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Rapid direct methods for enumeration of specific, active bacteria in water and biofilms

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1. SUMMARY

Conventional methods for detecting indicator and pathogenic bacteria in water may underestimate the actual population due to sublethal environmental injury, inability of the target bacteria to take up nutrients and other physiological factors which reduce bacterial culturability. Rapid and direct methods are needed to more accurately detect and enumerate active bacteria. Such a methodological advance would provide greater sensitivity in assessing the microbiological safety of water and food. The principle goal of this presentation is to describe novel approaches we have formulated for the rapid and simultaneous detection of bacteria plus the determination of their physiological activity in water and other environmental samples. The present version of our method involves the concentration of organisms by membrane filtration or immunomagnetic separation and combines an intracellular fluorochrome (CTC) for assessment of respiratory activity plus fluorescent-labelled antibody detection of specific bacteria. This approach has also been successfully used to demonstrate spatial and temporal heterogeneities of physiological activities in biofilms when coupled with cryosectioning. Candidate physiological stains include those capable of determining respiratory activity, membrane potential, membrane integrity, growth rate and cellular enzymatic activities. Results obtained thus far indicate that immunomagnetic separation can provide a high degree of sensitivity in the recovery of seeded target bacteria (*Escherichia coli* O157:H7) in water and hamburger. The captured and stained target bacteria are then enumerated by either conventional fluorescence microscopy or ChemScan®, a new instrument that is very sensitive and rapid. The ChemScan® laser scanning instrument (Chemunex, Paris, France) provides the detection of individual fluorescently labelled bacterial cells using three emission channels in less than 5 min. A high degree of correlation has been demonstrated between results obtained with the ChemScan and traditional plate counts of mixed natural bacterial populations in water. The continuing evolution of these methods will be valuable in the rapid and accurate analysis of environmental samples.

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2. INTRODUCTION

For over 100 years, microbiologists have used agar-based media for the detection and enumeration of bacteria in clinical and environmental samples. Pure cultures of bacteria in the laboratory appear to respond well in these procedures. In contrast, bacteria in environmental samples are usually exposed to various forms of stress which impair or restrict their growth response. Chemical and physical heterogeneities in 'real world' environments and the variability of microbial niches and populations may further affect the reliability of bacterial detection techniques.

Winogradsky (1949) recognized the failure of some bacteria to form colonies on solid media and subsequently others have

confirmed these concerns (McFeters *et al.* 1994). Only a small proportion, possibly less than 1%, of the number of viable bacteria may be enumerated in some samples (McFeters 1990). These observations have led to developments such as direct microscopic methods, particularly in the last decade, which may ultimately provide microbiologists with simple, rapid methods for the detection of specific viable bacteria. The concepts of bacterial injury (McFeters 1990) and 'viable but non-culturable' cells (Roszak and Colwell 1987; Desmonts *et al.* 1990, 1992) have been proposed as explanations for discrepancies between methods.

2.1. Direct counts

Direct count (DC) is a general term which refers to direct microscopic methods for enumerating bacteria. The most common of these is the acridine orange direct count (AODC), in which bacteria in a sample of water are collected by membrane filtration and stained with acridine orange (Hobbie *et al.* 1977). This stain is a fluorochrome which causes bacteria to fluoresce green or shades of red through orange. The wavelength of fluorescence depends on the staining conditions and the physiological state of the bacteria (McFeters *et al.* 1991) but the stain colour does not reliably reflect cell viability or activity in all bacteria and circumstances.

Stains other than acridine orange, including euchrysin, fluorescein isothiocyanate, rhodamine B, and acid fuchsin (Pettipher 1983), ethidium bromide (Williamson and Palfarman 1989), or 4′,6-diamidino-2-phenylindole (DAPI) (Kepner and Pratt 1994) may be preferred for the direct count as these may give better fluorescence while not staining other material to the same extent. Direct count methods can be used to monitor changes in total bacterial numbers but none provide discrimination between living and dead or active and inactive bacterial cells.

2.2. Direct viable counts

The direct viable count (DVC) is a procedure in which viable bacteria are enumerated by microscopy rather than routine cultural methods. Several approaches have been developed. In the method of Kogure *et al.* (1979), bacteria are incubated for a few hours with a quinolone antibiotic, usually nalidixic acid, which prevents cell division and causes viable (nutrient responsive) cells to elongate or enlarge. The nutrient and antibiotic concentrations should be optimized for the target organisms. The technique is applicable to both healthy and injured cells, and can be automated using image analysis (Singh *et al.* 1990). When applied to the direct assessment of disinfection of bacteria in biofilms, the DVC technique may indicate a larger number of survivors than would be detected by traditional plate counts (Yu *et al.* 1993).

Bacterial viability has also been measured by short-term incubation followed by counting of microcolonies. Postgate *et al.* (1961) incubated *Aerobacter aerogenes* (now *Enterobacter aerogenes*) on films of agar composed of continuous culture medium supplemented with yeast extract, casamino acids, and tryptic meat broth. After incubation for 2–4·5 h at 37 °C, microcolonies were counted by phase contrast microscopy. Although the technique has not been widely employed, it has been used to assess bacterial injury (Zaske *et al.* 1980) and modified for use with polycarbonate membranes incubated on selective agar media. The method 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 c.f.u. g^{-1} (Roderigues and Kroll 1988, 1989). The addition of a 3–5 h resuscitation step in tryptone soy broth permitted reliable estimates of injured bacteria in frozen and heat-processed foods (Roderigues and Kroll 1989). For this microcolony epifluorescence microscopy (MEM) method, microcolonies on membranes were stained with acridine orange before examination by epifluorescence microscopy. Image analysis has been used for enumeration of microcolonies formed by bacteria from pharmaceutical grade water, although background material resulted in overestimated counts for some samples (Newby 1991).

Both the DVC method and MEM rely on a growth response which is very similar to the requirements for colony or turbidity formation on solid or broth media. However, both have the advantage of relative rapidity compared to traditional culture methods.

3. PHYSIOLOGICAL ACTIVITY

Among microbiologists, the concepts of viability and physiological activity are a matter of continued philosophical debate (McFeters *et al.* 1994). In some environments there may be viable bacteria which are not detected using known cultural methods, as described above. Many of these bacteria demonstrate physiological or metabolic activity. It may be concluded that specific measurements of cellular activities provide a better estimate of the significance of bacteria in a particular environment than measurements of growth or colony formation on particular media. Furthermore, measurements which detect the activity of a whole community may not indicate differences between cells of different types or those located in distinct physicochemical situations within an environment. Examples include the distinction between planktonic and sessile cells, and the gradients which may occur within biofilms. For these reasons an increasing emphasis is being placed on direct determination of the activity of single cells.

3.1. Tetrazolium reduction

An electron transport indicator, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) has been widely

used to indicate respiratory activity (Zimmerman *et al.* 1978). In metabolically active cells, the electron transport system of the cell reduces INT to INT-formazan crystals which accumulate in the cell and are visible under bright-field microscopy as red deposits. The use of a counterstain such as acridine orange, malachite green (Dutton *et al.* 1986), DAPI (King and Parker 1988), or ethidium bromide (Swannell and Williamson 1988) facilitates counting of cells which contain INT-formazan. In some cases with fluorochrome staining, it is difficult to see the INT-formazan crystals. A filter-transfer-freeze procedure (King and Parker 1988) or a gelatin impression transfer (Kidd-Haack *et al.* 1985) may overcome this difficulty.

3.2. Fluorgenic physiological probes

In our laboratory, we are currently evaluating rapid methods which directly indicate physiological activities in bacteria using various fluorogenic probes of physiological activities (Yu and McFeters 1994a,b; Huang *et al.* 1995, 1998). These methods include 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a fluorescent compound which is similar to INT in that it also indicates respiratory activity (Rodriguez *et al.* 1992; Smith and McFeters 1997). The medium composition, particularly with respect to phosphate, may affect CTC reduction (Pyle *et al.* 1995a; Smith and McFeters 1996).

Determining the presence or absence of a membrane potential is another approach to assess physiological activity. Rhodamine 123 (Rh123) is a lipophilic cationic stain that is preferentially accumulated in cells possessing a membrane potential (Haugland 1996). Conversely, the lipophilic anionic stain, bis-(1,3-dibutylbarbituric acid) trimethine oxanol [$DiBAC_4(3)$], is excluded from cells with a membrane potential but accumulates intracellularly once the membrane potential is dissipated. Accordingly, $DiBAC_4(3)$ is used to detect the absence of a membrane potential (Haugland 1996).

Using multiple fluorochromes to assess physiological activity in healthy cultures of *Klebsiella pneumoniae*, found DVC, CTC and Rh123 provided comparable results which were always at least 1 log greater than those obtained by plate counts (Yu and McFeters 1994b). Thus, when cells are stressed or injured, the rapid direct methods using physiological probes may provide a more conservative and possibly more realistic assessment of bacterial survival. CTC was also used to follow the spatial and temporal progression of the effects of chlorine on bacterial respiration within intact biofilms (Huang *et al.* 1995). A gradient of respiratory activity developed, with exposure to disinfectant, and progressively moved deeper into the biofilm community structure over time.

Rh123 and CTC have also been used in conjunction with flow cytometry (Diaper *et al.* 1992; Kaprelyants and Kell 1992, 1993), and CTC has been used to quantify planktonic and sessile bacteria in drinking water (Schaule *et al.* 1993). All of these methods are non-specific. Hence, although they may be used with pure cultures, they will not differentiate between bacterial species when applied to mixed populations.

Fluorogenic stains for alkaline phosphatase, an enzyme produced in response to phosphate deprivation, were also used in mature biofilms to demonstrate spatial physiological heterogeneity with respect to gene expression (Huang *et al.* 1998). Following the withdrawal of phosphate from the bulk fluid medium, a discrete band of enzyme expression was observed in the top strata of mature biofilms that were exposed to nutrient media. This expression was directly related to the penetration of oxygen in biofilms composed of the obligate aerobe, *Pseudomonas aeruginosa*.

Other physiological probes are discussed elsewhere (McFeters *et al.* 1994, 1995).

3.3. Effect of environmental stress on rapid detection methods

We have used a suite of these stains and probes, in conjunction with viable plate counts, to assess the effect of chlorine disinfection on membrane potential [Rh123 and $DiBAC_4(3)$], membrane integrity (Live/Dead *Bac*Light kit), respiratory activity (CTC) and substrate responsiveness (DVC) in the pathogen *E. coli* O157:H7. After a 5-min exposure to the disinfectant, physiological indices were affected in the following order: viable plate counts > substrate responsiveness > membrane potential > respiratory activity > membrane integrity. *In-situ* assessment of physiological activity using a multi-phasic approach, as demonstrated in this study, permits more comprehensive decisions to be made in regard to determining the site and extent of injury in bacterial cells.

In related experiments, cultures of the pathogen *E. coli* O157:H7 were starved in M9 minimal medium, to which no carbon source had been added, for 14 days at 21–23 °C. Samples were removed on the day of inoculation and subsequently on days 2, 5, 10 and 14. Assays were performed to assess the effects of starvation on membrane potential [Rh123 and $DiBAC_4(3)$], membrane integrity (Live/Dead *Bac*Light kit), respiratory activity (CTC), intracellular esterase activity (ChemChrome $V3^R$) and substrate responsiveness (DVC) in conjunction with viable plate counts (R2A agar). Additional assays were performed to demonstrate the influence of starvation on susceptibility to disinfection with 0·5 p.p.m. chlorine (5 min at 21–23 °C, pH 7·5). Results indicated that during the time interval between culture inoculation and day 5, all assays used in this study demonstrated increased levels of various activities which remained relatively constant through day 14. However, there was a slight decrease in membrane potential and substrate responsiveness during this time interval. Interestingly, resistance to chlorine disinfection showed

a significant increase, as measured by per cent injury. The degree of injury from exposure to this concentration of disinfectant decreased from 92·6% to 44·3% by day 5 and remained relatively constant through day 14 (35·3%). These results indicate the starvation conditions used in this study permit this strain of *E. coli* to maintain an active physiological state while increasing resistance to disinfection with chlorine.

4. DIRECT METHODS WHICH DIFFERENTIATE SPECIES

4.1. Fluorescent antibody methods

Antibodies may be used to detect specific bacteria with several techniques, including the fluorescent antibody (FA) method. For this approach, an antibody which has been labelled with a fluorescent molecule is incubated with cells in the sample. Cells to which the FA attaches are detected by epifluorescent microscopy.

To enumerate specific bacteria by direct count methods in public health and water microbiology, FA techniques have been used to detect coliform bacteria, salmonellae, shigellae, enterococci, *Legionella*, nitrogen cycling organisms (Bohlool and Schmidt 1980), and more recently *Vibrio* spp. (Brayton and Colwell 1987) and enterobacteria (Hubner *et al.* 1992). A drawback of this procedure for environmental samples is that each antibody is highly specific so that polyvalent sera for a wide range of strains of the target species would be needed (Herbert 1990). The limitations of specificity, autofluorescence and nonspecific adsorption, antigen stability, and quantification are discussed in detail by Bohlool and Schmidt (1980).

Fluorescent antibody methods may be performed directly on membrane filters to avoid the need for fixation on glass slides and to permit sample concentration. The genus *Thermus* was detected directly on black polycarbonate membranes using a membrane filter-fluorescent antibody (MF-FA) technique (Cochran-Stafira and Strazak 1989). The MF-FA procedure included gelatin treatment to block non-specific binding of the fluorescent antibody.

Legionella pneumophila in an aquatic biofilm were visualized by episcopic differential interference contrast (Nomarsky) microscopy following reaction with monoclonal antibodies which were conjugated with immunogold or fluorescein isothiocyanate (Rogers and Keevil 1992). This permitted simultaneous visualization of the total biofilm flora and gold-labelled legionellae. The occurrence of *Legionella* microcolonies within the biofilm in the absence of amoebae was observed.

4.2. Molecular techniques

The proliferation of molecular biology methods in recent years has led to the polymerase chain reaction (PCR) technique. While the PCR has been employed for detection of natural bacterial populations in water, (e.g., Bej *et al.* 1991), the procedure is tedious and lengthy, requiring specialized thermal cycling equipment. Furthermore, no PCR method has been proven reliable for the detection, enumeration and examination of viable cells, because nucleic acid fragments from cells which may have been alive or dead, metabolically active or inactive, or even from previously lysed cells, may be amplified. This limitation appears to be pervasive in all of the molecular methods although a recent study has suggested that methods to detect mRNA represent a promising approach for distinguishing bacterial viability (Sheridan *et al.* 1998).

Oligonucleotide probes have been developed for a wide range of bacteria (Ward *et al.* 1992), and fluorescently-labelled oligonucleotide (FO) probes have been investigated using an *E. coli* 16s rRNA sequence and two eubacterial sequences in conjunction with DAPI for *in-situ* total direct counts in water samples (Hicks *et al.* 1992). Similarly, single bacterial cells have been identified using digoxigenin-labelled rRNA probes (Zarda *et al.* 1991), and probes have been used for detection of micro-organisms in soil (Hahn *et al.* 1992). Direct microscopic examination was employed following staining with DAPI, gel transfer, and hybridization and rhodamine-labelled probes. The cellular concentration of RNA has also been used as an indirect measure of growth rate through the use of molecular probes (Delong *et al.* 1989) and acridine orange (Wentland *et al.* 1996).

Although the FA and FO techniques are rapid and specific, they are of little value in establishing cell viability or physiological activity. The combination of these specific detection methods with rapid techniques for the assessment of viability or activity has the potential for further development. For example, to detect viable cells of specific bacteria, microcolony formation has been combined with immunofluorescence for the detection of viable *Listeria* (Sheridan *et al.* 1991) and *Salmonella* (Roderigues and Kroll 1990) in meat. 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 has also been successfully combined with immunofluorescence for the detection of viable *V. cholerae* (Brayton and Colwell 1987), and *E. coli* and *S. enteritidis* (Roszak and Colwell 1978). At various stages of cell survival in laboratory microcosms, good correlation was obtained for cell metabolic activity (measured by microautoradiography) and substrate responsiveness. Desmonts *et al.* (1990) employed the DVC-FA method to detect viable *Salmonella* spp. in chlorinated wastewater. They later modified the procedure by performing the DVC incubation directly on polycarbonate membranes to detect *Salmonella* in seawater (Desmonts *et al.* 1992).

5. COMBINED METHODS

The combination of the DVC and MEM procedures with FA techniques suggests the possibility of combining other approaches such as direct assessment of physiological activity with either FA or FO probes. As these methods are refined and modified for use with concentration techniques such as membrane filtration, an array of simple, rapid and direct methods for the detection and enumeration of specific viable bacteria will emerge. If these direct microscopic methods are to be adapted for automation by image analysis, attention may need to be paid to sample concentration and clarification. Likely sources for solutions to these problems may include the food and dairy industries. Developments in microscopy, including scanning confocal laser systems, may also permit enhanced discrimination and spatial evaluation (McFeters *et al.* 1994).

Recent developments in our laboratory have led to a combination method that has great potential for the rapid detection and quantification of specific, active bacteria in environmental samples (Pyle *et al.* 1995b). This technique takes advantage of the specificity of fluorescent-antibodies to detect specific bacteria combined with a fluorogenic stain (CTC) to discriminate actively respiring cells. The application of other probes to this basic strategy, including fluorescent molecular signals to distinguish individual organisms or individual bacteria and a range of fluorogenic stains capable of revealing other physiological processes characteristic of viable bacteria, will provide greater methodological versatility in the rapid detection of viable organisms in water and other environmental systems. In addition, the recent availability of the ChemScan® instrument provides a unique rapid capability to detect and enumerate bacteria. This unique instrument uses a high-speed laser scanning system that is capable of enumerating all fluorescent bacterial signals on a 25 mm membrane filter within 5 min. This exciting combination of methods and instrument could make obsolete our historical dependence on the highly inaccurate practice of plate counting within the next decade or two.

Recent work examined the efficiency of bacterial detection by immunomagnetic separation (IMS) and the compatibility of IMS with CTC incubation to determine respiratory activity, using the pathogen *E. coli* O157:H7. Counter-staining with DAPI or a specific fluorescein-conjugated anti-O157 antibody following CTC incubation was used to allow visualization of bacteria by epifluorescence microscopy. Broth-grown *E. coli* O157:H7 were used to inoculate fresh ground beef (< 17% fat) or sterile 0·1% peptone. Inoculated meat was mixed with 0·1% peptone and homogenized in a stomacher. Extraction buffer (Tween 8, trypsin, and collagenase) was added, incubated 0·5 hours at room temperature (21–23 °C), and stomached. The suspension was filtered to remove coarse fibrous material. Super-paramagnetic beads (Bangs), ~ 0·6 μm diameter, coated with Difco anti-O157 antibody were added and incubated for 1 h (21–23 °C, 60 rev min^{-1}). MacConkey Sorbitol agar plate counts indicated that 96% of the cells were removed from the meat suspensions containing 10^1–10^6 c.f.u. ml^{-1}. With inoculated meat, regression results for log-transformed respiring (CTC-positive) cell counts *vs* sorbitol-positive plate counts were: intercept = 1·37; slope = 0·77; r^2 = 0·95 (n = 12). Comparable results for inoculated liquids were: intercept = 0·74; slope = 0·86; r^2 = 0·98 (n = 22). Thus, within less than 8 h, the IMS/CTC/FA method detected essentially the same numbers of *E. coli* O157 cells as the plate count results which required 24 h of incubation before enumeration.

6. MODIFIED MEMBRANE FILTRATION METHODS

Although not considered rapid, the versatility of conventional membrane filtration may be coupled with an enzyme specific assay medium (e.g., Colisure, Millipore) for the detection of both total coliform bacteria and *E. coli*. This technique might be attractive for some applications.

A similar approach was described by Reasoner and Geldreich (1989) where the bacteria were concentrated by membrane filtration followed by incubation in the presence of ^{14}C-labelled mannitol. This method allowed the detection of faecal coliforms in 4·5 h using a two-temperature incubation (35 °C for 2 h then 44·5 °C for 2·5 h). Another approach might be to concentrate the waterborne bacteria by membrane filtration followed by detection, and possibly quantification, by the measurement of ATP. However, both of these techniques require specialized instrumentation.

The introduction of substrate-specific, presence/absence media coupled with changes in the drinking water regulation has had a significant effect on the way the drinking water industry determines potability in the USA. Only two such media have been approved by the USEPA; Colisure (Millipore) and Colilert (IDEXX). These media are generally regarded as superior to conventional methods for the routine analysis of indicator bacteria in potable water since the end points are easily observed and no confirmation is necessary. In both of these media, the hydrolysis of specific chromogenic (ONPG) and fluorogenic (MUG) substrates by unique enzymes provides a method to demonstrate the presence of total coliforms and/or *E. coli*, respectively. In our opinion, Colisure is preferred in many applications because the total coliform end-point colour is a bright magenta colour as opposed to a pale yellow in the case of Colilert. Since the temperature of incubation has also been optimized for the detection of *E. coli* at 41·5 °C (Sarhan and Foster 1991), results can be obtained in less than the standard 24 h incubation time. Another paper (Berg and Fiksdal 1988) reported that the addition of 0·02% sodium lauryl sulfate enhanced

MUG hydrolysis. These findings suggest that further optimization might be possible to enhance a more rapid response. Berg and Fiksdal (1988) also demonstrated the feasibility of using fluorimetry to follow MUG hydrolysis as a rapid assay for *E. coli* in water. It might be imagined that several litres of water could be passed through a membrane filter that would be inserted into a small volume of Colisure broth (possibly modified) medium and incubated. The macroscopic endpoints for both indicator bacteria could be easily observed. As additional benefits of this approach, reagent volumes would be small plus the medium and materials are currently available.

There are numerous applications for rapid methods. These include water and wastewater treatment, the food and dairy industries, pharmaceutical and electronic manufacturing, and medicine. Using image analysis and robotics, the possibility of rapid, automated monitoring for specific bacteria may be possible in the foreseeable future.

Despite the numerous shortcomings of traditional growth-dependent methods, they remain the cornerstone of microbiological quality assessment in most applied fields. Recently, Millipore Corporation has marketed a novel instrument called the Microstar®, capable of reducing the cultivation by a half to three-quarters the time required to observe macroscopic colonies on membrane filters following incubation on semisolid media. This instrument uses a highly sensitive photon counting system to detect the bioluminescent signal generated from ATP in bacterial microcolonies following lysis and the addition of luciferin-luciferase reagents. While this approach is attractive and will likely be useful in many applications, it still appears to be limited by many of the same constraints inherent in the more traditional growth-dependent methods. None the less, this instrument and approach has been applied to the accelerated detection of coliforms bacteria using the accepted media and methods from Japan (Tanaka *et al.* 1997).

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