Introduction

Dinoroseobacter shibae DFL12T is a model organism for the Roseobacter clade. It is a Gram-negative aerobic anoxygenic phototroph (AAP) and distributed over marine habitats world-wide. Versatile metabolic properties of this clade suggest it to be a major part in biogeochemical processes, in the carbon cycle and in phototrophic energy conservation (Buchan et al. 2005, Kolber et al. 2000). Recently it was shown that D. shibae loses major parts of its phosphorylated adenylates during short periods of anaoxia, but is able to regenerate quickly after air flushing and light exposure (Holert et al. 2011). In addition to that, it was found that light increases viability of D. shibae during nutrient limitation and starvation (Soora et al. 2013). Furthermore, variations in Life/Dead staining pattern between the different energetic states could be visualized. We suggest the membrane potential (ΔΨ) as the key factor for the ATP-level drop and regeneration, but also for the variation in Life/Dead staining. Current work focuses on what happens to the membrane potential during the phases of anaoxia since it is core element of ATP regeneration. The membrane potential sensitive dye DiOC₃(3) is used to track changes resulting from different energetic levels.

Results

The intracellular pH of D. shibae
- Intracellular pH (pHi) of D. shibae was determined after perforation of the cell membrane with 5% butanol (Scholes and Mitchell 1970)
- pH(i) was narrowed down to lie between 7.2 – 7.3, which is comparatively low
- pH(i) even lower then the growth medium after 48 hours of cultivation, early stationary phase

![Fig.2 Narrowing down the pH(i) with a constant measured cell suspension during butanol perforation. Starting with pH 7.4 or 7.2 the measurement will drift towards 7.3 after butanol addition](image.png)

- pH(i) was stable throughout short-term anaoxia, while the extracellular medium underwent slight alkalinization

![Fig.3 Experimental setup for determination of pH(i). pH electrode within highly concentrated cell suspension (OD 20) on a magnetic stirrer. Foreground: HG and KDH solutions for manual pH titration and butanol for permeabilization](image.png)

Variation in Life/Dead staining pattern
- Staining was performed with SYBR Green I and propidium iodide (PI)
- Life/Dead staining reveals differences in staining pattern between fresh de- and re-energized cells (Fig. 4 A-C)

![Fig.4 Life/Dead staining of D. shibae. A. Fresh D. shibae cells in early stationary phase, very few cells take up propidium iodide. B: Via N₂-gassing de-energized cells take up PI significantly more often. Also intermediate states (yellow cells) can be observed. C: Via aeration re-energized cells seem to recover, PI-positive cells are significantly more rare. Pictures enhanced with PICOLAY ©](image.png)

- While the uptake of PI usually indicates cell death, here the cells seem to recover and lose their permeability for the dye in connection with their energetic state

Visualizing ΔΨ with DiOC₃(3)
- Membrane potential sensitive dye DiOC₃(3) for tracking changes in membrane potential
- Fig. 5 illustrates the uptake of DiOC₃(3) into Gram-positive Bacillus subtilis
- The dye is taken up ΔΨ-dependent. The stronger the membrane potential, the more dye accumulates within the cell → fluorescent shift from green to red
- Due to fast photobleaching pictures have to be taken at extremely short exposure time

![Fig.5 DiOC₃(3) uptake in Bacillus subtilis, visualized with fluorescent microscopy (excitation 488 nm). Different cells can possess different membrane potential, even if still connected. Arrows indicate different intensities in dye accumulation within the cells. Picture enhanced with PICOLAY ©](image.png)

- First results revealed differences in the DiOC₃(3) uptake of D. shibae between the different energetic states (Fig.6)
- Cells that were N₂-flushed for 2 hours in darkness appeared more red due to increased dye accumulation inside the cytoplasm (Fig. 6, B) → indication for a shift in membrane potential
- N₂-flushed cells that were aerated for 10 minutes with light exposure showed decreased dye uptake (C), similar to the fresh cells before any treatment (A)
- Also the dye uptake was determined with a microplate reader via red fluorescence intensity, which produced similar results

![Fig.6 DiOC₃(3) uptake of D. shibae varies with the energetic state of the cells. A, B and C treated as described as in Fig.4. Pictures enhanced with PICOLAY ©](image.png)

Discussion and Outlook

- If the pH(i) of Dinoroseobacter shibae is lower than the the extracellular pH (growth medium), ΔpH can not contribute to the proton-motive force [see Fig.1]
- Fast ATP regeneration after anaoxia was already shown (Holert et al. 2011)
- Since ΔΨ is main part of the PMF, antagonizing ΔpH can be balanced. It emphasizes the essential importance of ΔΨ in ATP regeneration

- Differences in Life/Dead staining pattern between energetically depleted and regenerated cells as first hint for variations in ΔΨ
- Cells seem to recover from PI-permeability, which is usually indicator for cell death. Membrane potential is likely to be involved

- Staining with ΔΨ-sensitive dye reveals changes in membrane potential during the different energetic states
- Quantification of ΔΨ is still under investigation
- First metabolic analysis between the differently energized cells gave no indication for a directed shift of certain metabolites. 59 metabolites detected, mainly citric acid cycle metabolites focused. (In collaboration with the group Biotechnology and Bioinformatics of Prof. Dr. Schomburg at the University of Braunschweig, special thanks to Nelli Beil and Sarah Kleist.)

References


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