Cytochrome-c based Resonance Raman Microscopy: analyzing bacterial phylogeny *in vivo*

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We present a proof of concept to spectroscopically determine the phylogeny of four bacterial strains based on the similarity of their cytochrome-c resonant Raman spectra recorded *in vivo* from individual cells in planktonic culture.

1 Introduction

Biofilms are made of dense, highly-hydrated, highly-structured clusters of bacterial cells bound together by extracellular polymeric substances excreted by the cells upon biofilm formation. These clusters – so called microcolonies – differ in the bacterial species or phenotype of the species contained in them [1]. Therefore, bacterial phylogeny, i.e. the determination of the relative relatedness of different bacterial species, is crucial for understanding the process of biofilm formation. However, all standard methods for bacterial phylogeny – DNA-DNA hybridization, 16S rRNA sequencing, and even Single-Cell-Genomics – are all invasive, requiring the removal of the examined cells from the sample [2]. Clearly, a non-invasive method allowing consecutive analysis of an undisturbed, growing biofilm is essential.

Confocal Raman microscopy tuned for the porphyrin lattice vibrations of the heme-c in cytochromes-c may provide such a method.

Cytochromes-c (cyt-c) are ubiquitous heme proteins, essentially functioning as electron transfer proteins. They are found in the majority of lifeforms on Earth, with few exceptions among archaean extremophiles. The heme moiety of cyt-c, a porphyrin-structure embedded in the protein moiety (see Figs. 1 and 2), is a firm evolutionary constant; any change to this part of the molecule results in severe perturbation of the protein function. However, since bacteria often form multiple, specialized cyt-c dedicated to specific tasks involving electron transport, evolutionary change is abundant in the protein moiety of bacterial cyt-c.

2 Methodology

Cytochrome-c resonant Raman spectra recorded from individual bacteria cells *in vivo* and *in situ* already allow an in-depth analysis of native biofilms at cell level, including distribution, tracking, and identification of the bacterial species present in the sample [3][1]. Hierarchical Cluster Analysis (HCA) categorizes the Raman spectra into clusters based on their spectral similarity, thus determining the relative similarity of the cells' cyt-c content and therefore, how evolutionary close the analyzed bacteria cells are to each other [4][5].

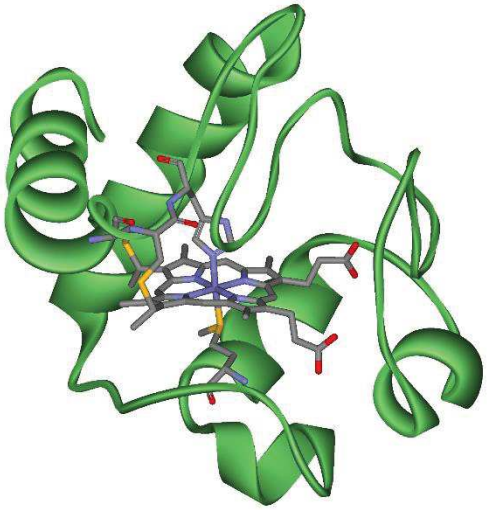

***Fig. 1** Schematic of cytochrome-c with the heme embedded in the protein moiety.*

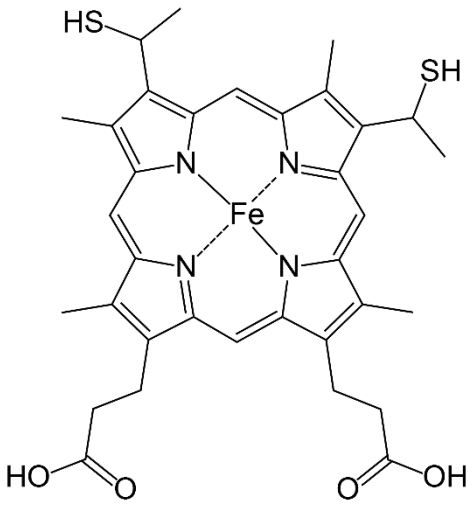

***Fig.2** Structure of the heme-c moiety.*

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3 Proof of Concept: Phylogeny *in vivo*

In a proof of concept, we determined the relative relatedness (phylogenetic distance) of three strains of native *Nitrosomonas* bacteria (*N. communis* Nm-02, *N. europaea* Nm-50, *N. eutropha* Nm-53) and the carotenoids-free mutant *Rhodobacter sphaeroides* DSM 2340^T based on the similarity of their cyt-c resonant Raman spectra recorded *in vivo* from individual cells kept in several planktonic cultures on different days. Spectra recorded from cells of the same strain had a spectral similarity of over 95% independent of strain, sample, day, or sampled culture. The spectra of Nm-50 and its evolutionary close relative Nm-53 showed an overall spectral similarity of 84% to each other, whereas the spectral similarity dropped to 77% when either of them was compared to their more distant relative Nm-02. A comparison between *Rhodobacter* and the tested *Nitrosomonas* strains returned a spectral similarity of merely 46%. As can be seen by comparing Figs. 3 and 4, the cyt-c resonant Raman analysis returns qualitatively the same species relations as established by the standard procedures, although further work is required to determine correlation factors, the valid phylogenetic range, and the applicability of the method to complex environmental samples [5].

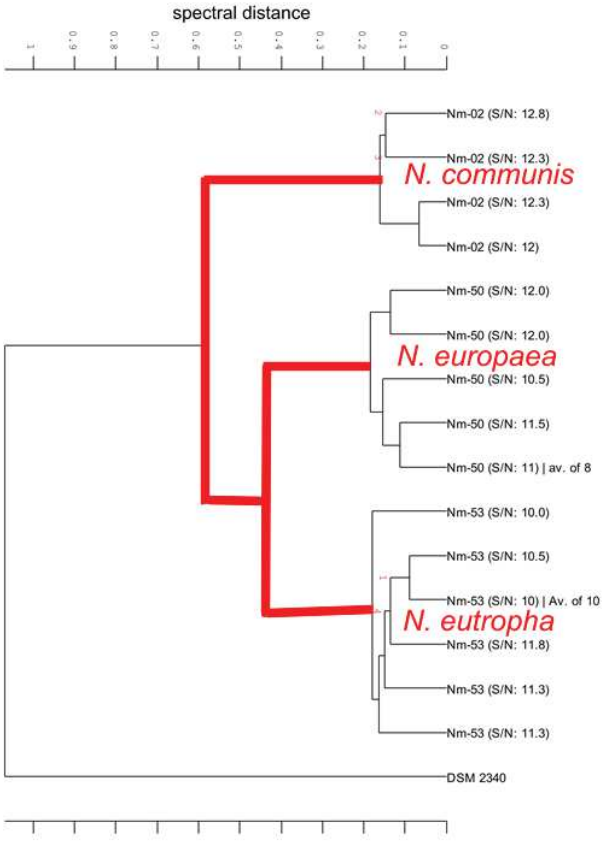

Fig. 3 *HCA of cyt-c resonant Raman spectra recorded from individual cells of Nm-02, Nm-50, Nm-53, DSM 2340T. Spectra were cut and vector-normalized to 600 - 1800 cm^{-1} prior HCA. HCA-Algorithm: Weighted-Average-Linkage. S/N-range: 10 - 13. Software: OPUS 5.5 by Bruker.*

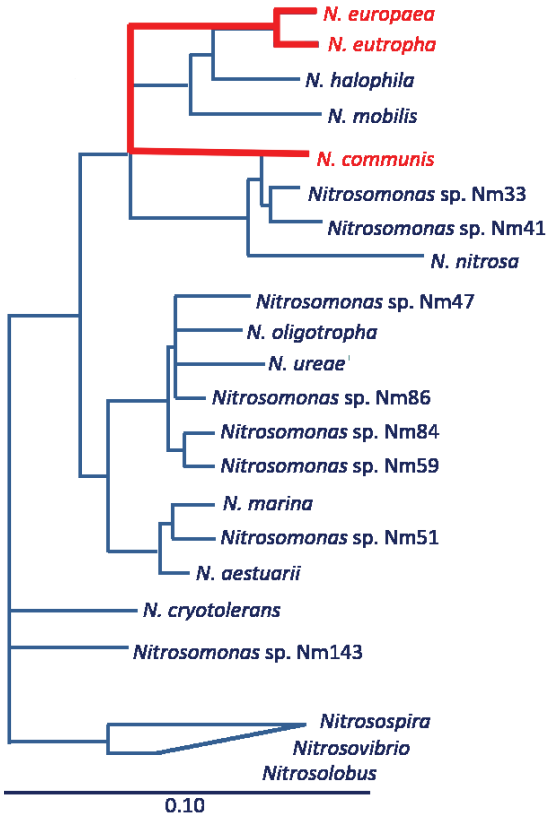

Fig. 4 *Phylogenetic distance tree of the genus Nitrosomonas as determined by DNA-DNA hybridization. source: PD Dr. A. Pommerening-Röser (priv. comm.)*

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