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Interplay between carotenoids, hemoproteins and the "life band" origin studied in live *Rhodotorula mucilaginosa* cells by means of Raman microimaging

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Abstract

Raman microimaging of live cells of *Rhodotorula mucilaginosa*, cultured in aerobic and anaerobic conditions, showed striking differences in composition and distribution of cell components. Analysis of these differences and recovery of oxidative phosphorylation upon environmental changes enabled to interrelate carotenoids, hemoproteins and the unknown species to be considered as "Raman signature of life".

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Introduction

The fundamental question "what is life" was formulated probably at the dawn of humanity and still is an inspiration for scientists in all fields of research, equally as for philosophers and physicists. Therefore, the finding of the Raman band that reflects the metabolic activity in a mitochondrion known as the "Raman signature of life"[1] provoked a great interest in the scientific community. The "Raman signature of life" was quite deeply characterized in fission yeast cells.[1-9] It was proven that this unique spectroscopic feature is directly related to respiration, as it vanishes upon KCN, NaN_3 and H_2O_2 treatment.[1-3, 5, 6] The signal at 1602 cm^{-1} is also closely related to heme functions as HEM1 knockout cells do not show traces of its presence.[3] Moreover, the band intensity is positively correlated with the nutrient increase and negatively with the increase of oxygen or nitrogen to 100% in the yeast culture[6] and undergoes quite rapid, but reversible, photobleaching under laser irradiation with the 632.8 nm wavelength.[8] It was also shown that the isotope substitution pattern indicates the C=C stretching vibration[9] rather than a vibration of a small molecule containing the oxygen atom to be responsible for the appearance of the 1602 cm^{-1} band.[6] Previously, several candidates for the origin of the "Raman signature of life" have been proposed including the semiubiquinone radical anion[7] and, most recently, ergosterol.[4] None of this assignments is confirmed, particularly ergosterol may not be the origin of the "Raman signature of life" as this feature was observed also in the mitochondria isolated from animals as well as in animal cells.[10]

Flavohemoglobins are widely presented in yeast and localized in the mitochondrial matrix.[11] Most probably yeast flavohemoglobins play a key role in an NO-detoxifying system under hypoxic conditions. By controlling levels of NO, flavohemoglobins prevent inhibition of cytochrome c oxidase and therefore suppression of oxidative respiration.[11]

Carotenoids, synthesized *de novo* in yeast cells, play primarily a role in defense against oxidative stress. Yeasts of *Rhodotorula* genus biosynthesize β and γ-carotene, torulene and torularhodin.[12] Torularhodin is a unique carotenoid, containing the carboxylic group, that shows considerable antioxidant activity, being a more potent peroxyl radicals scavenger than β-carotene.[13] In *Rhodotorula glutinis*, torularhodin is abundantly synthesized upon illumination (even with the light of weak intensity) indicating that increased production of torularhodin plays an important role in defending against peroxyl radicals and singlet oxygen.[13] It was also concluded that accumulation of torularhodin is an important mechanism that improves the resistance of yeast to UV-B radiation.[14]

To complement some of the previous findings concerning hemoproteins, carotenoids and the band at 1602 cm^{-1}, we decided to study a wild strain of yeast *Rhodotorula mucilaginosa* cultured in the aerobic and anaerobic environment. Our study enabled us to shed some light on the role of these key components: hemoproteins, carotenoids and the species manifested by the band at 1602 cm^{-1} and demonstrate their interrelation in respect to the process of the oxidative phosphorylation.

Experimental

Rhodotorula mucilaginosa was cultured in the aerobic conditions (Sabouraud's Dextrose Agar and potato-dextrose medium for *Culture 1* and *Culture 2*, respectively) or in the anaerobic environment (peptone-dextrose medium, *Culture 3*).

For Raman measurement, cells were placed on the CaF_2 slide and dried (*Culture 1*) or placed on the CaF_2 slide, covered with a drop of the sodium chloride 0.9% solution (Polpharma) and a microscopic slide (*Culture 2* and *3,* measurements of live cells).

Raman imaging was done with a Confocal Raman Imaging system WITec alpha 300 with the application of 100×air objective (Olympus, MPlan FL N, NA=0.9) for *Culture 1* or for 100xoil objective (Nikon, EPlan 100x, NA=1.25) for *Culture 2* and *3*. The laser excitation wavelength of 532 nm, laser power of ca. 2-8 mW and the integration time of 0.2 second per spectrum were used in all cases. Areas of minimally 5×5 μm to the edge length maximally 25×30 μm were measured with the 0.12 μm step in both x and y dimensions for *Culture 1*. Images of the edge length 15 × 15 μm (100 × 100 pixels) were recorded for *Culture 2* and *3*. The spectral resolution was 3 cm^{-1}.

The standard compounds: L-α-phosphatidylcholine, β-carotene and hemoglobin (all from Sigma Aldrich) were measured by collecting 100 scans with 0.5-1 s integration time with the application of 100×air objective (Olympus, MPlan FL N, NA=0.9) and 532 nm laser excitation wavelength.

Data matrices were evaluated using the WITec Project Plus software: background subtraction using a autopolynomial of the optimal degree and the automatic procedure for cosmic rays removal. The analysis of the spectra was supported by a Cluster Analysis (K-means, Manhattan distance, WITec Project Plus).

Results and Discussion

Rhodotorula mucilaginosa is one of yeast species able to synthesize carotenoids *de novo*. As carotenoids are intrinsically related to protection against oxidative stress – a natural question appears if and how the carotenoid synthesis can be related to aerobic

and anaerobic environment. Raman spectroscopy and mapping were previously demonstrated to be a suitable methods to investigate distribution and structure of carotenoids in the single algal cell.[15, 16] Therefore, high spatial resolution (lateral *ca.* 260 nm) Raman microscopy was used to image subcellular content of the *Rhodotorula mucilaginosa* cells grown in the aerobic and anaerobic conditions (Fig. 1).

Fig. 1 reflects striking differences in the phenotype of the *Rhodotorula* cells grown in aerobic and anaerobic conditions (*Culture 2 and 3*, respectively).

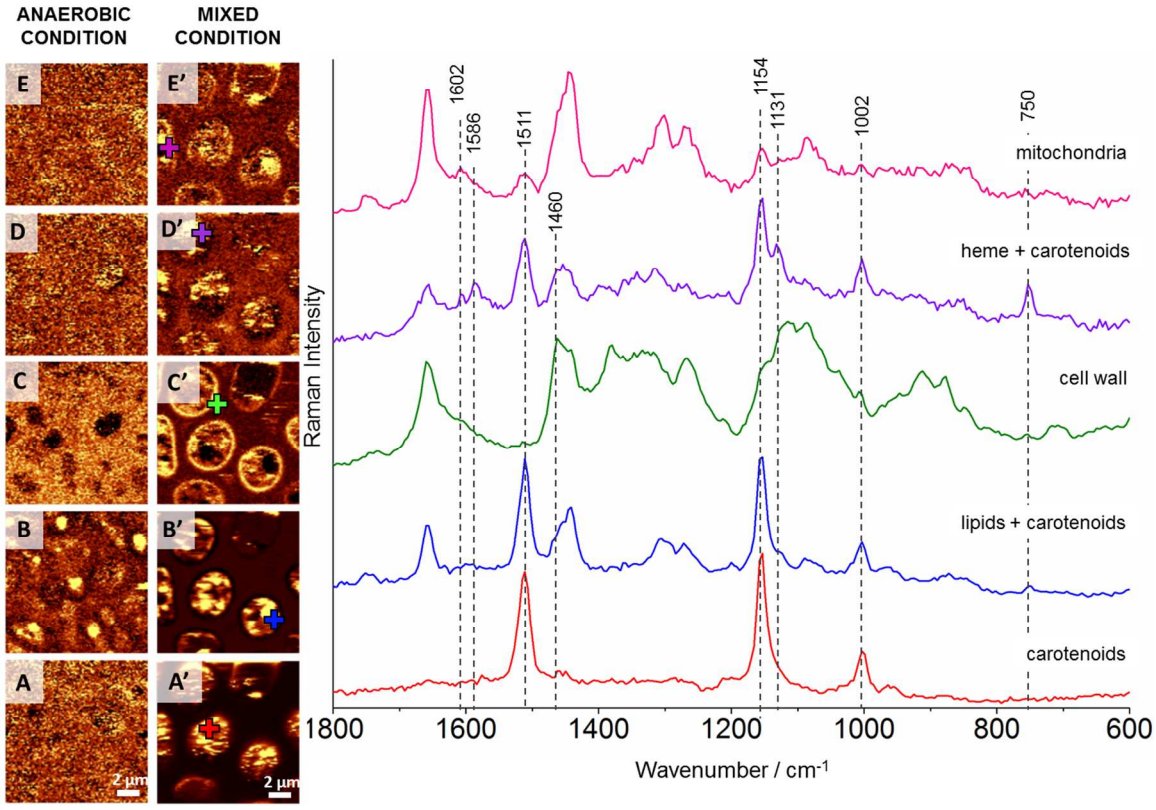

Fig. 1. The representative images showing distribution of carotenoids (integration over the band at 1156 cm^{-1}, **A** and **A'**), lipid bodies (2857 cm^{-1}, **B** and **B'**), cell wall (1460 cm^{-1}, **C** and **C'**), hemoproteins (750 cm^{-1}, **D** and **D'**) and the "life band" (1602 cm^{-1}, **E** and **E'**) in yeast cells cultured in the aerobic (*Culture 2*) and anaerobic (*Culture 3*) conditions. Single spectra (extracted from cross-marked point on the maps A' to E') showing the Raman features of the above-mentioned components for the aerobic culture (**F**).

Our results show that there is practically no resemblance in the distribution of the key components in cells cultured in these two different environments. Marker bands due to carotenoids (1511, 1154 and 1002 cm^{-1}), the cell wall (most probably related to oligosaccharides 1460 cm^{-1}), heme (1586, 1131 and 750 cm^{-1}) and the "life band" (1602 cm^{-1}) are not observed in the representative images of anaerobically-cultured cells (Fig. 1A and C-E). Contrarily, the intense signal of carotenoids is clearly visible in the cells grown in aerobic conditions (Fig. 1A'). These pigments are dissolved in lipid bodies as

indicated by the apparent co-localization of these components in Raman images (Fig. 1A' and B').Carotenoids have a high cross-section for Raman scattering upon excitation with the light in the visible range due to the resonance enhancement. Similarly, hemoproteins excited with 532 nm wavelength should yield high intensity resonantly-enhanced Raman signals. The obtained images of yeast grown in aerobic environment show only traces of hemoproteins (maker bands at 1586, 1131 and 750 cm^{-1}) and no hemoproteins signal was observed for cells grown in the anaerobic environment meaning that their concentration is below the sensitivity limit of the method. Moreover, the compound related with the "Raman signature of life" appears in the images of cells grown in aerobic, but not anaerobic conditions.

A very informative observation was made for the anaerobically-cultured cells imaged several hours after sample preparation. In such a case, both "life band" and features assigned to hemoproteins appeared in the spectra (Fig. 2A-D).

Quite high intensity of the appearing "life band" was observed for some cases (Fig. 2B) and correlated with the lipid signal of membranes, resembling the signal of phosphatidylcholine[5]. The signal due to hemoproteins appeared about 2 h after sample preparation and increased with time (the example of the heme signal after 4.5 h given in Fig. 2C), contrarily to the signal due to carotenoids that was observed in only one image per 6 images measured in total (Fig. 2F). The intensity of signal due to hemoproteins is much stronger relatively to what was observed for aerobic conditions (compare Fig. 1F and Fig. 2). Additionally, a very intense signal from hemoproteins was observed in the yeast cells grown in anaerobic conditions and measured 12 h after the transfer to aerobic conditions. These findings can be rationalized as follows: upon the influence of oxygen *Rhodotorula* cells adapt to the aerobic conditions and change the phenotype converting into oxidative phosphorylation. It was shown before that anoxic cells are able of oxygen respiration as they have a functional respiratory chain.[17] Additionally, it is known that the cellular heme concentration is lower under anaerobic growth conditions compared to aerobic ones.[18] The switch from the anaerobic to aerobic environment is related with the activation of mitochondria that can be seen in the Raman images indirectly via the "life band" at 1602 cm^{-1}. This phenotype change is closely associated with the production of hemoproteins that play a key role in NO detoxification thereby enabling the mitochondrial respiration, and work as ROS scavengers. Most probably, in aerobically cultured cells, the latter role is fulfilled mostly by carotenoids. We hypothesize that the carotenoid productions requires longer time to be initiated (in fact the yeast colony did not show nor orange color neither traces of carotenoids in Raman images even 12 h after the transfer from anaerobic to aerobic conditions) and before it occurs the ROS scavenging function is overtaken by hemoproteins.

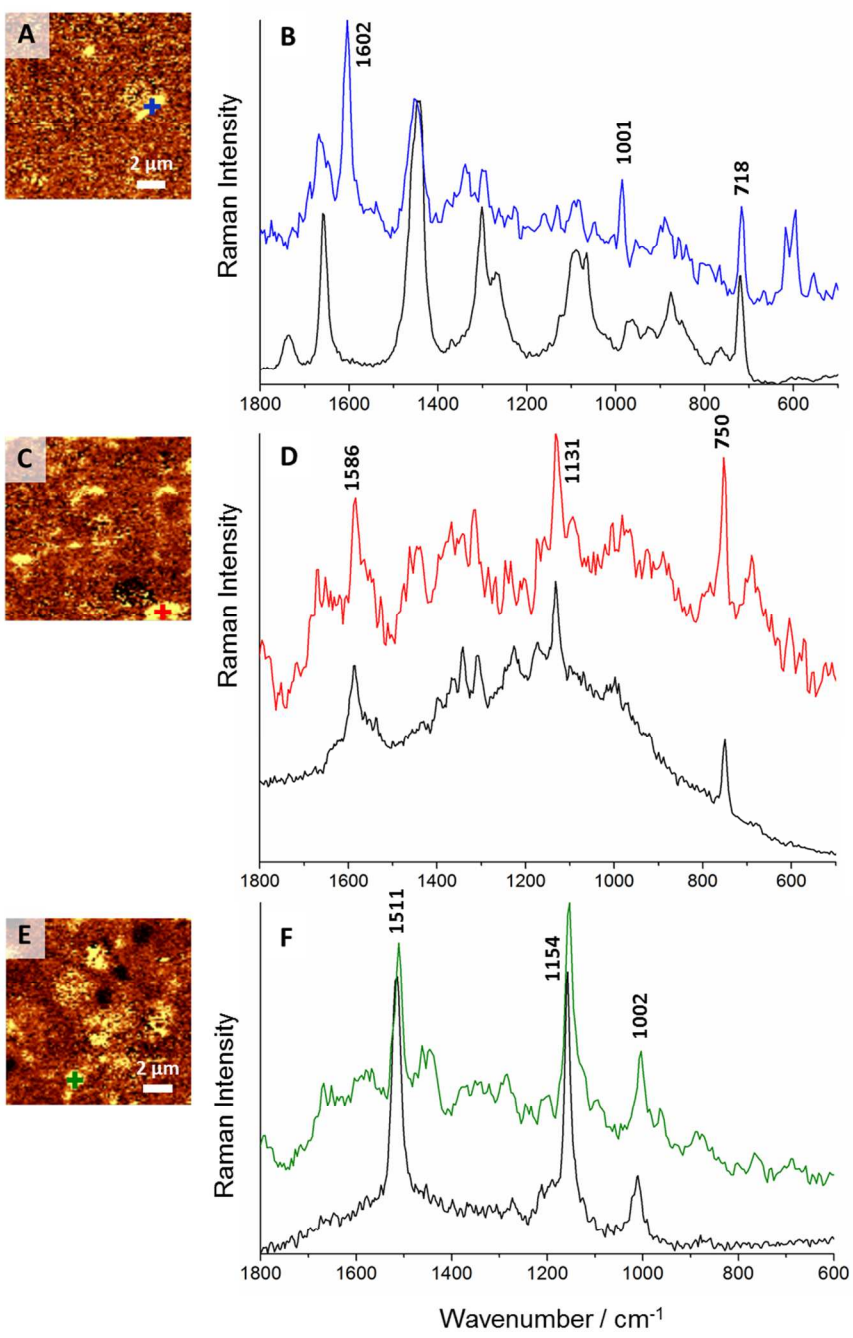

Fig. 2. The distribution images obtained by integration of: the "life band" (1602 cm^{-1}, **A**, measured 2.5 h after sample preparation), the marker band of heme (750 cm^{-1}, **C**, 4.5 h after sample preparation) and the marker band of carotenoids (1154 cm^{-1}, **E**, 2.5 h after sample preparation) in yeast cells cultured in the anaerobic conditions (*Culture 3*). The Raman spectra extracted from the point marked by the color crosses on the images are compared with the spectra of the respective standards: (black lines, phosphatidylcholine – **B**, hemoglobin – **D** and β-carotene – **F**).

The above observations are complemented with the results of *in vitro* measurements of cells grown in the aerobic environment (*Culture 1*) and submitted to stress conditions (drought, Fig. 3) to provoke ROS scavenger generation.

The signal of carotenoids in these cells is so intense that covers the contribution of practically all other components. Nevertheless, the repetitive imaging enables photobleaching of carotenoids and acquiring information about other cell constituents. As expected, no traces of the band related to the Raman signal of life was observed. Nevertheless, the images obtained after carotenoids photobleaching show significant amount of hemoproteins distributed quite uniformly in the cells. It indicates that carotenoids occurring in the cell grown in the aerobic conditions, upon stress conditions are accompanied by hemoproteins.

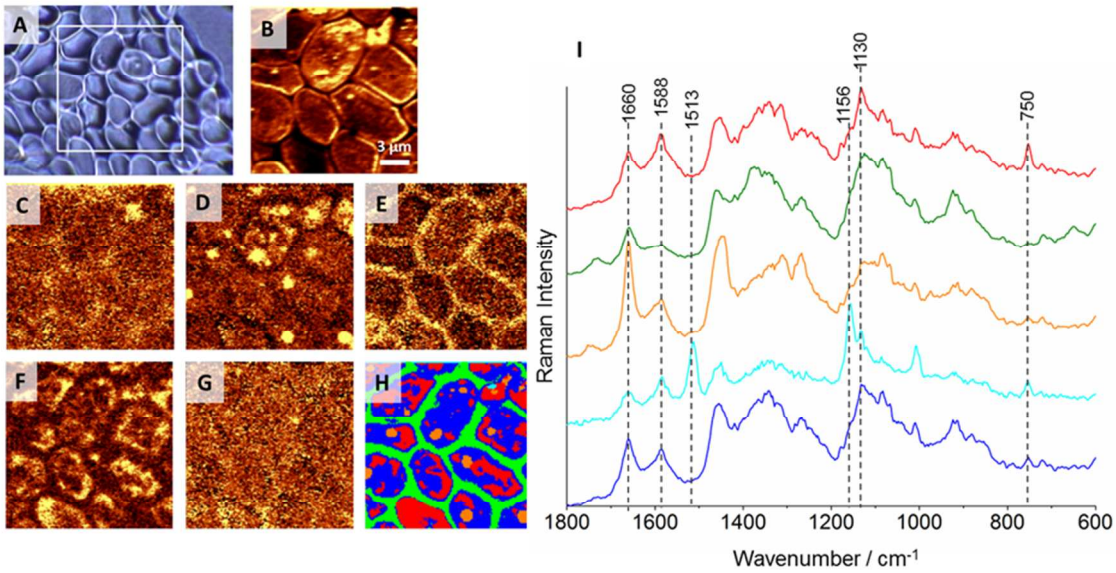

Fig. 3. The visual image (100x, **A**) and the representative images showing distribution of carotenoids before (**B**) and after photobleaching (**C**) (integration over the band at 1156 cm^{-1}), lipid bodies (2857 cm^{-1}, **D**), cell wall (1460 cm^{-1}, **E**), heme (750 cm^{-1}, **F**) and the "life band" (1602 cm^{-1}, **G**) and the Cluster Analysis image with the average spectra (**H**, K-means, Manhattan distance, 5 classes: lipid bodies – orange, heme – red, cell wall – green, carotenoids – turquoise and other constituents – blue) of yeast cells cultured in the aerobic conditions (*Culture 1*). Images **C**-**H** were obtained based on the repetitive measurement after photobleaching of carotenoids.

Conclusions

It is known that the abiotic stress, including drought, stimulates ROS generation.[19] ROS, apart from their destructive potential, play a role in cellular signaling. NO molecules interacts with ROS and may also acts as an antioxidant upon some circumstances, therefore balance between these species has to be maintained in the cell.[19] As flavohemoglobins are directly related to the NO level, while carotenoids are linked with

ROS, it is not unexpected that the ROS/NO balance can be represented by the ratio of carotenoids and hemoproteins. Using high-resolution Raman imaging of *Rhodotorula mucilaginosa* cells we directly demonstrated that these two key compounds are interlinked in the yeast cells upon stress conditions. Flavohemoproteins rather than carotenoids are also produced in the *Rhodotorula* cells, upon the change the phenotype to adjust from an anaerobic to an aerobic environment, while carotenoids are dominating in the cells cultured of the oxygen environment. We also demonstrated that the mitochondrial process associated with the appearance of the "life band" is closely related to key cellular antioxidants, most probably hemoproteins that appear in the cell in response to the anaerobic-aerobic switch. The recovery of the ability of aerobic respiration by the *Rhodotorula* cells exhibited in the presence of the oxygen, manifested by the appearance of the "Raman signature of life" confirms that this signal is directly related to oxidative phosphorylation.

The "life band" is absent in alive *Rhodotorula* cells grown in anaerobic conditions. It is observed in cells cultured in aerobic conditions, but also appears in yeast cells cultured in anaerobic environment upon the phenotype change related to the activation of mitochondria. The conversions of cells to oxidative phosphorylation is related with the production of hemoproteins that play the key role in NO detoxification thereby enabling the mitochondrial respiration. We hypothesize that upon aerobic conditions carotenoids are the major compounds that fulfill a detoxifying role as in this case the hemoprotein cellular concentration is rather low. Nevertheless, our experiments demonstrate that carotenoids and flavohemoproteins can interchange in their protective roles in the yeast cells and their ratio is dependent on environmental conditions.

Acknowledgements

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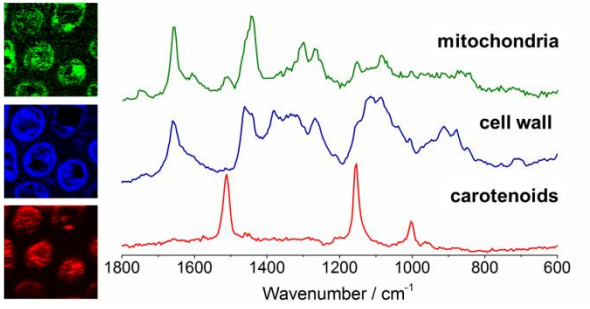

Raman microimaging of live cells of *Rhodotorula mucilaginosa* enabled to interrelate carotenoids, hemoproteins and the unknown species related to “Raman signature of life”.