin
Hb haemoglobin
Hmp FHb from *E. coli*
HSP heat-shock protein
Mb myoglobin
Ngb neuroglobin
***Ph*TAC125** *Pseudoalteromonas haloplanktis* TAC125
PTS phosphoenolpyruvate-dependent phosphotransferase system
RNS reactive nitrogen species
ROS reactive oxygen species
SDgb single-domain globin
TBDTs TonB-dependent transport systems
TF trigger factor

1. THE POLAR ENVIRONMENTS

Although many cold-ocean species have been studied, we still have limited knowledge about adaptation to low temperatures in sea water. In the light of the ongoing climate change, there is growing interest in polar marine organisms and how they have evolved at constantly cold temperatures. The rate of the impact of current climate change in relation to the capacity of species to acclimate or adapt is a crucial study area for managing polar ecosystems in the future.

While the early biological works focussed on specific aspects of molecular adaptation of single genes and proteins to cold, progress in genomics and postgenomics, as well as the availability of genomic sequences of several model species, allowed highlighting the adaptation mechanisms permitting species evolution in polar regions (Peck, 2011; Somero, 2010).

The planet is currently losing sea ice, most notably in the Arctic region, because of warming trends over the last century (Moritz, Bitz, & Steig, 2002). The dramatic sea-ice decrease is progressing from the Barents and Bering Seas to the central Arctic Ocean.

Isolation of the two polar oceans has occurred to a different extent. The land masses surrounding the Arctic Ocean have partially limited water exchanges with other oceans for the last 60 million years or so. The Southern Ocean was isolated much more thoroughly by the Antarctic Circumpolar Current, the strongest current system in the world, since 20–40 million years ago (Eastman, 2005).

Geography, oceanography and biology of species inhabiting Arctic and Antarctic polar regions have often been intercompared (see Dayton, Mordida, & Bacon, 1994) to detect and outline differences between the two ecosystems. The northern polar region is characterised by extensive, shallow shelf sea areas of the land masses that surround a partially land-locked ocean; in contrast, the Antarctic region comprises a dynamic open ocean that surrounds the continent, and a continental shelf (Smetacek & Nicol, 2005) that is very deep because of the enormous weight exerted on the continent by the covering ice sheet, which has a thickness of 2–4000 m. Although the climate drivers acting on the biota are relatively similar, the two polar environments are quite different from each other. One of the main differences is the freshwater supply. Arctic surface waters are modified by the

input of large rivers that influence the nutrient regimes and their differences. In the Southern Ocean, the influence of freshwater, mostly from glaciers, is much smaller.

The Arctic is currently experiencing some of the most rapid and severe climate changes on Earth. Some effects of increasing temperature on marine ecosystems are already evident (Rosenzweig et al., 2008). Over the next 100 years, warming is expected to accelerate, contributing to major physical, ecological, social and economic changes. In fact, all models forecast reduction of cold areas and expansion of the warmer ones, with consequent threat for cold-adapted organisms. However, there is still little understanding regarding how the loss of a species or groups of species will affect ecosystem services.

The most important Arctic characters include seasonality in light, cold temperatures with winter extremes, and extensive shelf seas around a deep central ocean basin. The Arctic comprises a vast ocean surrounded by the northern coasts of three continents, open to influx of warm water from the Atlantic and, to a lesser extent, from the Pacific. The "permanent cap" of ice, composed of multi- and first-year ice that forms annually and extends and retreats seasonally, is probably the most important feature of Arctic marine systems (Polyak et al., 2010). The major decline in sea ice that began to take place in the Arctic since 2000 is the most important climate-change signal (Comiso, Parkinson, Gersten, & Stock, 2008). Expectations are that summer sea ice will continue to decline in the future. Climate models have indicated that the Arctic Ocean might essentially be ice-free during summer by the later half of the twenty-first century (Overpeck et al., 2006; Wang & Overland, 2009), with dramatic and potentially devastating effects on a number of species associated with the sea ice (Moline et al., 2008) and significant biological consequences (Clarke et al., 2007).

In terms of constantly low temperatures, the southern polar environment is considered the most extreme on our planet. Antarctica has the capacity to influence the Earth's climate and ocean-ecosystem function, and from this standpoint, it is the world's most important continent. As such, its palaeo- and current geological and climatic history, physical and biological oceanography, as well as marine and terrestrial ecosystems have been—and are—the target of a wealth of studies. As a frozen deep mass of ice, most of Antarctica reflects the sun's radiation, buffering global warming trends. The current lack of warming is also due to the shielding effect produced by the stratospheric winds driven by the human-induced Ozone Hole. These winds generate a Polar Vortex extending to the surface that acts as

a strong barrier, keeping warm and moist air away (Turner et al., 2009). When and if the Ozone Hole closes, such a protection will no longer be efficient, and warming caused by greenhouse gases, another anthropogenic contribution, will take the lead. However, shielding by the Polar Vortex is not taking place in the Antarctic Peninsula, especially on the western side. Similar to the Arctic, the Peninsula is thus experiencing one of the fastest rates of warming on the planet (Convey et al., 2009; Turner et al., 2009). The consequences are already being seen on land (Convey, 2006, 2010), where ice loss leads to new land becoming available for rapidly occurring colonisation. Reduction of sea ice causes displacement of key invertebrate and fish species, whose reproductive processes, closely associated with sea ice, are upset (Moline et al., 2008). Such migrations have consequences on the whole food chain.

The Southern Ocean is the planet's fourth largest ocean. Over geological time, environmental conditions and habitats in the Antarctic have changed dramatically. Two key events allowed the establishment of the powerful Antarctic Circumpolar Current: (i) the opening of the Tasman Seaway, which occurred approximately 32–35 million years ago, according to tectonics and marine geology (Kennett, 1977; Lawver & Gahagan, 2003); and (ii) the opening of the Drake Passage, between South America and the Antarctic Peninsula, dated between 40 and 17 million years ago (Scher & Martin, 2006; Thomson, 2004). The Antarctic Polar Front, a roughly circular oceanic feature running between 50°S and 60°S, is the northern boundary of the Circumpolar Current. Along the Front, the surface layers of the north-moving Antarctic waters sink beneath the less cold and less dense sub-Antarctic waters, generating almost permanent turbulence. Just north of the Front, the surface water temperature is ca. 2–3 °C warmer. Separating warm northern waters from cold southern waters, the Front acts as a barrier for migration of marine organisms between Antarctica and the lower latitudes.

Thus, Antarctica is a closed system, shielded by the Antarctic Circumpolar Current from the influence of waters from latitudes lower than 60° (Eastman, 2005). These conditions promoted adaptive evolution to low temperature and extreme seasonality to develop in isolation, and led to the current composition of the Antarctic marine biota (Eastman, 2005). The earliest cold-climate marine fauna is thought to date from the late Eocene–Oligocene (about 35 million years ago). The water column south of the Front is close to O_2 saturation at all depths. O_2 solubility increases with temperature decrease, thus the cold seas are an O_2-rich habitat.

The dominant feature of the modern continent is its ice sheet, covering most of the land area. In the sea, during wintertime, ice coverage currently extends from the Antarctic coastline northward to approximately 60°S.

Antarctic marine environments are thus considered the most extreme on Earth in combining the globally lowest and most stable temperatures with the highest O_2 content, and at the same time great variability in light intensity, ice cover and phytoplankton productivity (Peck, Convey, & Barnes, 2006). Antarctic marine habitats include sea water and sediments at near −1 °C and the sea ice, where internal fluids remain liquid to −35 °C during winter.

2. PHYLOGENY AND BIOGEOGRAPHY OF COLD-ADAPTED MARINE MICROORGANISMS

Global warming is expected to increase the microbial activity and decrease the availability of energy and food for organisms that are at higher levels in the food chain (Kirchman, Morán, & Ducklow, 2009). The role of microorganisms in polar waters is essential. Microbial processes in polar ecosystems are highly sensitive to small environmental changes and influence ecosystem functioning. Cold marine environments are colonised by a wide diversity of microorganisms including bacteria, archaea, yeasts, fungi and algae (Margesin & Miteva, 2011; Murray & Grzymski, 2007). Sea-ice microbial communities at the two poles display closely related organisms (Brinkmeyer et al., 2003) and are continually seeded by alien microorganisms, including mesophilic species that contribute to the potential environmental pool of DNA (Cowan et al., 2011).

In the past, understanding bacterial, archaeal and viral diversity in polar marine environments has been somewhat impaired by the difficulty of accessing sampling locations year-round. In comparison, rapid advances in microbial ecological theory have been achieved through results from temperate oceans, particularly, with respect to long-term microbial observatories such as BATS (Bermuda Atlantic Time Series) (Steinberg et al., 2001) and Station HOT (Hawaii Ocean Time Series) (DeLong et al., 2006).

Some of the drawbacks in data acquisition have been overcome with the advent of culture-independent molecular tools and large-scale community sequencing. Studies based on denaturing gradient gel electrophoresis (Abell & Bowman, 2005; Giebel, Brinkhoff, Zwisler, Selje, & Simon, 2009) have revolutionised our views of diversity in Antarctic waters, making the Southern Ocean one of the focal regions of microbial ecology.

However, the applicability of these investigations to the peculiar characteristics of the polar microbiota is largely unknown. Deeper understanding is necessary in light of the important role that polar waters play in global carbon cycling. The microbial component represents up to 90% of cellular DNA (Paul, Jeffrey, & DeFlaun, 1985) and is estimated to be responsible for up to 80% of the primary carbon production (Douglas, 1984; Ducklow, 1999; Li et al., 1983) and for most of the carbon flux between the sea water and the atmosphere (Azam, 1998; Azam & Malfatti, 2007).

Environmental genomics revealed that heterotrophic bacteria play a key role in controlling carbon fluxes within oceans. These bacteria dominate biogeochemical cycles and are part of the microbial loop which, at least in part, causes the response of oceanic ecosystems to climate change (Kirchman et al., 2009). Recent diversity studies that employed sequencing of ribosomal RNA genes (Galand, Casamayor, Kirchman, & Lovejoy, 2009; Ghiglione & Murray, 2012; Kirchman, Cottrell, & Lovejoy, 2010), and metagenomics and metaproteomics (Grzymski et al., 2012; Wilkins et al., 2013; Williams et al., 2012, 2013) have clarified some of the aspects of the interactions between microorganisms and the polar environment, which is unique in terms of environmental parameters such as temperature, day length and trophic interactions.

Antarctic marine waters harbour taxa of heterotrophic microbes similar to those found in temperate and tropical waters. Among these, the most dominant are α-Proteobacteria and, in particular, specific phylotypes of SAR11 (Brown et al., 2012), γ-Proteobacteria, Flavobacteria and ammonia-oxidising Marine Group I Crenarchaeota (Grzymski et al., 2012; Wilkins et al., 2013; Williams et al., 2012). However, the emerging view is that, while the taxa present might be distributed worldwide, there are clear signatures of allopatric speciation, which are only evident at a finer phylogenetic scale (Brown et al., 2012).

The geographic separation necessary for such evolutionary events is provided by sharp transitions in chemicophysical parameters that mark and isolate water masses (Agogué, Lamy, Neal, Sogin, & Herndl, 2011). The Antarctic Polar Front provides one of the most dramatic examples of such transitions. Here, the water drops ~3 °C in temperature over a space of less than 30 miles which results in abrupt shifts in the microbial community composition and functional gene distribution (Wilkins et al., 2013).

Moreover, certain taxa become transiently dominant in response to particular seasonal changes in environmental parameters such as the Marine Group I Crenarchaeota which show a dramatic increase in relative

abundance and activity during the winter in the Antarctic Peninsula (Grzymski et al., 2012; Williams et al., 2012). Similarly, bacterial clades within the Rhodobacteraceae, uncultivated γ-Proteobacteria and Bacteriodetes show large seasonal variations between samples from summer and winter from both the Antarctic Peninsula and the sub-Antarctic Kerguelen Islands (Ghiglione & Murray, 2012). On top of these oscillations, over shorter time and spatial scales, Flavobacteria can become dominant in response to algal blooms (Ghiglione & Murray, 2012; Grzymski et al., 2012; Williams et al., 2013).

The composition of the sea-ice microbiota is also unique as a result of its seasonal nature and physicochemical environment (Bowman et al., 2012; Brown & Bowman, 2001). It has an important role in providing the "seed populations" for the productive springtime microbial communities. It is still unclear whether the selective pressure within the winter sea ice generates significant genetic bottlenecks on different microbial species (Connelly, Tilburg, & Yager, 2006; Junge, Imhoff, Staley, & Deming, 2002). What is clear is that, when compared to surrounding sea water, the species richness in sea ice is lower than in surrounding waters (Bowman et al., 2012), which in turn has been shown to decrease when moving from lower to higher latitudes (Sul, Oliver, Ducklow, Amaral-Zettler, & Sogin, 2013). This lower richness might not provide enough resilience in case of future climatic changes.

Compared to the heterotrophic community, the latitudinal distribution and temporal variation of primary producers are even more extreme. Cyanobacteria, for example, *Synechococcus* sp. and *Prochlorococcus* sp., are fundamental in carbon fixation and responsible for more than half of primary production in oligotrophic ocean waters (Liu et al., 1998; Liu, Nolla, & Campbell, 1997). A consistent trend is the progressive disappearance of *Prochlorococcus* populations south of the Polar Front and the appearance of specific clades of *Synechococcus* which dominate at higher latitudes (Scanlan et al., 2009). This trend holds true at both poles. In fact, bipolar distribution of organisms is the rule rather than the exception amongst microbial taxa (Sul et al., 2013). In microeukaryotes, the observation of pheromone cross signalling amongst Arctic and Antarctic strains of the polar protozoan ciliate *Euplotes nobilii* suggests mechanisms for recent genetic exchange (Di Giuseppe et al., 2011). If associated with the strong bipolar biogeographical patterns, this could be true for all classes of organisms living at low Reynolds numbers, with the caveat that deep-sea currents, in particular those associated with thermohaline circulation, are allowing an ongoing genetic exchange between the poles (Lauro, Chastain, Blankenship, Yayanos, & Bartlett, 2007).

Complementing these culture-independent studies, the last few years have seen an increase in genomic sequences of cultured isolates. The study of individual genomes facilitates the characterisation of physiological adaptations to the specific polar conditions. Nevertheless, in contrast with the large diversity observed with molecular techniques, the phylogenetic breadth of the taxa with at least one representative genome sequence is limited to a few genera (Fig. 8.1).

In view of the high degree of temporal and spatial variability observed in polar environments, which positively correlates with changes in microbial community structure and function, there is a pressing need for increasing culturing efforts and single-cell genomic analysis targeted at underrepresented phyla. These should be integrated within the larger framework of global organismal biogeography and ocean models.

3. THE ROLE OF TEMPERATURE IN EVOLUTIONARY ADAPTATIONS

The bulk of the Earth's biosphere is cold (e.g. 90% of the ocean is below 5 °C), sustaining a broad diversity of microbial life. Evolution under extreme conditions has been marked by a suite of adaptations (evolutionary gains) including the development of proteins that function optimally in the cold. A commonly accepted view for protein cold adaptation is the activity/stability/flexibility relationships. Although active sites are generally highly conserved among homologous proteins, adaptive changes may occur at recognition site(s). These alterations in the strength of subunit interactions may affect thermal stability and energy changes associated with conformational transitions due to ligand binding (D'Amico, Collins, Marx, Feller, & Gerday, 2006).

Comparative genome analysis indicates that the cold-adapted lifestyle is generally conferred by a collection of changes in the overall genome content and composition. The flexible structures of enzymes from cold-adapted bacteria compensate for the environment's low kinetic energy.

In cold environments, challenges to cellular function and structural integrity include low rates of transcription, translation and cell division, inappropriate protein folding and cold denaturation, as well as intracellular ice formation (D'Amico et al., 2006). The ability of an organism to survive and grow in the cold is dependent on a number of adaptive strategies (Table 8.1) to maintain vital cellular functions at cold temperatures (Rodrigues & Tiedje, 2008). These strategies include the synthesis of

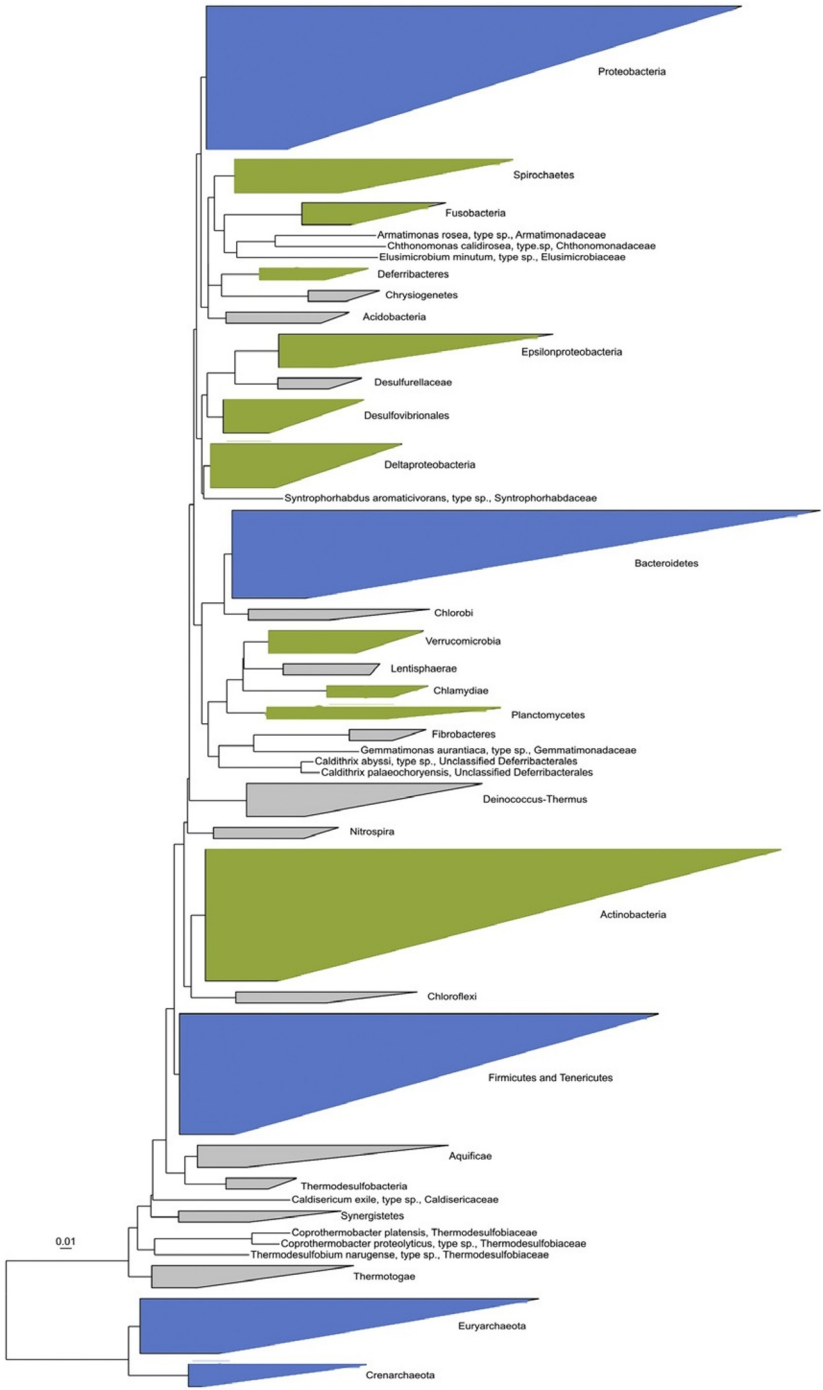

Figure 8.1 Bacterial and Archaeal phylogenetic tree adapted from the Silva comprehensive ribosomal RNA database (http://www.arb-silva.de/). Blue: phyla containing at least one sequenced polar genome. Green: phyla containing polar isolates or numerically relevant in culture-independent surveys of polar regions.

Table 8.1 Molecular adaptations in cold-adapted bacteria

Molecular adaptations	Explanation or consequence	Reference
Protection against reactive oxygen species (ROS): lower frequency of oxidisable amino acids; oxidoreductases, superoxide dismutases, catalases, peroxidases	Due to increased solubility of O_2 at low temperatures forming increased ROS	Rabus et al. (2004), Médigue et al. (2005), Methé et al. (2005), Bakermans et al. (2007), Duchaud et al. (2007), Ayub, Tribelli, and Lopez (2009) and Piette et al. (2010)
Enzymes	Maintain catalytic efficiency at low temperatures	Georlette et al. (2004)
Membranes: increased unsaturation and decreased chain length of fatty acids, carotenoids, desaturases	Increase the fluidity of membranes	Jagannadham, Rao, and Shivaji (1991), Chauhan and Shivaji (1994) and Ray et al. (1998)
Synthesis of specific elements: cold-shock proteins, molecular chaperones, compatible solutes	Maintain vital cellular functions at cold temperatures	Motohashi, Watanabe, Yohda, and Yoshida (1999), Cavicchioli, Thomas, and Curmi (2000), Watanabe and Yoshida (2004) and Pegg (2007)
Molecular mechanisms involved in protein flexibility	**Consequence**	**Reference**
Decreased number of H bonds and salt bridges	Increased flexibility	Feller and Gerday (1997)
Reduced proline and arginine content	Increased molecular entropy	Ray et al. (1998), Russell (2000), D'Amico et al. (2002), Cavicchioli, Siddiqui, Andrews, and Sowers (2002) and Rodrigues and Tiedje (2008)
Reduced frequency of surface, inter-domain and inter-subunit ionic linkages and ion-network	Increased conformational flexibility and reduced enthalphic contribution to stability	D'Amico et al. (2006)

Adapted from Casanueva, Tuffin, Cary, and Cowan (2010).

cold-shock proteins (CSPs) (Cavicchioli et al., 2000) and molecular chaperones (Motohashi et al., 1999), solutes (Pegg, 2007) and structural modifications for maintaining membrane fluidity (Chintalapati, Kiran, & Shivaji, 2004; Russell, 1998). Moreover, cold-adapted organisms must develop an effective and intricate network of defence mechanisms against oxidative stress: an increasing number of oxidoreductases, superoxide dismutases, catalases and peroxidases can be seen in this perspective (Ayub et al., 2009; Bakermans et al., 2007; Duchaud et al., 2007; Médigue et al., 2005; Methé et al., 2005; Piette et al., 2010; Rabus et al., 2004).

In addition to adaptations at the cellular level, a key adaptive strategy is the maintenance of adequate reaction rates at thermal extremes; therefore, adequate features of catalytic processes become crucial. Enzyme catalytic rates at low temperatures depend on increased protein flexibility and concomitant increase in thermolability (Georlette et al., 2004).

In this respect, a suite of factors contribute to maintaining enzyme molecular flexibility in all cold-adapted organisms. In bacterial enzymes, Ser, Asp, Thr and Ala are over-represented in the coil regions of secondary structures. On the other hand, in the helical regions, aliphatic, basic, aromatic and hydrophilic residues are generally under-represented (Cavicchioli et al., 2002; D'Amico et al., 2002; Ray et al., 1998; Rodrigues & Tiedje, 2008; Russell, 2000). Moreover, a reduction of surface, inter-domain, inter-subunit ionic linkages and a decreased number of hydrogen bonds and salt bridges are key mechanisms to induce an increasing of conformational flexibility of psychrophilic enzymes (D'Amico et al., 2006; Feller & Gerday, 1997).

Cold adaptation is also strongly linked to the capacity of the organism to sense temperature changes, perhaps by virtue of mechanisms linked with the lipid composition of the cell membrane and alterations in the DNA and RNA topology. The latter may enhance (or halt) the replication, transcription and translation processes (Eriksson, Hurme, & Rhen, 2002). Although increased unsaturation and decreased chain length of fatty acids are the major modifications of cell membranes, other membrane-associated components may well play important roles in adaptation to low temperatures (Jagannadham et al., 1991; Ray et al., 1998). Studies of Antarctic psychrotrophic bacteria *in vitro* have shown that carotenoids may have a function in buffering membrane fluidity (Jagannadham et al., 1991).

4. BACTERIAL GLOBINS

The traditional view of the exclusive role of haemoglobin (Hb) as O_2 carrier in vertebrates is obsolete. The discovery of globin genes in prokaryotic

and eukaryotic microorganisms, including bacteria, yeasts, algae, protozoa and fungi, suggests that the globin superfamily is exceptionally flexible in terms of biological roles and possible functions. The number of "globin-like" proteins is currently increasing as different genomes from microorganisms are sequenced and annotated (Vinogradov et al., 2005; Vinogradov & Moens, 2008; Vinogradov, Tinajero-Trejo, Poole, & Hoogewijs, 2013).

Non-vertebrate globins display high variability in primary and tertiary structures, which probably indicates their adaptations to additional functions with respect to their vertebrate homologues (Vinogradov et al., 2005; Vinogradov & Moens, 2008; Vinogradov et al., 2013).

The most recent bioinformatic survey of globin-like sequences in prokaryotic genomes revealed that over half of the more than 2200 bacterial genomes sequenced so far contain putative globins (Vinogradov et al., 2013). A new global nomenclature including prokaryotic and eukaryotic globins has been proposed, and globins have been classified within three families: (i) myoglobin (Mb)-like family (M) (displaying the classical three-on-three (3/3) α-helical sandwich motif) containing flavohaemoglobins (FHbs) and single-domain globins (SDgb); (ii) sensor globins family (S); and (iii) truncated haemoglobins family (T), showing the two-on-two (2/2) α-helical sandwich motif (Vinogradov et al., 2013).

Although there are still some uncertainties about the evolutionary relationship between these three classes of microbial globins, it has been proposed that prokaryotic and eukaryotic globins, including vertebrate α/β globins, Mb, neuroglobin (Ngb), cytoglobin (Cygb), and invertebrate and plant Hbs, emerged from a common ancestor (Vinogradov et al., 2005).

4.1. Flavohaemoglobins

Chimeric globins seem to have kept their original enzymatic functions in prokaryotes, plants and some unicellular eukaryotes. Therefore, the FHb sub-family has been the only one able to adapt to different functions more extensively than the other two globin families. Moreover, the presence of Hbs in unicellular organisms suggests that O_2 transport in metazoans is a relatively recent evolutionary acquisition and that the early Hb functions have been enzymatic and O_2 sensing (Vinogradov & Moens, 2008).

FHbs are widely present in bacteria, yeasts and fungi and belong to the ferredoxin reductase-like protein family. They consist of an N-terminal haem-globin domain fused with C-terminal reductase domain binding NAD(P)H and FAD (Bolognesi, Bordo, Rizzi, Tarricone, & Ascenzi, 1997; Bonamore & Boffi, 2008).

Sequence alignments in Gram-negative and Gram-positive bacteria and unicellular eukaryotes indicate that the FHbs family is a very homogeneous group of proteins that share highly conserved active sites in both domains. Amino acid residues building up the haem domain and the flavin-binding region are widely conserved, and the architecture of FHb domains is closely similar to those of globins and flavodoxin-reductase proteins. This finding suggests that the haem domain displays globin-like functional properties and that the flavin moiety acts as an electron-transfer module from NADH to the haem. However, sequence alignments on separate domains strongly diverge towards the homologous proteins, suggesting that the rise of FHbs comes from fusion of a protoglobin ancestor and a flavin-binding domain (Bonamore & Boffi, 2008).

Details of the structure, function and reaction mechanism of purified native or recombinant FHbs from bacteria and yeast are available (Lewis, Corker, Gollan, & Poole, 2008). FHb from *Escherichia coli* (Hmp) is the best characterised member of the family. Hmp is distributed into both the cytoplasmic and periplasmic space, although the biochemically active protein is exclusively found in the cytoplasmic fraction (Vasudevan, Tang, Dixon, & Poole, 1995). It is subject to complex control (reviewed by Spiro, 2007; Poole, 2008), being dramatically up-regulated in response to NO and nitrosating agents (Membrillo-Hernández et al., 1999; Membrillo-Hernández, Coopamah, Channa, Hughes, & Poole, 1998; Poole et al., 1996). In particular, the *hmp* gene is predominantly regulated at the transcriptional level by NO-sensitive transcription factors, especially NsrR and Fnr (Spiro, 2007). Remarkably, the fine-tuning of Hmp synthesis appears critical for *E. coli* survival. In fact, the constitutive expression of Hmp in the absence of NO generates oxidative stress because of partial O_2 oxidation by the haem to superoxide and peroxide anion; accumulation of O_2 radicals has been stressed both in kinetic studies on the purified protein (Orii, Ioannidis, & Poole, 1992; Poole, Rogers, D'mello, Hughes, & Orii, 1997; Wu, Corker, Orii, & Poole, 2004) and by detecting the superoxide-generating activity of Hmp *in vivo* (Membrillo-Hernández, Ioannidis, & Poole, 1996). Similar results have also been obtained in *Salmonella enterica* where the constitutive expression of Hmp makes cells hypersensitive to paraquat and H_2O_2 (Gilberthorpe, Lee, Stevanin, Read, & Poole, 2007), as well as to peroxynitrite ($ONOO^-$) (McLean, Bowman, & Poole, 2010).

FHbs display a pivotal role in NO detoxification (Poole & Hughes, 2000). NO, involved in many beneficial and/or dangerous physiological and pathological processes, is a signalling and defence molecule of great

importance in biological systems, and nowadays has an important role in contemporary medicine, physiology, biochemistry and microbiology. The behaviour of NO is made particularly complex by its ability to be oxidised to the nitrosonium cation (NO^+) or reduced to the nitroxyl anion (NO^-) and to react with O_2 to form nitrite (NO_2^-). Moreover, the reactions of NO led to production of reactive nitrogen species (RNS) (reviewed by Bowman, McLean, Poole, & Fukuto, 2011; Poole & Hughes, 2000), such as $ONOO^-$, formed by the reaction of NO with superoxide anion.

Extensive literature deals with NO and related species, especially considering that NO plays vital anti-microbial roles in innate immunity (Granger, Perfect, & Durack, 1986; Green, Meltzer, Hibbs, & Nacy, 1990; Iyengar, Stuehr, & Marletta, 1987; Liew, Millott, Parkinson, Palmer, & Moncada, 1990; Marletta, Yoon, Iyengar, Leaf, & Wishnok, 1988; Stuehr, Gross, Sakuma, Levi, & Nathan, 1989) and that microorganisms have evolved a large number of NO-sensitive targets and defence mechanisms against its toxic effects.

FHbs catalyse reaction of NO with O_2 to yield the relatively innocuous NO_3^- (Gardner, 2005; Mowat, Gazur, Campbell, & Chapman, 2010; Poole & Hughes, 2000) by a dioxygenase (Gardner et al., 2006, 2000; Gardner, Gardner, Martin, & Salzman, 1998) or denitrosylase (Hausladen, Gow, & Stamler, 1998, 2001). Anaerobically, Hmp shows low NO-reductase activity, converting NO to N_2O (Kim, Orii, Lloyd, Hughes, & Poole, 1999; Liu, Zeng, Hausladen, Heitman, & Stamler, 2000; Poole & Hughes, 2000; Vinogradov & Moens, 2008).

Deletion of the *hmp* gene alone abolishes the NO-consuming activity (Liu et al., 2000) and is sufficient to render bacteria hypersensitive to NO and related compounds, not only *in vitro* (Membrillo-Hernández et al., 1999) but also *in vivo* (Stevanin, Poole, Demoncheaux, & Read, 2002).

Similar to *E. coli* (Stevanin, Read, & Poole, 2007), the *S. enterica* serovar Typhimurium mutant defective in FHb shows enhanced sensitivity to mouse and human-macrophage microbicidal activity (Gilberthorpe et al., 2007; Stevanin et al., 2002), suggesting that the globin contributes to protection from NO-mediated toxicity in macrophages.

FHbs from several other bacteria show protective functions against RNS, such as those from *Ralstonia eutropha*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Deinococcus radiodurans*, *S. enterica* and *Klebsiella pneumoniae* (reviewed by Frey, Farres, Bollinger, & Kallio, 2002).

Although FHbs protect pathogenic microorganisms from the host immune systems, they also defend non-pathogenic organisms from

endogenous NO generated by nitrate and nitrite reduction, under anaerobic conditions (Rogstam, Larsson, Kjelgaard, & von Wachenfeldt, 2007). Vinogradov and Moens (2008) also propose that FHb may be involved in the coordination of intracellular NO concentration with extracellular O_2 levels, as suggested by the mitochondrial generation of NO in *Saccharomyces* exposed to hypoxia, with concomitant localisation of FHb in the pro-mitochondrial matrix (Castello, David, McClure, Crook, & Poyton, 2006).

Although FHbs are involved in NO detoxification, other physiological functions have been identified; for instance, Hmp has alkyl hydroperoxide reductase activity under anaerobic conditions, suggesting that, together with the unique lipid-binding properties, this globin is capable of catalysing the reduction of lipid-membrane hydroperoxides into the corresponding alcohols using NADH as electron donor and may thus be involved in the repair of the lipid-membrane oxidative damage generated during oxidative/nitrosative stress (Bonamore & Boffi, 2008; D'Angelo et al., 2004).

4.2. Single-domain globins

The first identified and sequenced SDgb was the Hb of *Vitreoscilla* (Vgb), whose expression is significantly increased under microaerobic conditions. It comprises a single domain, unmistakably globin-like, and the protohaem; it lacks the reductase domain seen in FHbs. Despite evidence that its expression in heterologous hosts can provide some protection from nitrosative stress, the generally accepted view is that Vgb facilitates O_2 utilisation, perhaps by directly interacting with a terminal oxidase (Wu, Wainwright, Membrillo-Hernández, & Poole, 2004, Wu, Wainwright, & Poole, 2003).

Another example of SDgb is offered by the microaerophilic, foodborne, pathogenic bacterium *Campylobacter jejuni*, exposed to NO and other nitrosating species during host infection. This globin (Cgb) is dramatically up-regulated in response to nitrosative stress (Elvers et al., 2005, Elvers, Wu, Gilberthorpe, Poole, & Park, 2004; Monk, Pearson, Mulholland, Smith, & Poole, 2008; Smith, Shepherd, Monk, Green, & Poole, 2011) and provides a specific and inducible defence against NO and nitrosating agents (Poole, 2005); it detoxifies NO and presents a peroxidase-like haem-binding cleft. In contrast to Vgb, there is no evidence that Cgb functions in O_2 delivery.

Since a detailed overview of SDgbs is beyond the goal of this contribution, the reader is directed elsewhere (Bowman et al., 2011; Frey et al., 2002;

Frey & Kallio, 2003, Frey, Shepherd, Jokipii-Lukkari, Haggman, & Kallio, 2011; Vinogradov et al., 2005, 2013).

4.3. Truncated haemoglobins

Members of the T family are found in eubacteria, cyanobacteria, protozoa and plants but not in animals (Milani et al., 2005; Wittenberg, Bolognesi, Wittenberg, & Guertin, 2002). The N-termini of these globins are 20–40-residue shorter; these globins display the 2/2 α-helical sandwich fold (composed of helices B, E, G and H), which has been recognised as a subset of the classical 3/3 α-helical sandwich (Milani et al., 2005; Wittenberg et al., 2002). The 2/2 α-helical sandwich fold results in four α-helices (B/E and G/H) arranged in antiparallel pairs connected by an extended polypeptide loop that replaces the α-helix F (Fig. 8.2).

It is noteworthy that the haem-proximal helix F is replaced by a polypeptide segment (Milani et al., 2001; Pesce et al., 2000). The residues comprising the F loop affect the orientation of the proximal HisF8 modulating the O_2-binding properties (Milani et al., 2005; Pathania, Navani, Gardner, Gardner, & Dikshit, 2002). Helix E is very close to the haem distal site; therefore, residues at positions B10, CD1, E7, E11, E15 and/or G8 (Table 8.2) modulate ligand binding (Milani et al., 2005).

On the basis of phylogenetic analysis, the T family can be further divided into three distinct sub-families: TrHbI (or N), TrHbII (or O) and TrHbIII (or P); specific structural features that depend on residues of the distal haem pocket distinguish each group (Table 8.2). In group I, the hydrogen bond network stabilising the haem-bound ligand involves the B10, E7 and E11 residues. Strongly conserved Tyr B10 plays a key role in ligand stabilisation through OH pointing directly to the heam-bound ligand. Normally, complete stabilisation by the H-bond network is provided by Glu located at E7 or E11, or at both positions. In group II, TrpG8 is fully conserved, contributing to ligand stabilisation by the H bond linking the indole nitrogen atom and the ligand.

Further, Tyr at CD1 in some TrHbIIs drastically modifies the interaction network (Pesce et al., 2000). In group III, for example, *C. jejuni* TrHbIII (Ctb), the hydrogen bond network stabilising the haem-bound ligand involves TyrB10 and TrpG8 (Nardini et al., 2006). Interestingly, the affinity of cyanide for the ferrous derivative of *C. jejuni* Ctb is higher than that reported for any known (in)vertebrate ferrous globin and is reminiscent

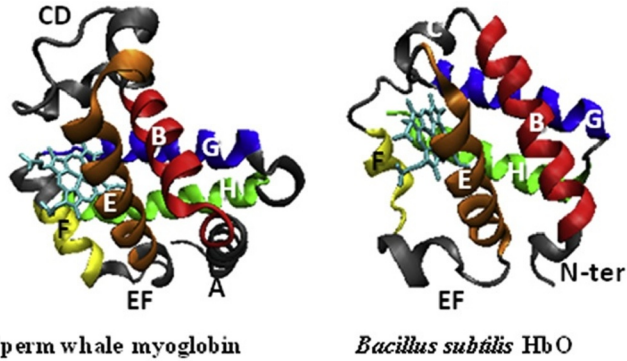

Figure 8.2 A stereo view of sperm whale Mb (3/3) (pdb:1JP6, Urayama, Gruner, & Phillips, 2002) and *B. subtilis* TrHbII (HbO) (pdb:1UX8, Ilari, Giangiacomo, Boffi, & Chiancone, 2005) tertiary structures, including the haem group and helices. Modifications of the conventional 3/3 Mb fold occur particularly at helix A and in the CD-D regions, which are virtually absent, and in the EF-F regions of TrHbs. A very short segment linking helices C and E forces the haem-distal helix E very close to the haem distal side.

of that of ferrous horseradish peroxidase, suggesting that this globin may participate in cyanide detoxification (Bolli et al., 2008).

Strikingly, TrHbs belonging to group III (or N) host a protein matrix tunnel system offering a potential path for ligand diffusion to and from the haem distal site. The apolar tunnel/cavity system, extending for approximately 28 Å through the protein matrix, is conserved in TrHbs belonging to group N, although with modulation of its size and/or structure (Milani et al., 2001; Pesce, Milani, Nardini, & Bolognesi, 2008). It has been proposed that in *Mycobacterium tuberculosis* HbN, the haem-Fe/O_2 stereochemistry and the protein matrix tunnel may promote O_2/NO chemistry *in vivo*, as a *M. tuberculosis* defence mechanism against macrophage nitrosative stress (Milani et al., 2001).

Unlike HbN, *M. tuberculosis* HbO does not host the protein matrix tunnel but two topologically equivalent matrix cavities. Moreover, the small apolar Ala E7 residue leaves room for ligand access to the haem distal site through the conventional E7 path (Pesce et al., 2008), as proposed for Mb.

In contrast to TrHbs I and II (Milani et al., 2004, 2001; Nardini et al., 2006; Pesce et al., 2008), Ctb does not display protein matrix tunnel/cavity systems at all (Nardini et al., 2006). Although the gating role of HisE7 in the modulation of ligand access into and out of the heam pocket is debated

Table 8.2 Amino acid residues building up the proximal (F8) and distal (B10, CD1, E7, E11, E15 and G8) haem pocket of sperm whale Mb and TrHbs belonging to groups I, II and III

Protein	Group	B10	CD1	E7	E11	E15	F8	G8
Sperm whale Mb		L	F	H	V	L	H	I
Nostoc commune		H	F	Q	L	L	H	I
Paramecium caudatum		Y	F	Q	T	L	H	V
Chlamydomonas eugametos	I	Y	F	Q	Q	L	H	V
Synechocystis		Y	F	Q	Q	L	H	V
Mycobacterium tuberculosis N		Y	F	L	Q	F	H	V
Mycobacterium avium N		Y	F	L	Q	F	H	V
Mycobacterium avium O		Y	Y	A	L	L	H	W
Mycobacterium tuberculosis O		Y	Y	A	L	L	H	W
Mycobacterium smegmatis O		Y	Y	A	L	L	H	W
Mycobacterium leprae	II	Y	Y	A	L	L	H	W
Thermobifida fusca		Y	Y	A	L	L	H	W
Bacillus subtilis		Y	F	T	Q	L	H	W
Arabidopsis thaliana		Y	F	A	Q	F	H	W
Thiobacillus ferrooxidans		Y	F	H	L	W	H	W
Mycobacterium avium P		Y	F	H	M	W	H	W
Campylobacter jejuni	III	Y	F	H	I	W	H	W
Bordetella pertussis 1		Y	F	H	L	W	H	W

Adapted from Milani et al. (2005).

(Lu, Egawa, Wainwright, Poole, & Yeh, 2007; Nardini et al., 2006), this mechanism appears to be operative in the *C. jejuni* TrHbIII (Nardini et al., 2006).

The sequence identity between TrHbs belonging to the three phylogenetic groups is very low (<20%) (Nardini, Pesce, Milani, & Bolognesi, 2007; Vuletich & Lecomte, 2006; Wittenberg et al., 2002), but may be higher than 80% within a given group. Analysis of the distribution of TrHbs suggests a scenario for the evolution of the different groups where the group II gene is ancestral and group-I and group-III genes are the results of duplications and transfer events (Vuletich & Lecomte, 2006).

TrHbs belonging to the three groups may coexist in some bacteria, suggesting distinct functions. These globins have been hypothesised to store ligands and/or substrates, to facilitate NO detoxification, to sense O_2/NO, to display (pseudo)enzymatic activities and to deliver O_2 under hypoxic conditions (Vinogradov & Moens, 2008; Wittenberg et al., 2002). The high O_2 affinity suggests that some TrHbs may function as O_2 scavengers rather than O_2 transporters (Ouellet et al., 2003; Wittenberg et al., 2002).

5. THE ANTARCTIC MARINE BACTERIUM *PSEUDOALTEROMONAS HALOPLANKTIS* TAC125: A CASE STUDY

5.1. General aspects

Despite the fact that the Antarctic marine environment is characterised by permanent low temperatures, the surface water and the sea-ice zones host a surprisingly high level of microbial activity.

A typical representative of γ-Proteobacteria found in the Antarctic is the marine cold-adapted psychrophile *P. haloplanktis* TAC125 (*Ph*TAC125), a Gram-negative bacterium, isolated in Antarctic coastal sea water in the vicinity of the French station Dumont d'Urville, Terre Adélie (66°40′S; 140°01′E). As in many marine γ-Proteobacteria, its genome is made up of two chromosomes (Médigue et al., 2005). This strain thrives between −2 °C and 4 °C, but is also able to survive long-term frozen conditions when entrapped in the winter sea ice. *Ph*TAC125 can grow in a wide temperature range (4–25 °C) (Fig. 8.3A) and achieve very high cell density even under uncontrolled laboratory conditions (Fig. 8.3B). In a marine broth, *Ph*TAC125 displays a doubling time of about 4 h at 4 °C and 5 h 15 min at 0 °C. At higher temperatures, the bacterium divides actively and the generation time decreases moderately (e.g. 1 h 40 min at 18 °C), with increase in the biomass produced at the stationary phase (Piette et al., 2011). In contrast, higher temperatures cause a drastic reduction in cell density at the stationary phase, suggesting that the heat-induced stress affects the growth (Piette et al., 2011).

The doubling time of *Ph*TAC125 at 16 °C is approximately 2 h, almost three times faster than that of *E. coli* under similar growth conditions (Piette et al., 2010). Consistent with the high growth rate, at room temperature, *Ph*TAC125 shows a very efficient chemotactic response, 10 times faster than that of *E. coli*, allowing it to exploit nutrient patches in the marine

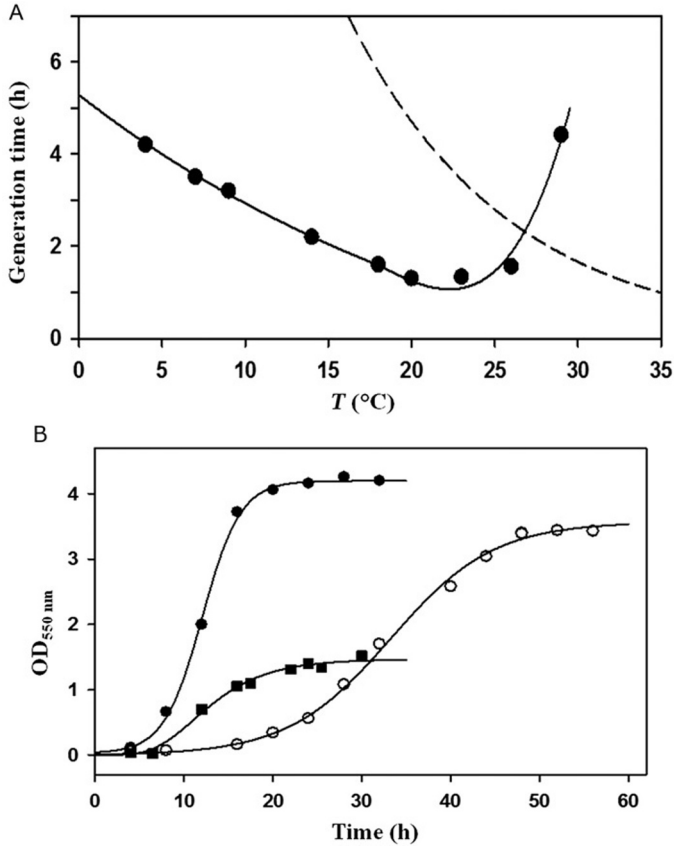

Figure 8.3 (A) Temperature dependence of the doubling time of Antarctic bacterium *Ph*TAC125, grown in marine broth (solid line and circles), compared to a typical growth curve of *E. coli* RR1 strain, obtained in LB broth (dashed line). (B) Growth curves of *Ph*TAC125, performed at 4 °C (open circles), 18 °C (filled circles) and 26 °C (filled squares). *Adapted from Piette et al. (2011).*

environment before they dissipate (Stocker, Seymour, Samadani, Hunt, & Polz, 2008). *Ph*TAC125 grows with extremely high growth rates in defined sea water medium, with peptone as the only carbon and nitrogen source, suggesting that these growth conditions resemble the favoured natural environment of the marine bacterium, which can be easily isolated from damaged tissues of fishes or molluscs, where such substrates are available (Wilmes et al., 2011). *Ph*TAC125 lacks a cyclic AMP (cAMP)–catabolite activator protein complex, that regulates carbon availability in related organisms, and a phosphoenolpyruvate-dependent phosphotransferase system (PTS)

for the transport and first metabolic step of so-called PTS sugars (Médigue et al., 2005; Wilmes et al., 2011), making the bacterium unable to grow on glucose.

Approximately 13% of the identified proteins in the periplasmic protein fraction of *Ph*TAC125 are transport-related proteins, mostly belonging to TonB-dependent transport systems (TBDTs) (Wilmes et al., 2011). The high amount of TBDTs in the genome (Médigue et al., 2005) and in the periplasmic proteome (Wilmes et al., 2011) support the idea that these transporters permit efficient use and scavenge the large variety of substrates found in the marine environment, probably representing an important prerequisite for fast growth under nutrient-rich conditions. Besides TBDTs, three ABC transporters, four porins and the transporter TolB are the other detected putative substrate-transport-related proteins (Médigue et al., 2005; Wilmes et al., 2011).

*Ph*TAC125 is also able to grow in anaerobiosis, although with lower yields (Médigue et al., 2005). It is worth noting that lower duplication rate and poor growth of *Ph*TAC125 in micro-aerobiosis have been observed (Parrilli, Giuliani, Giordano, et al., 2010). Due to lower O_2 solubility at 15 °C than at 4 °C, $OD_{600,\ max}$ is approximately 7.2 at 15 °C, and 4.3 at 4 °C, in extreme aerobiosis; moreover, $OD_{600,\ max}$ is approximately 1.25 at 4 °C and 0.38 at 15 °C, in micro-aerobiosis.

5.2. Genomic and post-genomic insights

Over the last decade, several genomes from psychrophilic bacteria and Archaea have been sequenced (Casanueva et al., 2010). Some of these (Allen et al., 2009; Ayala-del-Rio et al., 2010; Duchaud et al., 2007; Médigue et al., 2005; Methé et al., 2005; Rabus et al., 2004; Riley et al., 2008; Rodrigues et al., 2008; Saunders et al., 2003) have been analysed with respect to cold adaptation through proteomic and transcriptomic approaches (Bakermans et al., 2007, Bergholz, Bakermans, & Tiedje, 2009; Campanaro et al., 2011; Goodchild, Raftery, Saunders, Guilhaus, & Cavicchioli, 2005; Goodchild et al., 2004; Kawamoto, Kurihara, Kitagawa, Kato, & Esaki, 2007; Piette et al., 2011, 2010; Qiu, Kathariou, & Lubman, 2006; Ting et al., 2010; Williams et al., 2010; Wilmes et al., 2011; Zheng et al., 2007).

Through the MaGe annotation platform (http://www.genoscope.cns.fr/agc/mage/wwwpkgdb/Login/log.php?pid=7#ancreLogin), and by *in silico* and *in vivo* analyses, several exceptional genomic and metabolic features have been identified in *Ph*TAC125 (Médigue et al., 2005).

*Ph*TAC125 is an interesting model for investigating strategies adopted to survive at low temperature (Médigue et al., 2005). The available genome sequence, combined with remarkable versatility and fast growth (Duilio, Tutino, & Marino, 2004), makes *Ph*TAC125 an attractive model to study protein-secretion mechanisms in marine environments, in addition to its use as a non-conventional host for recombinant production of thermal-labile and aggregation-prone proteins at low temperature (Cusano et al., 2006; Gasser et al., 2008; Parrilli, Duilio, & Tutino, 2008; Parrilli, Giuliani, Pezzella, et al., 2010; Vigentini, Merico, Tutino, Compagno, & Marino, 2006). Actually, low temperatures improve the quality of the products, removing the negative effects of high temperatures on protein folding, due to the strong temperature dependence of hydrophobic interactions that mainly drive the aggregation (Kiefhaber, Rudolph, Kohler, & Buchner, 1991). The growth of *E. coli* at lower temperatures to minimise aggregation has not been successful, probably because sub-optimal temperatures act negatively on cell performance (Gasser et al., 2008).

The efficiency of the cold-adapted *Ph*TAC125 expression system was demonstrated by the production of biologically active soluble products, for example, a yeast α-glucosidase, the mature human nerve growth factor and a cold-adapted lipase (de Pascale et al., 2008; Parrilli et al., 2008).

At the genome level, a relatively large number of rRNA genes (nine rRNA gene clusters) and tRNA genes (106 genes, organised in long runs of repeated sequences) have been observed in *Ph*TAC125 (Médigue et al., 2005), similar to *Colwellia psychrerythraea* (Methé et al., 2005) and *Psychromonas ingrahamii* (Riley et al., 2008). This finding may be explained as a response to the limited speed of transcription/translation at low temperature, allowing fast growth in the cold. However, it has recently been speculated that a high number of rRNA genes may reflect an ecological bacterial strategy to improve the response to perturbations in nutrient resources (Klappenbach, Dunbar, & Schmidt, 2000). Moreover, *Ph*TAC125 contains 19 genes presumably encoding known RNA-binding proteins or RNA chaperones. An unexpected feature is the prominent absence of *hns*, an RNA/nucleoid-associated cold-shock gene found in all γ-Proteobacteria. In contrast, the presence of many RNA helicases (three copies of *rhlE*, and probably a fourth one, *PSHAa0641*, and two copies of *srmB*) instead of the single one in *E. coli*, suggests that the control of RNA folding and degradation is important at low temperature (Médigue et al., 2005). In fact, RNA helicases have been found to be over-expressed at low temperature in many other psychrophilic microorganisms, such as *Methanococcoides burtonii*

(Lim, Thomas, & Cavicchioli, 2000), *Exiguobacterium sibiricum* (Rodrigues et al., 2008), *Sphingopyxis alaskensis* (Ting et al., 2010) and *Psychrobacter arcticus* (Bergholz et al., 2009; Zheng et al., 2007). This feature may reflect the need for help to unwind the RNA secondary structures for highly efficient translation in the cold (Cartier, Lorieux, Allemand, Dreyfus, & Bizebard, 2010).

Sequence analyses using genomic and metagenomic data clearly show that different mechanisms of adaptation to cold, including a bias towards specific residues, occur. The analysis of the amino acid composition of γ-Proteobacteria from different biotopes reveals a similar trend in the various genomes, Leu being most abundant, and Trp, Cys, His and Met most unusual. The proteome of thermophilic microorganisms shows several differences when compared to those of mesophilic and psychrophilic species, for example, Gln is poorly represented in thermophilic species, whereas mesophilic and psychrophilic species prefer Ala (except in *Oceanobacillus iheyensis*). A few remarkable differences have been identified between mesophilic and psychrophilic species, in particular, that some Asn and Gln are pivotal for bacteria growth in cold environments.

Specifically, proteins from *Ph*TAC125 are rich in Asn (Médigue et al., 2005) and contain few hydrophilic uncharged residues bearing an often thermolabile amide group (Stratton et al., 2001; Weintraub & Manson, 2004; Zhou, Cocco, Russ, Brunger, & Engelman, 2000), which undergo deamidating cyclisation, a process extremely sensitive to temperature (Daniel, Dines, & Petach, 1996). The richness in Asn in the *Ph*TAC125 proteome makes this Antarctic bacterium an organism of choice for foreign protein production when deamidation ought to be at a minimum (Weintraub & Manson, 2004).

Other aspects of cold-adapted proteins (CAPs) are (i) the significantly high level of non-charged polar Gln and Thr, and the low content of hydrophobic residues (particularly Leu) in the archaeal psychrophiles *Methanogenium frigidum* and *M. burtonii* (Saunders et al., 2003); (ii) the low content of polar residues such as Ser, the replacement of Asp with Glu, and the general decrease in charged residues in the proteins of *C. psychrerythraea* (Methé et al., 2005); (iii) the reduction of Pro and Arg codons in the *P. arcticus* genome, in particular, in the cell-growth and reproduction genes (Ayala-del-Rio et al., 2010); and (iv) decrease in Ala, Pro and Arg in *Shewanella halifaxensis* and *S. sediminis* (Zhao, Deng, Manno, & Hawari, 2010). These findings support the hypothesis of the increased flexibility and reduced thermostability of CAPs (Methé et al., 2005). However,

no substitution promoting cold adaptation has been found in the *Desulfotalea psychrophila* genome (Rabus et al., 2004).

The proteomes expressed by *Ph*TAC125 at 4 and 18 °C were compared (Piette et al., 2010, 2011) to identify the cold-acclimation proteins, that is, those continuously over-expressed at high level at low temperatures, and to highlight the numerous down-regulated cellular functions (Piette et al., 2011). Interestingly, three proteins (Pnp, TypA and Tig, involved in distinct functions such as degradosome, membrane integrity and protein folding, respectively) have been identified as CAPs in *Ph*TAC125 (Piette et al., 2010) and as CSPs in mesophiles. Moreover, several CSP homologues have been reported in other cold-adapted bacteria (Bakermans et al., 2007; Bergholz et al., 2009; Kawamoto et al., 2007), suggesting striking similarities between CSPs in mesophiles and CAPs in psychrophiles. In agreement with these results, it has been proposed that "from an evolutionary point of view, one of the adaptive mechanisms for growth in the cold is to regulate the cold-shock response, shifting from a transient expression of CSPs to a continuous synthesis of at least some of them" (Piette et al., 2010, 2011).

The proteomic analyses of *Ph*TAC125 revealed that 30% of the identified CAPs are directly related to protein synthesis, covering all essential steps, from transcription (including RNA polymerase RpoB) to translation (i.e. methionyl-tRNA synthetase MetG that can be connected to the need of an increased pool of initiation tRNA to promote protein synthesis) and folding (i.e. the trigger factor—TF—Tig that acts on proteins synthesised by the ribosome; and PpiD, involved in the folding of outer membrane proteins). The genes *pnp* and *rpsA* also encode components of the degradosome that regulate transcript lifetimes and two putative proteases (*PSHAa2492* and *PSHAa2260*), identified as CAPs, likely to be involved in the proteolysis of misfolded proteins (Piette et al., 2010). Other identified CAPs are both components and regulators of the outer membrane architecture (Piette et al., 2010). In particular, the TonB-dependent receptor is indicative of sensing and exchanges with the external medium, and TypA (involved in lipopolysaccharides core synthesis) and Pal (a peptidoglycan-associated protein) are involved in the outer membrane stability and integrity (Abergel, Walburger, Chenivesse, & Lazdunski, 2001).

These results strongly suggest that low temperatures impair protein synthesis and folding, resulting in up-regulation of the associated functions and indicating that both cellular processes are limiting factors for bacterial development in cold environments (Piette et al., 2010).

Similar amounts of ribosomal and translation-specific proteins have also been revealed in mesophilic fast-growing bacteria, such as *B. subtilis* or *B. licheniformis*, under optimal growth conditions (Buttner et al., 2001; Voigt et al., 2004; Wilmes et al., 2011), suggesting that also the expression of these proteins, directly related to protein synthesis, is likely growth-rate dependent (Klumpp, Zhang, & Hwa, 2009).

Based on the cytoplasmic proteome and the available genome sequence, the analysis of the amino acid degradation pathways showed that all the common degradation routes are present in *Ph*TAC125, with the exception of those involved in Trp and Lys catabolism (Wilmes et al., 2011). Since the Antarctic genome contains coding sequences of biosynthetic enzymes for all 20 proteinogenic amino acids, it is likely that the degradation of Trp and Lys occurs by reversal of the biosynthetic routes. For instance, an alternative way to use Lys may be decarboxylation to cadaverine via *PSHAa1094* (annotated as a putative basic amino acid decarboxylase) (Wilmes et al., 2011). Further, a relatively high abundance of the tricarboxylic acid cycle enzymes in the cytoplasmic proteome analysed at 16 °C, needed for efficient catabolism of the peptone-based amino acids, is in line with the extremely high growth rate (maximal rate being 0.35 h^{-1}) of the Antarctic bacterium (Wilmes et al., 2011).

The major CAP, 37-fold over-expressed at 4 °C (Piette et al., 2010), is the TF Tig, a CSP in *E. coli* (Kandror & Goldberg, 1997). TF is the first molecular chaperone interacting with virtually all newly synthesised polypeptides on the ribosome; it assists folding by delaying premature chain compaction and maintaining the elongating polypeptide in a non-aggregated state until adequate structural information for correct folding is available, and later promotes protein folding (Hartl & Hayer-Hartl, 2009; Martinez-Hackert & Hendrickson, 2009; Merz et al., 2008). TF also possesses a peptidyl-prolyl *cis–trans* isomerase activity (Kramer et al., 2004), the rate-limiting step in the folding of a wide range of proteins (Baldwin, 2008). In *Ph*TAC125, the peptidyl-prolyl *cis–trans* isomerase involved in protein folding is up-regulated at low temperature (Piette et al., 2010).

The major heat-shock proteins (HSPs), such as the chaperone DnaK, the chaperonin GroEL/ES and the chaperone Hsp90, are cold-repressed in the proteome of *Ph*TAC125. However, their expression is up-regulated when the bacterium is grown at higher temperature, indicating heat-induced cellular stress (Goodchild et al., 2005; Rosen & Ron, 2002). Synthesis of HSPs is also repressed during growth at low temperatures (Kandror & Goldberg, 1997) in *E. coli*. Accordingly, the observed cold repression of HSPs would be beneficial not only to *Ph*TAC125 but also to *E. coli*.

TF is transiently expressed in mesophilic bacteria but continuously overexpressed in psychrophiles to achieve cold adaptation, rescuing the chaperone function at low temperatures (Piette et al., 2010, 2011).

Either PPiases or TF act as potential CAPs in the proteome of most cold-adapted microorganisms analysed so far (Goodchild et al., 2005, 2004; Kawamoto et al., 2007; Qiu et al., 2006; Suzuki, Haruki, Takano, Morikawa, & Kanaya, 2004; Ting et al., 2010), suggesting that the constraint imposed by protein folding at low temperature and the cellular responses are common traits in most psychrophiles (Piette et al., 2010). In contrast, an almost inverse regulation was found in *P. arcticus* where GroEL/ES chaperonins and repression of TF are up-regulated under cold conditions (Bergholz et al., 2009; Zheng et al., 2007). Increased synthesis of chaperonins has also been reported in *S. alaskensis* (Ting et al., 2010) possessing two sets of *dnaK–dnaJ–grpE* gene clusters; proteomic analysis suggests that one of these sets functions as a low-temperature chaperone system whereas the other functions at higher temperatures (Ting et al., 2010).

At low temperature, in accordance with reduced biomass, almost half of the down-regulated proteins are involved in general bacterial metabolism. Most of these proteins are involved in oxidative metabolism, including glycolysis, the pentose phosphate pathway, the Kreb's cycle and the electron chain transporters (Piette et al., 2011; Wilmes et al., 2011).

The *Ph*TAC125 genome contains genes putatively involved in NO metabolism, such as NO reductase, *PSHAa2417*, and NO_2^- reductase, *PSHAa1477* (Médigue et al., 2005). In this context, the presence of multiple genes in distinct positions on chromosome I encoding three TrHbs (annotated as *PSHAa0030*, *PSHAa0458*, *PSHAa2217*) and a FHb (*PSHAa2880*) (Giordano et al., 2007; Médigue et al., 2005) may be pivotal for cell protection (see Section 6).

5.3. Excess of O_2 and metabolic constraints

Gases (e.g. O_2) and radicals (e.g. NO) are highly soluble and stable at low temperature with visible consequences in genome annotations in cold-adapted bacteria, having developed responses to strong oxidative stress (see Casanueva et al., 2010).

The apparent benefits of easier O_2 supply are contrasted by the adverse effects of low temperature on (macro)molecular functions and on the increased production of RNS and reactive oxygen species (ROS) (Casanueva et al., 2010; D'Amico et al., 2006). In fact, although RNS and

ROS could act as signalling molecules during cell differentiation and cell cycle progression, and in response to extracellular stimuli (Sauer, Wartenberg, & Hescheler, 2001), they are potentially toxic for cells (Finkel, 2003), being involved in a large number of pathological mechanisms.

Several mechanisms to cope with RNS and ROS have been proposed for cold-adapted bacteria. They include slightly lower frequency of oxidisable residues in protein sequences, occurrence of specific reductases, presence of dioxygenases and deletion of RNS- and ROS-producing metabolic pathways (see Casanueva et al., 2010). Interestingly, acyl desaturases (that introduce a double bond into fatty-acyl chains, using O_2 as substrate) combine the elimination of toxic O_2 with the improvement of membrane fluidity (Zhang & Rock, 2008). Therefore, the augmented capacity in antioxidant defence is likely an important component of evolutionary adaptation to a cold and O_2-rich environment (Ayub et al., 2009; Bakermans et al., 2007; Duchaud et al., 2007; Médigue et al., 2005; Methé et al., 2005; Piette et al., 2010; Rabus et al., 2004).

The cold environment raises the question of how *Ph*TAC125 can cope with RNS and ROS. We have evidence proving that, in order to prevent significant damage to cellular structures, *Ph*TAC125 improves the redox buffering capacity of the cytoplasm, and glutathione synthetase is strongly up-regulated at low temperature (Piette et al., 2010). These adjustments in antioxidant defences are needed to maintain the steady-state concentration of ROS and may be important components in evolutionary adaptations in cold and O_2-rich environments (Chen et al., 2008).

The main adaptive strategy used by *Ph*TAC125, exposed to permanent oxidative stress, is expected to be increased production of enzymes active against hydrogen peroxide and superoxide. Surprisingly, in the genome of *Ph*TAC125, only two genes, encoding an iron superoxide dismutase (*sodB*; *PSHAa1215*) and a catalase (*katB*, with the possible homologue *PSHAa1737*), have been identified. This catalase has very high similarity to catalases from other α-, β- and γ-Proteobacteria, for example, *Psychrobacter*, *Mannheimia*, *Haemophilus* and *Neisseria* (Médigue et al., 2005). Moreover, while the O_2-responding OxyR control has been found in *Ph*TAC125, SoxR regulation is absent (Médigue et al., 2005).

Proteomic analyses of *Ph*TAC125 reveal that oxidative stress-related proteins, such as catalase, glutathione reductase and peroxiredoxin (Piette et al., 2011), are repressed at 4 °C. However, because *Ph*TAC125 metabolism is stimulated at 18 °C, it should be mentioned that, although these proteins would be repressed at 4 °C, they would most likely be induced at 18 °C (Piette et al., 2011).

In contrast, the Arctic bacterium *C. psychrerythraea* has developed an enhanced antioxidant capacity owing to the presence of three copies of catalase genes as well as two superoxide-dismutase genes, one of which codes for a nickel-containing superoxide-dismutase, never reported before in proteobacteria (Methé et al., 2005).

*Ph*TAC125 copes with increased O_2 solubility by deleting entire metabolic pathways that generate ROS as side products. It is worth noting that, despite the availability of molybdate in sea water (Hille, 2002), *Ph*TAC125 not only lacks the molybdate biosynthetic and transport genes but also genes encoding enzymes using molybdate as cofactor, for example, trimethylamine N-oxide reductase, xanthine oxidase, biotin sulphoxide reductase and oxido-reductase YedY (Loschi et al., 2004).

The *Ph*TAC125 genome also contains all genes required for the pentose phosphate pathway (Médigue et al., 2005); moreover, glucose-6-phosphate dehydrogenase (Zwf; *PSHAa1140*), transketolase (TktA; *PSHAa0671*) and transaldolase (TalB; *PSHAa2559*) have been found in the cytoplasmic proteome (Wilmes et al., 2011). This feature increases the concentration of NADPH, which in turn provides high levels of reduced thioredoxin that can help to protect against the toxic effects of O_2. Therefore, to develop better oxidative stress adaptation in the cold, *Ph*TAC125 can use the pentose phosphate pathway for carbohydrate inter-conversion.

In order to cope with the improved stability of ROS at low temperatures, iron-related proteins are down-regulated at 4 °C (Piette et al., 2011), presumably to avoid oxidative cell damage induced by the deleterious Fenton reaction (Valko, Morris, & Cronin, 2005). Cell protection may be achieved by dioxygenases that are coded in large number in both chromosomes (Médigue et al., 2005). Moreover, O_2-consuming lipid desaturases protect against toxic O_2 by increasing the membrane fluidity at low temperature (Médigue et al., 2005).

A further tool, possibly related to the peculiar features of the Antarctic habitat, may be the synthesis of globins facilitating several biological functions, including protection from nitrosative and oxidative stress (see Section 6.2.4).

5.4. Biotechnological applications

Microorganisms are an interesting source of cold-active enzymes endowed with biotechnological potential. Enzymes from psychrophiles have recently received increasing attention, because they offer novel opportunities in

several industrial processes where high enzymatic activity or peculiar stereospecificity at low temperature is required. The high specific activity of cold-adapted enzymes is due to the lack of a number of non-covalent stabilising interactions, providing improved flexibility of the conformation (Feller, 2010; Feller & Gerday, 2003; Siddiqui & Cavicchioli, 2006); this is a key adaptation to compensate for the exponential decrease in chemical reaction rates at lower temperatures. For instance, cold-active esterases and lipases found in the genome of *Ph*TAC125 (Aurilia, Parracino, Saviano, Rossi, & D'Auria, 2007; de Pascale et al., 2008; Médigue et al., 2005) can be added to detergents for use at low temperatures and to biocatalysts for biotransformation of heat-labile compounds (Margesin & Schinner, 1994). Cold-active Lip1 lipase (de Pascale et al., 2008), encoded by the *PSHAa0051* gene, was functionally over-expressed in *Ph*TAC125 at 4 °C (Duilio et al., 2004). In contrast, in mesophilic *E. coli*, the recombinant production was always found associated with the inclusion bodies and refolding was unsuccessful (de Pascale et al., 2008).

Engineered *Ph*TAC125 expressing a toluene-*o*-xylene mono-oxygenase from mesophilic *Pseudomonas* sp. OX1 (Bertoni, Bolognese, Galli, & Barbieri, 1996), combined with the endogenous laccase-like protein induced by copper, can convert several aromatic compounds into non-toxic metabolites (Papa, Parrilli, & Sannia, 2009; Parrilli, Papa, Tutino, & Sannia, 2010; Siani, Papa, Di Donato, & Sannia, 2006). This strategy endows *Ph*TAC125 with degrading capabilities and wide potentiality in bioremediation applications, for example, removal of organic pollutants from chemically contaminated marine environments and cold effluents of industrial processes (Parrilli, Papa, et al., 2010).

6. *P. haloplanktis* TAC125 GLOBINS

6.1. General aspects

Three TrHbs were identified in *Ph*TAC125: one TrHbI (encoded by the *PSHAa0458* gene) and two TrHbsII (encoded by *PSHAa0030* and *PSHAa2217*) (Giordano et al., 2007). The sequence identity between TrHbs from different groups is generally low but may be higher within a given group. The identity between the two TrHbs belonging to group II is 24%, suggesting that these proteins may have different function(s) in bacterial cellular metabolism. Moreover, a FHb, annotated as *PSHAa2880*, has been found in *Ph*TAC125 (Giordano et al., 2007).

The distribution of globins in polar bacteria is very different (Table 8.3).

Table 8.3 Distribution of FHb and TrHbs in polar bacteria

Polar bacteria	Strain origin	FHb	TrHbs
Colwellia psychrerythraea 34H	Arctic marine sediments	1	–
Shewanella frigidimarina NCMB400	Sea ice, sea water, Antarctica	1	2
Psychrobacter arcticus 273-4	Siberian permafrost	–	–
Psychrobacter cryohalolentis K5	Siberian permafrost	1	–
Oleispira antarctica RB-8	Rod Bay, Ross Sea Antarctica	–	–
Pseudoalteromonas haloplanktis TAC125	Coastal Antarctic sea water, Terre Adélie	1	3
Desulfotalea psychrophila LSv54	Arctic marine sediments, Svalbard	–	–
Exiguobacterium sibiricum 255-15	Siberian permafrost	1	1
Psychroflexus torquis ATCC 700755	Sea-ice algal assemblage Prydz Bay, Antarctica	–	–
Polaribacter filamentous 215	Surface sea water, north of Deadhorse, Alaska	–	–
Polaribacter irgensii 23-P	Nearshore marine waters off Antarctic Peninsula	–	–
Psychromonas ingrahamii 37	Sea ice, off Point Barrow, Northern Alaska	1	1
Marine actinobacterium PHSC20C1	Nearshore marine waters of Antarctic Peninsula	1	1

Interestingly, the *C. psychrerythraea* and *P. cryohalolentis* K5 genomes do not possess genes encoding TrHbs, but contain a gene for a single FHb; *E. sibiricum* 255-15 (Rodrigues et al., 2008), *P. ingrahamii* 37 (Riley et al., 2008) and *Marine actinobacterium* PHSC20C1 genomes contain one gene encoding a TrHb and one encoding an FHb; *S. frigidimarina* NCMB400 contains two genes encoding TrHbs and one encoding a FHb; *P. arcticus* 273-4, *Oleispira antarctica* RB-8, *D. psychrophila* LSv54, *Psychroflexus torquis* ATCC 700755, *Polaribacter filamentous* 215 and *Polaribacter irgensii* 23-P do not possess globin genes (Table 8.3).

The presence of multiple genes encoding globins may be considered a mechanism of defence against oxidative and nitrosative stress also in mesophilic organisms.

M. tuberculosis carries both a TrHbI (HbN) and a TrHbII (HbO) (Milani et al., 2005). It is worth noting that HbN efficiently protects *M. tuberculosis* from nitrosative damage, contributing to its survival in the host macrophage (Bidon-Chanal et al., 2006), whereas HbO may have an oxidation/reduction function because it has peroxidase activity with formation of ferryl intermediates (Ouellet et al., 2007). On the other hand, *Mycobacterium leprae* only displays HbO, which is capable of protecting against NO as well as oxidative stress (Ascenzi, De Marinis, Coletta, & Visca, 2008). *B. subtilis* (Ouellet et al., 2007) and *Thermobifida fusca* (Bonamore et al., 2005) encode both a TrHbII and an FHb. To our knowledge, *Ph*TAC125 is the first example of coexistence of genes encoding a FHb and three TrHbs (see Section 6.2.2).

A transcriptional analysis of the *PSHAa0030*, *PSHAa0458* and *PSHAa2217* genes encoding the TrHbs and the FHb-encoding gene was carried out on *Ph*TAC125 wild type and on a mutant strain in which *PSHAa0030* was inactivated. In *Ph*TAC125 wild-type cells, *PHSAa0030* is expressed at 4 °C and 15 °C. *PSHAa0458* and *PSHAa2217* encoding the other TrHbs are expressed in both strains under all conditions, whereas transcription of the FHb-encoding gene is detectable only in mutant cells grown at 4 °C in micro-aerobiosis (Parrilli, Giuliani, Giordano, et al., 2010) (see Section 6.2.4).

To date, only group II of the TrHbs encoded by *PSHAa0030* (hereafter named *Ph*-2/2HbO) has been thoroughly investigated from the structural and functional viewpoints (Coppola et al., 2013; Giordano et al., 2007, 2011; Howes et al., 2011; Parrilli, Giuliani, Giordano, et al., 2010; Russo et al., 2013). The gene was selected as the first of the three because its position on chromosome I is very close to the origin of replication of the bacterium, indicating an important physiological role (Giordano et al., 2007).

Since transcription of FHb-encoding genes is usually directly or indirectly induced by NO (Hausladen et al., 1998; Spiro, 2007), the observed FHb-gene expression only in a *Ph*TAC125 mutant strain is suggestive of occurrence of an NO-induced stress related to the absence of the TrHb encoded by *PSHAa0030* (Parrilli, Giuliani, Giordano, et al., 2010; see Section 6.2.4).

6.2. Structure–function relationships of *Ph*-2/2HbO

6.2.1 Structure

Group II of TrHbs is by far the most populated of the three and is characterised by specific residues building up the haem cavity (Vuletich &

Lecomte, 2006; Wittenberg et al., 2002). The crystal structures of TrHbsII from *B. subtilis* (Giangiacomo, Ilari, Boffi, Morea, & Chiancone, 2005), *T. fusca* (Bonamore et al., 2005), *Geobacillus stearothermophilus* (Ilari et al., 2007) and *M. tuberculosis* (Milani et al., 2005, 2003) show a network of interactions between polar residues and the haem-Fe atom that may explain the high O_2 affinity of these globins (Bonamore et al., 2005; Giangiacomo et al., 2005; Ilari et al., 2007; Milani et al., 2005; Mukai, Savard, Ouellet, Guertin, & Yeh, 2002; Ouellet et al., 2003).

Ph-2/2HbO displays structural features typical of TrHbII (Giordano et al., 2007). In particular, *Ph*-2/2HbO has Trp at G8, and Tyr at both CD1 and B10 (Fig. 8.4; Howes et al., 2011). These three positions are pivotal for the stabilisation of the haem-bound O_2 in TrHbsII (Milani et al., 2005). It is worth noting that CD1 Phe, that wedges the haem into its pocket, is considered a conserved residue among globins, unlike members of TrHbsII from *M. tuberculosis*, *Mycobacterium avium*, *M. leprae*, *Mycobacterium smegmatis*, *Streptomyces coelicolor*, *Corynebacterium diphtheriae* and *T. fusca*, which host Tyr instead (Table 8.2; Bonamore et al., 2005; Milani et al., 2005).

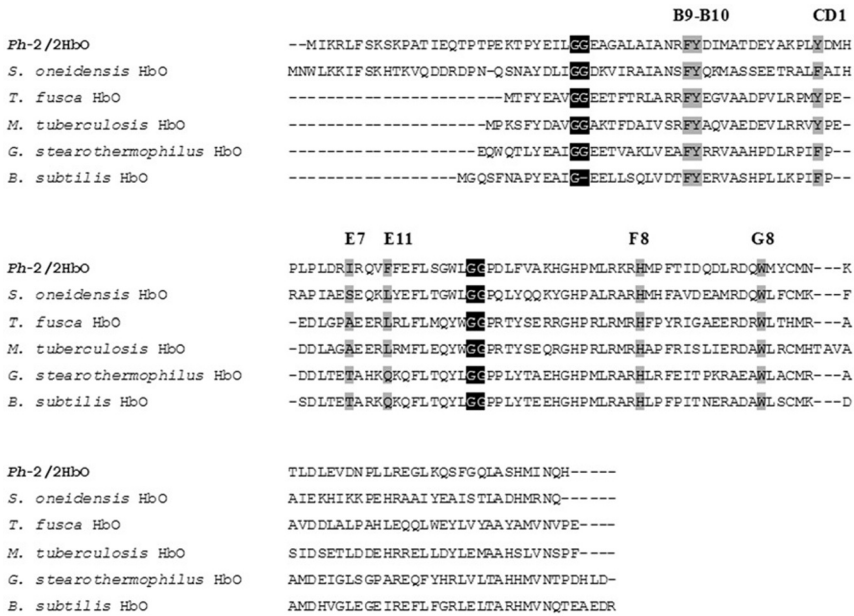

Figure 8.4 Sequence alignment of some representative TrHbs of group II. Identical functionally important residues of the distal haem pocket (B9, B10, CD1, E7, E11 and G8) and the proximal His F8 are highlighted in grey. The Gly-Gly motifs typical of TrHbs are highlighted in black. *Adapted from Howes et al. (2011).*

Ph-2/2HbO shows structural differences with respect to other TrHbsII. In particular, the insertion of three residues in the CD loop (Howes et al., 2011; Fig. 8.4) confers higher flexibility that may facilitate its action at low temperature, providing greater freedom for the correct positioning of ligand(s) (Feller & Gerday, 2003; Siddiqui & Cavicchioli, 2006). In contrast to TrHbsI, the E7 and E11 positions are occupied by non-polar residues, Ile and Phe, respectively, precluding haem-bound ligand stabilisation. On the proximal side, HisF8, conserved in all members of the globin superfamily (Howes et al., 2011), is coordinated to the haem-Fe atom (Table 8.2 and Fig. 8.4).

Ph-2/2HbO shows an unusual extension of 15 residues at the N-terminus (pre-helix A), similar to *M. tuberculosis* HbN and to many slow-growing species of *Mycobacterium*, such as *M. bovis*, *M. avium*, *M. microti*, *M. marinum* (Lama et al., 2009) and *Shewanella oneidensis* (Vuletich & Lecomte, 2006). The pre-A motif of *M. tuberculosis* HbN does not significantly contribute to the structural integrity of the protein, protruding out of the compact globin fold (Milani et al., 2001). However, the deletion of this motif reduces the ability of *M. tuberculosis* to scavenge NO (Lama et al., 2009). Unlike in *M. tuberculosis* HbN, the deletion of the N-terminal extension of *Ph*-2/2HbO does not seem to reduce the NO scavenging activity (Coppola et al., 2013) (see Section 6.2.4).

6.2.2 Hexacoordination

Ph-2/2HbO displays hexacoordination of the ferric and ferrous haem-Fe atom (Giordano et al., 2011; Howes et al., 2011). Hydrostatic pressure enhances hexacoordination in both oxidation states of the haem-Fe atom, as previously shown in other haem proteins (Hamdane et al., 2005), indicating that a flexible protein allows structural changes (Russo et al., 2013).

Binding of O_2 to Mb and Hb occurs on the distal side of the pentacoordinated haem-Fe atom, where O_2 establishes a sixth coordination bond to the Fe atom, whereas the fifth coordination position is occupied by invariant HisF8 (Fig. 8.5). The haem-Fe-bound O_2 is generally stabilised by interaction(s) with distal residues. The main O_2 stabilising interaction is usually provided by an H bond donated by HisE7 (Fig. 8.5).

In hexacoordinated globins, where, in the absence of external ligands, the sixth position is taken by an internal residue, exogenous ligands (e.g. O_2, CO and NO) compete with the internal ligand to bind Fe, this behaviour being the basis of the control of Fe reactivity (Smagghe, Trent, & Hargrove, 2008; Trent, Hvitved, & Hargrove, 2001).

Haem-Fe hexacoordination is widespread in globins, having been found in unicellular eukaryotes (Wittenberg et al., 2002), plants (Watts et al.,

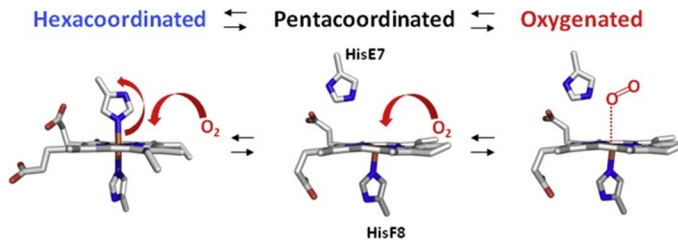

Figure 8.5 Schematical representation of the iron coordination in hexacoordinated, pentacoordinated and oxygenated forms. The protein is in hexacoordinated conformation when, in the absence of external ligands, there is an amino acid residue as internal ligand. The sixth ligand is usually provided by His. Upon addition of gaseous ligands, for example, O_2, there is competition between the external ligand and the sixth ligand, with replacement of the internal ligand with O_2.

2001), invertebrates (Dewilde et al., 2006), but only a few cases have been reported in bacterial TrHbs (Falzone, Christie Vu, Scott, & Lecomte, 2002; Razzera et al., 2008; Scott et al., 2002; Vinogradov & Moens, 2008; Visca et al., 2002). Hexacoordination has also been found in higher vertebrates, for example, in ferric β-chains of tetrameric Antarctic fish Hbs (Riccio, Vitagliano, di Prisco, Zagari, & Mazzarella, 2002; Vergara et al., 2007; Vergara, Vitagliano, Verde, di Prisco, & Mazzarella, 2008; Vitagliano et al., 2004, 2008) and in the ferric and ferrous states of mammalian (Pesce et al., 2003; Vallone, Nienhaus, Brunori, & Nienhaus, 2004) and Antarctic fish (Giordano et al., 2012) Ngbs and Cygbs (de Sanctis et al., 2004; Alessia Riccio et al., unpublished results). The occurrence of ferrous (haemochrome) and ferric (haemichrome) oxidation states in members of the Hb superfamily is not uniform, suggesting that the functional roles of these states are multiple, possibly being a tool for modulating ligand-binding or redox properties (Vergara et al., 2008; Vitagliano et al., 2008). Exchange between haemichrome and pentacoordinated forms may play a physiological role in Antarctic fish due to higher peroxidase activity (Vergara et al., 2008; Vitagliano et al., 2008).

Over the years, haemichromes in tetramers have been considered precursors of Hb denaturation (Rifkind, Abugo, Levy, & Heim, 1994); however, haemichromes can be obtained under non-denaturing as well as physiological conditions (Vergara et al., 2008). It has also been suggested that haemichromes can be involved in Hb protection from peroxide attack (Feng et al., 2005), given that the haemichrome species of human α-subunits complexed with the α-helix-stabilising protein do not exhibit peroxidase activity (Feng et al., 2005).

Hexacoordination of the haem-Fe atom may suggest a common physiological mechanism for protecting cells against oxidative chemistry in response to high O_2 concentration. Several roles have been hypothesised for hexacoordinated Ngb and Cygb, for example, as O_2 scavengers under hypoxic conditions (Burmester, Ebner, Weich, & Hankeln, 2002; Burmester, Weich, Reinhardt, & Hankeln, 2000), as terminal oxidases (Sowa, Guy, Sowa, & Hill, 1999), as O_2-sensor proteins (Kriegl et al., 2002), and in NO metabolism (Smagghe et al., 2008). It was recently reported that Ngb over-expression and intracellular localisation confer protection to neurons, both *in vitro* and *in vivo*, against oxidative stress and enhance cell survival under anoxia and ischaemic conditions (Fiocchetti, De Marinis, Ascenzi, & Marino, 2013). However, their physiological role is still a matter of debate.

Hexacoordinated globins are characterised by specific electronic absorption bands in the UV–visible spectra, clearly indicating the electronic structure of the Fe atom and its axial ligands (Dewilde et al., 2001).

The electronic-absorption spectrum of ferric *Ph*-2/2HbO is characterised by hexacoordinated high-spin (bands at 503 nm and charge-transfer transition at 635 nm) and low-spin forms (bands at 533 and 570 nm) (Fig. 8.6A), the latter being characteristic of a Tyr coordinated

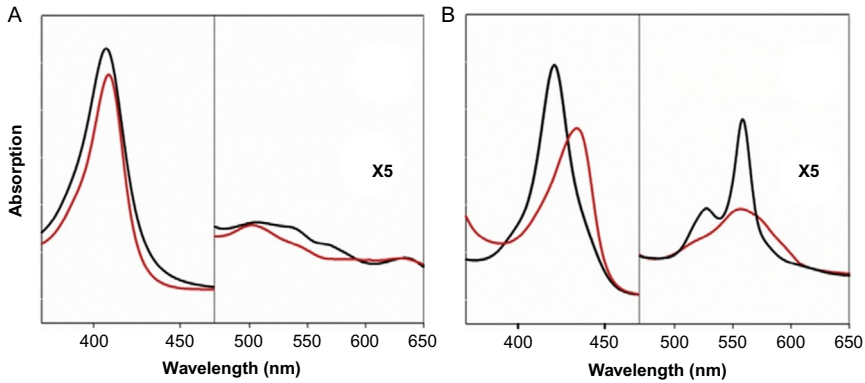

Figure 8.6 Overlay of absorption spectra of (A) ferric hexacoordinated *Ph*-2/2HbO (black line) with ferric Mb (red line) and (B) deoxy ferrous hexacoordinated *Ph*-2/2HbO (black line) with ferrous Mb (red line). All measurements were at pH 7.6 and 25 °C. The ferrous samples were prepared by adding 2 μl of sodium dithionite (10 mg ml^{-1}) to 600 μl of deoxygenated buffered solution of ferric globins, obtained flushing the ferric forms with nitrogen. The protein concentration was 10 μM on a haem basis.

to the Fe atom (Howes et al., 2011). The ferrous state shows a mixture of a predominant hexacoordinated low-spin state (Soret band at 421 nm and Q bands at 528 and 559 nm) and a pentacoordinated high-spin state (shoulder at 440 nm) (Fig. 8.6B; Giordano et al., 2011). These spectra are in marked contrast to those of monomeric Mb, in which the Fe atom is pentacoordinated. In fact, the deoxygenated ferrous form has a broad peak at 556 nm (Fig. 8.6B), whereas the ferric form exhibits two peaks at 504 and 632 nm (Fig. 8.6A; Antonini & Brunori, 1971).

Based on the spectroscopic data and molecular-dynamics simulation (Howes et al., 2011), it has been shown that either TyrCD1 or TyrB10 can coordinate the ferrous atom. Although His is the most common residue that coordinates the Fe atom, Tyr coordinates Fe of ferrous *Herbaspirillum seropedicae* Hb (Razzera et al., 2008) and of ferrous and ferric *Chlamydomonas* Hb (Couture et al., 1999; Das et al., 1999; Milani et al., 2005).

6.2.3 Reactivity

Reversible hexa- to pentacoordination of the haem-Fe atom modulates the reactivity of *Ph*-2/2HbO; in fact, the cleavage of the haem distal Fe-TyrCD1 or Fe-TyrB10 bonds is the rate-limiting step for the association of exogenous ligands (e.g. O_2, CO and NO) and (pseudo)enzymatic activities (Russo et al., 2013).

CO binding to *Ph*-2/2HbO displays a rapid spectroscopic phase independent of CO concentration, followed by standard bimolecular recombination. CO-rebinding kinetics show an unusually slow geminate phase, which becomes dominant at low temperature. While geminate recombination usually occurs on the ns timescale, *Ph*-2/2HbO displays a component of about 1 μs that accounts for half of the geminate phase at 8 °C, indicative of a relatively slow internal ligand binding (Russo et al., 2013).

After ligand escape, bimolecular recombination takes place. Second-order rebinding indicates two major conformations at 25 °C, characterised by CO-association rates that differ by a factor of 20, with pH-dependent relative fractions. A dynamic equilibrium was found between a predominant hexacoordinated low-spin state and a pentacoordinated high-spin state. A shift in the equilibrium between the two conformations may also provide a large change in the ligand affinity. The second-order rate constant of the fast phase (Russo et al., 2013) is of the order of $10^7\ M^{-1}\ s^{-1}$ and closely similar to that of human Ngb (Uzan et al., 2004), whereas the second-order rate constant of the slow process (Russo et al., 2013) is compatible with that of Mb (Table 8.4), being in the range of $10^5\ M^{-1}\ s^{-1}$ (Springer, Sligar,

Table 8.4 Values of kinetic parameters for O_2 and CO binding to penta and hexacoordinated Hbs.

haem protein	$k_{on}O_2$ (μM^{-1} s^{-1})	$k_{off}O_2$ (s^{-1})	$k_{on}CO$ (μM^{-1} s^{-1})	$k_{off}CO$ (s^{-1})	$P_{50}O_2$ (Torr)	Reference
Hexacoordinated						
Ph-2/2HbO (8 °C)	0.9	1.0	0.35	–	1	Russo et al. (2013)
Ph-2/2HbO (25 °C)		4.2	0.69 (slow) 12.0 (fast)	–	–	Giordano et al. (2011)
Ngb	170	0.7	40	–	6.8 (0.9 S–S)	Uzan et al. (2004)
Pentacoordinated						
Sperm whale Mb	14	12	0.51	0.019	0.51	Springer et al. (1994)
M. tuberculosis HbO*	0.11	0.0014	0.014	0.004	–	Ouellet et al. (2003)
	(80%) 0.85 (20%)	(78%) 0.0058 (22%)	(79%) 0.18 (21%)	(60%) 0.0015 (40%)	–	

*The relative percentage of the two rate constants are reported in parentheses.

Olson, & Phillips, 1994). The relatively fast CO-dependent kinetic process is unusual for a TrHbs of group II; the second-order rate constant of the fast phase for carbonylation of *M. tuberculosis* HbO is in the range of 10^5 M^{-1} s^{-1}, whereas the second-order rate constant of the slow phase is in the range of 10^4 M^{-1} s^{-1} (Ouellet et al., 2003). Thus the proteins displays two conformations that greatly differ in the ligand association rate, suggesting that they may switch between two distinct functional levels.

At 8 °C, 85% of the CO bimolecular recombination occurs on the ms timescale at a rate similar to that of a 3/3 Mb (3.5×10^5 M^{-1} s^{-1}), whereas the remaining kinetic component is faster (10^7 M^{-1} s^{-1}) (Russo et al., 2013), as observed at 25 °C (Giordano et al., 2011; Table 8.4).

Hexacoordination of the haem-Fe atom of *Ph*-2/2HbO via distal Tyr is only partial (the Tyr equilibrium affinity is close to 1), indicating a weak interaction between Tyr and the Fe atom under atmospheric pressure (Russo et al., 2013). The fast binding and dissociation of Tyr from the Fe atom can be a molecular event that triggers the shift of the globin between two

conformations (penta- vs. hexacoordinated haem) with different redox potentials. This behaviour may be considered as an ancestral mechanism for modulating a conformational switch between two functional species (Russo et al., 2013).

Ph-2/2HbO is quickly oxidised in the presence of O_2, probably due to the superoxide character of the haem-Fe-O_2 adduct, affected by the presence of the surrounding hydrogen-bond donor residues (Milani et al., 2001, 2003).

The O_2 affinity, poorly affected by competition with Tyr, is about 1 Torr at 8 °C, pH 7.0 (Table 8.4). The O_2 affinity of *Ph*-2/2HbO is compatible with the *in vivo* conditions (Fig. 8.7A), considering that the *Ph*TAC125 bacterial metabolism must cope with high O_2 concentration and high-salinity conditions at low temperature. However, Mb-like functions do not seem to be possible for *Ph*-2/2HbO, requiring a still unknown efficient reducing system, and a local high globin concentration (Russo et al., 2013).

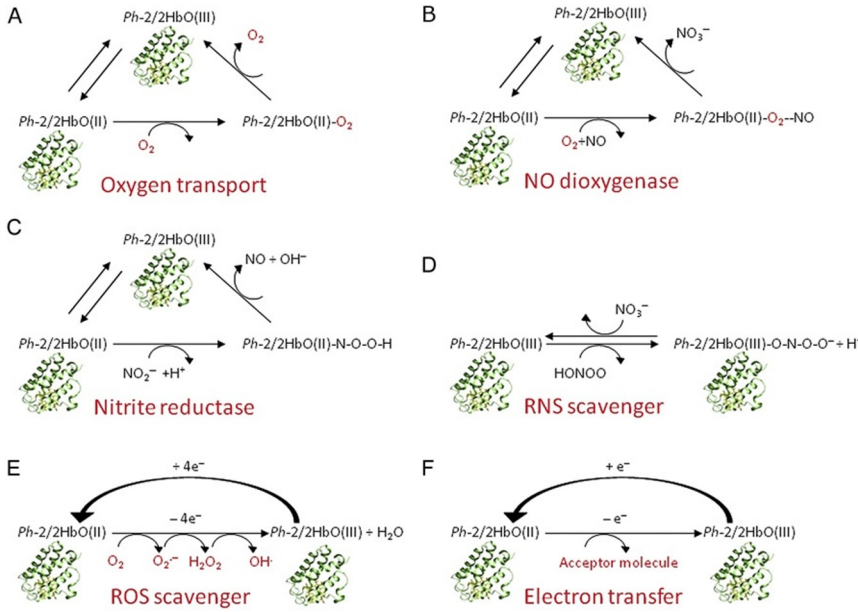

Figure 8.7 Some postulated functions of *Ph*-2/2HbO. (A) O_2 carrier even though this function requires the presence of a still unknown coupled reductase system for high turnover reaction; (B) *Ph*-2/2HbO may convert NO to nitrate and (C) nitrite to NO; (D) it may detoxify RNS and (E) ROS; (F) it may function as electron transfer coupled to an acceptor molecule. *The model of* Ph-*2/2HbO was kindly provided by L. Boechi.*

Thus *Ph*-2/2HbO is likely to be involved in a redox reaction(s) associating diatomic ligands and their derived oxidative species. Such a reaction is not unusual, since globins generally display NO dioxygenase activity leading to nitrate synthesis (Flögel, Merx, Gödecke, Decking, & Schrader, 2001; Gardner et al., 2006; Fig. 8.7B). It is noteworthy that *Ph*-2/2HbO provides protection against NO and related reactive species (Fig. 8.7D), under aerobic conditions (Coppola et al., 2013; see Section 6.2.4). Moreover, *Ph*-2/2HbO exhibits a twofold higher nitrite-reductase activity than horse Mb at pH 7.0, 25 °C. This evidence suggests (Fig. 8.7C) that, during an anaerobic phase, *Ph*-2/2HbO may supply NO via nitrite reduction (Russo et al., 2013).

Other reactions (Fig. 8.7E) may involve complex ROS chemistry (Flögel, Gödecke, Klotz, & Schrader, 2004). O_2 is necessary for bacterial metabolism, but can become poisonous if it is responsible for oxidative-stress burst. A number of reactions take place between ROS and the haem-Fe atom since O_2 is reduced by four e^- before yielding a water molecule with three ROS intermediates, whereas the haem-Fe atom is susceptible to oxidation from +2 to +4. In general, pentacoordinated globins are more prone to ROS oxidation than hexacoordinated forms (Herold, Kalinga, Matsui, & Watanabe, 2004; Lardinois, Tomer, Mason, & Deterding, 2008). However, in *Ph*-2/2HbO, due to the low affinity of the haem for distal Tyr (weak protection) in ferrous and ferric states, the protection against deleterious oxidation at high ROS concentration (autocatalytic oxidations leading to irreversible haem oxidation and globin degradation) is not expected (Russo et al., 2013).

The redox state of *Ph*-2/2HbO *in vivo* will depend on the presence of specific reductases and on the O_2 levels. The redox state could be involved in an electron-transfer reaction or in a regulatory mechanism with the protein acting as a redox sensor. In fact, at high O_2 concentration, in the presence of a reducing system that can compensate for autoxidation, the globin will be mainly ferrous, but under intermediate conditions it could be in the ferric form. This behaviour has been observed in pentacoordinated sperm whale and pig Mb (II), probably upon nucleophile attack such as that mediated by water (Brantley, Smerdon, Wilkinson, Singleton, & Olson, 1993), and in hexacoordinated bis-His form of the globin GLB-26 of the nematode worm *Caenorhabditis elegans* and of human Ngb, which promotes O_2 reduction (Kiger et al., 2011). By analogy, the hexacoordinated His-Fe-Tyr adduct of *Ph*-2/2HbO (Fig. 8.7F) may be involved in electron transfer (Russo et al., 2013).

6.2.4 NO detoxification

The remarkably high number of TrHbs in the *Ph*TAC125 genome strongly suggests that these globins fulfil important functional roles associated with the extreme features of the Antarctic habitat. The involvement of cold-adapted *Ph*-2/2HbO in detoxification of RNS and ROS may be a mechanism associated with high production of toxic species upon cold stress. A similar function has been reported in HbN of *M. tuberculosis* (Pathania et al., 2002) and *M. bovis* (Ouellett et al., 2002) and *M. leprae* HbO (Fabozzi, Ascenzi, Renzi, & Visca, 2006), which protect pathogenic bacteria from the toxic activity of macrophage-generated RNS and ROS.

The physiological role of *Ph*-2/2HbO has been investigated using a genomic approach, by the construction of a *Ph*TAC125 mutant strain in which the *PSHAa0030* gene was inactivated by insertional mutagenesis. The mutant strain was grown under controlled conditions and its growth behaviour was compared to that of wild-type cells, changing O_2 pressure in solution and growth temperature (4 and 15 °C). Regardless of temperature, growth of the mutant strain in extreme aerobiosis is lower than that of the wild type, also in terms of biomass. The presence of *Ph*-2/2HbO in wild-type cells is thus an advantage when cells are grown at high O_2 concentration. In micro-aerobiosis, both strains slow down their replication kinetics. At 4 °C, the wild-type cells appear better suited to the challenging conditions, reaching higher biomass than the mutant cells (Parrilli, Giuliani, Giordano, et al., 2010).

The inactivation of the *Ph*-2/2HbO gene makes the mutant strain sensitive to high O_2 levels, hydrogen peroxide and nitrosating agents (Parrilli, Giuliani, Giordano, et al., 2010), suggesting involvement of the protein in protection from oxidative and nitrosative stress. Moreover, the transcription of the FHb-encoding gene occurs only in the mutant in which *PSHAa0030* is inactivated, when grown in micro-aerobiosis at 4 °C, suggesting that the occurrence of the NO-induced stress is probably related to the absence of *Ph*-2/2HbO (Parrilli, Giuliani, Giordano, et al., 2010). In micro-aerobiosis, *Ph*TAC125 may endogenously produce NO, due to a gene encoding a nitrite reductase (*PSHAa1477*), as reported in other Gram-negative bacteria (Corker & Poole, 2003; Ji & Hollocher, 1988). Further, NO may accumulate when its spontaneous oxidation is limited by low O_2 availability. In micro-aerobiosis, O_2 is further reduced when the biomass is increased, that is, in the late exponential phase, and NO accumulation may become a serious threat for cell viability. Induction of the FHb gene may be viewed as a suitable strategy aimed at counteracting NO-induced stress due to the absence of *Ph*-2/2HbO (Parrilli, Giuliani, Giordano, et al., 2010).

An up-to-date approach set up by Coppola et al. (2013) to establish the participation of *Ph*-2/2HbO in RNS detoxification, tested the influence of heterologous expression of the *PSHAa0030* gene *in vivo* on protection from NO toxicity in a NO-sensitive *E. coli* strain (*E. coli hmp*, defective in the FHb) (see Section 4.1).

The growth properties and O_2 uptake of *E. coli hmp* having the *PSHAa0030* gene was analysed in an attempt to demonstrate that *Ph*-2/2HbO offers resistance to nitrosative stress. Wild-type *E. coli* and a *hmp* mutant, carrying the *PSHAa0030* gene or not, were grown at 25 °C under aerobic conditions and treated with either the NO-releaser DETA-NONOate or the nitrosating agent GSNO. As expected, exposure to these sources of NO has no effect on the growth of wild-type *E. coli* or the expression of Hmp from the complemented plasmid, and a comparable level of resistance is evident in cells expressing *Ph*-2/2HbO. In contrast, in the absence of *Ph*-2/2HbO, expression results in severe growth inhibition (Coppola et al., 2013).

Moreover, Coppola et al. (2013) demonstrated that upon addition of NO, *E. coli hmp* not expressing *Ph*-2/2HbO shows prolonged inhibition of O_2 uptake (Fig. 8.8A) until the NO level falls steadily. In contrast, in the mutant strain carrying hmp^+, the addition of NO does not inhibit O_2 uptake (Fig. 8.8B), confirming that Hmp is able to detoxify NO, as reported previously (Membrillo-Hernández et al., 1999; Mills, Sedelnikova, Søballe, Hughes, & Poole, 2001; Stevanin et al., 2000). Upon addition of NO to the *E. coli* mutant carrying *Ph*-2/2HbO, only very short periods of inhibition of respiration are observed and, again, the disappearance of NO is very fast (Fig. 8.8C). When NO reaches negligible levels, the O_2 uptake is brought back to a rate similar to that occurring before NO addition, unlike in the cells bearing the empty vector (Fig. 8.8A). Following exhaustion of O_2, further addition of NO results in a larger signal and a slower rate of consumption (Coppola et al., 2013).

Under aerobic conditions, over-expression of *Ph*-2/2HbO provides significant resistance to NO and nitrosating agents and distinct NO consumption ability to the NO-sensitive *E. coli hmp* mutant. In contrast, growth curves and cellular respiration are strongly inhibited in *E. coli hmp* not expressing the Antarctic globin gene. These results are clear evidence of a very important physiological role of *Ph*-2/2HbO in *Ph*TAC125.

In vitro kinetic studies of peroxynitrite isomerisation by the ferric protein support the NO and O_2^- detoxification activity as a possible functional role of the cold-adapted globin, thus confirming the involvement of *Ph*-2/2HbO in the protection from nitrosative stress. The high reactivity of

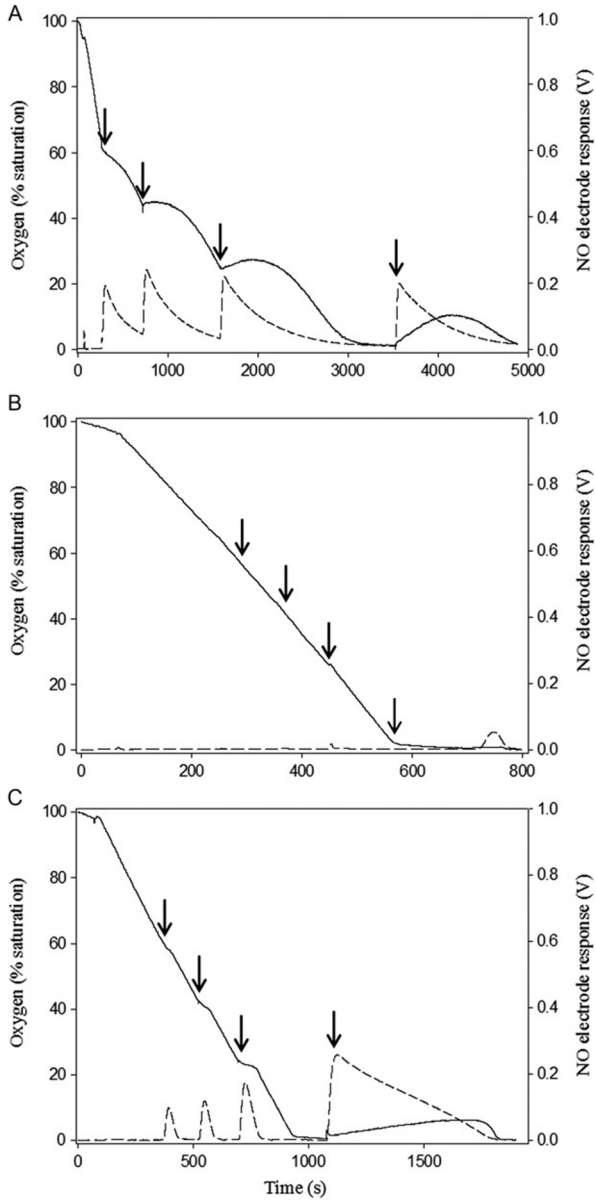

Figure 8.8 NO uptake and respiration of *E. coli hmp* cells (A) carrying the empty vector pBAD/HisA, or (B) expressing Hmp, or (C) *Ph*-2/2HbO. Respiration was followed in a Clark-type O_2 electrode (solid lines) upon addition of 1 μM Proli-NONOate (arrows). NO uptake was measured simultaneously with NO electrode (dashed lines). *Adapted from Coppola et al. (2013).*

ferric *Ph*-2/2HbO towards peroxynitrite at low temperature ($k_{on} = 3.5 \times 10^4\ M^{-1}\ s^{-1}$ at 5 °C and $2.9 \times 10^4\ M^{-1}\ s^{-1}$ at 20 °C) suggests that the protection of the psychrophile *Ph*TAC125 against RNS and ROS may happen also in the cold Antarctic environment (Coppola et al., 2013). The k_{on} values for the ferric *Ph*-2/2HbO-mediated peroxynitrite isomerisation are similar to those reported for catalysis of the ferric equine-heart Mb ($2.9 \times 10^4\ M^{-1}\ s^{-1}$, Herold & Kalinga, 2003), sperm-whale Mb ($1.6 \times 10^4\ M^{-1}\ s^{-1}$, Herold et al., 2004) and human Hb ($1.2 \times 10^4\ M^{-1}\ s^{-1}$, Herold & Kalinga, 2003), whereas they are lower than those of horse-heart native and carboxymethylated cytochrome *c* in the presence of saturating cardiolipin (3.2×10^5 and $5.3 \times 10^5\ M^{-1}\ s^{-1}$, respectively), horse-heart carboxymethylated cytochrome *c* ($6.8 \times 10^4\ M^{-1}\ s^{-1}$), and human serum haem-albumin ($4.1 \times 10^5\ M^{-1}\ s^{-1}$) (Ascenzi, Bolli, et al., 2011; Ascenzi, Bolli, Gullotta, Fanali, & Fasano, 2010; Ascenzi, Ciaccio, Sinibaldi, Santucci, & Coletta, 2011a, 2011b; Ascenzi et al., 2009). As reported for several haemoproteins, the acceleration of the peroxynitrite-isomerisation rate by ferric *Ph*-2/2HbO seems to be caused by reaction of peroxynitrite with the ferric penta-coordinated derivative only (Ascenzi, Bolli, et al., 2011, 2010; Ascenzi et al., 2011a, 2011b, 2009; Goldstein, Lind, & Merényi, 2005; Herold & Kalinga, 2003; Herold et al., 2004).

Taken together, the *in vivo* and *in vitro* evidence suggests that, under aerobic conditions, *Ph*-2/2HbO supplies cell protection against RNS and ROS, compensating for the defect in NO detoxification of *E. coli hmp*, which lacks the major NO-scavenging protein.

Finally, attempting to ascertain whether the N-terminal motif of *Ph*-2/2HbO is a requirement for efficient NO scavenging, similar to *M. tuberculosis* HbN (Lama et al., 2009; see Section 6.2.1), Coppola et al. (2013) investigated the effect of deletion of the first 20 residues of the N-terminus of the protein on the ability of *E. coli hmp* strain to deal with nitrosative stress; while full-length *Ph*-2/2HbO restores the ability to survive and grow under nitrosative-stress conditions, the protein without the N-terminal extension does not significantly contribute to NO detoxification, since its deletion does reduce the globin NO-scavenging ability in the heterologous host, unlike in *M. tuberculosis* HbN.

7. CONCLUSION AND PERSPECTIVES

The Antarctic exhibits stable living conditions due to substantial isolation, also by virtue of the Polar Front. The evolutionary processes of

Antarctic organisms and the time scales in the context of geological and climatic changes have been extensively analysed and discussed (Peck, 2011).

The structure and function of proteins are the basis for understanding the evolutionary forces operating at sub-zero temperature, and—in this context—the knowledge gained at the molecular level is also crucial for predictions of the evolutionary consequences of global warming. In fact, at all analysed levels, the functional adaptation to permanently low temperature appears to require maintenance of flexibility of molecules in order to adequately support the cellular functioning. Proteins are one main factor of the ensuing mechanisms of adaptation.

Temperature is the prime driver that shaped the current structure and function of polar communities. Amongst abiotic factors influenced by temperature, O_2 and CO_2, and their concentrations, play an important role in life-sustaining processes. Due to low temperature, their concentrations are several-fold higher than in temperate and tropical marine habitats. The temperature-dependent balance between O_2 demand/supply and the associated functional capacity for specific functions of macromolecules shape the performance window in polar species (Pörtner et al., 2007). In polar environments, the benefits of O_2 levels (high by default) are indeed largely apparent, because they are counterbalanced by the kinetics of biological processes operating at low temperature (D'Amico et al., 2006) which decreases the rates, and by increased production of ROS. O_2 is obviously necessary for aerobic bacterial metabolism, but it can become poisonous in triggering oxidative-stress bursts.

In view of these considerations, in all Antarctic organisms, biological processes envisaging O_2 (respiration, transport/release, scavenging, reactive species, etc.) and other gases are bound to attract the interest of biologists.

In the realm of microbial life, the cold-adapted bacterial protein *Ph*-2/2HbO displays hexacoordination of the ferric and ferrous haem-Fe atom (Giordano et al., 2011; Howes et al., 2011). Investigating the features of this globin, in an attempt to shed light on possible multiplicity of functions, has been an important task. For instance, *Ph*-2/2HbO appears to exhibit a pseudo-enzymatic function in which O_2 is involved (Russo et al., 2013) and is available for reactions with NO to produce nitrate anions. In a single molecule, multiple conformations (penta- vs. hexacoordinated states) may account for multiple functions: under aerobic conditions, on one hand, *Ph*-2/2HbO provides cells with protection against NO and related RNS; on the other, during the anaerobic phase, *Ph*-2/2HbO may provide NO via nitrite reduction. The evidence summarised here indicates that

Ph-2/2HbO displays unique adaptive structural properties ensuring higher flexibility, thus facilitating its function at low temperatures, for example, by enhancing the capacity for correct positioning of ligand(s), which would be made more difficult by a rigid structure. In summary, this globin is a notable case study of relationship between molecular structure, cold adaptation and a wide range of equally important biological functions.

Hexacoordinated globins in Antarctic microorganisms call for efforts in shedding light on their place and role in the context of evolution. Knowledge of the range of their functions and physiological role *in vivo* is still incomplete and a matter of lively debate. However, modern concepts of biological sciences appear more and more to support the idea that the physiological role of a given molecule is not restricted to a single aspect, although one aspect may well be predominant. Based on this assumption, the current knowledge summarised in this review seems to be a useful starting point to achieve progress by further investigations aimed at increasing our albeit incomplete understanding of the biological function of a fundamentally important class of macromolecules such as globins, not only *Ph*-2/2HbO—a valuable case study—but also other globins, for example, Ngb and Cygb, whose biomedical significance is steadily growing.

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