PH.D THESIS

Structural and functional changes of photosynthetic apparatus of bacteria to environmental stresses

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Introduction

Photoheterotrophically growing purple bacteria represent the most thoroughly investigated species that show remarkable versatility and metabolic elegance. It is capable of growth by aerobic and anaerobic respiration, fermentation, and anoxygenic photosynthesis. Therefore, it provides an excellent model system for simultaneous study of both photosynthesis and membrane development and for addressing significant problems in many areas of interest in cell biology, physiology, and bioenergetics. The basic batch culture growth model draws out and emphasizes aspects of bacterial growth that can be divided into four different phases: lag phase, log phase or exponential phase, stationary phase, and death phase (Frankhauser 2004). Conventional steady state and pulsed absorption and fluorescence techniques of intact cells open new possibilities to reveal up to now hidden aspects of cell aging. After several generations, the division of the cells can be synchronized. The advantage of the synchronization is the possibility to directly track the insertion of the photosynthetic apparatus into the old and newly formed intracytoplasmic membrane (ICM). The bacterium contains three distinct membrane systems: the ICM, cytoplasmic membrane, and outer membrane with their own unique macromolecular composition and structure. The ICM houses the photosynthetic apparatus. The ICM adapts to alterations in light intensity and oxygen tension (Woronowicz et al. 2013; Niederman 2013). The ICM originates from the invagination of the CM that occurs at low (around 3 %) oxygen concentration (Koblížek et al. 2005). The organization of the complexes resulting in the physiological function of the apparatus has been characterized by high atomic resolution and at an unprecedented level of biochemistry and physical chemistry. There are, however, a number of open questions that are as yet unanswered. One of the major problems currently involves the location and the stoichiometry of the partners in the photosynthetic membrane (Cartron 2014).

Many bacteria can move using a variety of mechanisms: flagella are used for swimming through fluids; bacterial gliding and twitching motility move bacteria across surfaces; and changes of buoyancy allow vertical motion (Bardy and Jarell 2003). Phototrophic organisms can orient themselves with active movement most efficiently to receive light for photosynthesis. Photosynthetic bacteria can switch from planktonic lifestyle to phototrophic biofilm in mats in response to environmental changes (Steonou et al. 2013). A biofilm is group of bacteria in which stick to each other and often these bacteria adhere to a surface. These bacteria are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). The bacteria growing in a biofilm are physiologically distinct

from planktonic cells of the same organism, which, by contrast, are single-cells that may float or swim in a liquid medium. Regulatory pathways involved in the transition from planktonic to biofilm lifestyles have not yet been identified. The surface attached biofilms increase resistance to antimicrobial agents, heavy metals and toxins compared to the resistance of free-swimming organisms probably due to decreased metabolic activity within the depths of a biofilm and to binding and sequestration of dangerous substances by biofilm components. The mechanisms of phototrophic biofilm formation are, however, not characterized. The central to sensing and responding to environmental signals in purple bacterium *Rubrivivax gelatinosus* is a two-component control system (Wuichet and Zhulin 2010). Biofilms profoundly affect industrial productivity and human health. However, it is very important to point out that biofilms are an integral part of the natural environment. They can also serve very beneficial purposes, such as in the treatment of drinking water, wastewater and detoxification of hazardous waste.

Metal ions (mercury, lead, cromium) of environmental contamination may constitute one the most important factors of toxicity. Essential metal ions of low concentrations play an integral role in the life processes of microorganisms: they function as catalysts for biochemical reactions, are required for maintenance of osmotic balance, drive redox processes (iron, copper), and stabilize various enzymes (magnesium) and DNA. Microorganisms have to face with and accommodate to several stress factors of either natural or anthropogenic origins in their environment. The scientists have the task to work out useful applications in conservation of the environment including the protection of the biodiversity of aqueous habitats and monitor and remediation of pollution in the environment (Giotta et al. 2006). To limit the extent of metals metabolism of photosynthetic bacteria, the first step is the understanding of passive and active pathways of uptake of the metal in the bacteria (Mehta and Gaur 2005). Very little is known regarding the mechanism of uptake of inorganic Hg(II) by photosynthetic organisms, in part because of the inherent difficulty in measuring the intracellular mercury concentration. It has been revealed that Hg(II) uptake is an active transport process requiring energy and not a passive process as commonly perceived (Schaefer et al. 2011). The question can be asked whether cellular Hg uptake is specific for Hg(II), or accidental, occurring via some essential metal importer. The evaluation of mercury binding mechanism of highly resistant marine bacteria (Deng and Wang, 2012) and genetically engineered photosynthetic bacteria (Deng and Jia, 2011) and different heavy metal uptake and resistance mechanisms have been identified (Bruins and Kapil 2000).

Aims:

- The structure and function of photosynthetic membrane of bacteria are different during the early exponential and stationary phases of growth. What are these differences attributed to and more specifically, what differences can be revealed in the energetic coupling of the photosynthetic units during membrane development?
- Synchronized bacterial cultures provide additional information on the highly organized cell structures. What photosynthetic processes show cell cycle dependent development and which are independent on the cell division?
- Aerobic-anaerobic transitions induce dramatic effects in the structure and function of the membrane. What can we learn from transition studies? How does the photosynthetic membrane assemble from its components? Is the building up sequential or concerted?
- Photosynthetic bacteria (*Rba. sphaeroides*, *Rsp. rubrum*, *Rvx. gelatinosus*) indicate different sensitivity to heavy metal ions (mercury, lead, chromium). How can heavy metal resistance mechanisms be developed in bacteria? How can photosynthetic bacteria be used for biomonitoring and/or remediating the polluted environment?
- *Rvx. gelatinosus* cells are able to adsorb and uptake large amount of mercury ions. What factors control the kinetics and stoichiometry of bioaccumulation?
- The *Rvx. gelatinosus* cells carry out two different lifestyles: planktonic and biofilm. The transition is expressed in form of abrupt and collective sinking of the bacteria. The mode of sedimentation resembles of critical phenomenon widely occurring in the physics of nature. What factors lead to sudden sedimentation of the cells?

Materials and Methods

Bacterial strains and growth conditions

The photosynthetic purple bacteria (*Rhodobacter sphaeroides* 2.4.1, *Rhodospirillum rubrum*, *Rubrivivax gelatinosus*) were grown in Siström's medium. The cells were inoculated from a dense batch culture in 1:100 dilution and were illuminated by tungsten lamps that assured 13 W m^{-2} irradiance on the surface of the growth vessel.

Aerob-semianerob growing: the half filled Erlenmeyer-flask was purged with a mixture of air and nitrogen. The oxygen-to-nitrogen volumetric ratio of the gas mixture was adjusted

by calibrated flow rate meters (rotameters). The oxygen tension balanced with N_2 could be changed between 21 % (air) and 0 % (anaerobic condition).

Synchronization of the cells: the cells in the logarithmic phase of growth were the inoculum (1:100) source for 3 times repeated dark–light periods (3.5–3.5 h).

The total cell number was determined by calibrated Bürker counting chamber under light microscope.

Steady-state absorption spectroscopy

The steady-state near infrared absorption spectra of the cells during the growth were recorded at room temperature by a single beam spectrophotometer (Thermo Spectronic Helios). The baselines were corrected for light scattering, and the spectra were decomposed into Gaussian components by least square Marquardt procedure.

Flash-induced absorption kinetics

The kinetics of absorption changes of the whole cells induced by Xe flash or by laser diode (Roithner Laser- Technik LD808-2-TO3, wavelength 808 nm and power 2W) were detected by a home-constructed spectrophotometer (Maróti and Wraight 1988). The oxidized bacteriochlorophyll dimer (P^+) and the electrochromic shift (ECS) of the carotenoids in the photosynthetic membrane were detected at 798 nm and 525 nm (with reference to 510 nm), respectively. The optical density of the samples was kept low [OD (808 nm) < 0.1] and weak monochromatic detection light was used to keep the secondary effects negligible.

Induction and relaxation of BChl fluorescence

The induction and subsequent decay of the BChl a fluorescence of intact cells were measured by a home built fluorometer (Kocsis et al. 2010). The light source was a laser diode (808 nm wavelength and 2 W light power) that produced rectangular shape of illumination and matched the 800 nm absorption band of the LH2 peripheral antenna of the cells. The BChl *a* fluorescence (centered at 900 nm in mature cells) was detected in the direction perpendicular to the actinic light beam, with a near infrared sensitive, large area (diameter 10 mm) and high gain Si-avalanche photodiode (APD; model 394-70-72-581) protected with an 850-nm high-pass filter (RG-850) from the scattered light of the laser. The induction was measured during the actinic laser light and the dark-relaxation was tested by attenuated short (3 μs) laser pulses in geometrical series.

Extraction and assay of molecular components of the cells

BChl: The BChl was extracted from the cells by acetone/methanol (7:2 v/v) mixture using the extinction coefficient of 75 $mM^{-1}cm^{-1}$ at 770 nm (Clayton and Clayton 1981). *Phospholipids*: The phospholipids from the cell suspension were extracted by the method of Bligh and Dyer (1959) and the quantitative (colorimetric) determination of the inorganic phosphate was based on the Bartlett assay (Bartlett 1959). *Carotenoids*: The carotenoids were extracted from the cells by acetone/methanol (7:2 v/v%) mixture using the extinction coefficient of 128 $mM^{-1}cm^{-1}$ at 484 nm (Clayton 1966).

Determination of Hg(II) with dithizone.

The amount of Hg(II) in aqueous solution of bacteria was determined by use of indirect spectrophotometric measurements of the Hg(II)-dithizone complex that has high stability constant (Théraulaz and Thomas 1994). Mercury concentrations were determined from the difference of the absorbances (*A*) measured at 480 nm (Hg(II)-dithizonate) and 585 nm (dithizone) based on calibration of the method: $R=(A_{585}–A_{480})/A_{585}$ (Greenberg et al.1992).

Imaging

*Electron microscopy:*The bacteria were filtered with high grade filter paper and fixed with 4% glutaraldehyde. The specimens were embedded and 70-nm thin sections were prepared with an Ultracut S ultra-microtome. After staining with uranyl acetate and lead citrate, the sections were observed with a Phillips CM10 electron microscope equipped with a Mega-view G2 digital camera and iTEM imaging analysis software (Olympus, Münster, Germany).

Time-lapse video: The aggregation *Rvx. gelatinosus* cells was recorded using webcam (time lapse mode).

Videos (20 frame per secundum) and Nomarski pictures (magnification: 60x, oil immersion) were taken (Olympus Fluoview FV1000 LSM, Olympus Life Science Europa GmbH, Hamburg, Germany) from *Rvx. gelatinosus* cells.

Diffusion coefficient

Diffusion coefficient of *Rvx. gelatinosus* during cell growth and with Ficoll 400 polymer (1-10%) were determined by statistical methods. ImageJava Software was used to find the location of the selected bacteria (500 points during 25 seconds). The displacements of 5-10 cells were taken to determine the mean values of squares of displacements that was

plotted versus time. The points were fitted by a straight line with slope of four times the diffusion coefficient ($4 \cdot D$) in the plane.

Results, thesis

1.) Photosynthetic bacteria in the stationary phase of growth demonstrated closer packing and tighter energetic coupling of the photosynthetic units (PSU) than in their early logarithmic stage of development. [1], [4]

The development of photosynthetic membranes of intact cells of *Rhodobacter sphaeroides* was tracked by light-induced absorption spectroscopy, induction and relaxation of the bacteriochlorophyll fluorescence. 1) The dominance of the core light harvesting complex LH1 compared to the peripheral complex LH2 was stronger in old cells, than in young cells. 2) The fluorescence maximum of the old cells showed red shift of about 5 nm and similar size but blue spectral shift was observed for the B875 pigment. 3) The variable fluorescence was larger by 10% in old bacteria than in young bacteria and the photochemical rise time showed also slight variation (200→140 ms) indicating moderate changes of the size or connectivity of the antenna system during aging. 4) The old cells performed more stable membrane potential after flash excitation than the younger cells (Fig.1.).

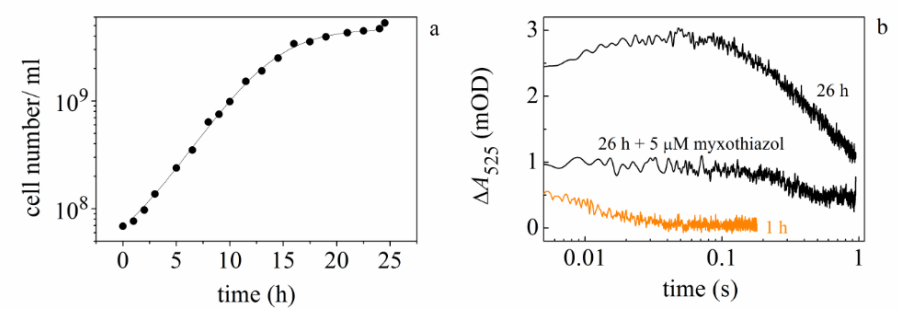

Fig.1. *Rba. sphaeroides* 2.4.1 growth curve (a) and energetization of membrane (b) at 1 h (lag phase) and 26 hours (early stationary phase) cells.

1 h after inoculating a fresh culture, the cells have a small abrupt increase of electrochromism followed by fast decay. The intact cells in stationary phase of growth (26 h) have additionally a second and slower increase followed by a much slower relaxation. 5.) The dark decay of the fluorescence after relatively long (1 ms) illumination shows marked difference between the cells at different growth phases. The mature (26 h) cells keep the high level of fluorescence longer (ten times) than the lag phase (1 h) cells. In stationary phase cells, the shuttle time of the electrons via mobile redox species between the complexes becomes longer probably due to rearrangement of the membrane resulting in slower diffusion.

2.) While the production of the bacteriochlorophyll and carotenoid pigments and the activation of light harvesting and reaction center complexes showed cell-cycle independent and continuous increase, the accumulation of phospholipids and the energetization of the membrane exhibited stepwise increase controlled by cell division. [2], [4]

Photosynthetic membranes of *Rhodobacter sphaeroides* steady-state synchronised culture were characterized by light-induced absorption spectroscopy, induction of bacteriochlorophyll fluorescence and molecular components of cells (phospholipids, carotenoids and bacteriochlorophyll). In steady-state synchronous culture, the number of cells increases stepwise as all of the cells are in the same stage of their development. The production and insertion of bacteriochlorophyll (0.26 μM h^{-1}) and carotenoid (0.38 μM h^{-1}) pigments into the ICM and activation of the photosynthetic complexes (light harvesting (F_{max}: 0.23 h^{-1}), RC (ΔA_{798}: 0.67 mOD/h) and cytochrome bc_1 complex) are cell-cycle independent processes. They do not follow a stepwise but rather a continuous process with well-defined lag phase (~3 h) at the beginning.

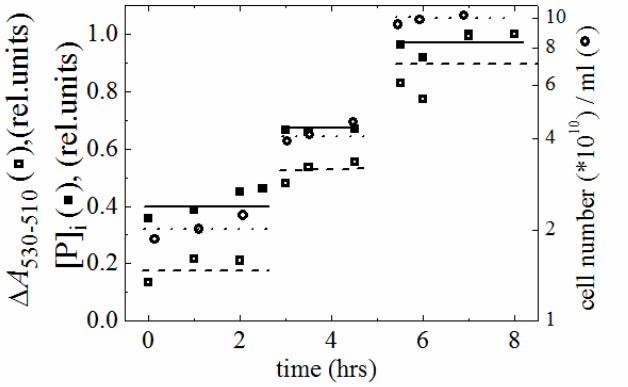

Fig.2. Cell-cycle dependent changes at synchronized *Rba. sphaeroides* 2.4.1 culture. The cell doubling time was three hours (3 cycles), followed by the total amount of inorganic phosphate $[P]_i$ and absorption changes at 530 nm (ref. 510 nm).

In contrast to the continuous production of the pigments and the RC protein, the phospholipid synthesis and the electrochromic signal due to the cytochrome bc_1 complex showed clear cell-cycle dependence. Upon division of the cell, the area of the total membrane surface of the cell should increase significantly due to the resulting daughter cells with their own outer, cytoplasmic and ICM membrane systems. This is why a burst of phospholipid synthesis occurs prior to cell division (between 3-4 hours $[P]_i$: 0.4→0.7 (rel. units)) and the phospholipids will be inserted into the replicating ICM as it is being partitioned to daughter cells. The electrochromic change that is connected to the energetization of the membrane, demonstrates cell-cycle-dependent increase ($\Delta A_{530-510}$: 0.2→0.5 (rel. unit)) upon cultivation. The changes observed during the cell cycle can be attributed either to shorter distance of diffusion (membrane bilayer crowding) or to increased diffusion coefficient (increased fluidity of the membrane) or to both effects.

3.) The photosynthetic apparatus during aerob-anaerob transition (greening) is assembled in functional unit. [3], [4]

The disintegration and assembly of photosynthetic membrane of intact cells of *Rba. sphaeroides* and *Rvx. gelatinosus* were tracked by light-induced absorption spectroscopy and induction and relaxation of the bacteriochlorophyll fluorescence. The morphological changes were recognized in electron micrographs. As the ICM formation is repressed by high oxygen tension, increasing the oxygen partial pressure results in disruption of ICM assembly together with disintegration of the photosynthetic complexes. The B800 component of the LH2 complex exhibits fast drop at 20 % oxygen tension, while no loss of the B850 component is observed, i.e. RC-LH1 core (875 nm) dominates. The ratio of BChl variable fluorescence to maximum fluorescence (from 0.70 to 0.10) and the photochemical rate constant (from $1.2 \cdot 10^5$ s^{-1} to $2 \cdot 10^4$ s^{-1}) exhibits marked decreases. The rate of fluorescence relaxation showed slight increase from $6 \cdot 10^3$ s^{-1} to $1.5 \cdot 10^4$ s^{-1}, because the protein complexes (RC and cyt bc_1) are readily accessable to the mobile redox species (cyt c_2 and Q) that assures high electron transfer rate.

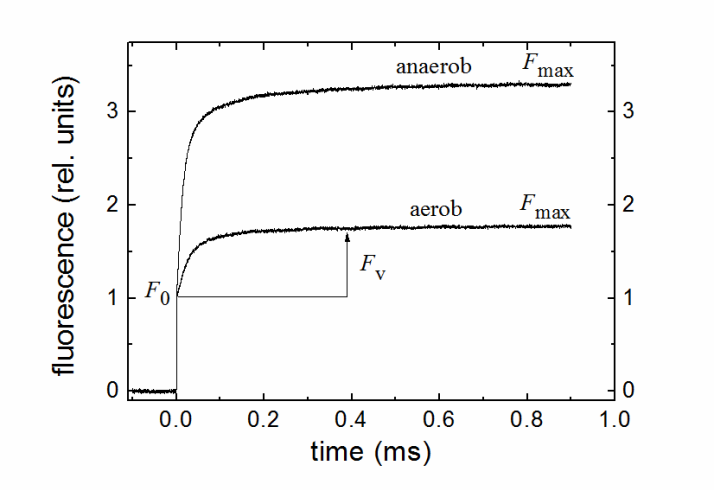

Fig.3. Temporal changes of fluorescence induction of *Rba sphaeroides* aerobic and anaerobic culture.

The greening resulted in rapid (within 0–4 h) induction of BChl synthesis accompanied with a dominating role for the peripheral light harvesting system (up to LH2/LH1~2.5), significantly increased rate (~$7 \cdot 10^4$ s^{-1}) and yield (F_v/F_{max} ~0.7) of photochemistry and modest (~2.5-fold) decrease of the rate of electron transfer ($6 \cdot 10^3$ s^{-1}). At the beginning of adaptation to the anaerobic conditions, the peripheral light harvesting antenna (LH2) starts to surround the core complex that improves the yield and rate of photochemical conversion. The initially loose structure of the supercomplex facilitates the access of cyt c_2 to the RC and cyt bc_1 complexes that makes fast cyclic electron transfer available. Upon synthesis and insertion of more and more LH2 complexes, the membrane becomes more densely packed and the diffusion of mobile redox species will be partially hindered. After 3–4 h, the regular anaerobic photosynthetic competence is achieved with optimal organization of the cyclic electron transport chain.

4.) The photosynthetic bacteria were vulnerable to the prompt effect of Pb^{2+}, showed weak tolerance to Hg^{2+} and proved to be tolerant to Cr^{6+}. Due to the biofilm lifestyle *Rubrivivax gelatinosus* shows higher resistance to heavy metals than strains with planktonic lifestyle. [5]

Heavy metal ion pollution is major environmental risks for microorganisms in aqueous habitat. The potential of purple non-sulfur photosynthetic bacteria for biomonitoring and bioremediation was assessed by investigating the photosynthetic capacity in heavy metal contaminated environments. Cultures of bacterial strains *Rhodobacter sphaeroides*, *Rhodospirillum rubrum* and *Rubrivivax gelatinosus* were treated with heavy metal ions in micromolar (Hg^{2+}), submillimolar (Cr^{6+}) and millimolar (Pb^{2+}) concentration ranges. Functional assays (flash-induced absorption changes and bacteriochlorophyll fluorescence induction) and electron micrographs were taken to specify the harmful effects of pollution and to correlate to morphological changes of the membrane. The aggressive nature of Pb(II) is expressed by severe prompt effects on near IR steady state absorption spectra and BChl fluorescence induction. The lead treatment decreases both the initial (F_0) and the maximum (F_{max}) fluorescence levels without modifying the variable fluorescence (the F_v/F_{max} value) significantly.

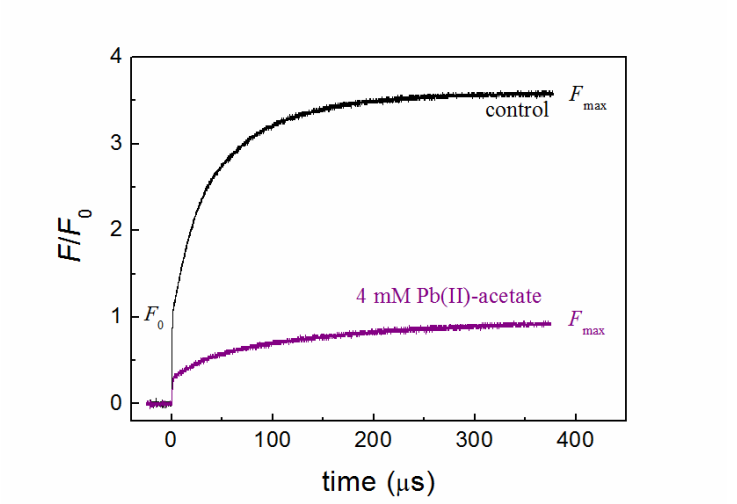

Fig.4. Prompt effect in fluorescence induction of *Rsp. rubrum* whole cell under lead treatment.

Besides Pb(II) decreases the magnitude of light-induced electrochromism, it prohibits the fast component of discharge of the energetized membrane. Addition of Cr(III) ion of high (up to 20 mM) concentration to the culture, the kinetics will be not modified to that of the intact cells (control) and supports the harmless nature of the trivalent form of chromium. The treatment with 0.8 mM Cr(VI) modify of electron and proton transfer between RC and cyt bc_1 complexes. While 20 μM Hg^{2+} bleaches the cells of *Rps. rubrum* within 2–3 h and the rate of growth of *Rba. sphaeroides* culture is halved at 2 μM Hg^{2+} concentration, in cells of *Rvx. gelatinosus* orders of magnitude larger concentration (200 μM Hg^{2+}) does not cause significant damage. *Rvx. gelatinosus* with biofilms were more resistant to Hg^{2+} than planktonic cells (without biofilms) because the

biofilm increases the mercury binding capacity further by a factor of about five. *Rvx. gelatinosus* is able to evolve biofilm during the growth and is expected to affect mercury availability in several ways, including 1) changes in mercury speciation with steep chemical gradients within the biofilm, 2) the formation of an additional diffusive layer surrounding cells, and 3) adsorption of mercury by the biofilm.

5.) The mercury uptake by photosynthetic bacteria consists of a rapid passive adsorption and a slower active metabolic step. It is most effective at neutral pH and can be activated by light. Two distinct binding sites were identified with 1 $(\mu M)^{-1}$ (strong) and 1 $(mM)^{-1}$ (weak) equilibrium binding constants. [6]

Mercury bioaccumulation is studied in intact cells of *Rvx. gelatinosus* by use of analytical (dithizone) assay and physiological photosynthetic markers (pigments, fluorescence induction and membrane potential) to determine the amount of mercury ions bound to the cell surface and taken up by the cell. The Hg(II) uptake 1.) has two kinetically distinguishable components. The prompt (subminute time scale) uptake is passive, reversible, relatively nonspecific with respect to the metal species and independent of cellular metabolisms. The slow (1-30 minutes) kinetic phase, however, reflects active processes and depends on the cellular metabolism. 2.) It includes co-opted influx through heavy metal transporters since the slow component is inhibited by high Ca^{2+} concentrations and Ca^{2+} channel blockers. 3.) It describes complex pH-dependence demonstrating the competition of ligand binding of Hg(II) with H^+ ions (low pH) or hydroxyl ions (high pH). 4.) It is energy dependent as evidenced by light-activation and inhibition by protonophore.

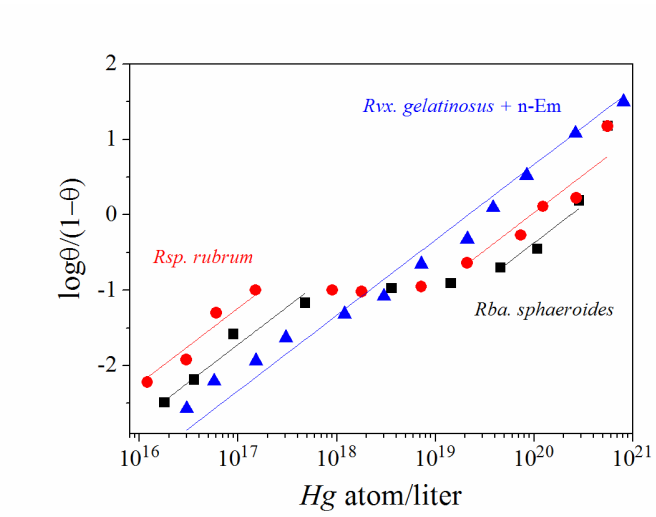

Fig. 5. Hill-plot of *Rvx. gelatinosus* mercury uptake.

Photosynthetic bacteria can accumulate Hg(II) in amounts much (about 10^5) greater than their own masses by strong and weak binding sites with equilibrium binding constants in the range of 1 $(\mu M)^{-1}$ and 1 $(mM)^{-1}$, respectively. The strong binding sites are attributed to sulfhydryl groups as the uptake is blocked by use of sulfhydryl modifying agents and their number is much (two orders of magnitude) smaller than the number of weak binding sites.

It is a remarkable experimental conclusion that despite of the large number of binding sites and their large affinity of mercury ions, the binding sites are independent, i.e. their binding status does not influence the binding properties of the neighbors.

6.) The main factors of sudden and collective sinking of *Rubrivivax gelatinosus* cells upon biofilm formation are the significant decrease of the diffusion coefficient and the aggregation of the bacteria. [7]

Rvx. galatinosus cells (particularly in the stationary phase of growth) are able to increased polymer production that excrete the extracellular compartment. Full polymer network (biofilm matrix) is formed, that will embed the cells. The integration of the bacteria into the dense matrix will increase the net mass via aggregation and decrease the diffusion coefficient (D) of the cells. Both factors lead to significant reduction of the mobility of the bacterium. These are the main factors resulting in a sudden and collective sinking that shows up physiological significance: it is a form of bacterial motility to search for new sources of food. The bacteria perform active random movements (twitching) that prevent the individual planktonic bacteria (in the early exponential phase) from sinking. In stationary phase, however, the cell can be found both in planktonic (D = 13.5 μm^2/s) and biofilm (D < 1 μm^2/s) lifestyles. In biofilm, not only the diffusion of the cells is reduced but its aggregation number is increased, as well. It was found, that even a relatively small (< 5) aggregation number can decrease the diffusion coefficient below the limit of measurement.

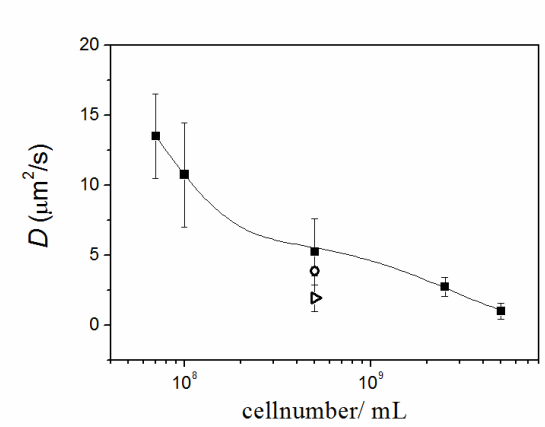

Fig.6. *Rvx. gelatinosus* diffusion coefficient (■) decreases upon increase of the cell concentration (during growth of the culture). The presented range of cell numbers corresponds to those in the stationary phase of cell growth. The diffusion coefficients decrease also upon association of 3-5 (○) and 10-15 (▷) cells in a culture of selected mean cell number (follow a vertical direction).

The behavior of the cells in natural biofilm can be modeled by planktonic cells in Ficoll 400 polymer solution. In low concentration of bacteria ($c < 1 \cdot 10^9$ cells/mL), the lateral diffusion coefficient of the cells (D = 2.25 μm^2/s at 5% Ficoll) decreased in a similar manner as in biofilm under natural conditions. It can be concluded that not the slight increase of the viscosity but rather the strong connectivity of the components can be made responsible for the sudden and collective sinking of the bacteria.

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IF: 3.15

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