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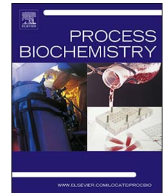

Characterization of *Phormidium lacuna* strains from the North Sea and the Mediterranean Sea for biotechnological applications

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ABSTRACT

In biotechnological applications, cyanobacteria are employed for conversion of CO_2 into bioproducts with sunlight as sole energy source. We describe the isolation of motile filamentous cyanobacteria from rockpools of the North Sea or the Mediterranean Sea and their characterization by physiological assays and genome sequencing. The five isolated lines are genetically highly similar, we regard them as strains of the same species. Phylogenetic studies placed the strains in the genus *Phormidium*; the species is termed *Phormidium lacuna*. Under liquid media growth conditions or in photobioreactors, *Phormidium* growth rates were comparable with the single celled model cyanobacterium *Synechocystis* PCC6803. However, *Phormidium* strains tolerate different media that can contain up to 3.7× the salt concentration of seawater and grows at temperatures up to 50 °C. Growth in medium free of NH_3 or NO_3^- suggests that *Phormidium* can fix atmospheric dinitrogen by nitrogenase even in the presence of light. Genome data confirmed the presence of nitrogenase and revealed its evolutionary position close to anoxygenic δ-proteobacteria. Genes for photosynthesis, photoreceptors, nitrogen metabolism, hydrogenases, tryptophan synthesis, glucose uptake, and fermentative pathways are discussed in the context of biotechnological applications.

1. Introduction

The use of microalgae for biomass production is increasing. Microalgae can use sunlight as the only energy source and have the potential to replace crop plants for biofuel production [1]. In a collaborative 18 years (1978–1996) research project, the "Aquatic Species Program" of the US Department of Energy, members of the order Oscillatoriales were among the microalgal species with the highest growth rates [2]. Oscillatoriales are unbranched, filamentous cyanobacteria, which are composed of cells of the same type. These species are thereby distinguished from the filamentous Nostocales, which have two different cell types, heterocysts and normal cells [3]. Often, the use of single celled species is proposed for biotechnological applications [4], because filaments form aggregates in standard bioreactors. However, the filamentous *Arthrospira platensis* is propagated in large open ponds worldwide as food supplement. Since *Arthrospira* grows in alkaline conditions [5], there is no difficulty with contaminating organisms. Other biotechnological applications of Oscillatoriales deal with heavy metal detoxification of waste water [6,7]. The filamentous growth can also be an advantage for other biotechnological applications. Most often, the desired product is released into the growth medium. Filaments are easier to be separated from the growth medium as compared to single cells. Specific growth reactors, for example membrane reactors [8], may be designed in which aggregation of filaments does not lead to difficulties. Biofilm formation can be useful for applications which require high cell densities [9]. The continued motility of Oscillatoriales on surfaces [10,11] is another feature that might be useful for various biotechnological applications.

Besides carbon fixation through oxygenic photosynthesis, some cyanobacteria possess also the capacity to fix molecular nitrogen [12]. The key enzyme nitrogenase reduces molecular nitrogen into ammonium. Nitrogenase genes are found in most cyanobacterial genomes. Oxygen production during photosynthesis of cyanobacteria is expected to inhibit nitrogenase [12]. Two oxygen protection mechanisms are

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known from cyanobacteria. (i) Spatial separation between oxygen production and dinitrogen fixation by cellular differentiation in cyanobacteria of the order Nostocales. Nitrogen fixation takes place in heterocysts, which are larger than adjacent cells, lack photosystem II and have thick cell walls to keep oxygen away [13]. (ii) Temporal separation of photosynthesis and nitrogen fixation in single celled or filamentous cyanobacteria [8,14]. In these species, nitrogen fixation takes place at night. The mechanism, how nitrogenase is protected from oxygen in the remaining cyanobacteria including Oscillatoriales, is not clear. Members of *Trichodesmium*, a genus of the Oscillatoriales, are regarded as the most important nitrogen fixing organisms of the oceans [12].

Our group has collected and characterized microalgal samples from marine origins. We became interested in *Phormidium* strains (members of the order Oscillatoriales) from rockpools of the North Sea and the Mediterranean Sea, which could easily be propagated and brought into axenic culture. We reasoned that these robust cyanobacteria must be well adapted to moderate climate conditions and could be used for outdoor biotechnological applications in the future. In the present study, we characterized these strains using growth optimization techniques in order to explore their genome potential in terms of growth and survival. The functioning of nitrogenase was analyzed experimentally. We sequenced the genome of the strain HE10JO to explore its metabolic potential and compare it with other cyanobacterial species. To this end, we performed a comprehensive analysis and provided a schematic model of its energy and nitrogen metabolism. Further, systematic evolutionary analyses of its nitrogenase and bidirectional hydrogenase enzymes were carried out. Towards the phylogenetic positioning of this strain, a comprehensive analysis was performed using various taxonomic strategies. In addition, the industrial importance of this strain was explored to exploit and scale up high-value products of the Oscillatoriales at industrial level in future.

2. Materials and methods

2.1. Growth media

Growth medium f/2 [15] consists of artificial seawater (0.41 M NaCl, 53 mM $MgCl_2$, 28 mM Na_2SO_4, 10 mM $CaCl_2$, 9 mM KCl, 2.4 mM $NaHCO_3$, 0.84 mM KBr, 0.49 mM H_3BO_3, 90 µM $SrCl_2$, 72 µM NaF), which is complemented by 0.88 mM $NaNO_3$, 0.11 mM Na_2SiO_3, 36 µM NaH_2PO_4, trace metals (12 µM $FeCl_3$, 12 µM EDTA disodium, 0.91 µM $MnCl_2$, 77 nM $ZnSO_4$, 42 nM $CoCl_2$, 39 nM $CuSO_4$, 26 nM Na_2MoO_4), and vitamins (0.30 µM Thiamin, 2.1 nM Biotin, 0.37 nM Vitamin B12).

BG11 medium (Sigma-Aldrich, Germany) contains 18 mM $NaNO_3$, 0.3 mM $MgSO_4$, 0.25 mM $CaCl_2$, 0.23 mM K_2HPO_4, 0.19 mM Na_2CO_3, 48 µM boric acid, 31 µM citric acid, 23 µM ferric ammonium citrate, 9.2 µM $MnCl_2$, 2.8 µM EDTA disodium magnesium, 1.6 µM Na_2MoO_4, 0.77 µM $ZnSO_4$, 0.32 µM $CuSO_4$, 0.17 µM $Co(NO_3)_2$. For BG11/TES medium, autoclaved TES buffer from a 10× stock solution (100 mM, pH 8) were mixed with the 50× BG11 stock and H_2O for final 1× concentration. For salt variations, the concentrations of "artificial seawater" in f/2 medium were adjusted accordingly, other compounds were kept constant. For solid cultures, 1% bacto agar (BD, USA) was added to the growth medium.

2.2. Isolation of species

The *Phormidium lacuna* strains were isolated from rockpools of the islands Giglio, Italy and Helgoland, Germany. The samples were filtered through 70 µm mesh, transferred into culture flasks, and stored at 4 °C for up to 8 weeks. For preparation of axenic cultures, all cells were initially collected on a sterile filter, suspended in 10 ml medium, and transferred into new culture flasks. Mixed cultures were brought onto agar medium from which the strains of interest were isolated by repeated subcultivation. The cells were cultivated under continuous white light.

2.3. Sample preparation and cultivation

For subcultivation and optical density measurements (OD_{750nm}) the filaments were homogenized for 3 min using an Ultraturrax (Silent Crusher M, Heidolph, Germany) with the dispersion tool 18F at 10 000 rpm. For inoculation, 1 ml culture was added to 9 ml fresh medium. Typically, 10 ml cyanobacteria culture were cultivated in 50 ml filter cap cell culture flasks (Greiner Bio-One, Germany) at 25 °C and 32 $\mu mol\ m^{-2}\ s^{-1}$ white light from fluorescent tubes (Lumilux-DeLuxe L 18/954, Osram, Germany). *Phormidium* strains were cultivated in f/2 and *Synechocystis* PCC 6083 in BG11/TES medium unless indicated otherwise. Cultures were either shaken at 100 rpm or incubated without shaking. The length and diameter of the cells and filaments were determined with an Axioscope microscope (Zeiss, Germany).

2.4. Photobioreactor

For photobioreactor batch experiments, a stirred tank reactor with a working volume of 1.7 L from BioEngineering (KLF200, Switzerland) equipped with an LED illumination shell was employed. Cells were mixed by two 6-blade agitators and baffles rotating at 250 rpm. The LED illumination shell was developed by the Institute of Bioprocess Engineering (KIT, Germany). The arrangement of LEDs around the cylindrical bioreactor focused the light towards the reactor center, compensating for the mutual shading of microalgae cells and therefore resulting in a nearly homogeneous light field inside the reactor [16]. Photon flux density (photosynthetic active radiation) was measured inside the reactor vessel by a planar quantum sensor (LI-250A, Li-Cor) prior to cultivation. Online parameters like temperature (PT100, Pico-Technologies, UK), pH (Polylite Plus 225, Hamilton, Switzerland), and the exhaust gas (N_2 and CO_2, gas analyzer M610 Maihak AG, Germany) were measured and controlled using a LabView (National Instruments, USA) based process control system. Prior to inoculation, the photobioreactor was sterilized by autoclaving and culture medium was saturated with 3% CO_2 in air at 25 °C and pH 7.5. HCl (1 M) was used for pH regulation. Mass flow controllers (MKS instruments, USA) were used to adjust the gas flow (0.05 vvm).

2.5. Temperature variation

For temperature tests, 2 ml cell suspension was cultivated in reaction tubes in a thermoblock (Digital Dry Block Heater CL-206, Omega, Germany) without shaking at the relevant temperatures for 5 days.

2.6. Growth under nitrate limitation

Samples were cultivated without shaking for 6 days in day-night cycles in f/2 medium with differing nitrate concentrations with or without additional glucose as given in the results section. The culture flasks were filled completely with 50 ml medium to reduce oxygen. Dark controls were placed in black boxes. To calculate the dry mass of *Phormidium*, we used the empirical value of one $OD_{750nm} \triangleq 0.95$ g/l dry mass.

2.7. Preparation of genomic DNA

Genomic DNA was prepared from a 9 ml *Phormidium* culture ($OD_{750nm} = 0.4$). Cells were homogenized as above, centrifuged at 7000 g for 15 min, suspended in 6 ml phosphate buffer (10 mM NaH_2PO_4, pH 6.8) and centrifuged again. The pellet was suspended in 850-µl sucrose-Tris solution (25% sucrose, 10 mM Tris, and pH 8.0) and 100 µl Lysozyme (10 mg/ml) were added. The solution was incubated at 37 °C for 15 min. Following the addition of 1.25 ml sucrose-Tris

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