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Review Article

Physiological assessment of bacteria using fluorochromes

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1. Introduction

Microbiologists involved in ecological and environmental studies are often faced with the challenge of detecting or enumerating bacteria and assessing specific physiological activities in complex natural or engineered systems. Although these tasks appear almost trivial from the perspective of pure-culture studies in the laboratory, autochthonous communities and allochthonous organisms exposed to environmental stressors present some real analytical challenges. In addition to the inadequacies associated with most of the methods available, the patchiness and non-homogeneous chemical and physical character of most natural habitats further complicate the analytical aspect of such studies. Although these factors are of importance and must be considered when dealing with planktonic organisms, they are of particular significance in studies of biofilms, sediments and particulates where spatial relationships are inherently complex and important in the ecology of the community or system.

The failure of bacteria to form colonies is a widely acknowledged problem when using plate counting procedures. This concern was clearly recognized by Winogradsky [1] and more recently by others [2–4] due to the failure of known cultural methods when examining parameters such as species diversity or physiological activity of many microbial communities. As a result, our ability to describe microbial populations using methods based on colony formation is flawed

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and it has been estimated that only 20% of the bacteria within autochthonous communities are known [5]. In addition, allochthonous as well as some autochthonous bacteria exposed to potentially lethal environmental conditions including nutrient restriction, ultraviolet irradiation or sublethal concentrations of antibacterial compounds undergo physiological and morphological alterations that complicate the detection and accurate enumeration of such stressed bacteria using available cultural approaches [6–10]. As a result, the use of most established media often leads to serious underestimations of the complexity and bacterial population density of natural and engineered systems. Although these considerations present very real and practical constraints on the reliability of established media, the possibility of developing novel cultural approaches with appropriate nutrients for the detection of bacteria that have been affected by environmental exposure [11–13] should be acknowledged. Hence, bacteria that are sometimes regarded as non-culturable in the literature might be more realistically considered uncultivated, as suggested by Ward et al. [4].

A spectrum of alternative methodological approaches is now either under development or available to detect and quantify microorganisms or their activities in ecological and environmental studies. A variety of microscopic methods have been successfully adapted to such studies including traditional microscopy [14] and more recently, scanning confocal laser microscopy (SCLM) [15]. A range of different molecular approaches has also been developed and these are used in microbial ecology and environmental microbiology [4,16,17]. A number of other current approaches, including immunological methods, flow cytometry, image analysis technology, cellular biochemical signatures, microcalorimetry and microelectrodes, have also been reviewed recently [18–21]. However, most of these technologies also carry with them disadvantages that must be recognized in the design and interpretation of experiments where they are used. For example, most of these techniques provide data that are estimates averaged over the entire community so that physiological gradients and differences between cells or heterogeneities in space cannot be resolved. This characteristic is particularly germane in communities where defined spatial relationships can be crucial, such as biofilms.

Finally, the relationship between the concepts of viability and physiological activity requires brief consideration. Although philosophical debates will likely continue for some time on this issue, it is relatively clear that bacteria (i.e. the vast majority in some environments) are viable, since they are capable of reproduction in that habitat yet they are not detected using known cultural methods. At the same time, many such bacteria are demonstrably active when tested using one or more of the specific available tools to detect different measures of physiological activity. Therefore, the working hypothesis–that specific measures of physiological or metabolic activity provide more descriptive information concerning the potential physiological activity of bacteria and their dynamics within natural or engineered microbial communities than the ability of the cells to grow and form colonies on a specific medium– has been adopted

throughout the remainder of this review. Further, use of the term 'viable' is minimized herein, since it embodies a concept that is less definitive and is usually equated to reproduction in/on a given medium.

This minireview focuses on the application of fluorogenic compounds in the detection of bacteria with particular emphasis on the assessment of physiological activity using epifluorescence microscopy. Microbiological applications of several related methods will also be reviewed. Because of space limitations this article is not exhaustive but is intended to provide an overview of this topic, with representative examples and some suggestions concerning the potential for the fruitful development of fluorogenic probes in the future.

2. Fluorescence microscopy and staining in microbiology

Fluorescence microscopy and fluorescent stains are widely and productively applied in microbiology. The use of incident or epi-illumination fluorescence microscopy is of particular utility in microbiology, since objects may be viewed on opaque surfaces. This design alternative, often called epifluorescence microscopy, has recently been reviewed by Ploem [25].

Well before the development of the fluorescence microscope in the early years of this century, a range of fluorescent dyes became available. As a result, compounds like fluorescein and acriflavine were used in a number of microscopic applications and shortly thereafter the field of vital staining with fluorescent compounds was borne, as reviewed by Kasten [26]. Most of these early applications were limited to studies of plant and histological samples. By the late 1930s, microbiologists were using fluorescence microscopy in a number of applications. Early microbiological applications of autofluorescence and induced fluorescence have been reviewed [27]. Currently, direct microscopic enumeration with acridine orange is one of the most commonly used methods in microbial ecology [28,29]. However, it is worth noting that the selection of optical excitation and barrier filters is a critical step in adapting a fluorescence microscope for each fluorochrome and that filters from different microscope manufacturers lack uniformity.

3. Fluorochromes and fluorometric methods in use

Fluorescent reagents are currently used in an impressive range of biological applications where the response of individual cells can be observed microscopically [30,31]. The application of these probes has played a central role in many recent extraordinary developments in cellular biology. However, this approach has been exploited in only a limited number of microbiological applications.

3.1. Acridine orange

Acridine orange (AO) has been available for over 100 years and is one of the most commonly used fluorogenic dyes in microbial ecology and environmental microbiology as a part of the acridine orange direct count (AODC) or the direct total microbial count method [32]. The notable disadvantage of this technique is the inability of AO to distinguish between physiologically active and dead cells. However, some have suggested that the reaction of bacteria with AO will allow this discrimination. That hypothesis is based on the well documented difference in the way AO reacts with double-stranded versus single-stranded nucleic acids. Specifically, AO complexes with single-stranded nucleic acids in a manner that results in red to orange fluorescence while the product of AO and double-stranded nucleic acids yields green fluorescence. However, this metachromatic effect is influenced by the ratio of AO to nucleic acid [33]. The classical interpretation of this red versus green AO metachromatic effect is based on the relative abundance of ribosomal and other forms of RNA in rapidly growing bacteria as contrasted with the significantly lower RNA:DNA ratio found in a starved cells [34–36]. Consequently, bacteria that appear green after AO staining have been considered inactive while red or orange cells are thought to be active according to the classical interpretation. However, little effort has been devoted to confirming this putative relationship under controlled experimental conditions. Tempered confirmation of this concept was obtained [33] using purified DNA, ribosomes, bacteriophage-infected cells and *Escherichia coli* under a range of defined physiological circumstances, although the outcome of the AO staining reaction was shown to be influenced by a number of variables and by drying of the specimen [37]. These observations collectively indicate that investigators applying the AO staining reaction as an indicator of physiological activity should understand the relevant variables and validate their conclusions using independent indices of physiological activity. Additional general information on the application of AO as a probe for molecular and cell biology is available [38].

The sensitivity of the AODC technique may be enhanced, with concurrent determination of bacterial viability, by short-term incubation in the presence of growth promoting nutrients followed by staining and counting microcolonies. A microcolony method which included incubation on selective agar media gave reliable estimates of coliforms, pseudomonads and staphylococci in foods after incubation at 30°C for 3 or 6 h, at contamination levels above 10^3 CFU/g [39]. The addition of a 3 to 5 hour resuscitation step in tryptone soy broth permitted reliable estimates of injured bacteria in frozen and processed foods [39]. For this epifluorescence microscopy (MEM) technique, microcolonies on membranes were stained with acridine orange before examination by epifluorescence microscopy. This method has been applied to the detection of *Pseudomonas fluorescens* in soil [13]. Image analysis has been used for the enumeration of microcolonies formed by bacteria from pharmaceutical grade water, although background material resulted in overestimated counts for some samples [40].

Stains other than acridine orange, including euchrysin, fluorescein isothiocyanate, rhodamine B and acid fuchsin [41] or ethidium bromide [42], which is toxic, may be preferred for the direct count as these may give better fluorescence while not staining other material to the same extent. The latter report [42] also suggested the use of aluminum oxide membranes (Anopore) instead of polycarbonate when staining with ethidium bromide.

3.2. Other fluorescent nucleic acid stains

An extensive list of fluorescent nucleic acid stains has been used by cell biologists [31]. A limited number of these have been applied by microbiologists to determine a 'total bacterial count' in a range of circumstances. The compound 4',6-diamidino-2-phenylindole (DAPI), for example [43–45], has been used in this way and as a counterstain in procedures where contrasting fluorochromes are used [46]. Other stains including acriflavine, bisbenzimide [47,48], Hoechst 33258 [49], Hoechst 33342 [50,51] and ethidium bromide [52] have also been used in microbiological applications. Another fluorometric technique has been used to determine spatial and temporal patterns of DNA replication in bacteria within alginate gel beads [53]. In this method, pulse labeling with bromouracil is followed by detection of the polymerized analog by immunofluorescence as a measure of newly synthesized DNA. However, the dependence of this technique on bacteria that are auxotrophic for thymine could limit its application in the study of natural or complex biofilm communities. Specific RNA staining with pyronin Y combined with scanning microfluorimetry has recently been applied to demonstrate gradients in the RNA-to-DNA ratio of bacteria entrapped in gel beads [54].

3.3. Fluorescent potentiometric membrane dyes

Not surprisingly, cell biologists have extensively used indicators of membrane potential for nearly 20 years [55,56]. Resorufin is such a probe that has been used to distinguish between active and dead facultatively anaerobic bacteria although several factors complicate the interpretation of the results [15]. The use of rhodamine 123 (Rh-123), which has also been extensively employed by cell biologists, was reported in studies of bacteria [46,57] and more recently by microbiologists using flow cytometry [58–60] and spectrofluorocytometry [61]. This dye has also been found comparable to other probes in the rapid microscopic assessment of bacterial physiological status within growing, pure culture biofilms in situ [46]. However, the Gram-negative envelope is only slightly permeable to Rh-123 and a permeation procedure is used to obviate that problem [46,60]. The intracellular fluorescence of *N*-phenyl-1-naphthylamine [62], merocyanine [63] and dansyl galactoside [64] have also been proposed as general indicators of membrane energy level or energy-linked parameters in bacteria and vesicles.

3.4. Fluorescein diacetate

This compound (FDA) has been suggested as a 'vital' stain because its accumulation and hydrolysis depends upon an intact membrane and active metabolism in bacteria, mycobacteria and fungi [65–69]. FDA can be transported across the bacterial envelope and deacetylated by nonspecific esterases, resulting in the intracellular accumulation of fluorescein. This index of activity has been closely correlated with cellular ATP concentration and glucose-stimulated respiration rate in one report [70], while that relationship was less clear in a study of bacteria in wastewater and activated sludge [71]. Further, the low level of active bacteria, when compared with those cells demonstrating electron transport activity in some systems, may be attributed to the low permeability of the FDA fluorogen through the Gram-negative envelope. Treatment to reduce that limitation results in higher proportions of bacteria demonstrating activity [66]. The low level of fluorescent emission in FDA-positive bacteria and fungi is a limitation for conventional microscopy that may be solved through the use of a sensitive ISIT camera or a laser microscope [15].

3.5. Fluorescent stains for inactive bacteria

Dansyl lysine (DL), a nontoxic fluorescent membrane dye, has been used for assessing heat-induced killing and thermotolerance in eukaryotic cells [72]. Preliminary results obtained in our laboratory (unpublished) indicated that this stain has potential to be used for assaying inactive bacteria. Hydroethidine (HE), which is a reduction product of ethidium bromide, is found to be decomposed to a bright red-fluorescent compound by a range of microorganisms [73]. However, HE seems to be decomposed biologically by a number of microorganisms and also abiotically by ultraviolet light to a red fluorescent material. As a result, this compound has limited potential for assessing microbial activity.

3.6. Intracellular pH sensitive stains

Since intracellular pH may be influenced by the environment in relation to cell envelope integrity, its measurement may provide an indication of cell injury and potential viability. A pH-sensitive probe, 2′,7′-bis-(2-carboxyethyl)-5-(an-6)carboxyfluorescein (BCECF) was used to determine the intracellular pH of *Proprionibacterium acnes* [74]. Good correlation was obtained between the pH measured by BCECF and that determined by ^{31}P-NMR, although it was necessary to take into account BCECF leakage. Other intracellular pH indicators, such as benzo[c]-xanthene dyes including seminaphthorhodafluors (SNARFs) and seminaphthofluoresceins (SNAFLs) [75] may also prove useful in the microscopic indication of bacterial pH, although they have been developed for use with eukaryotic cells [76].

3.7. Fluorescent antibody techniques

Fluorescent antibody (FA) techniques have been used in environmental and ecological studies to detect a range of different bacteria including coliforms,

salmonellae, shigellae, enterococci, legionellae, nitrogen cycling organisms [77] and more recently, *Vibrio* spp. [78] and members of the family *Enterobacteriaceae* [79]. To discriminate active cells of specific bacteria, microcolony formation has been combined with immunofluorescence for the detection of *Listeria* spp. [80] and *Salmonella* spp. [81]. In the latter study, the fluorescent antibody-microcolony technique was used after preliminary enrichment, and the results were in good agreement with those obtained by conventional methods. The direct viable count method (DVC) has also been successfully combined with immunofluorescence for the detection of viable *Vibrio cholerae* [78] as well as *E. coli* and *Salmonella enteritidis* [9]. Good correlation was obtained for cell metabolic activity, measured by autoradiography, and substrate responsiveness at various stages of bacterial survival in laboratory microcosms. Others [11] employed the DVC/FA method to detect viable *Salmonella* spp. in chlorinated wastewater. They later modified the procedure by performing the DVC incubation on polycarbonate membranes to detect *Salmonella* spp. in seawater [82].

4. Recently developed fluorescent probes of physiological activity

4.1. CTC

5-cyano-2,3-ditolyl tetrazolium chloride (CTC) has recently been applied to visualize respiring aerobic and facultative bacteria in environmental samples [83]. This compound is related to 2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) which has been extensively used to microscopically discriminate actively respiring bacteria in a wide range of ecological and environmental studies [70,83,84]. Both CTC and INT act as artificial electron acceptors and INT is converted to insoluble red (nonfluorescent) crystals of INT-formazan within metabolically active bacteria. Results obtained with INT correlated well with both cellular ATP content and cell density in a study of pure and mixed microbial cultures [70]. However, the microscopic visualization of formazan crystals from INT is virtually impossible when cells are on opaque surfaces or mixed with particulate material, since the transmission of visible light through the specimen is required. CTC has also been used recently with flow cytometry to determine respiratory activity [85] and dormancy in individual *Micrococcus luteus* cells [86] as well as respiring autochthonous bacteria in drinking water and biofilms [87].

4.2. Fluorogenic assays of intracellular enzymatic activity

A combination of reporter gene technology and a fluorogenic enzyme substrate has recently been used to study the activity of an alginate promoter gene within biofilms of *Pseudomonas aeruginosa* [88]. This approach utilized a plasmid (pNZ63) in which the promoter for *algC* was fused to promoterless *lacZ* (β-galactosidase) as the reporter gene. This study, which demonstrated substratum

activation of alginate gene expression, also showed that the activity of specific enzymes within individual bacteria could be observed microscopically when the fluorogenic substrate, methylumbelliferyl β-D-galactoside was used. Another group also reported the successful application of a different fluorogenic substrate for β-galactosidase to discriminate the fraction of enzymatically active *E. coli* in a population using flow cytometry [89]. They found that fluorescein di-β-galactopyranoside bearing a C_{12} fatty-acyl chain (ImaGene Green C_{12}FDG, Molecular Probes, Eugene, OR) [31] was taken up by *E. coli* and that the fluorescent product was retained within the bacteria. Other fluorogenic substrates are available for many enzymes [31,69].

4.3. Fluorogenic probes for ribosomal RNA

In the course of developing molecular methods for the detection and characterization of microorganisms, oligonucleotide probes have been produced for a wide range of bacteria [4]. While these probes have been typically used for detection by polymerase chain reaction (PCR), fluorescently labeled oligonucleotide probes have been applied successfully for the in situ detection and identification of individual microbial cells [12]. The use of fluorescent oligonucleotide (FO) probes targeted to ribosomal RNA may avoid some of the problems of fluorescent antibodies and can be used to detect previously uncultured species. This technology has been largely devoted to the detection of microorganisms, without respect to physiological status, although recent developments might afford this capability. For example, image analysis microscopy has been used to quantify the cellular content of ribosomes of sulfate-reducing bacteria in multispecies biofilms [90]. This approach was used to infer growth rates in young and established biofilm communities. Provided that background fluorescence and autofluroescence can be minimized, cells have sufficiently high ribosome content and the bacteria are permeable to the probes, methods employing FO probes hold great promise in future microbial ecology investigations of both planktonic and sessile populations.

5. Future prospects

There is an impressive array of fluorogenic reagents utilized by biologists studying eukaryotic cells [31] in a wide variety of physiological applications [30]. By comparison, there are only a few microbiological uses of this analytical approach. These observations suggest that there is a great potential for the expanded application of fluorescent probes to determine bacterial physiological and metabolic activities. This position is supported by recent advances in scanning confocal laser microscopy, digital image analysis and the availability of highly sensitive cameras [15]. Such a strategy provides information describing the physiological status of individual bacteria and assessment of specific metabolic activities and gene expression in situ, as well as enumerative data and positioning

within communities. Some of these assays may also be done nondestructively and in real time. The resulting information provides exciting new physiological capabilities to complement the recently developed genetic/molecular analyses that are currently used in many areas of microbiology [15,16,19]. However, such applications need to be verified with independent methods to assure that the purported physiological process or cellular activities are being monitored.

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