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Research paper

The role of a 2-on-2 haemoglobin in oxidative and nitrosative stress resistance of Antarctic *Pseudoalteromonas haloplanktis* TAC125

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ABSTRACT

The 2-on-2 haemoglobins, previously named truncated, are monomeric, low-molecular weight oxygen-binding proteins that share the overall topology with vertebrate haemoglobins. Although several studies on 2-on-2 haemoglobins have been reported, their physiological and biochemical functions are not yet well defined, and various roles have been suggested. The genome of the psychrophilic Antarctic marine bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) is endowed with three genes encoding 2-on-2 haemoglobins. To investigate the function played by one of the three trHbs, *Ph*HbO, a *Ph*TAC125 genomic mutant strain was constructed, in which the encoding gene was knocked-out. The mutant strain was grown under controlled conditions and several aspects of bacterium physiology were compared with those of wild-type cells when dissolved oxygen pressure in solution and growth temperature were changed. Interestingly, inactivation of the *Ph*HbO encoding gene makes the mutant bacterial strain sensitive to high solution oxygen pressure, to H_2O_2, and to a nitrosating agent, suggesting the involvement of *Ph*HbO in oxidative and nitrosative stress resistance.

1. Introduction

Life at low temperature imposes a wide array of challenges to marine bacteria. One of them is related to the increase of gas solubility in cold water. Indeed, at low temperatures the enhanced O_2 solubility significantly increases the production rate of toxic reactive O_2 species (ROS). From the genome analysis of *Colwellia psychroerythraea* [1] and *Desulfotalea psychrophila* [2], a common strategy to face this environmental challenge can be envisaged, consisting in developing enhanced antioxidant capacity owing to multiple genes that encode catalases and superoxide dismutases. By contrast, *in silico* analysis of the *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) genome [3] 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 which generate ROS as side products. The remarkable deletion of the ubiquitous molybdopterin-dependent metabolisms in the *Ph*TAC125 genome [3] can be regarded in this perspective. The microorganism is also notably resistant to H_2O_2 [3], and this ability was *in silico* correlated to the presence of: i) several enzymes involved in scavenging chemical groups affected by ROS (such as peroxiredoxins and peroxidases); and ii) to the presence of one catalase-encoding gene (*katB*) and a possible homologue (PSHAa*1737*) [3].

In this context, our interest was focused on oxygen-binding proteins involved in oxygen scavenging and/or transport, eventually contributing to *Ph*TAC125 adaptation to high oxygen concentration in cold sea water.

Bacteria may produce three types of haemoglobins (Hbs), namely 2-on-2 or truncated Hbs (trHbs), monomeric Hbs and flavohaemoglobins (flavoHbs). 2-on-2 haemoglobins are monomeric, low-molecular weight O_2-binding haemoproteins and their fold is based on a "2 on 2" α-helical sandwich [4]. Based on sequence clustering, three main trHb groups have been identified i.e. group I (also named HbN), group II (also named HbO) and group III (also named HbP) [4–6]. All these trHbs are able to bind diatomic ligands such as O_2, CO, and NO, with different affinities [7]. The high oxygen affinity displayed by most trHbs makes their role as O_2 transporters

Abbreviations: GSNO, *S*-nitrosoglutathione; SNP, sodium nitroprusside; RT-PCR, reverse transcriptase PCR.

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very unlikely [4,8], while several other physiological functions have been proposed [4,9,10].

The *Ph*TAC125 genome contains three genes encoding trHbs and one that codes for a flavoHb [11]. The presence of several 2-on-2 haemoglobins (2-on-2 Hbs) encoding genes raises the question whether their function can be related to *Ph*TAC125 ability to cope with the high oxygen concentration of cold sea water. This hypothesis and the availability of several genetic tools [12] suitably developed for *Ph*TAC125 prompted us to use this organism to investigate the functional role of 2-on-2 Hbs in Antarctic cell physiology. Indeed some microorganisms with interesting truncated globins (i.e. *Mycobacterium tuberculosis*, *Nostoc commune*, *Chlamydomonas eugamentos* and *Paramecium caudatum*) are not readily amenable to genetic manipulation; as a consequence, the experimental demonstrations of proposed physiological hypothesis are limited [13].

We have recently reported the recombinant production and purification of *Ph*HbO [11], one of the three *Ph*TAC125 trHbs. To investigate its physiological role, a genomic mutant strain was constructed, in which the *Ph*HbO encoding gene (PSHAa*0030*) was inactivated by insertional mutagenesis. The mutant strain was grown under controlled conditions and its growth behaviour was compared with that of wild-type cells, when solution oxygen pressure and growth temperature changed. This approach allowed proposing a possible role of *Ph*HbO in the adaptation of *Ph*TAC125 to the Antarctic marine environment.

2. Materials and methods

2.1. Bacterial strains and growth conditions

*Ph*TAC125 [3] was isolated from Antarctic sea water. *Escherichia coli* DH5α was used as host for gene cloning and was routinely grown in Luria–Bertani broth [14] at 37 °C with the appropriate antibiotic selection.

*Ph*TAC125 was grown in minimal medium [15] supplemented with 0.5% yeast extract and 0.2% galactose. 100 μg/ml ampicillin, 50 μg/ml chloramphenicol were added to liquid cultures when transformed. Antarctic bacterium was made recombinant by intergeneric conjugation as previously reported [12]. Batch cultivations were performed in a computer-controlled bioreactor (Sixfors System, Infors) equipped with control units for pH, an oxygen sensing electrode measuring the solution oxygen pressure, temperature, and mechanical stirring (rpm) at 4 °C and 15 °C. pH was maintained at 7.0 ± 0.5 by the addition of 1% H_2SO_4 or 5% NH_4OH. Each medium filled fermentation unit was equilibrated at process temperature, then the stirring speed was set up at the maximum value to be used during the fermentation and sterile air supply was switched on. The system was let to stabilize for at least 30 min to guarantee the medium to be saturated with air. In these conditions the 100% of measured oxygen pressure was assigned. The zero-point set calibration was performed by saturating the medium with sterile nitrogen gas. Under extreme aerobiosis conditions, measured oxygen pressure was maintained always above 80% by modifying stirring speed and aeration rate. In microaerobiosis (measured oxygen pressure always below 5% saturation) air supply was stopped after inoculum. For each strain, the growth kinetics were followed in triplicate in at least two independent experiments.

2.2. Molecular methods and reagents suppliers

Standard methods were employed for DNA manipulation and isolation, amplification by PCR, and DNA sequencing [14,16]. Restriction enzymes, T4 DNA ligase, alkaline phosphatase, T4 polynucleotide kinase, Klenow fragment, *Taq* DNA polymerase were supplied from Boehringer-Roche, Amersham-Pharmacia Biotech, Promega, and New England Biolabs. DNA fragment purification was carried out with the QUIAEX II kit from Qiagen GmbH.

2.3. Vector pVS0030 and PhTAC125-30 mutant construction

The reaction of PCR [14] was employed to amplify a DNA fragment of PSHAa*0030* gene. *Ph*TAC125 genomic DNA was used as PCR template and two primers were designed to amplify a 241bp-long region of the PHSAa*0030* gene and to introduce two SphI sites (Oligo S0030fw 5′-TTGCATGCATACTAGGTGGAGAAGC-3′ and Oligo S0030rv 5′-AAGCATGCGCGCAAGTCTTGATC-3′). The amplified DNA fragment was digested by SphI, and inserted into the pVS plasmid [17] corresponding site, thus obtaining the pVS*0030* vector. The vector was mobilized by intergeneric conjugation into *Ph*TAC125 cells and insertion mutants were screened on plates at 4 °C containing carbenicillin (30 μg/ml) as selection agent.

2.4. Construction of vector pUC0030

pUC*0030* was constructed starting from pUCC vector, a plasmid deriving from the pUCLOriT/R plasmid [18], containing the T/R box, the transcription termination signal from the *Ph*TAC125 *aspC* gene and the chloramphenicol-resistance gene. The nucleotide region including the PSHAa*0030* gene and its upstream region was amplified to introduce HindIII and BamHI restriction sites using oligo 0030 fw (5′-TTAAGCTTTAGCTCCCTTACCGCC-3′) and oligo 0030 rv (5′-AAGGATCCGTGCCAGCTTTAAGGC-3′). The PCR product was subjected to double HindIII and BamHI digestion, purified, and inserted into pUCC vector corresponding sites. The amplified fragments were cloned and checked by nucleotide sequencing to rule out the occurrence of mutations during synthesis.

2.5. RNA preparation and RT-PCR

Total RNA was isolated (RNeasy Mini kit, Qiagen) from 500 μl aliquots of *Ph*TAC125-*30* mutant and *Ph*TAC125 wild-type cell cultures withdrawn at different growth times at 4 °C and 15 °C, in both microaerobiosis and extreme aerobiosis, and subjected to in-column DNase treatment (Rnase-Free Dnase Set, Qiagen). Reverse-transcription (RT) reactions using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) were performed using 100 pmol of specific primers. In detail, reverse transcriptase analyses were performed on approximately 5 μg of purified RNA using PHSAa*0030* specific primers 0030Sfw (5′-TTGCATGCATACTAGGTGGAGAAGC-3′) and 0030Xrv (5′-AAGCATGCGCGCAAGTCTTGATC-3′), *Ph*TAC125 PHSAa*0458* specific primers 0458Erv (5′-CTTTGCTCGAGCATAGCA TTAATTAG-3′) and 0458Sfw (5′-AATGGGCATGCCGGCATAGAAA AAC-3′), PSHAa*2880* specific primers 2880Srv (5′-GAGTATTTTCGCA AGCATGCAAATAC-3′) and 2880Efw (5′-ATTTTGCTCGAGCACTACCC AATTG-3′), PSHAa*2217* specific primers 2217Srv (5′-GCTTTTTGCA TGCATAATAGCCAAGC-3′) and 2217Efw (5′-TACCCAGCTCGAGGATG CATTTTATG-3′). PCR amplifications were performed using 2 μl of RT reaction sample as a template, Taq DNA polymerase (Promega) and specific target primer pairs. For each reverse transcriptase amplification, an additional PCR reaction on DNA-free total RNA was performed as negative control (Supp Fig. 1).

2.6. Disk-diffusion assays

The sensitivity of the *Ph*TAC125-*30* mutant and *Ph*TAC125 wild-type cells to 100 mM GSNO, 100 mM SNP, 256 mM H_2O_2, and 100 mM spermidine NONOate was assayed at 15 °C in a plate diffusion assay [19].