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Coexistence of multiple globin genes conferring protection against nitrosative stress to the Antarctic bacterium Pseudoalteromonas haloplanktis TAC125

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Running title: The role of bacterial globins in the Antarctic environment

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Abstract

Despite the large number of globins recently discovered in bacteria, our knowledge of their physiological functions is restricted to only a few examples. In the microbial world, globins appear to perform multiple roles in addition to the reversible binding of oxygen; all these functions are attributable to the heme pocket that dominates functional properties. Resistance to nitrosative stress and involvement in oxygen chemistry seem to be the most prevalent functions for bacterial globins, although the number of globins for which functional roles have been studied via mutation and genetic complementation is very limited. The acquisition of structural information has considerably outpaced the physiological and molecular characterisation of these proteins.

The genome of the Antarctic cold-adapted bacterium Pseudoalteromonas haloplanktis TAC125 (PhTAC125) contains genes encoding three distinct single-chain 2/2 globins, supporting the hypothesis of their crucial involvement in a number of functions, including protection against oxidative and nitrosative stress in the cold and O_2-rich environment. In the genome of PhTAC125, the genes encoding 2/2 globins are constitutively transcribed, thus suggesting that these globins are not functionally redundant in their physiological function in PhTAC125. In the present study, the physiological role of one of the 2/2 globins, Ph-2/2HbO-2217, was investigated by integrating in vivo and in vitro results. This role includes the involvement in the detoxification of reactive nitrogen and O_2 species including NO by developing two in vivo and in vitro models to highlight the protective role of Ph-2/2HbO-2217 against reactive nitrogen species. The PSHAa2217 gene was cloned and over-expressed in the flavohemoglobin-deficient mutant of Escherichia coli and the growth properties and O_2 uptake in the presence of NO of the mutant carrying the PSHAa2217 gene were analysed. The ferric form of Ph-2/2HbO-2217 is able to catalyse peroxynitrite isomerisation in vitro, indicating its potential role in the scavenging of reactive nitrogen species. Here we present in vitro evidence for the detoxification of NO by Ph-2/2HbO-2217.

Keywords: Antarctic cold-adapted bacterium; bacterial globin; nitrosative/oxidative stress; Resonance Raman spectroscopy.

1. Introduction

Many bacterial genomes contain genes encoding more than a single globin and there is a strong correlation between the number of globin genes and the genome size (Giovannoni et al., 2005). Globins are classified in three families: (i) myoglobin (Mb)-like proteins (M), displaying the classical three-on-three (3/3) α-helical sandwich motif; (ii) sensor globins (S), and (iii) truncated (T) hemoglobins (Hbs), showing the two-on-two (2/2) α-helical-sandwich motif (Vinogradov et al., 2013). Members of the T family (also known as 2/2Hbs) are found in eubacteria, cyanobacteria, protozoa, and plants, but not in animals (Wittenberg et al., 2002; Milani et al., 2005; Vinogradov et al., 2013). On the basis of phylogenetic analysis, the T family can be further divided into three distinct sub-families/groups: HbI (or N), HbII (or O) and HbIII (or P), with a novel, small (4%) clade of sequences named HbIV (or Q) that contains only bacterial sequences (Bustamante et al., 2016).

Some of the organisms hosting the 2/2Hbs are pathogenic bacteria; others perform photosynthesis, fix nitrogen or may display distinctive metabolic capabilities (Pesce et al., 2013, and refs therein). Some proposed functions include protection from reactive oxygen and nitrogen species (ROS and RNS, respectively), O_2 and sulfide chemistry (Pesce et al., 2013, and refs therein; Boubeta et al., 2016). In fact, recent genome analyses (Vinogradov et al., 2013; Bustamante et al., 2016) reveal a preponderance of 2/2Hbs in cyanobacteria and green algae, and phylogeny supports the rise of these globins after the appearance of life about 3 billion years ago (Vinogradov et al., 2006). Since the 2/2Hbs scaffold probably evolved before the development of the current aerobic environment, a putative original role for these proteins could have been that of O_2 detoxification following the increase of O_2 levels and the evolution of photosynthesis (Crowe et al., 2013). The search for globin physiological functions is further driven by the evidence that many 2/2Hbs are capable of reacting with NO, nitrite, and peroxynitrite (Gardner, 2005; Ascenzi et al., 2009; De Marinis et al., 2009; Ascenzi et al., 2014; Pesce et al., 2016). Interestingly, an unusual occurrence of the concomitant presence of 2/2Hbs of group II and flavohemoglobin (FHb) in the same genome

has been demonstrated (Vinogradov et al., 2013). These findings might indicate that the function of the 2/2Hbs of group II can be intimately linked to the well-known function of FHb in NO detoxification (Gardner et al., 1998; Membrillo-Hernàndez et al., 1999; Mills et al., 2001; Stevanin et al., 2000). In some cases, 2/2Hbs from more than one group can coexist in the same organism, indicating diversification of their functions (Vinogradov et al., 2013). The 2/2Hbs have amino-acid sequences either shorter or longer than those of α and β globins and Mb (i.e. less than 130 and more than 160 residues, respectively).

The most striking differences between the 2/2 and the 3/3 globin folds are: (i) the drastically shortened helix A; (ii) the severe alteration of the C-E region; (iii) the presence of a long polypeptide segment (pre-F) in extended conformation, and (iv) a variable-length helix F that effectively supports the proximal HisF8 residue coordinated to the heme Fe atom (Pesce et al., 2013).

A distinct aspect of groups I and II is the presence of cavities inside the structure linking the protein surface to the distal heme, responsible for storage and diffusion of ligands to/from the heme. The 2/2Hbs generally display moderate to very low O_2-dissociation rates, and thus moderate to high O_2 affinity, due to the presence of at least one hydrogen-bond between the heme Fe-bound ligand and the protein matrix, most commonly provided by TyrB10, TrpG8, His or Tyr at CD1 and GlnE11 (Pesce et al., 2013; Bustamante et al., 2016).

The genome of the cold-adapted bacterium Pseudoalteromonas haloplanktis TAC125 (PhTAC125) contains multiple genes encoding three distinct 2/2Hbs (Giordano et al., 2007), supporting the hypothesis of their involvement in several functions, including protection against oxidative and nitrosative stress in the cold and O_2-rich environment of Antarctica. In particular, PhTAC125 also hosts one 2/2HbI (encoded by the PSHAa0458 gene), two distinct 2/2Hbs of group II (Ph-2/2HbO-0030 and Ph-2/2HbO-2217, encoded by the PSHAa0030 and PSHAa2217 genes, respectively), and one FHb, annotated as PSHAa2880 (Giordano et al., 2007). It is worth noting that

Ph-2/2HbO-0030 and Ph-2/2HbO-2217 are both endowed with hexa-coordination (Giordano et al., 2011; Howes et al., 2011; Russo et al., 2013; Giordano et al., 2015; this study).

Ph-2/2HbO-0030 has been extensively characterised by spectroscopic analysis, kinetic measurements, computer simulation and X-ray crystallography by some of the present authors (Howes et al., 2011; Giordano et al., 2011, 2013, 2015; Russo et al., 2013). The results indicate unique adaptive structural properties that enhance the overall flexibility of the protein (Giordano et al., 2015). Recent results on a genomic mutant strain highlight the involvement of cold-adapted Ph-2/2HbO-0030 in protection against stresses induced by high O_2 concentration (Parrilli et al., 2010) and RNS (Coppola et al., 2013).

In the genome of PhTAC125, two 2/2 globins Ph-2/2HbO-0030 and Ph-2/2HbO-2217 encoding genes are constitutively transcribed, thus suggesting that these 2/2Hbs are not functionally redundant in their physiological function in PhTAC125. Thus, the putative role of the Ph-2/2HbO-2217 globin was investigated in the present study by integrating in vivo and in vitro results, with the aim of shedding light on its physiological role, with special attention to involvement in the RNS detoxification mechanisms, in the context of analyzing specific functional hypotheses.

The PSHAa2217 gene was cloned and over-expressed in the FHb-deficient mutant of Escherichia coli and the growth properties and O_2 uptake in the presence of NO of the mutant carrying the PSHAa2217 gene were analysed. The ferric form of Ph-2/2HbO-2217 is able to catalyse peroxynitrite isomerisation in vitro, indicating its potential role in the scavenging of RNS. Here we present in vitro evidence for the detoxification of NO by Ph-2/2HbO-2217.

2. Materials and Methods

2.1. Sequence alignment

Sequence alignment was performed by the program CLUSTAL OMEGA and manual adjustments were based on known crystal structures. The 2/2Hbs belonging to Group II are: Ph-

2/2HbO-2217, Ph-2/2HbO-0030 (Giordano et al., 2015), Thermobifida fusca (Tf-2/2HbO) (Bonamore et al., 2005), Mycobacterium tuberculosis (Mt-2/2HbO) (Milani et al., 2003), M. leprae (Ml-2/2HbO) (Visca et al., 2002), Agrobacterium tumefaciens (At-2/2HbO) (Pesce et al., 2011), Bacillus subtilis (Bs-2/2HbO) (Giangiacomo et al., 2005), and Geobacillus stearothermophilus (Gs-2/2HbO) (Ilari et al., 2007). The homology model of Ph-2/2HbO-2217, using the 3D-structure (PDB ID 4UUR) of Ph-2/2HbO-0030 as template, was built with SwissModel (https://swissmodel.expasy.org/) (Arnold et al., 2006, 2011; Biasini et al., 2014).

2.2. Strains and culture conditions

Since the FHb (Hmp) from E. coli provides a highly effective detoxification mechanism for NO, we used strain RKP3036 (carrying a genomic hmp null mutation) for cloning and expressing the PSHAa2217 gene, to test cell survival and O_2 uptake in the presence of nitrosative stress. E. coli RKP3919 (E. coli RKP3036 carrying the empty vector pBAD/HisA) was used as a negative control. E. coli RKP3910 strain [E. coli RKP3036 transformed with the pPL341 vector carrying the wild-type hmp^+ gene (Vasudevan et al., 1991)] and E. coli RKP3036 carrying the PSHAa0030 gene (Coppola et al., 2013) were used as positive controls. The E. coli TOP10 strain was used for cloning and expressing the PSHAa2217 gene, and to purify the protein Ph-2/2HbO-2217. Cells were grown in Luria-Bertani (LB) medium, pH 7.0, at 25 °C, 180 rpm and under aerobic conditions. When required, ampicillin (Amp, 100 μg/mL) and kanamycin (Km, 35 μg/mL) were added.

2.3. Cloning and expression of the PSHAa2217 gene

The primer pairs forward (5′-TATGAGTGAGCCATGGATACTAAAGT-3′) and reverse (5′-GCGGGATCCCTAGCTACCCGATACCATTCT-3′) were designed on the basis of the PSHAa2217 gene sequence encoding Ph-2/2HbO-2217 (Médigue et al., 2005). The sequence corresponding to the NcoI site was introduced in the forward primer. The PSHAa2217 gene was retrieved from the genomic DNA of PhTAC125 using the PCR approach. The amplified fragment

was directly cloned into the pTZ57R/T vector and sequenced to verify its authenticity.

The NcoI-PstI digested fragment of the PSHAa2217 gene was further cloned into the corresponding sites of the L-arabinose-inducible, Amp-resistant, and His-tagged pBAD/HisA vector (Invitrogen, Carlsbad, CA, USA). The restriction enzyme cut-sites (NcoI and PstI) were designed for the insertion of the PSHAa2217 gene in pBAD/HisA without the His-tagged region. The construction was verified by sequencing and named pBAD-2/2HbO-2217.

For over-expression of the globin gene in the E. coli hmp mutant and in E. coli TOP10, the cells were transformed with plasmid construct pBAD-2/2HbO-2217 and inoculated into LB medium supplemented with Amp (100 μg/mL). For growth of the E. coli hmp mutant, Km (35 μg/mL) was also added to the medium. Cells were allowed to grow at 25 °C until A_{600} reached ~ 1 OD and then supplemented with 0.2 mM δ-aminolevulinic acid, 0.012 mM $FeCl_3$, and 0.06% L-arabinose, and further incubated for 5 h at 25 °C. Expression of the globin was monitored by running the cell lysate of recombinant strains on 15% SDS-PAGE followed by Coomassie Brilliant Blue staining.

2.4. Protein purification

Purification of Ph-2/2HbO-2217 was achieved by FPLC (GE Healthcare Biosciences, Amersham Biosciences Ltd, UK) anion-exchange chromatography, loading the cell lysate obtained from E. coli TOP10 expressing the PSHAa2217 gene on a Q Sepharose column (HiTrap™ QFF, GE Healthcare Biosciences, Amersham Biosciences Ltd, UK), equilibrated with 20 mM Tris-HCl pH 8.2. Ph-2/2HbO-2217 was eluted with a NaCl gradient from 0 to 1.0 M. The eluate was further purified by a second anion-exchange chromatography step on a Mono Q-Tricorn column, equilibrated with 20 mM Tris-HCl pH 8.2. The protein was eluted with a NaCl gradient from 0 to 250 mM. All buffers were prepared in MilliQ water. The protein obtained was > 98% pure on SDS-PAGE. The N-terminal sequence was determined by automatic sequencing performed with an Applied Biosystems Procise 494 automatic sequencer, equipped with on-line detection of

179 phenylthiohydantoin amino acids.

180

181 2.5. Samples for spectroscopic analysis

182 Ferric Ph-2/2HbO-2217 at pH 6.0, 7.6 and 9.9 was prepared in 50 mM MES [2-(N-
183 morpholino) ethanesulfonic acid], 20 mM Tris-HCl and 50 mM glycine, respectively. The hydroxyl
184 complex in isotopically enriched water was prepared by washing Ph-2/2HbO-2217 in 20 mM Tris-
185 HCl pH 7.6 with 0.1 mM glycine pD 10.2 prepared with D_2O (99.8%) (Merck AG Darmstadt,
186 Germany). Ferrous samples at pH 7.6 were prepared by addition of a freshly prepared sodium
187 dithionite solution (10 mg/mL) to the ferric forms previously flushed with nitrogen. The Fe(II)-CO
188 complex at pH 7.6 was prepared by flushing ferric Ph-2/2HbO-2217 firstly with nitrogen, then with
189 ^{12}CO or ^{13}CO (Rivoira, Milan, Italy), and reducing the heme by addition of a freshly prepared
190 sodium dithionite solution (10 mg/mL). All chemicals were of analytical or reagent grade and were
191 used without further purification.

192 Protein concentration in the range 10–30 μM was used for electronic absorption and
193 Resonance Raman (RR) spectroscopies at both room and low temperature. The concentration used
194 for Electron Paramagnetic Resonance (EPR) spectroscopy was 100 μM. The protein concentration
195 was estimated on the basis of the molar absorptivity of the ferric form at 408 nm, $\varepsilon = 131\ mM^{-1}$
196 cm^{-1}.

197

198 2.6. Electronic absorption measurements

199 UV-visible absorption spectra of whole cells of E. coli hmp carrying pBAD/HisA and pBAD-
200 2/2HbO-2217 were recorded using an SDB-4 dual-wavelength scanning spectrophotometer
201 (University of Pennsylvania Biomedical Instrumentation Group, and Current Designs, Inc.,
202 Philadelphia, PA) at room temperature (Kalnenieks et al., 1998). Samples were generally scanned
203 with a 0.5-nm step size. Data were analysed using SoftSDB (Current Designs) and Sigma Plot 11.0
204 (Systat Software, Inc., San Jose, CA, USA).

Aerobic cultures were grown overnight (for about 18 h) in flasks containing medium up to 1/5 of their volume with appropriate antibiotics and supplements in different concentrations. Cells were harvested by spinning at 5500 rpm for 15 min at 4 °C and the pellets were re-suspended in 6 mL of 0.1 M sodium phosphate buffer, pH 7.0. Spectra were recorded between 400 nm and 700 nm. All spectra were baseline-corrected.

UV-visible absorption spectra of cell lysates of E. coli hmp carrying either pBAD/HisA or pBAD-2/2HbO-2217 were measured with a double-beam Cary 300 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), using a 120-nm/min scan rate. Pellets, prepared as already described, were re-suspended in 50 mM Tris-HCl buffer pH 7.4, containing 2 mM $MgCl_2$ and 1 mM EGTA; cells were disrupted by sonication. The debris was pelleted by centrifugation at 12,000×g for 15 min and membranes recovered from the supernatant by ultracentrifugation for 1 h at 225,000×g, 4 °C. The absorption spectra of the supernatants were recorded between 400 and 700 nm.

2.7. Resonance Raman measurements

The RR spectra were obtained at 25 °C using a 5-mm NMR tube by excitation with the 406.7 and 413.1 nm lines of a Kr^+ laser (Innova 300 C, Coherent, Santa Clara, CA, USA), the 514.5 nm line of an Ar^+ laser (Innova 90/5, Coherent), and the 441.6 nm line of a He–Cd laser (Kimmon IK4121R-G). Back-scattered light from a slowly rotating NMR tube was collected and focused into a triple spectrometer with spectral resolution as reported elsewhere (Ciaccio et al., 2017). A cylindrical lens, which focuses the laser beam in the sample to a narrow strip rather than the usual point, was used to collect the spectra of both the Fe(II)-CO complex and the ferric sample at pH 7.6 in order to avoid photolysis and minimize sample degradation induced by irradiation.

The RR spectra were calibrated with indene, n-pentane and carbon tetrachloride as standards to an accuracy of 1 cm^{-1} for intense isolated bands. All RR measurements were repeated several times under the same conditions to ensure reproducibility. To improve the signal-to-noise ratio, a

number of spectra were accumulated and summed only if no spectral differences were noted. All spectra were baseline-corrected.

For the low temperature experiments, a 1.5-cm diameter quartz crucible positioned in a THMS600 cryostat (Linkam Scientific Instruments, Surrey, UK) containing ~ 100 µL frozen samples at 80 K was used.

Absorption spectra recorded using a 5-mm NMR tube (300 nm/min scan rate) or a 1-cm cuvette (600 nm/min scan rate) at 25 °C by means of a Cary 60 spectrophotometer (Agilent Technologies, Glostrup, Denmark) (resolution of 1.5 nm), were measured both prior to and after RR measurements to ensure that no degradation occurred under the experimental conditions used. ~~All spectra were baseline-corrected.~~

For the low temperature experiments, a 1.5-cm diameter quartz crucible positioned in a THMS600 cryostat (Linkam Scientific Instruments, Surrey, UK) containing ~ 100 µL frozen samples at 80 K was used.

2.8. EPR measurements

EPR spectra were recorded with an Elexsys E500 (Bruker, Rheinstetten, Germany), equipped with an NMR gaussmeter and a microwave frequency counter. An ESR 900 cryostat (Oxford Instruments, Abingdon, UK), was used to obtain low temperatures. Spectra were recorded under non-saturating conditions at 5 K, 1-mW microwave power and 1-mT modulation amplitude. The g values were determined by careful visual inspection of the spectra.

2.9. S-nitrosoglutathione and NO-donors

Three different agents of nitrosative stress were used, according to the experimental design. For growth experiments, a source of NO gas was compared with a nitrosating agent. As a source of NO, DETA-NONOate (Enzo Life Science, Farmingdale, NY, USA) with a half-life of 20 h at 37 °C and 56 h at 22–25 °C in 0.1 M phosphate buffer pH 7.4 was used; thus NO release and provision to

the bacteria were prolonged over several hours. The nitrosating agent S-nitrosoglutathione (GSNO) was prepared as previously reported (Hart, 1985). It is widely used in microbial growth experiments because it is moderately stable in aqueous solutions; however, a derived nitrosated dipeptide, S-nitroso-L-cysteinylglycine, is transported inwards (via the Dpp-encoded dipeptide permease in certain bacteria) and intracellular transnitrosation reactions ensue (Laver et al., 2012). In contrast, for short-term respiration experiments designed to test the addition of a bolus of NO, we used Proli-NONOate (Bioquote Limited, York, UK) with a half-life of 1.8 s at 37 °C in 0.1 M phosphate buffer pH 7.4. All experiments were performed in triplicate.

2.10. GSNO and NO susceptibility

Cultures of the E. coli hmp mutant, transformed with pBAD/HisA (negative control), pBAD-2/2HbO-0030 (positive control) (Coppola et al., 2013) and pBAD-2/2HbO-2217, were grown in plastic universal tubes in 2 mL of LB medium containing appropriate antibiotics and incubated for 2.5 h at 25 °C. The culture was then supplemented with: 0.2 mM δ-aminolevulinic acid, 0.012 mM $FeCl_3$, L-arabinose at the final concentration of 0.06% for E. coli hmp carrying either Ph-2/2HbO-2217 or the empty vector, and at the final concentration of 0.2% for the positive control E. coli hmp carrying Ph-2/2HbO-0030 (as in Coppola et al., 2013). GSNO and DETA-NONOate were later added to all tubes at different concentrations (GSNO: 0, 1, 3, and 5 mM; DETA-NONOate: 0, 0.5, 1, and 2 mM).

The cultures were incubated for approximately 18 h in the dark, at 25 °C with shaking, and then the optical density at 600 nm was recorded.

2.11. Growth curves

Cultures of the E. coli hmp carrying different plasmids were grown in the dark in 250 mL flasks containing 10 mL of medium with appropriate antibiotics. Induction supplements (for details

see section on GSNO and NO susceptibility), GSNO (3 mM) or DETA-NONOate (0.5 mM) were added to each flask, at t = 2.5 h.

2.12. NO uptake and cellular respiration

Cultures of the E. coli hmp mutant carrying pBAD-2/2HbO-2217, pBAD-2/2HbO-0030, and pBAD/HisA were grown in 250 mL flasks containing 40 mL of LB medium, supplemented with the appropriate antibiotics. Induction supplements were added when the cells reached an OD of 1.0. Cultures were grown overnight (around 18 h), at 25 °C. Cells were harvested at 5500 rpm for 15 min at 4 °C; the pellets obtained were washed twice with 10 mL 50 mM Tris-HCl buffer pH 7.5, then re-suspended in 5-10 mL of the same buffer to normalise the optical density (OD) of the suspensions.

The respiration rates of whole cells were measured using a Clark-type polarographic O_2 electrode (Rank Bros, Bottisham, Cambridge, UK) operating at a polarising voltage of 0.60 V. The apparatus consists of a Perspex chamber kept at 25 °C using a water jacket around the chamber and stirred magnetically with a membrane-covered electrode placed at the bottom of the chamber (Stevanin et al., 2000). The electrode was calibrated using air-saturated buffer, which was then treated with a small amount of sodium dithionite to achieve anoxia. Parallel measurements of O_2 and NO were made by housing a World Precision Instruments (Sarasota, FL, USA) ISO NOP sensor (2-mm diameter) in the same vessel (Mills et al., 2001); note that the ingress of air around the NO port results in slow backflow of O_2 into the chamber contents.

The NO electrode was calibrated as described by the manufacturer (World Precision Instruments, Sarasota, FL, USA). Briefly, sequential volumes of 50 μM $NaNO_2$ (e.g. 100, 200, 400, and 800 μL) were added under stirring to 20 mL of 0.1 M H_2SO_4/KI, in which the NO electrode was suspended.

After calibration of the O_2 and NO electrodes, the whole cell suspension was diluted with 50 mM Tris-HCl pH 7.5 in the O_2-electrode chamber to a final volume of 2 mL, and closed with a tight-fitting lid . The respiration was started using 25 mM glucose. Proli-NONOate (final

concentration, 1 μM) was added through a hole in the vessel lid using a Hamilton syringe, at progressively lower O_2 concentrations. Respiration was followed until the chamber became devoid of O_2.

2.13. Heme assay

Absorption spectra of sonicated samples (0.6 mL without clarification) containing 0.6 mL of reagent (0.4 M NaOH, 4.2 M pyridine) were taken in a quartz cuvette (with stopper) and analysed between 500 and 700 nm using a double-beam Cary 300 spectrophotometer with a 120-nm/min scan rate. Each sample was reduced by adding small amounts of sodium dithionite followed by gentle stirring. A sample was used to obtain the oxidised spectrum by addition of potassium ferricyanide. Difference spectra of reduced vs oxidised forms were obtained; the heme concentration was calculated from the absorbance difference at 556 and 539 nm for the dithionite-reduced and ferricyanide-oxidised samples, respectively.

2.14. Peroxynitrite isomerisation

Peroxynitrite was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). The concentration of peroxynitrite was determined spectrophotometrically by measuring the absorbance at 302 nm ($\varepsilon = 1.705\times10^3\ M^{-1}\ cm^{-1}$). The peroxynitrite stock solution (2.0×10^{-3} M) was diluted immediately before use with degassed 1.0×10^{-2} M NaOH to reach the desired concentration (Bohle et al., 1996; Koppenol et al., 1996; Herold and Shivashankar, 2003; Herold et al., 2004a; Ascenzi and Fasano, 2007; Goldstein and Merényi, 2008).

Kinetics of peroxynitrite isomerization, by ferric Ph-2/2HbO-2217 and Ph-2/2HbO-0030, in the absence and presence of cyanide (final concentration, 5.0×10^{-4} M), was recorded at 302 nm ($\varepsilon = 1.705\times10^3\ M^{-1}\ cm^{-1}$) by rapidly mixing the buffer solution (1.0×10^{-1} M bis-tris-propane buffer, pH 7.4) or the ferric globin solutions (final concentration, 4 to 16 μM; 1.0×10^{-1} M bis-tris-propane buffer, pH 7.4) with the peroxynitrite solution (final concentration, 2.5×10^{-4} M).

In the absence and presence of ferric globins and cyanide, values of the pseudo-first-order rate constant for peroxynitrite isomerization (i.e. k) were determined from the analysis of the time-dependent absorbance decrease at 302 nm, according to Eq. (1):

$$[\text{peroxynitrite}]_t = [\text{peroxynitrite}]_t \times e^{-k \times t} \quad (1)$$

Values of the second-order rate constant for peroxynitrite isomerization by ferric Ph-2/2HbO-2217 and Ph-2/2HbO-0030 (i.e., k_{on}) and of the first-order rate constant for the spontaneous conversion of peroxynitrite to nitrate (i.e. k_0) were determined from the dependence of k_{on} on the ferric globin concentration, according to Eq. (2):

$$k = k_{on} \times [\text{Ph-2/2HbO-2217-Fe(III)}] + k_0 \quad (2)$$

3. Results

3.1. Primary structure

Figure 1A shows the alignment of Ph-2/2HbO-2217 and Ph-2/2HbO-0030 with some representative 2/2Hbs belonging to group II. The sequence identity between the two Antarctic globins is only 24%, thus suggesting that these proteins may play different function(s) in bacterial physiology.

The main difference between Ph-2/2HbO-2217 and Ph-2/2HbO-0030 is the presence in the former of a longer sequence extension at the N terminus (19 residues in Ph-2/2HbO-0030 and 9 residues in Ph-2/2HbO-2217), rarely observed in 2/2Hbs. In Ph-2/2HbO-0030, the extension is proteolytically cleaved during protein purification (Giordano et al., 2007), and it does not appear to be a requirement for NO detoxification (Coppola et al., 2013).

By comparison with other group II globins , and taking Mt-2/2HbO as the reference, the two Antarctic globins show: (i) a three-residue insertion in the BC loop, (ii) one-residue deletion in the CE loop, (iii) three-residue deletion in the GH loop and at the C terminus (Giordano et al., 2015, this study), (iv) His and Trp residues at positions F8 and G8, respectively, (v) the Phe-Tyr motif at

positions B9-B10, and (vi) a His residue at position CD1. The analysis of all bacterial sequences available to date (~ 1100) demonstrated that the CD1 position is occupied predominantly by Phe and in some cases by His or Tyr (~ 20 and 15%, respectively) (Bustamante et al., 2016). His (a hydrogen bonding residue) at the topological position CD1 site is always matched by a hydrophobic E11 residue (Leu or Phe). Thus, one of the necessary hydrogen bonding elements involved in ligand stabilisation is alternatively located at opposite edges of the heme distal cavity, either at the CD1 or at the E11 sites, but never simultaneously. In Ph-2/2HbO-2217, the E11 residue is Leu, while it is Phe in Ph-2/2HbO-0030.

In Ph-2/2HbO-0030 the two Gly-Gly motifs, located in the AB and EF hinges of 2/2Hbs belonging to groups I and II, are present and help to stabilize the short helix A in a conformation locked onto helices B and E. In contrast, in Ph-2/2HbO-2217 the second Gly residue is replaced by Glu at the AB hinge.

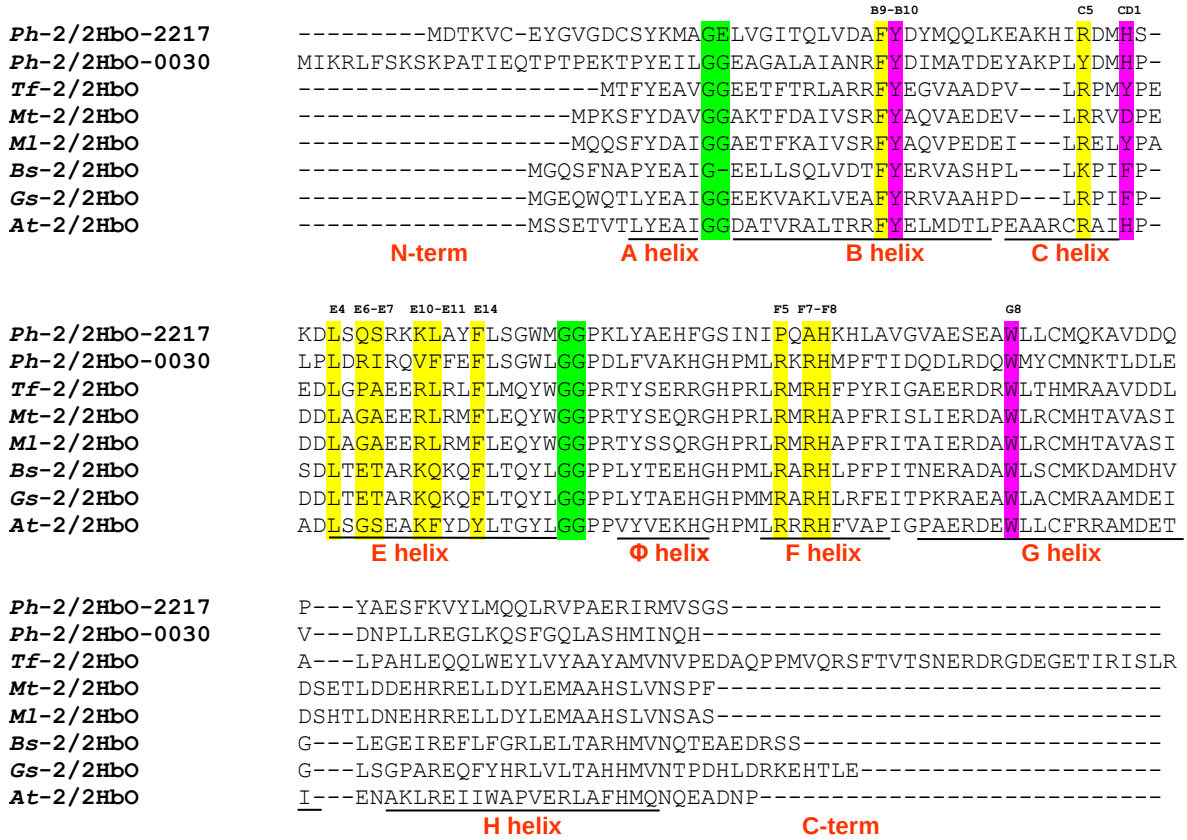


Figure 1A. Sequence alignment, carried out by Clustal Omega, of Ph-2/2HbO-2217 and Ph-2/2HbO-0030 compared with other members of group-II. Manual adjustments have been based on known crystal structures adapted from Giordano et al. (2015). Functionally important residues are shown in yellow; residues (B10, CD1 and G8) specific for 2/2Hbs of group-II are in purple; the Gly-Gly motifs are in green. Helical regions (A-H) are indicated by black bars and helix Φ, specific for 2/2Hb of group-II, is shown. The numbering of residues is based on the position of residues in the helices of sperm whale Mb, adapted from Giordano et al. (2015).

Position E7 is variable in group II globins and is usually occupied by a small residue (typically Ala, Ser or Thr), thus suggesting an E7 route entry path to facilitate the accessibility of diatomic ligands to the heme distal site (Milani et al., 2003; Vuletich and Lecomte, 2006; Nardini et al., 2007; Pesce et al., 2013). In Ph-2/2HbO-2217, E7 is occupied by Ser (**Figure 1B**), whereas in Ph-2/2HbO-0030 by Ile, separating the heme distal cavity from the solvent region (Giordano et al., 2015).

Figure 1B overlays the heme pocket of a Ph-2/2HbO-2217 homology model and the Ph-2/2HbO-0030 structure used as template, showing different residues involved in the stabilisation of the heme through Fe coordination. In Ph-2/2HbO-0030, the heme was found to be stabilized through direct Fe coordination to proximal His(96)F8, electrostatic interactions with the heme propionates, and van der Waals contacts (< 4.0 A) with 23 residues. In particular, propionate D is stabilized by an H-bonded salt bridge with Arg(95)F7, and propionate A is electrostatically coupled to Arg64; in addition, both propionates are H-bonded with a water molecule (Giordano et al., 2015). However, in Ph-2/2HbO-2217 the propionate-protein interactions are quite different. In Ph-2/2HbO-2217, position EF6 is occupied by Tyr (**Figure 1B**), highly conserved in 2/2Hbs of group II (Bustamante et al., 2016), but not present in Ph-2/2HbO-0030 (Phe was found instead) (Giordano et al., 2015). Therefore, the formation of an additional H bond with propionate D is predicted. However, Arg at F7, present in other 2/2Hbs and conserved in Ph-2/2HbO-0030, is replaced by Ala in Ph-2/2HbO-2217. Moreover, Arg(64)E6, electrostatically coupled to propionate A in Ph-2/2HbO-0030

(Giordano et al., 2015), in Ph-2/2HbO-2217 is replaced by Gln.

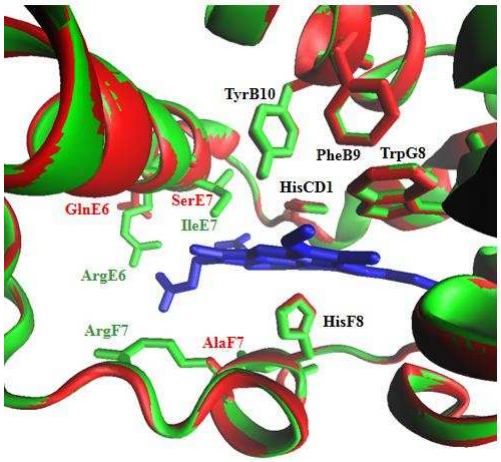
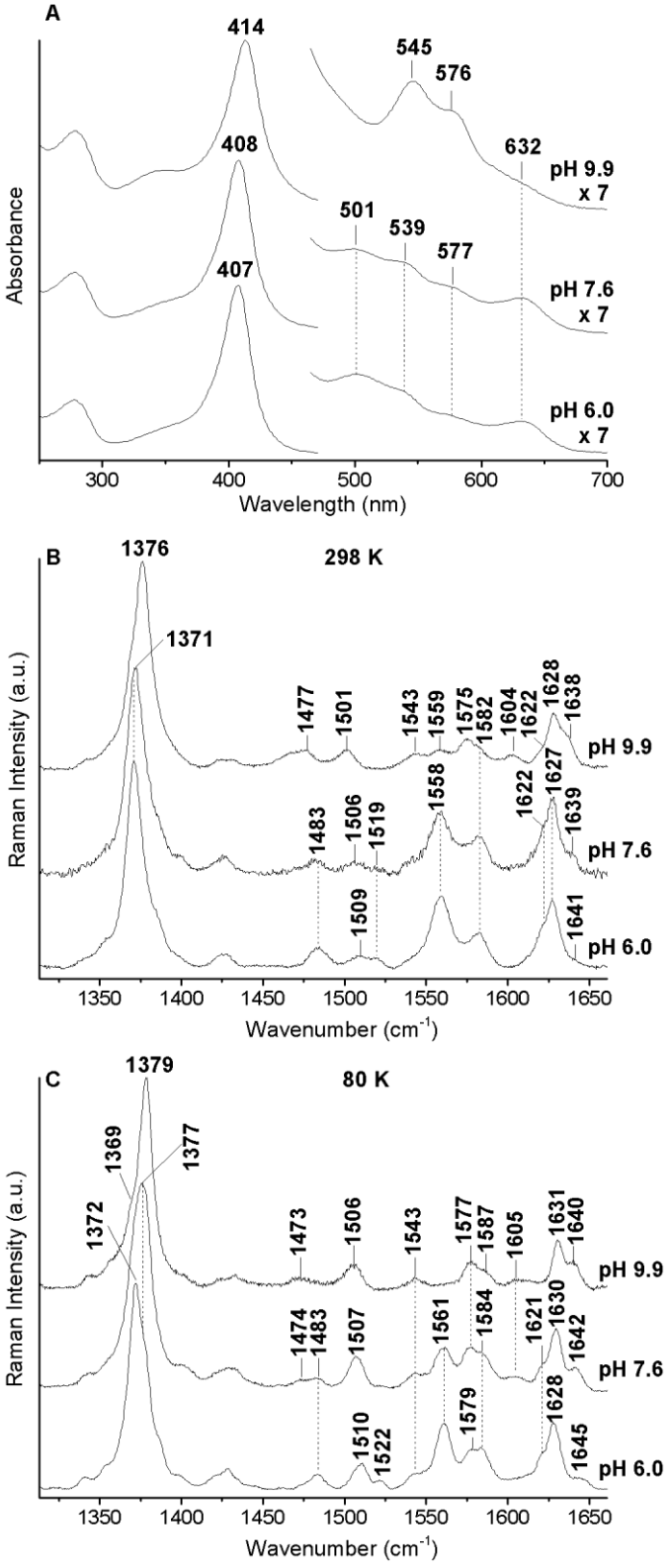


Figure 1B. Superimposition of the heme pocket of a Ph-2/2HbO-2217 homology model (red) and the Ph-2/2HbO-0030 template structure (green). The heme group is in blue. The residues involved in the stabilisation of the heme through Fe coordination are shown (B9, B10, CD1, G8, E6, E7, F7, F8), as reported in Giordano et al. (2015).

3.2. Spectroscopic characterisation

3.2.1. Ferric Form

Figure 2 compares the UV-vis and the RR high-frequency region (at 298 and 80K) spectra of ferric Ph-2/2HbO-2217 at different pH. At pH 6.0, room-temperature spectra (**panels A, B)** are characteristic of a predominantly hexa-coordinate (6c) high-spin (HS) (aquo) form [charge-transfer (CT1) band at 632 nm and RR bands at 1483 (ν_3), 1558 (ν_2) cm^{-1}]. However, a weak 6c low-spin (LS) form [α band at 577 nm, RR bands at 1509 (ν_3), 1641 (ν_{10}) cm^{-1}], is also present. As in Ph-2/2HbO-0030, Ph-2/2HbO-2217 undergoes an acid-alkaline transition.



418

419 **Figure 2**. Comparison of the UV-Vis (panel A) and RR spectra in the high frequency region at 298

420 K (panel B) and 80 K (panel C) of ferric Ph-2/2HbO-2217 at pH 6.0 (bottom), 7.6 (middle) and 9.9

421 (top). The spectra in all the panels have been shifted along the ordinate axis to allow better

422 visualisation. The 470–700-nm region of the UV-Vis spectra has been expanded 7-fold. RR

experimental conditions: 298 K: excitation wavelength 406.7 nm; (pH 6.0), laser power at the sample 5 mW, average of 4 spectra with 20-min integration time; (pH 7.6), laser power at the sample 4 mW, using a cylindrical lens and cooling the sample with a gentle flow of N_2 passed through liquid N_2, average of 13 spectra with 13-min integration time; (pH 9.9), excitation wavelength 413.1 nm, laser power at the sample 10 mW, average of 3 spectra with 15-min integration time; 80 K: (pH 6.0 and 7.6), excitation wavelength 406.7 nm, laser power at the sample 10 mW, average of 3 spectra with 30-min integration time; (pH 9.9), excitation wavelength 413.1 nm, laser power at the sample 5 mW; average of 12 spectra with 60-min integration time. The intensities of the RR spectra are normalised to that of the ν_4 band.

Hence, upon increasing pH, the 6cHS aquo and 6cLS forms decrease in intensity, and the new OH^--ligated forms, both 6cHS and 6cLS, grow in. At pH 9.9 (one unit lower than for Ph-2/2HbO-0030), the UV-Vis and RR spectra are typical of an OH^--ligated form, both 6cHS [1477 (ν_3), 1559 (ν_2) cm^{-1}] and 6cLS [1501(ν_3), 1575 (ν_2), 1638 (ν_{10}) cm^{-1}]; however, unlike for Ph-2/2HbO-0030, no 5cHS form is observed. The full assignment of the RR bands, based also on experiments carried out with excitation at 514.5 nm (**Figure S1**), is reported in Table S1.

The spectral dependence on pH is clearly observed in the RR spectra at low temperature (**Figure 2, panel C)**. In fact, upon lowering the temperature, the sharpening of the bands and the presence of only a 6cLS OH^- ligated form at alkaline pH facilitate the identification of different 6cLS forms at pH 6.0 and 9.9 characterised by different RR frequencies, which are, on the other hand, both present at intermediate pH 7.6. Accordingly, in addition to a 6cHS form ($g_\perp \sim 6$ and $g_\parallel$ 2.00), the EPR spectrum at pH 7.6 (**Figure S2**) displays two 6cLS forms: one with $g_1 = 2.95$, attributable to His–Fe–Tyr coordination, similar to Ph-2/2HbO-0030 (Giordano et al., 2015), and the other with $g_1 = 2.71$, typical of His–Fe–OH^- coordination, absent in Ph-2/2HbO-0030 at pH 7.6, but present at pH 10.7 (Giordano et al., 2015).

Figure 3 compares the low-frequency RR spectra of Ph-2/2HbO-2217 at alkaline pH in H_2O and D_2O buffered solutions, at 298 K (**panel A, left**) and 80 K (**panel B, left**) together with the difference spectra H_2O - D_2O (**Figure 3, right**).

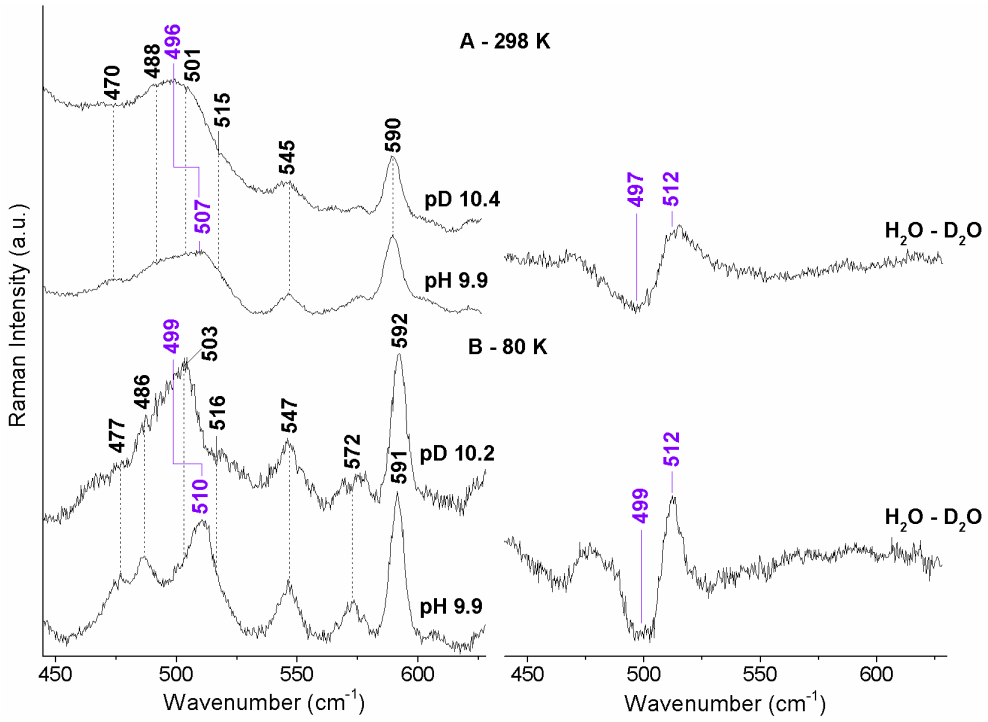


Figure 3. Comparison of the low-frequency region RR spectra at alkaline pH of ferric Ph-2/2HbO-2217 at room (panel A, left) and low temperature (panel B, left) in H_2O (bottom) and D_2O (top). The corresponding difference spectra H_2O - D_2O are also shown (panels A and B, right). The ν(Fe-OH) and ν(Fe-OD) mode frequencies are reported in purple. The spectra have been shifted along the ordinate axis to allow better visualisation. The intensity of the spectra is normalised to that of the ν_4 band. Experimental conditions: excitation wavelength 413.1 nm; pH 9.9; laser power at the sample 10 mW, average of 9 spectra with 45-min integration time (298 K) and of 3 spectra with 60-min integration time (80K); pD 10.2: laser power at the sample 10 mW, average of 7 spectra with 70-min integration time (298 K) and laser power at the sample 5 mW, average of 4 spectra with 40-min integration time (80 K).

The 450-530 cm^{-1} region of the spectra is quite complex, due to the porphyrin modes (see **Tables S1 and S2**). However, on the basis of the isotopic substitution, the bands at 507 and 510 cm^{-1} at 298 and 80 K, respectively, which shift to 496 and 499 cm^{-1} in D_2O at 298 and 80 K, respectively, have been assigned to the ν(Fe-OH) mode of a His–Fe–OH^- 6cLS form. Accordingly, the difference spectrum at 80 K shows narrow and well-defined bands at 499 and 512 cm^{-1}. The difference spectrum at 298 K shows two broad bands at 497 and 512 cm^{-1}, possibly due to the concomitant presence of a His–Fe–OH^- 6cHS form observed at room temperature in the RR high-frequency region (**Figure 2, panel B)**.The frequency of the Ph-2/2HbO-2217 ν(Fe-OH) mode is about 18 and 46 cm^{-1} lower than that observed for Ph-2/2HbO-0030 (525 cm^{-1} at 298 K, Giordano et al., 2015) and human Hb (553 cm^{-1} at 298 K, Feis et al., 1994), indicating the presence of strong H-bonds between the OH^- ligand and distal residues. In fact, with an increase of the H-bond strength, a decrease of the force constant of the Fe–OH bond, with concomitant decrease of the ν(Fe–OH) stretching frequency, is expected. However, no upshift of the frequency is observed in D_2O, as in Ph-2/2HbO-0030 (543 cm^{-1}, Giordano et al., 2015) or other heme proteins in the presence of strong H-bonds (Nicoletti et al., 2014; Howes et al., 2015). Moreover, due to the instability of Ph-2/2HbO-2217 in $H_2{}^{18}O$ buffer, the identification of any ν(Fe–OH) band on the basis of its sensitivity to ^{18}O substitution was not possible.

Therefore, in general, the spectroscopic features of ferric Ph-2/2HbO-0030 and Ph-2/2HbO-2217 are similar, the main differences being the lower pK_a for the alkaline transition (about one unit) of Ph-2/2HbO-2217 compared to Ph-2/2HbO-0030, and the absence of a 5cHS form in Ph-2/2HbO-2217 at alkaline pH. Accordingly, the low-frequency regions of the RR spectra of the two proteins are also very similar (**Figure S3**). The only notable differences concern the frequency and/or intensity of the vinyl and propionyl bending modes. The $\delta(C_\beta C_a C_b)$ bending modes of the vinyl groups of Ph-2/2HbO-2217 give rise to a broad band centered at 415 cm^{-1}, 5 cm^{-1} higher than in Ph-2/2HbO-0030. At present, the significance of these variations is not clear, but they possibly suggest

differences in vinyl orientation, in agreement with the 3-cm^{-1} difference of the ν(C=C) stretching modes (Smulevich et al., 1996; Marzocchi and Smulevich, 2003).

The relative intensity of the $\delta(C_\beta C_c C_d)$-propionyl bending modes is markedly different. In particular, the propionyl mode at 381 cm^{-1} is more intense in Ph-2/2HbO-2217 than in Ph-2/2HbO-0030. This behaviour is observed also in the ferrous carbonylated complex (see below). The frequency and intensity of this mode has been correlated with the hydrogen-bond strength between the heme-propionate and the nearby residues (Cerda-Còlon et al., 1998). As suggested by the different primary structure of the residues surrounding the heme propionyls, the intensity change of one propionyl mode indicates that the H-bonding interactions are different for the two proteins, and in particular much stronger in Ph-2/2HbO-2217.

3.2.2. Ferrous Form

Upon reduction, the UV-Vis and RR high-frequency-region spectra of Ph-2/2HbO-2217 clearly reveal the presence of a 6cLS form (Soret band at 420 nm and RR bands at 1493 (ν_3), 1577 (ν_2) cm^{-1}) and a 5cHS form (Soret band at 435 nm and RR bands at 1470 (ν_3), 1558 (ν_2) and 1603 (ν_{10}) cm^{-1}), (**Figure 4**), the latter being much more pronounced in Ph-2/2HbO-2217 than Ph-2/2HbO-0030 (**Figure S4**). The RR low-frequency-region spectrum of Ph-2/2HbO-2217 is characterised by a very strong band at 227 cm^{-1}, assigned to the ν(Fe-Im) stretching mode (**Figure 4**). The frequency of this band is 5-cm^{-1} higher than in Ph-2/2HbO-0030 (222 cm^{-1}) (Giordano et al., 2011), indicating a stronger proximal Fe-His bond. The frequency is similar to that of other 2/2Hbs (Egawa and Yeh, 2005), consistent with a staggered orientation of the imidazole ring of the proximal His with respect to the four pyrrole nitrogen atoms of the porphyrin ring, revealed by the crystallographic data of Ph-2/2HbO-0030 (Giordano et al., 2015), in contrast to the eclipsed orientation observed in human Hb.

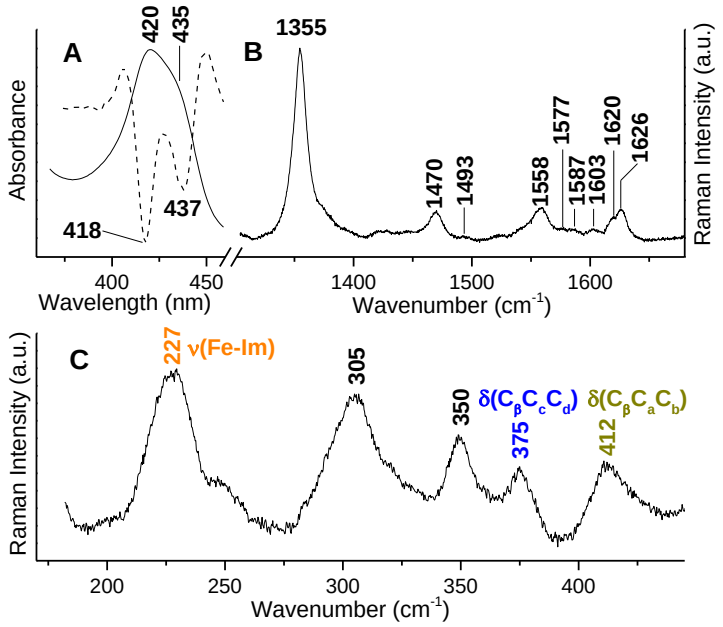
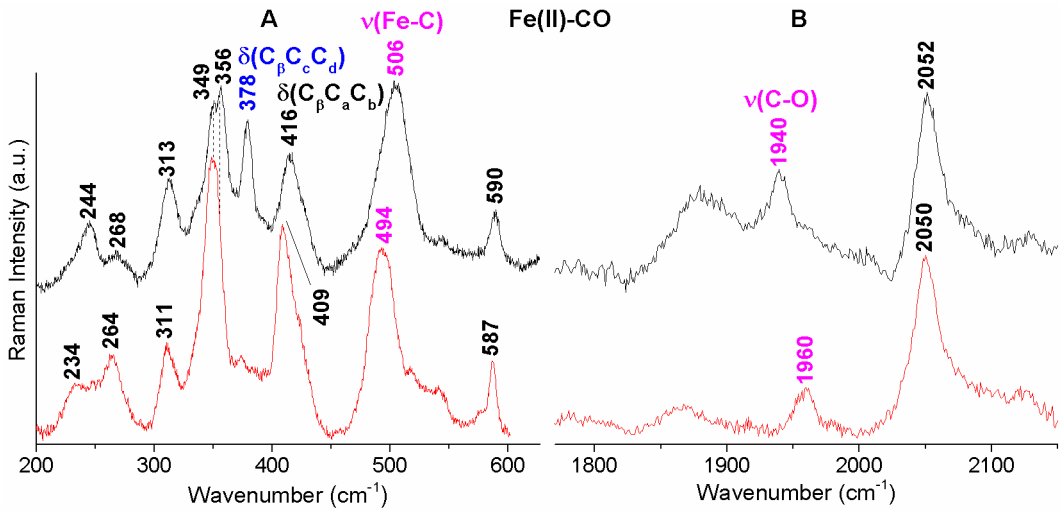


Figure 4. UV-Vis and its the second derivative spectrum (D^2) (dashed line) (panel A), RR spectra in the high- (panel B) and low-frequency region (panel C) of ferrous Ph-2/2HbO-2217 at pH 7.6. The spectra have been shifted along the ordinate axis to allow better visualisation. In panel C the ν(Fe-Im), $\delta(C_\beta C_c C_d)$ and $\delta(C_\beta C_a C_b)$ mode frequencies are reported in orange, blue and green, respectively. RR experimental conditions: excitation wavelength 441.6 nm; laser power at the sample 10 mW, average of 4 spectra with 20-min integration time.

Moreover, similar to the ferric form, slight differences compared to Ph-2/2HbO-0030 are evident in the vinyl stretching (1624-1626 cm^{-1}) and bending (409, 412 cm^{-1}) modes.

As in Ph-2/2HbO-0030 (Giordano et al., 2011), Ph-2/2HbO-2217 binds CO, giving rise to a 6cLS species. In the RR low-frequency region of the CO adduct (**Figure 5, panel A**), one isotope-sensitive band is identified in both Ph-2/2HbO-0030 (494 cm^{-1}) and Ph-2/2HbO-2217 (506 cm^{-1}), which shift to 489 and 501 cm^{-1}, respectively, in the case of ^{13}CO (**Figure S5**). These bands are assigned to the ν(Fe–C) stretching mode. Accordingly, a corresponding ν(C–O) stretching mode is observed at 1960 cm^{-1} (Ph-2/2HbO-0030) and 1940 cm^{-1} (Ph-2/2HbO-2217) (**Figure 5, panel B**), which shift to 1914 cm^{-1} and 1898 cm^{-1}, respectively, upon ^{13}CO substitution (**Figure S5**).



532

533 **Figure 5**. Comparison of the RR spectra of the Fe(II)-^{12}CO complexes of Ph-2/2HbO-0030 (red) and
534 Ph-2/2HbO-2217 (black) at pH 7.6. The low (panel A) and the high (panel B) frequency regions
535 show the ν(Fe–CO) and ν(C–O) stretching modes (in magenta), respectively. The δ($C_\beta C_c C_d$) mode
536 frequency is shown in blue. The spectra have been shifted along the ordinate axis to allow better
537 visualisation. RR experimental conditions: excitation wavelength 413.1 nm; a cylindrical lens was
538 used to focus the laser beam on the sample; laser power at the sample 2 mW, average of 4 spectra
539 with 10-min integration time (Ph-2/2HbO-0030, LF and HF); laser power at the sample 2 mW,
540 average of 3 spectra with 30-min integration time (Ph-2/2HbO-2217, LF); average of 9 spectra with
541 90-min integration time (Ph-2/2HbO-2217, HF).

542

543 Interestingly, unlike ferrous carbonylated Ph-2/2HbO-0030 CO, whose frequencies are
544 characteristic of a CO conformer in which polar interactions with the surrounding residues of the
545 distal cavity are absent, the corresponding CO frequencies for the Ph-2/2HbO-2217 CO complex
546 indicate polar interactions between the distal residues and the CO molecule. This finding is
547 consistent also with the presence of strong H-bonds between the OH^- ligand and distal residues, not
548 observed for Ph-2/2HbO-0030. Furthermore, the frequency shifts and marked intensity increase of
549 the δ($C_\beta C_c C_d$)-propionate bands upon formation of the CO-complex suggest a change in the
550 environment around the propionate group in Ph-2/2HbO-2217 when the hexa-coordinated species is

551 formed. This is likely consequent to a marked strengthening of the H-bonding interactions between
552 the heme propionates and the nearby residues. Interestingly, in Ph-2/2HbO-0030, CO binding causes
553 the complete disappearance of the propionyl bending modes (**Figure 3**), indicating changes in the
554 strength of hydrogen bonding from moderate to weak upon formation of the CO-complex. These
555 observations suggest a flexible heme rocking motion that contributes to the ligand-binding
556 mechanism in the two proteins that might also be influenced by the different H-bonding residues in
557 the vicinity of the propionates for Ph-2/2HbO-2217 and Ph-2/2HbO-0030.
558
559 3.3. Cloning and expression of the PSHAa2217 gene in E. coli TOP10 cells and in the hmp mutant
560 The PSHAa2217 gene from PhTAC125 was cloned into the commercial vector pBAD/HisA
561 (Invitrogen, Carlsbad, CA, USA), under control of an L-arabinose-inducible promoter. The construct
562 pBAD-2/2HbO-2217 was confirmed by sequencing, and later expressed both in E. coli TOP10 cells
563 to purify the protein, and in the E. coli hmp mutant to perform in vivo function experiments. Since
564 the mutant is very sensitive to NO and RNS, complementation in trans of sensitivity indicates that
565 the expressed globin is endowed with detoxification properties. Expression resulted in the
566 accumulation of heme protein inside the cell, giving a reddish brown colour to the recombinant E.
567 coli cells. SDS-PAGE demonstrated the presence of a ~15.5-kDa protein corresponding to the
568 expected size of Ph-2/2HbO-2217. Ph-2/2HbO-2217, cloned and over-expressed in E. coli TOP10,
569 was purified by two consecutive anion-exchange chromatography steps (**Figure S6**, **panels A-B**). In
570 agreement with the primary structure of Ph-2/2HbO-2217, a ~15.5-kDa protein was obtained
571 (**Figure S6**, **insert C**).
572 To confirm the expression of the PSHAa2217 gene in E. coli hmp, the UV-visible absorption
573 spectrum of lysate of the cells carrying pBAD-2/2HbO-2217 was compared with that of cells
574 transformed with the empty vector pBAD/HisA (**Figure S7**).
575
576 3.4 Growth of the NO-sensitive E. coli hmp strain carrying Ph-2/2HbO-2217 under nitrosative stress

577 The putative role of the globin Ph-2/2HbO-2217 was investigated in vivo using the E. coli hmp
578 mutant to identify NO-detoxification mechanisms. E. coli hmp cells carrying Ph-2/2HbO-2217 were
579 grown overnight with induction supplements at 25 °C, in the absence and presence of increasing
580 concentrations of either the nitrosating agent GSNO (0, 1, 3, and 5 mM) (**Figure 6A**) or of the NO-
581 releaser DETA-NONOate (0, 0.5, 1, and 2 mM) (**Figure 6B**). The slightly higher concentrations
582 required for GSNO than for DETA-NONOate activity presumably reflect the fact that GSNO
583 releases only low levels of NO (Bowman et al., 2011; Laver et al., 2012) that is detoxified by
584 globins. In fact, 500 μM GSNO releases less NO than 5 μM DEA-NONOate, a relatively fast-
585 releasing NO-donor liberating 1.5 mol per mol parent compound (Jarboe et al., 2008).
586 The effect of the expression of the PSHAa2217 gene (**Figure 6**) on the ability of E. coli hmp
587 cells to survive in the presence of nitrosative stress was compared to that of cultures of E. coli hmp
588 transformed with pBAD/HisA (negative control), or pBAD-2/2HbO-0030 (positive control)
589 (Coppola et al., 2013).
590 The growth of the hmp mutant was progressively inhibited at all GSNO and DETA-NONOate
591 concentrations tested (up to 5 mM GSNO and 2 mM DETA-NONOate). However, exposure to
592 nitrosative stress had no effect on the growth of E. coli hmp cells expressing Ph-2/2HbO-0030
593 (Coppola et al., 2013), reflecting complete restoration of NO-detoxifying properties endowed by the
594 FHb. The growth of the E. coli hmp mutant carrying the PSHAa2217 gene was also significantly
595 improved relative to the un-complemented mutant, at 3 mM GSNO (**Figure 6A**) or 0.5 mM DETA-
596 NONOate (**Figure 6B**). These data demonstrate the involvement of both cold-adapted globins in
597 protecting the heterologous host from NO toxicity.
598 The protein encoded by the PSHAa2217 gene appeared less efficient in protection from
599 nitrosative stress compared to Ph-2/2HbO-0030. However, we cannot exclude the possibility that the
600 lower efficiency of the Ph-2/2HbO-2217 compared to Ph-2/2HbO-0030 may be due to different
601 levels of expression of these globins. The heme content in the cells carrying the PSHAa0030 gene
602 was around 2-fold higher than that of E. coli hmp carrying the PSHAa2217 gene.

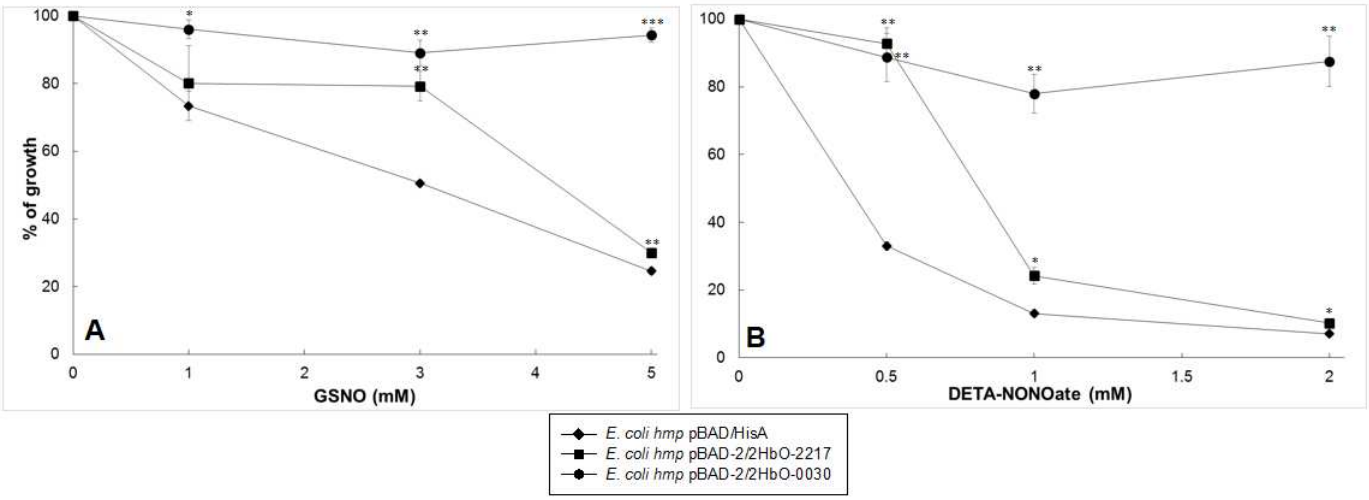


Figure 6. Susceptibility test of E. coli hmp expressing different plasmids supplemented with GSNO (A) and DETA-NONOate (B). Cultures of E. coli hmp carrying pBAD-2/2HbO-2217 after addition of 0.2 mM δ-aminolevulinic acid, 0.012 mM $FeCl_3$, 0.06% L-arabinose (squares), were grown for 18 h at 25 °C under aerobic conditions, and the optical density was recorded. The same strain, carrying pBAD/HisA (diamonds) and pBAD-2/2HbO-0030 (circles), grown in the presence of 0.2% and 0.06% L-arabinose, respectively, were included as controls. Values are means ± standard deviation. Errors bars of standard deviation were calculated by experiments carried out in triplicate. The significance of the data (E. coli hmp expressing Ph-2/2HbO-2217 and Ph-2/2HbO-0030 compared to E. coli hmp with pBAD/HisA) was estimated with a Student's t-test. ***P<0.001; **P<0.01; *P<0.05.

To confirm these results and demonstrate that, similar to Ph-2/2HbO-0030, Ph-2/2HbO-2217 also confers resistance during growth, cultures of the E. coli hmp mutant, carrying pBAD-2/2HbO-2217, pBAD/HisA, or pBAD-2/2HbO-0030, were grown at 25 °C under aerobic conditions and treated with either 3 mM GSNO (**Figure 7A**) or 0.5 mM DETA-NONOate (**Figure 7B**).

In the presence of GSNO, the ability of cells expressing Ph-2/2HbO-2217 to grow under nitrosative stress was also compared to that of cultures of E. coli hmp transformed with the plasmid

carrying the wild-type hmp+ gene from plasmid pPL341 (**Figure 7B**), as an additional positive control (Coppola et al., 2013).

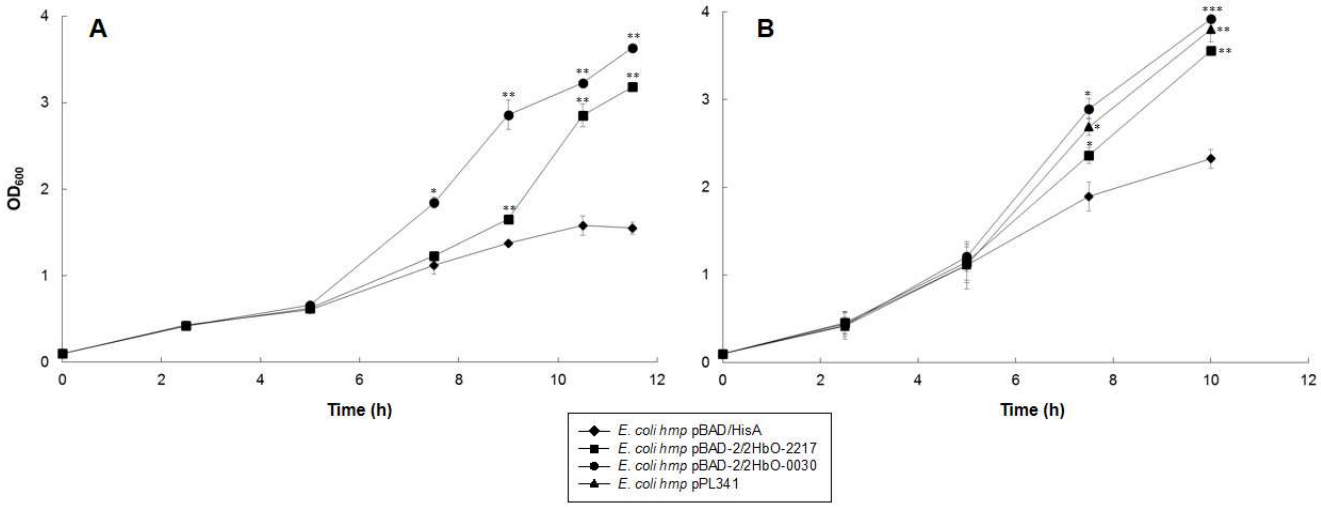


Figure 7. Growth profile of E. coli hmp expressing Ph-2/2HbO-2217 (squares), Ph-2/2HbO-0030 (circles), pBAD/HisA (diamonds), and pPL341 (triangles, Fig. 7B only), exposed to 3.0 mM GSNO (A) and 0.5 mM DETA-NONOate (B). Cultures were grown at 25 °C, under aerobic conditions, and supplemented with 0.2 mM δ-aminolevulinic acid, 0.012 mM $FeCl_3$, L-arabinose at the final concentration of 0.06% for E. coli hmp carrying either Ph-2/2HbO-2217, the empty vector or pPL341, and at the final concentration of 0.2% for the positive control E. coli hmp carrying Ph-2/2HbO-0030. Values are means ± standard deviation. Errors bars of standard deviation were calculated by experiments carried out in triplicate. The significance of the data (E. coli hmp expressing Ph-2/2HbO-2217 and Ph-2/2HbO-0030 compared to E. coli hmp with pBAD/HisA) was estimated with a Student's t-test. ***P<0.001; **P<0.01; *P<0.05.

Exposure to GSNO caused a slight decrease in growth of the E. coli hmp mutant carrying pBAD-2/2HbO-2217 compared to the mutant carrying Ph-2/2HbO-0030. In contrast, the growth profile of negative control cells bearing the empty vector pBAD/HisA was drastically reduced. Similar results were obtained in the presence of DETA-NONOate, even if the Antarctic globins

seem to be more effective in protecting growth from inhibition by DETA-NONOate than from GSNO. This may reflect the more complex toxic effects of GSNO (especially nitrosation reactions) (Laver et al., 2012) than NO. It is worth noting that globins detoxify NO but not GSNO per se (Laver et al., 2012). In the case of the NONOate, the complete alleviation of growth inhibition by expressing pPL341 encoding the E. coli flavohaemglobin Hmp, is clear.

Altogether, these results demonstrate that the globin Ph-2/2HbO-2217 provides substantial protection to the cells from NO toxicity in the heterologous host.

3.5. NO consumption and respiration rate of E. coli hmp expressing the PSHAa2217 gene

We noted that expression of Ph-2/2HbO-2217 was lower in the hmp mutant than that of Ph-2/2HbO-0030 and so, to eliminate the possibility that this contributed to interpreting measurements of growth and respiration in the presence of nitrosative stress agents, we performed experiments with suspensions of harvested cells in which protein and heme levels were quantified. Cells were grown at 25 °C in the presence of the induction supplements and under aerobic conditions and the respiration of E. coli hmp cells carrying the PSHAa2217 gene, the PSHAa0030 gene, or the empty vector, exposed to NO toxicity, was measured. Because GSNO is a poor NO donor (Jarboe et al., 2008) and is not a potent inhibitor of cell respiration, the course of O_2 and NO consumption was measured in the presence and absence of the fast NO-releaser Proli-NONOate (**Figure 8**). The toxicity of NO depends on O_2 concentration (Stevanin et al., 2000); thus, additions of NO were made at three different O_2 tensions after stimulating respiration by adding 25 mM glucose. At each NO addition, revealed by a rapid upward excursion of the NO electrode output, the O_2 uptake was abruptly stopped but resumed when the NO levels fell. When NO decreased to a minimal level, respiration continued until the chamber became anaerobic. In the absence of NO, all three strains consumed O_2 at a similar rate when respiration was normalised to total cell protein content, as assessed in the Markwell assay (6.4-8.4 nmol O_2/min/mg) (Markwell et al., 1978). However, when respiration rates were expressed relative to heme content, the strains expressing the globins showed

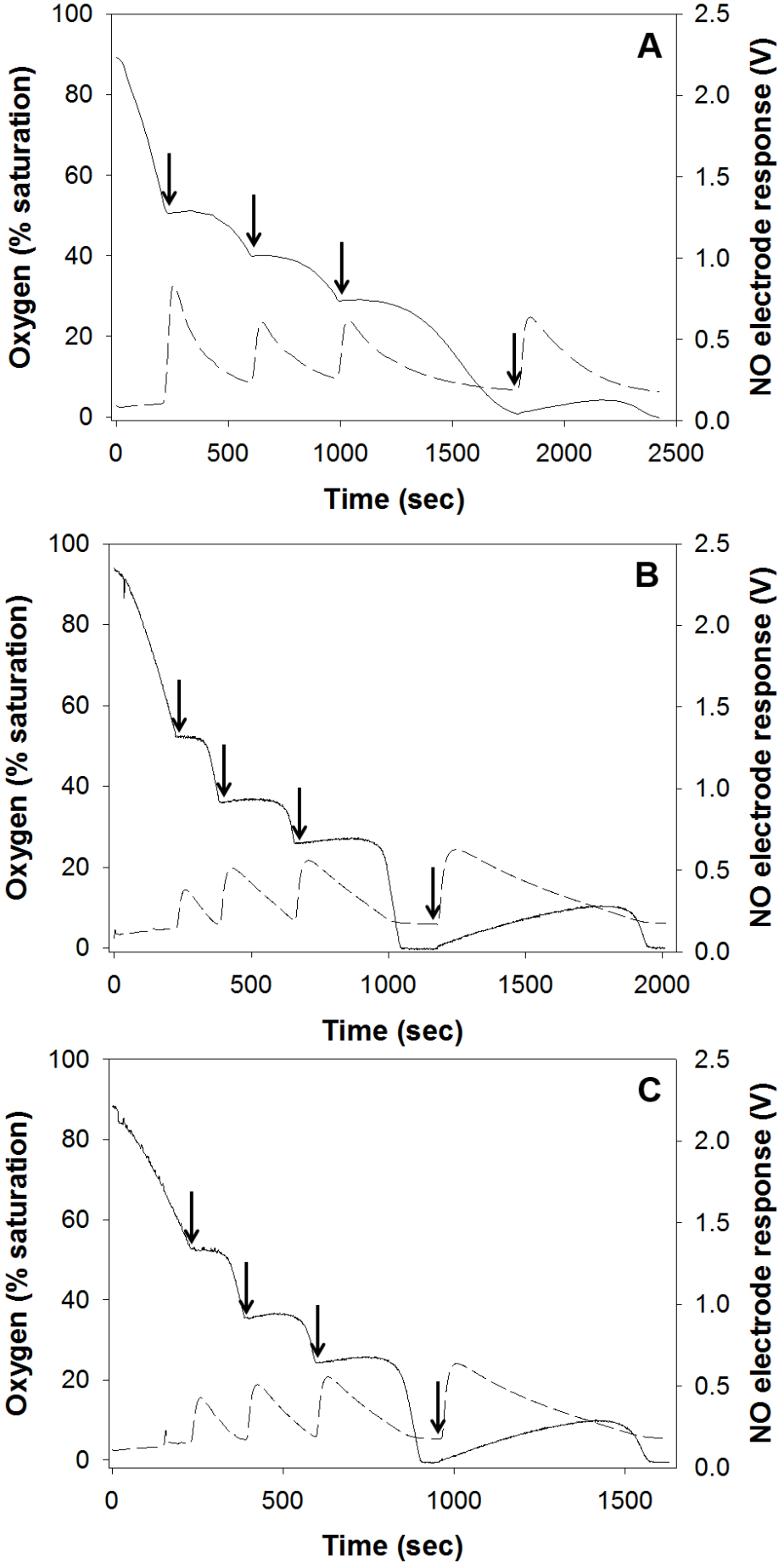
667 significantly lower respiration rates (6-10 nmol O_2/min/mM heme) than the strain containing the
668 control pBAD/HisA plasmid (15 nmol O_2/min/mM heme); this reflects the lack of globin-catalysed
669 O_2 uptake in the absence of NO.

670 To assess the roles of the two globins in NO detoxification, we measured the rates of O_2 uptake
671 after each NO addition and expressed this relative to the pre-NO rates of O_2 uptake. In Figure 8A,
672 the rate before adding NO (49 nmol O_2/min) was severely reduced by successive NO additions.
673 After each addition there was a transient, almost complete, inhibition of respiration, but activity
674 resumed as the NO concentration declined (as shown by the NO electrode traces). However, the
675 rates never regained the pre-NO rates: in Figure 8A, three successive NO additions resulted in
676 inhibition of >65-73% of the pre-NO rate. In marked contrast, in the case of the two globin-
677 expressing strains (Figure 8B, C), the resumption of O_2 uptake after NO additions regained
678 completely the pre-NO rates and, indeed, an acceleration of O_2 uptake. In the case of Ph-2/2HbO-
679 2217, the observed rates were 1.5-2.1-fold higher than the pre-NO rate and in the case of Ph-
680 2/2HbO-0030, the observed rates were 1.2-1.7-fold higher than the pre-NO rate.

681 We have already reported the ability of the E. coli hmp strain carrying the PSHAa0030 gene to
682 detoxify NO (Coppola et al., 2013); the present data show that both globins are able to restore
683 respiration to pre-NO rates. We attribute the final stimulation of respiration to the globin-catalysed
684 reaction between the remaining NO and O_2. When O_2 was depleted, further additions of NO resulted
685 in a larger NO signal and in its slower disappearance (**Figure 8A-C**), indicating O_2-dependent NO
686 consumption.

687 Taken together, these results indicate that Ph-2/2HbO-2217, like Ph-2/2HbO-0030, is able to
688 restore O_2 consumption in vitro after NO challenge, and probably involved in the bacterial defence
689 against nitrosative stress.

690


A
B
C
Oxygen (% saturation)
NO electrode response (V)
Time (sec)
0
20
40
60
80
100
0.0
0.5
1.0
1.5
2.0
2.5
0
500
1000
1500
2000
2500

691

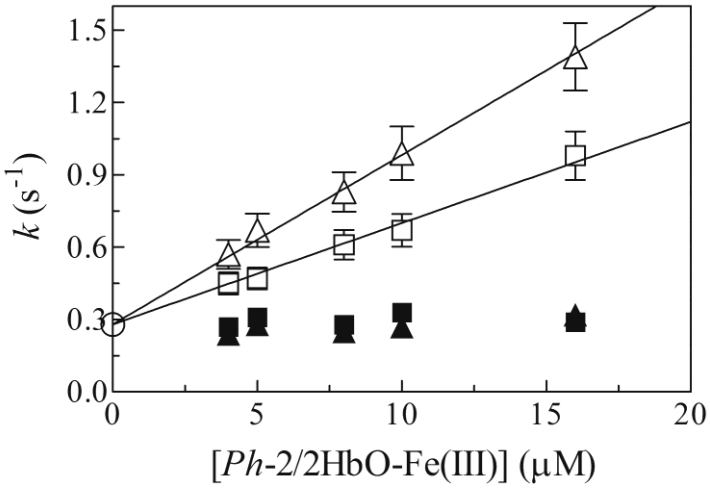
Figure 8. NO uptake and respiration of E. coli hmp carrying either the empty vector (pBAD/HisA) (A) or expressing Ph-2/2HbO-0030 (B), or Ph-2/2HbO-2217 (C). Respiration was followed in a Clark-type O_2 electrode (solid traces) upon additions of 1 μM Proli-NONOate (arrows). NO uptake was measured simultaneously with an NO electrode (dashed traces). After inhibition of respiration by the last aliquot of NO, the slight upward deflections of the O_2 traces probably reflect either the polarographic drift or the back-diffusion of O_2 into the chamber through the Hamilton syringe used to make NO additions. All experiments were performed in triplicate. The volumes of cell suspensions used were adjusted to give similar O_2 uptake rates in the absence of NO.

3.6. Peroxynitrite isomerisation

As shown in **Figure 9**, ferric Ph-2/2HbO-2217 catalyses peroxynitrite isomerisation, as reported for several globins, e.g. horse heart Mb (Herold and Shivashankar, 2003). On the other hand, cyanide-bound ferric Ph-2/2HbO-2217 does not facilitate peroxynitrite isomerisation, as previously reported in hexa-coordinated heme-proteins, e.g. human neuroglobin (Herold et al., 2004b) and horse heart cytochrome c (Ascenzi et al., 2011a, b), showing high affinity with intramolecular distal ligands.

Under all the experimental conditions, the time course of peroxynitrite conversion to nitrate is a monophasic process for more than 87% of its course. As shown in **Figure 9**, the first-order rate constant k increases linearly with the concentration of ferric Ph-2/2HbO-0030 and Ph-2/2HbO-2217. The analysis of data shown in **Figure 9** according to Eq. 2 allowed the determination of the k_{on} and k_0 values for peroxynitrite conversion to nitrate by the ferric globins. The values of k_{on} (corresponding to the slope of the linear plot) are 4.0×10^4 M^{-1} s^{-1} and 7.2×10^4 M^{-1} s^{-1} for Ph-2/2HbO-2217- and Ph-2/2HbO-0030-mediated isomerisation of peroxynitrite, respectively, at pH 7.4 and 20 °C.

716 The y intercept of the linear plot corresponds to 0.28 s^{-1}, matching with k_0 values (0.30 s^{-1})
717 obtained either in the absence of both ferric globins or in the presence of unreactive ferric globin-
718 cyanide adducts.
719 Interestingly, values of k_{on} for peroxynitrite scavenging by Ph-2/2HbO-2217 and Ph-2/2HbO-
720 0030 are similar to those reported for sperm whale Mb and human Hb (Herold and Shivashankar,
721 2003), representing the major targets of RNS in vivo (Herold and Fago, 2005).
722



723
724 **Figure 9.** Dependence of k for peroxynitrite isomerization on the concentration of ferric Ph-
725 2/2HbO-2217, in the absence and presence of cyanide (open and filled squares, respectively). Data
726 for peroxynitrite isomerisation by ferric Ph-2/2HbO-0030 in the absence and presence of cyanide
727 (open and filled triangles, respectively) are reported for comparison. All data were obtained at pH
728 7.4 and 20 °C. The circle on the ordinate indicates the value of k in the absence of globins. The
729 continuous lines were calculated according to Eq. 2 with $k_{on} = 4.0\times10^4$ M^{-1} s^{-1} (squares) and
730 7.2×10^4 M^{-1} s^{-1} (triangles), and $k_0 = 0.28$ s^{-1}. In the presence of saturating cyanide (5.0×10^{-4} M),
731 values of k are independent of the ferric-globin concentration and the average value of k =0.30 s^{-1}
732 corresponds to that of k_0 (circle). The peroxynitrite concentration was 2.5×10^{-5} M. When not
733 shown, the standard deviation is smaller than the symbol. For details, see text.
734

4. Discussion

Life at low temperature imposes a wide array of challenges to marine bacteria. At low temperatures, the enhanced O_2 solubility significantly increases the production rate of ROS. Therefore, bacteria must be able to adjust to temperature changes and availability of nutrients. A genome analysis of Colwellia psychroerythraea (Methé et al., 2005) and Desulfotalea psychrophila (Rabus et al., 2004) suggests that a common strategy to face environmental challenges consists of developing enhanced antioxidant capacity, resulting from multiple genes that encode catalases and superoxide dismutases.

By contrast, in silico analysis of the PhTAC125 genome (Médigue et al., 2005) suggests that this Antarctic marine bacterium may cope with increased O_2 solubility by multiplying O_2-scavenging enzymes (such as dioxygenases) and deleting entire metabolic pathways that generate ROS as side products. Moreover, its resistance to H_2O_2 is due to the presence of several enzymes involved in scavenging chemical groups affected by ROS (such as peroxiredoxins and peroxidases), and one catalase-encoding gene (katB) and a possible homologue (PSHAa1737) (Médigue et al., 2005).

Furthermore, in order to prevent significant damage to cellular structures, PhTAC125 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 defenses 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. In fact, PhTAC125 is able to thrive in pelagic form, where cells experience a high concentration of O_2 and other gases that characterise cold waters. In addition, although the strain thrives between 2 and 4 °C, it is also able to survive long-term frozen conditions when entrapped in the winter sea ice (Médigue et al., 2005).

The presence of multiple globin genes in distinct positions on chromosome I of PhTAC125 (Giordano et al., 2007) may be pivotal for cell protection. To our knowledge, PhTAC125 is the first example of coexistence of genes encoding a FHb and three 2/2Hbs (Giordano et al., 2013), of which

761 both Ph-2/2HbO-0030 and Ph-2/2HbO-2217 are endowed with hexa-coordination (Giordano et al.,
762 2011; Howes et al., 2011; Russo et al., 2013; Giordano et al., 2015; this study). Endogenous hexa-
763 coordination may be essential for proteins that function under high levels of oxidative stress
764 (Johnson and Lecomte, 2013).

765 When complementing this study with our earlier work (Coppola et al., 2013), it appears that
766 both Ph-2/2HbO-2217 and Ph-2/2HbO-0030 provide protection against NO and related reactive
767 species, under aerobic conditions. At first sight, two hexa-coordinated globins capable of
768 performing NO detoxification appear redundant. However, variations in physico-chemical features
769 of the marine environment may require diversified responses, which may be reflected in appropriate
770 modulation of gene expression in this bacterium. Our incomplete knowledge of the physiological
771 role of the two globins, which is probably multifaceted, is another aspect that needs to be
772 considered. For example, the genome of many fish species can express multiple Hbs having similar
773 Bohr and Root effects, which points to apparently similar mechanisms in O_2 binding and release.

774 Transcriptional analysis of the genes encoding globins in PhTAC125 wild type and in the
775 PhTAC125-0030 mutant showed that the transcription of the FHb-encoding gene (PSHAa2880)
776 was observed in the PhTAC125-0030 mutant when grown at 4 °C in microaerobiosis (Parrilli et al.,
777 2010). Since the transcription of FHb-encoding genes is linked to globin-mediated NO
778 detoxification (Membrillo-Hernàndez et al., 1999; Mills et al., 2001; Stevanin et al., 2000), the
779 observed FHb-gene expression is suggestive of the occurrence of NO-induced stress intimately
780 correlated to the absence of Ph-2/2HbO-0030. Although the PhTAC125 genome contains two
781 additional 2/2Hbs encoding genes, transcribed in all the experimental conditions tested in Parrilli et
782 al. (2010), mutation of the gene encoding Ph-2/2HbO-0030 is sufficient to obtain a strain with a
783 clear mutant phenotype. This suggests that the numerous globins in this bacterium are not
784 functionally redundant in PhTAC125 physiology.

785 The high reactivity of the ferric forms of Ph-2/2HbO-0030 (Coppola et al., 2013) and Ph-
786 2/2HbO-2217 towards peroxynitrite suggests that protection against RNS and ROS is a strong need

787 in the cold Antarctic environment. Low temperatures are known to decrease nitrate uptake among
788 bacteria, and nitrogen is fundamental for bacteria replication and synthesis of proteins.
789 Several procedures were attempted to purify the expressed Ph-2/2HbO-2217 to
790 homogeneity, but they were unsuccessful thus precluding the possibility to determine its physico-
791 chemical properties under physiological conditions. Therefore, two in vivo and in vitro models have
792 been developed to highlight the protective role of Ph-2/2HbO-2217 against RNS. However, the
793 nitrosative stress-sensitive E. coli is protected from NO by ferrous Ph-2/2HbO-2217, which is
794 involved in O_2- and NO-consumption (**Figures 6, 7 and 8**), and ferric Ph-2/2HbO-2217 which
795 catalyses in vitro peroxynitrite scavenging (**Figure 9**). These results suggest that ferrous and ferric
796 Ph-2/2HbO-2217 could be involved in the detoxification of RNS (i.e., NO and peroxynitrite,
797 respectively), thus protecting the bacterium from these nitrosative stress mechanisms.
798 The main features of Ph-2/2HbO-2217 and Ph-2/2HbO-0030 are the presence of a longer
799 sequence extension of the N-terminal region (19 residues in Ph-2/2HbO-0030 and 9 residues in Ph-
800 2/2HbO-2217), that in Ph-2/2HbO-0030 is proteolytically cleaved during protein purification
801 (Giordano et al., 2007), and does not appear to reduce the NO scavenging activity (Coppola et al.,
802 2013). Pesce et al. (2016) have recently demonstrated that removal of the pre-A region in M.
803 tuberculosis Mt-2/2HbN promotes the assembly of a stable dimer, both in the crystals and in
804 solution, hypothesising that the pre-A region may be essential for survival of the microorganism
805 because it significantly reduces the ability of Mt-2/2HbN to scavenge NO by interfering with ligand
806 diffusion. Accordingly, kinetic measurements of Mt-2/2HbN-DpreA indicate that the k_{on} values for
807 peroxynitrite isomerisation by the mutant protein were four-fold lower than in the wild-type protein
808 (Pesce et al., 2016).
809 Interestingly, also 2/2HbI, encoded by the PSHAa0458 gene in the PhTAC125 genome, is
810 characterised by an extension at the N terminus longer than that observed in Mt-2/2HbN; from
811 preliminary results, the protein shows endogenous hexa-coordination (Daniela Giordano, personal
812 communication), similar to the other genes of 2/2Hbs present in the genome of PhTAC125.

Although transcriptional regulation is the main mechanism in stress responses, regulation of translation is faster and consequently very important for species. Post-transcriptional regulation occurs at different stages and includes generation of proteins that need to be activated to perform their function (Varshavsky, 2011). The strategy allows cells to respond quickly to environmental stimuli by simply activating preexistent proteins. We cannot exclude that these extensions at the N terminus may play a role in the native host, namely the Antarctic bacterium, although the experiments performed by Coppola et al. (2013) in the mutant of E. coli as heterologous host did not show any involvement of the pre-A region of Ph-2/2HbO-0030 in NO detoxification. Altogether, these findings indicate the need of PhTAC125 to quickly react to the environment by implementing proteins that function under high levels of oxidative stress.

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