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ABSTRACT

In this paper we present a longitudinal study of bacteria metabolism performed with a novel Raman spectrometer system. Longitudinal study is possible with our Raman setup since the overall procedure to localize a single bacterium and collect a Raman spectrum lasts only 1 minute. Localization and detection of single bacteria are performed by means of lensfree imaging, whereas Raman signal (from 600 to 3200 cm^{-1}) is collected into a prototype spectrometer that allows high light throughput (HTVS technology, Tornado Spectral System). Accomplishing time-lapse Raman spectrometry during growth of bacteria, we observed variation in the net intensities for some band groups, e.g. amides and proteins.
The obtained results on two different bacteria species, i.e. *Escherichia coli* and *Bacillus subtilis* clearly indicate that growth affects the Raman chemical signature. We performed a first analysis to check spectral differences and similarities. It allows distinguishing between lag, exponential and stationary growth phases. And the assignment of interest bands to vibration modes of covalent bonds enables the monitoring of metabolic changes in bacteria caused by growth and aging. Following the spectra analysis, a SVM (support vector machine) classification of the different growth phases is presented.
In sum this longitudinal study by means of a compact and low-cost Raman setup is a proof of principle for routine analysis of bacteria, in a real-time and non-destructive way. Real-time Raman studies on metabolism and viability of bacteria pave the way for future antibiotic susceptibility testing.

Keywords: Raman spectroscopy; Lensfree imaging; Bacteria detection; Bacteria growth phases; Bacteria chemical fingerprint.

1. INTRODUCTION

The identification, analysis and characterization of single-cell bacteria are a challenging task on many fields, such as biomedical application, clinical diagnostic, environmental monitoring. Several methods have been developed so far aiming at fast and reliable bacterial identification, e.g. mass spectroscopy, fluorescence immuno-assay, flow cytometry and polymerase chain reaction. Analysis of micro-organisms can also be approached via vibrational methods, i.e. Raman spectroscopy, Raman spectroscopy has attracted much attention recently as emerging, powerful, nondestructive and high specific technique for rapid microbial detection and classification[2]. The technique relies on the acquisition of consolidated spectra on single bacterium which give unique molecular signature. Hence Raman spectroscopy is label-free, it eliminates the need for extensive sample preparation and use of labels[3,4]. Most important it allows identifying single bacterium and does not need culture of bacteria colonies to collect sufficient signal. But the acquisition of Raman spectra of a single bacterium put a strong constraint on the focalization of the excitation light. The required precision of a few cubic micrometers implies the use of large magnification microscopy (x50 up x100). But clearly this is at the price of larger cost and complexity. In order not to compromise with the overall simplicity of the methods, the bacterial localization is here performed by means of lensfree imaging technique. The latter offers many advantages in comparison with regular microscopy, mostly large field of view, simplicity and small for factor. Lensfree imaging serve already numerous biological applications[5,6]. Here it allows to localize single-cell bacteria over a large field-of-view of $24mm^2$, then to focalize the Raman spectroscopy excitation light precisely over the single bacteria. The acquisition of the Raman signature further determines the chemical bonds, given by inelastic scattered light following excitation, and allows the association of molecules such as proteins, carbohydrates, nucleic acids, organic and other functional groups[7].
The aim of the present study is to demonstrate the possibility of monitoring real-time changes in single-cell bacteria

metabolism during the growth phases thanks to our novel Raman spectroscopy system[8]. The study was performed using *Escherichia coli* and *Bacillus subtilis* bacterial cells which are respectively Gram negative and Gram positive. Characterization of their Raman spectra during the growth is investigated, in order to understand the metabolic variability included in the Raman spectra of the single-cell bacteria. Our system allows observing variation in the net intensities for some band groups which indicates that growth indeed affects the Raman bio-chemical fingerprint[17]. Further we used a support vector machine classification to further asses the metabolic changes in bacteria growth between lag, exponential and stationary phases. The study demonstrates the potential of our novel Raman spectrometer to provide information of the metabolism of the single bacteria during the growth phase and strengthens the validation of our innovative setup in the microbiological applications. Other works dealing with metabolism measured by Raman spectrometry can be found in J.Popp *et al.*, in which various vibrational spectroscopic techniques are applied to characterize *S. epidermidis* strain focusing on the exploration of the protein and nucleic-acid components change[17], and in Wei E. Huang *et al.*, which investigated the potential of Raman microscopy to detect differences in the physiological state of single species[21].

2. EXPERIMENTAL SECTION

2.1 Growth and culturing of bacteria

The microorganisms were chosen in order to characterize two different gram-types of bacteria: *E. coli* ATCC 11775 as *Enterobacteriaceae* family and Gram-negative model and *B. subtilis* ATCC 23857 as *Bacillaceae* family and Gram-positive model.

B. subtilis and *E. coli* were grown overnight in Trypcase Soja Broth (Fluka 22092) and Luria Broth (Sigma-Aldrich L2542) respectively at 30°C and 37°C. Colonies are transferred to 2 mL of Milli-Q ultrapure water in order to obtain a suspension with an OD (optical density) close to 0.1 at 580 nm. 1 mL of this suspension is then transferred into 100mL of TSB, homogenized and placed in a Thermomixer (Eppendorf) at 450 rpm and 30°C (for *B. subtilis*) or 37°C (for *E. coli*) for a new growth phase. Different samplings of 2mL are then performed during the microbial growth before stationary phase. Each sampling is centrifuged at 3500 rpm for 5 minutes at room temperature. The bacteria pellet is resuspended in 2 mL of Milli-Q ultrapure water. A second centrifugation at 3500 rpm for 2 minutes was done to ensure the complete removal of the medium. The pellet is then resuspended in 100 µL of Milli-Q water. The bacteria concentration obtained is 106 – 107 CFU/µL and following dilutions are performed in order to obtain OD of 0.2.

The growth curves were recorded by sampling the bacterial culture in different time steps, covering the lapse of time from the log phase to the stationary phase. Absorbance was measured at λ=580 nm (photospectrometer Uvicon923 – BioTek Kontron) and the values were plotted as a function of time to determine correctly the point of bacteria sampling.

2.2 Spectroscopic setup

The setup proposed here implements both Raman scattering and lensfree imaging in a single combined analytical platform. The system used in this study has been previously described in detail[8]. A single focused laser beam at 532 nm (Spectra Physics) with 50 mW of optical output power coupled by a beamsplitter (Semrock) and a microscope objective (Olympus 100x/air/0.98/wd) is delivered to the sample by a laser spot of about 1 µm and 34 mW (this power is adjusted by mean neutral optical densities), in order both to target the bulk of individual bacteria and to generate Raman scattering.

A double motorized linear stage (PI micos, minimal step size of 0.1 µm, LabView controlled) ensure x-y plane displacements of lensfree module allowing the precise targeting of single bacteria. The vertical adjustment of the focalization is assured by a third identical motorized linear stage mounted in the vertical axis.

The lensfree module acts too as sample holder, where the sample quartz coverslip is placed right above image sensor. It features a RGB CMOS image sensor (Aptina) with an active area of 24 mm^2 and a field of view of 2592x1944 pixels (2.2 µm pixel size).

The Raman signal scattered from single bacteria is filtered by a notch filter (Semrock) and then focalized into a multimode optical fiber (Thorlabs, 0.22 NA) by a short focal lens. The spectrometer used for this study (APEX-532) is a custom-built unit comprised of the best features from Tornado Spectral Systems' HyperFlux 532 spectrometer and Ocean Optics QE65Pro spectrometer. High throughput virtual slit (HTVS) technology allows this unit to generate both broadband (spectral range 475 nm to 650 nm) and high resolution (0.51 nm) spectra without compromising the optical throughput of the system. APEX-532 is equipped with Hamamatsu detector thermoelectric cooled to guarantee a very low noise level and a performing peak signal-to-noise ratio compared to commercial systems[9,10]. APEX-532 has been

calibrated with Hg lamp calibration and polystyrene has been used as reference sample.

2.3 Raman data acquisition

An amount of 5 µL for each bacteria solution is pipetted on top of a quartz coverslip (Ted Pella Inc. 19 mm^2 and 0.5 mm thickness) previously rinsed with ethanol solution at 70% (Sigma-Aldrich) and dried with nitrogen. Before starting the measurements, we let the liquid drop containing bacteria evaporate to create an investigation region of a few mm in diameter. After each analysis, the quartz coverslip is carefully cleaned in ultrasonic bath (Novatec) for 10 minutes.
Each Raman spectral acquisition has been performed with an integration time of only 10s. 30 different single-cell bacteria are recorded for each growth phase. The background signal of quartz coverslip, to be subtracted from Raman spectra of bacteria during data processing, is acquired at the same integration time on average 5 random surface points before the deposition of the bacteria solution drop. Spectra were cropped to spectral regions of interest (ROI) ranging from 650 to1800 cm^{-1} and 2600 to 3200 cm^{-1}, which cover the bio-chemical specific peaks of bacteria[20,21].

2.4 Raman data pre-processing and Support Vector Machine data classification

The analysis of Raman spectra was performed using R software environment in two steps: first a preprocessing of the spectra and second a following classification using SVM (support vector machine). The detailed spectrum pre-processing is reported elsewhere[19]. We just summarize the main stages here.
The cosmic spikes removal is the first step. At the difference of the usual method that proceeds by comparison between different spectra, spikes are detected using their spectral characteristics and removed from each spectrum treated individually[11]. The second stage of pre-processing is the subtraction of the background in the smoothed spectrum. Different steps are included for the subtraction of the background. The first one allows eliminating the quartz substrate contribution: the strategy consists in considering the mean quartz spectra and in fitting it on each bacteria spectrum for the large peak specific to quartz spreading from 200 to 650 cm^{-1}. The fitted mean quartz spectrum is then removed from the considered bacteria spectrum. Afterwards, the Clayton's algorithm (with a neighborhood window of 3 channels, 4000 iterations) is used to obtain a peak stripping on the resulting spectrum[13,14].
The last stage involves the normalization process. The signal is divided by its mean value on a chosen ROI, enabling to have the same scale for all spectra collected and erasing the contribution given by the variation of the experimental factors. It is also reduced to both regions from 650 cm^{-1} to 1800 cm^{-1} and from 2600 cm^{-1} to 3200 cm^{-1}, which contain the specific peaks of bacteria, to be employed as input of the classification algorithm in order to separate the different growth phases of a strain.
The classification algorithm used is the SVM. For the cross-validation, we proceeded as follows: all growth phases were represented in the reference spectra base. In order to avoid a too perfect match, a one-tenth of each growth phase of all phases was randomly chosen at each loop, and removed from the reference base to form the validation base. Thus, a 10-fold cross-validation enables to test all spectra. Moreover, this process was carried out ten times, so the mean and the standard deviation of the global correct identification rate for all phases, as well as a mean confusion matrix providing the results for each one, could be given.

3. RESULTS AND DISCUSSION

3.1 Raman investigation

The growth curve was recorded by sampling the bacterial culture at a total of different time steps (six points for *B. subtilis* and four for *E. coli* respectively), covering the lapse of time from the lag phase to the stationary phase. One point is measured for the lag phase, three point for the exponential phase and two points for the stationary phase.
Absorbance was measured at λ=580 nm (photospectrometer Uvicon923 – BioTek Kontron) and the values were plotted as a function of time to determine correctly the point of bacteria sampling. Figure 1 shows the *B. subtilis* (left side) and the *E. coli* (right side) growth curves in a semi-logarithmic representation as a function of the different samplings.

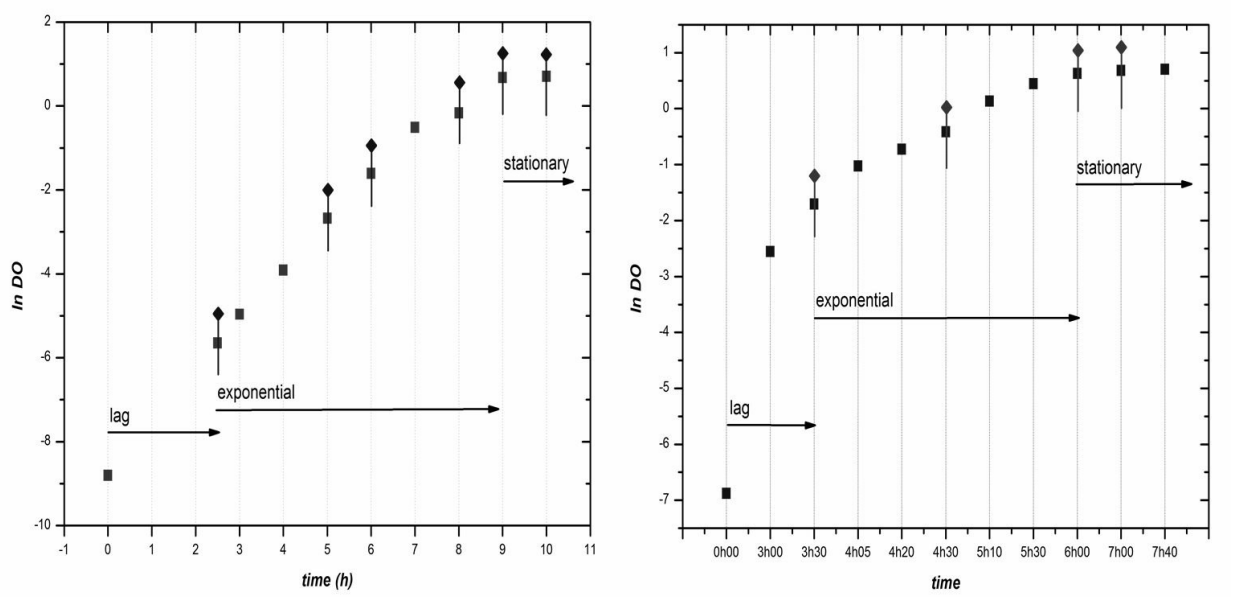

Figure 1. *B. subtilis* and *E. coli* optical densities (at λ=580 nm) growth curves measured by means of photospectrometer Uvicon923: the bars with dot indicate the sampling at different growth phase. Raman spectroscopy was then performed for each sampling.

Raman spectroscopy analysis has been performed for each bacterial sampling over 30 single bacteria, acquisition time is 10s per bacteria. Figure 2 shows the Raman spectra, as average of the 30 single acquisition as a function of the different growth phases. The rejected spectra, due to high-level of fluorescence or photobleaching effect, are in the order of 10% for each series collected.

The Raman spectra of *B. subtilis* single bacteria collected during lag (1 sampling), exponential (3 sampling) and stationary (2 sampling) phases are shown in the top of Figure 2. In the bottom, we represent those of *E. coli* for the three exponential sampling and the stationary one.

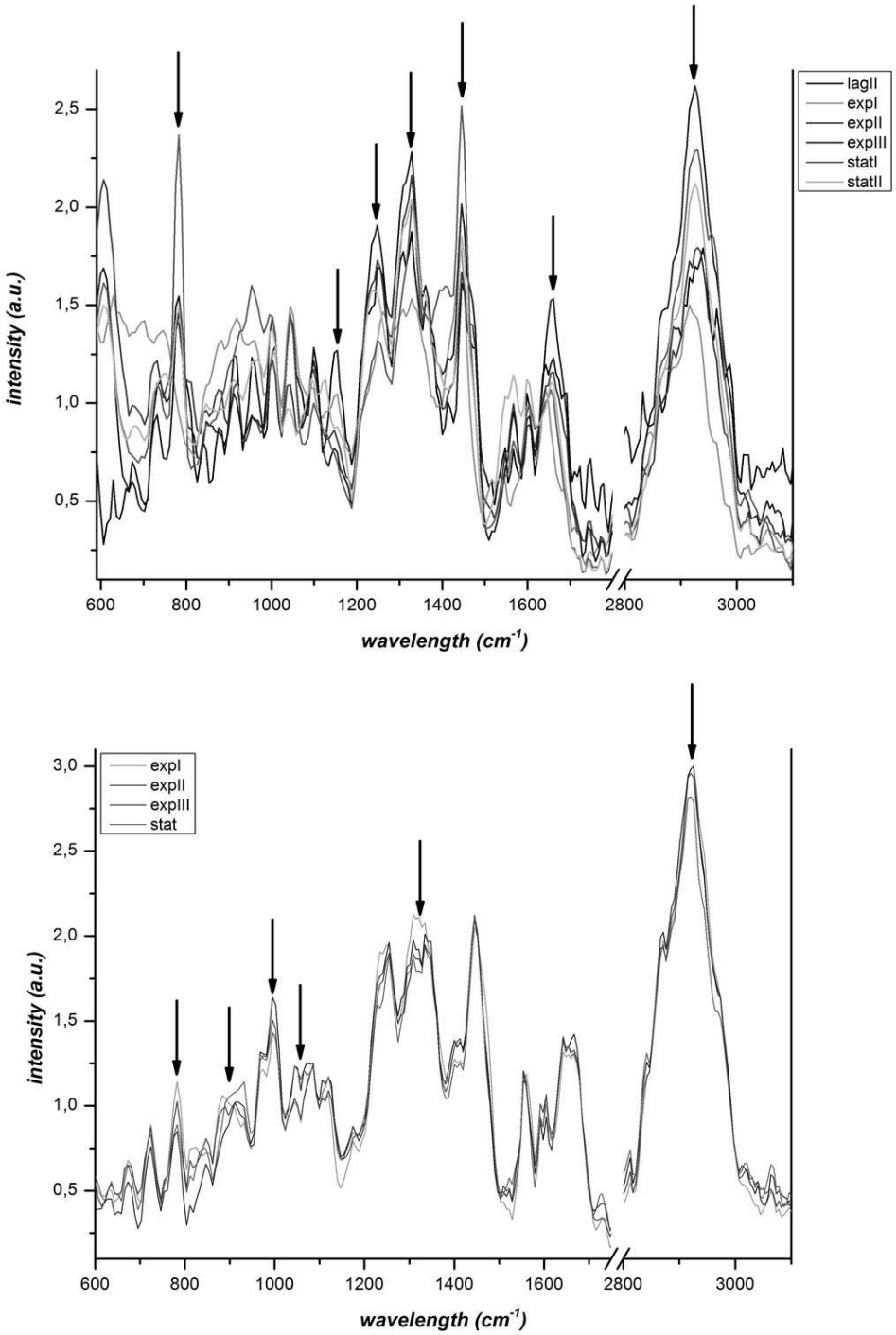

Figure 2. Comparison of normalized averaged Raman spectra of single bacteria collected at different sampling during the growth phase of *B. subtilis* (top) and *E. coli* (bottom) strain. Arrows show the peak with larger variations depending on the growth stage.

On Figure 2 one can notice that, according to the different growth change, the Raman spectra display significant changes in some peak intensities and ratio (as indicated by the black arrows). Raman peaks can be grouped to the same type of biological molecules in order to study the evolution of each group along the growth phase. The peak assignment, based on previous works[8,17], and the group division are shown in Table 1.

Table 1. Assignment of Raman bands detected for strains studied and peptides group identification.

Raman frequency (cm^{-1})	*Assignment*	*Group*
2915 - 2940	CH str	
1640 – 1665	Amide I	Peptides
1604	Phenylalanine	Peptides
1540 - 1570	Guanine, Adenine; Amide II	
1445	CH_2 scissoring	
1328	CH def	
1248 – 1261	Amide III	Peptides
1154	C-C, C-O, C-N	
1099	CC skel, COC a-str, PO2 str	
1044	C-C, C-O, C-N str, C-O-H	
1002	Phenylalanine	Peptides
782	Cytosine, Uracil	
606	Phenylalanine (skeletal)	Peptides

During the different growth phases, the bacterium contains different biomolecules that reflect its ability to increase the quantity of cellular constituents from the environment nutrients. Different Raman fingerprint are then generated and reflect the different metabolic states. During the lag phase, the bacteria do not increase the biomass (as observed above in Figure 1). The beginning of exponential phase is characterized by an increment of the growth rate until a constant value. The growth rate is a specific parameter of each bacterial strain and change depending on the growth conditions[18]. The exponential phase follow the bacteria generation time law: $N(t)=N_0 \times 2^{t/g}$, where N and N_0 are respectively the number of bacteria at time t and the number of bacteria at the beginning of the exponential phase and g the generation time. From the slope of the growth curves in Figure 1, we calculated g=37min for *B. subtilis* and g=39min for *E. coli*. The stationary phase occurs at the end of cell division, after that the density of bacteria population has become too high: the biological mass does not increase anymore, the cells start to dye and the lysis of the biomass occurs.

A preliminary analysis can be obtained plotting the trend of intensity changes during the growth phase time for different group of Raman peaks[16]. We decided to investigate the group of peptides in *B. subtilis*, as we shown in Figure 3: this group of Raman peaks has been identified both on according to the same type of biomolecules and on similarities in their time trace profiles. Raman peaks assignment of peptides group is specific to amino-acids and proteins. In according with biology knowledge, the sum of intensities of these peaks shows an increasing trend during the exponential phase, due to the augmentation of protein mass necessary for the cell division and replication. In fact, during the exponential phase and consequently the bacterial growth, new proteins are synthesized. This behavior ends in the stationary phase in which a decreasing effect of the intensity trend is observed, due to the bacterium division stop inducted by the unfavorable environmental conditions (inactive metabolism).

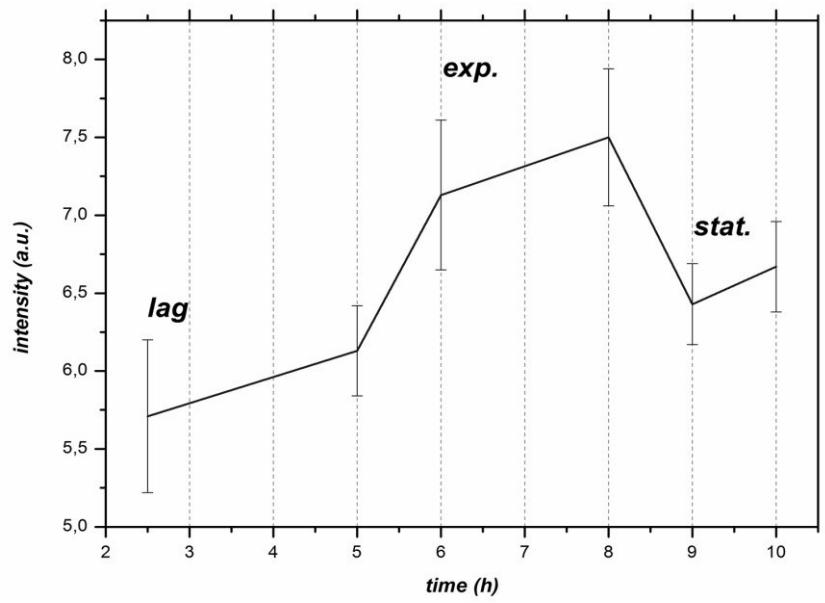

Figure 3. Trend of the sum of peptides peak contributions specific to nucleic acids related to the Raman spectra of *B. subtilis* strain. Error bars corresponds to the standard deviation measured over the 30 Raman spectra.

3.2 SVM classification

Raman spectroscopy is shown to be sensitive enough to detect changes in the bacterial cell during the different growth phases. The analysis of Raman spectra reveals that the contribution due to nucleic acids and proteins is the responsible of fingerprint changes during bacterial growth.

A complementary way to investigate the metabolic changes occurring during bacterial growth is the statistical analysis. The spectra we collected were subjected to SVM classification in order to obtain a recognition rate for each growth phase.

The confusion matrix generated by SVM (Figure 4) shows the results of growth phase identification of the bacterial spectra. This statistical method helps in to obtain characteristic changes in the bacterial composition from the Raman spectra.

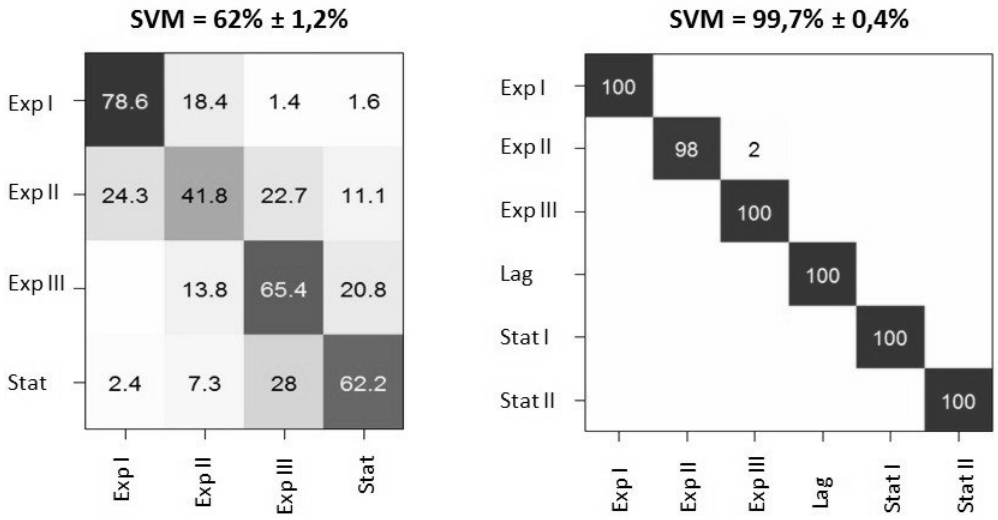

Figure 4. SVM classification rates of different growth phases for *E. coli* strain (left side) and *B. subtilis* strain (right side).

The total classification rate achieved a performance of 62% for *E. coli* and 99,7% for *B. subtilis*. In particular we can underline that the separation is lower for *E. coli* strain that was selected as model for Gram-negative bacteria. This can be explained observing that the SNR ratio is much better for *B. subtilis* (1.13) respect to *E. coli* strain (0.90). The SNR is a quality indicator of the individual spectra. It is calculated by the mean of the specific net signal in a region specific to bacteria, here the peak at 1445 cm^{-1}, divided by the standard deviation of the specific net signal in a region without bacteria signal, here the 2000-2500 cm^{-1} region. An impact on SVM classification for the physiological variation of single bacteria composition during the growth phase can be supposed considering the small growth-phase variations in membrane compounds, polysaccharides, proteins, lipids, and nucleic acids, as observed previously[21,22]. This approach demonstrates the potentiality to detect differences in fingerprint profiles according to individual physiological state of the single bacteria during the growth phase.

4. CONCLUSIONS

We have demonstrated that our compact Raman spectroscopy setup can be used to determine biochemical changes of the metabolic states that occur during single bacteria growth and to discriminate the different phases.
Detection and discrimination between the different growth phases (lag, exponential and stationary), via Raman spectral features, are primarily identified for the peaks of spectral fingerprint associated with peptides/amino acids contributions. Further, it was possible to characterize the bacterial cells with their overall chemical composition and to obtain more detailed information about some targeted group of peaks (i.e. peptides).
The statistical analysis of the Raman spectra during the growth phase revealed these significant changes. The SVM statistical classification technique allows achieving an average classification rate of 99% for *B. subtilis* and 62% for *E. coli*.
The differences observed on Raman spectra as a function of the growth phase are important. Hence they must be considered, especially when Raman spectroscopy is used for identification purpose. In other words we consider that the Raman acquisitions must be performed at supervised time in the growth phase, e.g. in the middle of the exponential phase or in the stationary phase.
In sum, our approach that combines Raman spectra analysis with SVM classification is sufficiently sensitive to facilitate the rapid recognition and the discrimination of different growth phases on single bacteria with unique metabolic responses.
Therefore, this compact platform provides new capabilities for many different real-time and non-destructive applications in micro-biology without extensive sample preparation (i.e. susceptibility of the bacteria towards antibiotics or response to drug treatment).
Future works will focus on studying the response of different bacterial species (or microorganisms) to different antibiotic treatments in order to consolidate the Raman spectra database we are building, as presented in parallel researches.

ACKNOWLEDGEMENTS

The authors thank the French trans-governmental CBRN-E R&D program for its financial support and Dr. Dorothée Jary and his staff for providing us bacteria samples and for their technical support.

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