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SHORT COMMUNICATION

Recent Advances in the Use of Intrinsic Fluorescence for Bacterial Identification and Characterization

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Abstract Live bacteria contain a variety of intracellular biomolecules that have specific excitation and emission wavelength spectra characterizing their intrinsic fluorescence. This paper reviews recent developed methods using bacterial intrinsic fluorescence for identification and characterization purposes. Potential applications of such methods at the industrial level are also addressed.

Keywords Fluorescence spectroscopy · Intrinsic fluorophores · Autofluorescence · Identification of bacteria · Bacterial metabolism · Chemometry

General introduction

Fluorescence spectroscopy has been extensively exploited for studies of molecular structure and function in chemistry and biochemistry. However, its effectiveness in microbial identification and characterization has only been recently recognized in the last two decades [1, 2].

Live bacteria own numerous intracellular biological molecules associated with energy yielding reactions. The fluorescent characters of these endogenous molecules at specific excitation and emission wavelengths make them very attractive probes for biological detection and characterization. Table 1 lists the biological molecules that exhibit endogenous fluorescence, along with their excitation and emission maxima. These endogenous fluorophores include protein' tryptophans, other amino acids (tyrosine and phenylalanine), nucleic acids, and co-enzymes. Their excitation maxima lie in the range 250–450 nm (spanning the UV/VIS spectral range), whereas their emission maxima lie in the range 280–540 nm (spanning the UV/VIS spectral range) [3]. The ideal target fluorophore should have an intrinsic fluorescence spectrum that distinguish it from other materials and have sufficient fluorescence intensity to produce strong signal [4]. In general, shorter excitation wavelengths engender higher energy and thus are likely to produce fluorescence in more types of material.

The goal of this paper is to succinctly review recent developed methods using the intrinsic fluorescence of bacteria for their identification or the study of their metabolism.

Bacterial detection and identification

Bacteria and other microorganisms are found widely throughout nature and the environment. Foods, water, and air own many types and high numbers of bacterial species. Some of them are associated with beneficial effects and are nowadays deliberately used as starter and/or probiotic cultures to bring about technological, safety and/or health benefits. However, some of them may represent a threat for the human health. In fact, a growing number of bacterial pathogens have been identified as important food-, water- and air-borne pathogens including *Escherichia coli* O157: H7 in ground beef products, vegetables, and juice, *Salmonella typhi* in chicken, eggs, and mayonnaise-based salads, *Cryptosporidium parvum* and *Vibrio cholerae* in water, *Legionella pneumophila* in air [5]. Hence a quick and accurate assessment of microbial contamination in a variety of settings is necessary.

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Table 1 Excitation and emission maxima of endogenous fluorescent bacterial molecules

Endogenous fluorophores	Excitation maxima (nm)	Emission maxima (nm)
Amino acids		
Tryptophan	280	350
Tyrosine	275	300
Phenylalanine	260	280
Enzymes and coenzymes		
FAD, flavins	450	535
NADH	290, 351	440, 460
NADPH	336	464

Bacterial identification by conventional phenotypic procedures based on biochemical, physiological, and morphological criteria are often tedious, time consuming, involves a large number of reagents, and are not reliable [6]. The recent development of molecular methods has been particularly fruitful for the reliable identification of micro-organisms. However, these methods are also reagent- and time-intensive, and require highly qualified technical staff, so their applications in industrial settings are not yet widespread.

Intrinsic-fluorescence spectroscopy has been shown to offer direct (labelling-free), rapid, simple, low-cost and reliable tool for the identification of bacteria. The technique has been successfully applied on food-associated bacteria and proved to distinguish bacteria from yeast based on the differences in emission (λexc=290 nm, λemi=[310, 420 nm]) and excitation (λexc=[200, 320 nm], λemi= 340 nm) spectra of proteins' tryptophan residues [7]. The indole groups of tryptophan residues are well-known to be the dominant source of UV absorbance and emission in proteins. The fluorescence of tryptophan has been shown to be highly sensitive to its local environment, and spectral shifts in its emission have been observed as a result of a range of phenomena, such as binding of ligands and protein–protein association [8]. The intrinsic fluorescence of tryptophan collected between 305–400 nm following excitation at 270 nm allowed also to accurately discriminate (0% of error rate) between *Lactococcus lactis*, *Pediococcus pentosaceus*, *Kocuria varians*, *Pseudomonas fluorescens* and *Listeria innocua*. Hence demonstrating the ability of the technique to discriminate between microorganisms belonging to different taxonomical families [9]. Moreover, differences in the emission spectra of the three aromatic amino acids (tryptophan, tyrosine and phenylalanine) and nucleic acids (DNA and RNA) (AAA+NA) collected between 280 and 480 nm, following excitation at 250 nm have been shown to allow more than 97% of accurate identification of meat-borne lactic acid bacteria (LAB) and human bifidobacteria at the genus, species and subspecies level [10, 11]. These finding could be of precious help in large starter and/or probiotic surveys.

Alternatively, the technique has also been fruitfully applied to clinical bacteria and proved to discriminate four common pathogens associated with otitis media, namely *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *Haemophilus influenzae* [12] and a noninvasive method using fluorescence spectroscopy (λexc=[250, 550 nm], λemi=[265, 700 nm]) and fiber optic cable allowed the *in situ* identification of these pathogens in an animal model (chinchilla) of acute otitis media [13]. Similarly, a method based on differences that occur within the excitation spectra collected between 330 and 510 nm (increment 20 nm) and the emission spectra ranging from 410 to 430 nm (increment 2 nm) has been developed for the identification of the most clinically significant species *Escherichia coli*, *Enterococcus faecalis*, and *Staphylococcus aureus*. Thus suggesting a rapid, ease to perform and low cost method compared to standard clinical diagnostic tests [14].

Furthermore, intrinsic fluorescence spectroscopy has been shown to allow detecting and differentiating viable but nonculturable (VBNC) bacteria in the presence of culturable bacteria, hence overcoming to the limitations of most actually used techniques in the diagnosis of VBNC Gram-negative pathogens. Roselle et al. observed changes in the intrinsic fluorescence spectra of *Escherichia coli* starved for 210 days in nutrient-free saline solution. While the fluorescence from tryptophan turn out to be insignificant, the measured fluorescence from a new band absorbing near 300 nm and emitting near 400 nm increased. A soluble byproduct is also produced, as *Escherichia coli* are starved, that absorbs near 350 nm and emits near 440 nm [15].

As well, the fluorescence band with an emission peak at 410 nm and excitation peak at 345 m from dipicolinic acid, which is produced during spore formation, has been found to serve as a spectral signature for the detection of bacterial spores [16]. These findings would be highly valuable for the dairy industry in the manner that they can avoid using milk batches containing spores in the manufacturing of hard cheeses.

It has to be raised that AAA+NA, NADH and FAD are the main fluorophores that have been investigated for their ability to reliably discriminate bacteria at the family, genus, species, and the subspecies level. However, better results were obtained when targeting AAA+NA [10, 11, 17]. In fact, NADH and FAD are metabolic coenzymes (the reduced form of a pyridine nucleotide and the oxidized form of flavine adenine dinucleotide) and hence their fluorescence intensities are closely related to the metabolic state. While these fluorophores show dramatic variation in their fluorescence intensities according to their metabolic activity, thus on their physiological age, and growth

conditions, those of AAA and NA are unaffected until cell death [10, 11, 18]. Nevertheless, these coenzymes fluorophores have been shown as reliable indicators of cells viability, which makes them a good candidate for discriminating particles of bacterial origin from inanimate material [4].

The promising aforementioned results, which suggest that a fluorescence spectrum afford a 'fingerprint', and the ability of intrinsic-fluorescence spectroscopic methods to *in-situ* measure with no sample contact and without requiring a step of cellular outgrowth promoted the development of some prototypes allowing a real-time, quick and accurate detection of microbial contamination in food, water, air, and on surfaces. In this respect, Powers et al. developed a hand-held prototype combining microbe capture chips with a fluorescence detector, which is capable of statistically sampling the environment for pathogens (including spores), identifying the specific pathogens/exotoxins even at low levels (≈20 cells/cm^2 or cm^3 of microbes in seconds), and determining cell viability where appropriate [5, 18].

With the increasing threat of bioterrorism (*Bacillus anthracis:* anthrax and *variola major*: smallpox) and the spread of infectious diseases such as severe acute respiratory syndrome (SARS), the real-time detection and identification of such harmful microorganisms became an urgent civilian and military objectives. This has motivated development of several ultraviolet laser-induced fluorescence (UV-LIF) based systems as front-ends or "triggers" for biological agent aerosol detectors, e.g.,: the Ultraviolet-Aerodynamic Particle Sizer (UV-APS) manufactured by TSI [19], Inc., the Fluorescence Aerodynamic Particle Sizer (FLAPS) developed by TSI Inc. and the Canadian Defense Ministry [20, 21], the Biological Agent Warning Sensor (BAWS) [22], developed by MIT Lincoln Laboratory, and the Single Particle Fluorescence Analyzer (SPFA) developed at the Naval Research Laboratory [23]. Typically, these types of systems have employed laser excitation sources that are now readily available commercially; utilizing either the third harmonic wavelength (349 nm) of a Nd:YLF (UVAPS and FLAPS) targeting NADH and riboflavin which have characteristic broad emission bands peaked at 450 and 560, or 266 nm light from quadrupled Nd:YAG (BAWS and SPFA) targeting aromatic amino acids, tyrosine, tryptophan, and phenylalanine that each have characteristic emission bands between 300 and 400 nm.

Others intrinsic fluorescence-based prototypes have been also developed for bioaerosols detection and discrimination from organic interferents emitted by combustion (traffic related urban aerosols) [24–26]. Although these apparatus can lack of specificity (limitation of the reference spectral database, cross-sensitivity to the non-bacterial organic materials, counting efficiency, upper limit of detection) compared to biochemical assays, they are commonly expected to be a first alert stage, prior to more precise analysis.

Bacterial tracing

Bacterial intrinsic fluorescence has been also shown to allow tracing for sources of bacterial contamination. Indeed, Leriche et al. assessed several *Pseudomonas* spp. isolated from milk, water, cheese centre and cheese surface in two dairy small-scale facilities manufacturing raw milk St. Nectaire' cheese and succeeded to trace, using intrinsic fluorescence spectroscopy, routes of cheese contaminations [27]. Additional investigations need however to be performed in order to confirm the contamination tracing capability of the technique. Such finding would be of valuable help for industries in order to quickly go back up in the processing line, detect sources of microbial contaminations in the process and thus improve the product safety.

Bacterial metabolism

Cellular fluorescence provides a sensitive index of the functional state of a living cell and hence may be used to determine their metabolic status. Indeed, it has been shown that differences in the AAA+NA (λexc=250 nm, λemi=[280, 480 nm]) and tryptophan (λexc=270 nm, λemi=[305, 400 nm]) emission spectra recorded for three different bacterial species, i.e. *Lactococcus lactis*, *Staphylococcus carnosus* and *Escherichia coli*, allowed their classification in three groups corresponding to the three main phases of the growth profile, i.e., lag phase, exponential phase and stationary phase [17]. Meanwhile, NAD(P)H fluorescence with excitation wavelengths of 340±20 nm and emission wavelengths of 400–480 nm has been shown to provide a powerful tool for online monitoring of microaerobic metabolism occurring during the transition conditions in which the organism performs simultaneous aerobic and anaerobic respiration or fermentation [28, 29].

As well, the tryptophan fluorescence collected in the range of 300–500 nm, following excitation at 275 nm has been used to investigate the bactericidal effect of some disinfecting agents exerted on *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. While the application of sodium hypochlorite resulted in an almost total loss of fluorescence for the five bacterial species, which indicated that total destruction of proteins and amino acids have occurred, the application of hydrogen peroxide decreased the fluorescence intensity and shifted tryptophan maxima of emission to shorter wavelengths, except in *S. aureus*, which is

resistant to oxidizing agents [30]. Similar results were obtained while using the intrinsic fluorescence of bacterial tryptophan and NADH excited at 280 and at 350 nm and monitored at 350 and at 430 nm, respectively, to study the influence of the potassium iodate on the bacterial growth of *E. coli*. The stop of bacterial growing at a defined amount of KIO_3, combined to a significant decrease in the fluorescence intensity of tryptophan and NADH suggested that KIO_3 has great inhibiting effects on the growth of *E. coli* through the pathway of protein synthesis and respiratory chain [31]. Therefore, bacterial intrinsic fluorescence may be used for investigating the bactericidal or bacteriostatic effect of disinfectant, but also for detecting disinfection-resistant bacteria. Such approach may be very helpful in the setting of a directed microbial ecology approach in food processing plants. Moreover, such approach would be of great interest for pharmaceutical industries in the rapid assessment of the effective range of newly developed antimicrobials, i.e. range of bacteria that a particular antibiotic is effective against. In the contrary, it will allow fast and reliable determination of resistant bacteria to antibiotics or bile salts. Such resistances are now considered as one of the main criteria for the selection of probiotics or starter cultures [32].

Chemometric methods

The huge extent of spectral databases, the high correlations occurring between the wavelengths and the close similarity of bacterial spectra make very difficult to analyze collected data. Chemometric methods allow overcoming theses limitations. For instance, the main chemometric methods that have been applied on intrinsic fluorescence spectroscopic databases are the Principal Component Analysis (PCA) and the Factorial Discriminant Analysis (FDA) [7, 9–11, 17, 27]. PCA allow transforming the large number of potentially correlated factors into a smaller number of uncorrelated factors (i.e., principal components), and thus reducing the size of the data set. This multivariate treatment allows score plots of the samples to be drawn that represent the spectral patterns [33]. Neighboring points on these score plots represent similar spectra. Alternatively, the aim of the FDA is to predict the likelihood of an observation (spectrum data) belonging to a previously-defined qualitative group [10].

Conclusions

Intrinsic fluorescence spectroscopy is a prime candidate for the detection and identification of bacteria since it can provide a remotely (no sample contact) real-time, reagentless, reliable, fast, simple and low-cost tool for the detection and identification of bacteria, and for tracing their routes of contamination. However, additional investigations need to be performed in order to enlarge bacterial spectral databases, simplify and homologate bacterial identification prototypes.

Furthermore, intrinsic fluorescence has the advantage over the extrinsic fluorescence of an unperturbed environment during investigation, especially in complex systems such as biological cells and hence offers a rapid, noninvasive and simple way to study the metabolism of bacteria.

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