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High hydrostatic pressure increases amino acid requirements in the piezo-hyperthermophilic archaeon *Thermococcus barophilus*

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Abstract

We have established a defined growth medium for the piezophilic hyperthermophilic archaeon *Thermococcus barophilus*, which allows growth yields of ca. 10^8 cells/ml under both atmospheric and high hydrostatic pressure. Our results demonstrate a major impact of hydrostatic pressure on amino acid metabolism, with increases from 3 amino acids required at atmospheric pressure to 17 at 40 MPa. We observe in *T. barophilus* and other *Thermococcales* a similar discrepancy between the presence/absence of amino acid synthesis pathways and amino acid requirements, which supports the existence of alternate, but yet unknown, amino acid synthesis pathways, and may explain the low number of essential amino acids observed in *T. barophilus* and other *Thermococcales*. *T. barophilus* displays a strong metabolic preference for organic polymers such as polypeptides and chitin, which may constitute a more readily available resource of carbon and energy in situ in deep-sea hydrothermal vents. We hypothesize that the low energy yields of fermentation of organic polymers, together with energetic constraints imposed by high hydrostatic pressure, may render de novo synthesis of amino acids ecologically unfavorable. Induction of this metabolic switch to amino acid recycling can explain the requirement for non-essential amino acids by *Thermococcales* for efficient growth in defined medium.

Keywords: Amino acids metabolism; *Archaea*; *Thermococcales*; Piezophily; Deep biosphere

1. Introduction

In the deepest parts of oceans, hydrothermal vent ecosystems are subjected to extremely high hydrostatic pressure (HHP), which can reach 110 MPa, e.g. 1100 times the atmospheric pressure [35]. HHP is known to impact the structure of several cellular components and functions, such as membrane fluidity, protein activity and structure [10,25]. Physically, the impact of pressure bears resemblance to both a lowering of temperature, since it will reinforce the structure of some molecules such as membrane lipids, and an increase in temperature, since it will also destabilize other structures such as proteins [12]. Piezophilic microorganisms, e.g. microorganisms that have optimal growth conditions under high hydrostatic pressure, have been isolated from various deep-sea hydrothermal vents [19,22,23,36]. One of the first piezophilic isolates was *Thermococcus barophilus* strain MP, isolated from the Snake Pit hydrothermal vent system on the Mid-Atlantic Ridge at 3550 m depth [23]. *T. barophilus* grows optimally at 40 MPa, 85 °C and 3% salinity and is able to grow at temperatures up to 103 °C and pressures up to 80 MPa. Low hydrostatic pressure is perceived as a stress [24]; which makes this strain one of the best model organisms for studying genetic and structural adaptation to life under high hydrostatic pressure. However, more than a decade after its description,

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our knowledge about the structures and strategies underlying its high hydrostatic pressure adaptation remains nascent. One bottleneck for the study of this organism has been the lack of a suitable defined growth medium to allow genetic manipulations and detailed physiological studies.

The archaeal order *Thermococcales* is currently composed of three genera, *Palaeococcus*, *Pyrococcus* and *Thermococcus*. Members of the three genera are genetically and metabolically very similar. They are obligate heterotrophs growing on complex organic substrates, with a preference for polypeptides, usually in the presence of elemental sulfur. While there is evidence that individual amino acids are taken in [1] and assimilated [2,13] by *Thermococcales*, complex polypeptide-containing media are usually chosen for the apparent inability of these organisms to utilize mixtures of single amino acids as sole carbon and/or energy sources [6,11]. This may arise from an inability of the organisms to transport certain amino acids into the cell, competition between the uptake of several essential amino acids or the greater thermal instability of single amino acids compared to their stabilization in peptides. Although first attempts met with limited success, which generated only a fraction of the growth observed in peptide-containing rich media [2,13,20]; defined media for studying the growth physiology of hyperthermophilic microorganisms in which suitable growth rates and yields can be reached have been obtained for a few *Thermococcales*, such as *Pyrococcus furiosus* [27] and *Pyrococcus abyssi* [33] or *Thermococcus litoralis* [28]; *Thermococcus celer* [17] and *Thermococcus kodakarensis* [9]. Although the organisms are closely genetically related, their amino acid requirements differ widely. A minimal medium containing 9 amino acids (e.g. Arg, His, Iso, Leu, Met, Phe, Thr, Tyr, and Val) supported the growth of *P. abyssi* to the same extent as a rich peptide-containing medium [33]. Several species of *Thermococcales*, such as *P. sp*. GB-D [18] and *P. sp*. KS1, KS2, KS3 or *T. celer* [17] have requirements close to those of *P. abyssi*. For example, in addition to these 9 amino acids, *T. celer* also requires Lys and Trp, but does not require His. In contrast, only four amino acids, Gly, Iso, Thr and Val, are essential for the growth of *T. litoralis* [28]; and only two, Cys and Pro or Iso and Val, are required for growth in continuous or batch culture of *P. furiosus*, respectively [2,13,18,27]. Surprisingly, efficient growth of the latter two species cannot be achieved in minimal medium containing only these essential amino acids. In fact, only 4 amino acids, Ala, Asp, Gln and Glu, could be omitted from the growth medium of *T. celer* [17].

We explored the amino acid requirements of *Thermococcus barophilus* strain MP as a function of growth pressure in order to identify a defined growth medium suitable for growth and genetic studies in this strain. We show that this strain has different growth requirements under atmospheric or optimal growth pressures. Only three amino acids, alanine, glutamine, and proline, were not essential for growth at 40 MPa. In contrast, only three amino acids, glutamate, cysteine and tyrosine, were essential for growth at atmospheric pressure.

2. Materials and methods

2.1. Strain and growth conditions

T. barophilus strain MP was isolated from the Snake Pit hydrothermal vent located on the Mid-Atlantic Ridge at a water depth of 3550 m. Strain MP displays optimal pressure, temperature and salinity at 40 MPa, 85 °C and 3% of NaCl, respectively. *Thermococcales* Rich Medium (TRM) was used as the rich medium [36]. The mineral base of TRM was used as the mineral base of our minimal medium (*Thermococcales* Basic Medium, TBM). The mineral base composition of TBM is given in Table S2. TBM was supplemented with filter-sterilized Wolfe's mineral solution (10 ml l^{-1}) and Wolfe's vitamins solution (10 ml l^{-1}) [34] and a mix of 20 amino acids (20aa: Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, each at a concentration of 0.1 g l^{-1}). In most experiments, acetate (2 g l^{-1}) was added as a carbon source. To test for growth on peptone or yeast extract, TRM was prepared by omitting either peptone (TRM-P), yeast extract (TRM-Y) or both carbon sources (TRM-YP). All cultures were incubated at 85 °C. Cells were grown anaerobically in sealed 50 ml serum vials at ambient pressure or in sterile syringes sealed with a rubber stopper under high hydrostatic pressure as described previously. Final anaerobiosis was achieved by adding neutral sterile $Na_2S \cdot 9H_2O$. Powdered sulfur was used as the sulfur source (10 g/l).

2.2. Amino acid requirement and carbon source assays

For each substrate utilization assay, 100 µl of a glycerol stock of strain MP, stored at −80 °C, was used to inoculate 10 ml of TRM and incubated in a sterile syringe at 40 MPa and 85 °C for 9 h until late exponential phase. The culture was used to inoculate 10 ml of fresh TBM medium supplemented with the 20 aa mixture (0.1 g/l) and acetate (2 g/l) at a final concentration of 5×10^5 cells ml^{-1} and incubated at 85 °C and ambient pressure until mid-exponential phase for 15 h to adapt the cultures to minimal medium conditions. The adapted cells were used to inoculate fresh TBM medium (to a final concentration of 5×10^5 cells ml^{-1}) supplemented with either: 1) the 20 aa mixture and a carbon source (glucose, sucrose, rhamnose, maltose at 4 g/l, cellulose, starch, chitin, at 2 g/l); or 2) acetate (2 g/l) and a mixture of amino acids lacking 1 of the 20 different amino acids. Assay cultures were incubated at 85 °C and ambient or 40 MPa pressure for up to 72 h. Growth was measured by direct cell counting on a Thoma chamber using a light microscope, at 24 h, 48 h and 72 h. All experiments were performed in triplicate at minimum.

2.3. Amino acid intake experiments

Cells were grown in TBM supplemented with acetate (2 g/l) as a carbon source, yeast extract (0.02 g l^{-1}) to provide vitamins and oligoelements, and a mixture of the 20 amino acids (Asp, Asn, Gln, Val at 50 mg/l; Ala, Met, Phe, Ser, Trp at