Letters in Applied Microbiology 1996, **22**, 283–287

Variations in the fluorescence intensity of intact DAPI-stained bacteria and their implications for rapid bacterial quantification

J. Ross, P.I. Boon[1], R. Sharma and R. Beckett

Water Studies Centre and Cooperative Research Centre for Freshwater Ecology, Monash University, Melbourne, and [1]Department of Environmental Management, Victoria University of Technology, Melbourne, Australia

PJB/12: received 11 September 1995 and accepted 18 September 1995

J. ROSS, P.I. BOON, R. SHARMA AND R. BECKETT. 1996. As current techniques for the quantification of bacteria are laborious and often imprecise, instrumental approaches such as sedimentation field-flow fractionation (SdFFF) are attractive. In this technique, fluorogenic dyes specific for nucleic acids are used to identify bacterial cells. Bacterial biomass can be quantified directly with SdFFF if the specific fluorescence of bacterial cells is constant. The effect of different growth conditions on the specific fluorescence of one strain each of *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis* and *Staphylococcus epidermidis* stained with 4′,6-diamidino-2-phenylindole was examined. Specific fluorescence varied over a 500-fold range, from 0·22 to 103 arbitrary fluorescence units per cell. Specific fluorescence was highest when cells were in log phase, and lowest when cells were in stationary phase. Specific fluorescence decreased when cells harvested in log phase were starved for 7 d in a carbon-free minimal medium, and increased rapidly (within 2 h) after cells were relieved from carbon limitation. Such variations in specific fluorescence must be considered when using gross fluorescence as a direct indicator of bacterial numbers in the SdFFF technique for quantifying bacterial biomass. Moreover, they have serious implications for the application of fluorescence techniques in other instrumental approaches for bacterial enumeration in environmental samples.

INTRODUCTION

Quantifying the presence of bacteria is a fundamental aspect of almost all bacteriological studies. Plate counts are used widely for enumeration, but have severe disadvantages when applied to complex environmental samples. Moreover they indicate only viable bacterial numbers, not *in situ* bacterial biomass. Direct microscopy techniques, generally combining eipfluorescence microscopy and nucleotide-specific dyes, are now a favoured technique for determining bacterial abundance, especially with environmental samples (Fry 1988). These methods, however, are laborious and there are severe limits on the number of samples that can be processed in a given time. The counting itself is also subjective to some extent. Such factors affect the precision and accuracy of the enumeration. If bacterial biomass needs to be quantified, dimensions of individual cells are measured, biovolumes calculated, and conversion factors applied to arrive at a total biomass value (e.g. Boon 1991). This involves a number of steps, each of which is subject to a variety of potential errors (Karl 1986; Fry 1988; Krambeck *et al.* 1990). Overall errors of up to sixfold can be expected if bacterial biomass is estimated in this way (Sharma *et al.* 1993).

These limitations have prompted a search for alternatives that are faster and more accurate. Sedimentation field-flow fractionation (SdFFF) is an instrumental approach that can be used to determine bacterial biomass in samples from complex or natural environments (Sharma *et al.* 1993; Sharma 1994). It is a chromatography-like analytical separation technique that uses a thin ribbon-shaped channel subjected to centrifugal force. Particles, such as bacteria, elute at different volumes according to their effective mass (see Giddings *et al.* 1980 and Beckett *et al.* 1988 for details). Before injection, the bacteria are stained with a nucleic-acid specific fluorogenic

Correspondence to: Dr R. Beckett, Water Studies Centre, Monash University, PO Box 197, Caulfield East, Victoria 3145, Australia.

dye (such as 4′,6-diamidino-2-phenylindole; DAPI) and are detected after separation by measuring the fluorescent signal generated. If fluorescence per cell has a constant and known value, the measurement of biomass is direct, rapid and automated. If specific fluorescence varies, the number of cells must be manually determined, usually with epifluorescence microscopy. This introduces an additional step, detracting significantly from the power and elegance of the SdFFF method. Sharma *et al.* (1993) found that for single cultured species harvested at a specific time, specific fluorescence was constant across cell diameter in the sample. It is not clear, however, whether bacteria from complex or natural environments (where cells may be growing slowly or rapidly, or be starved or dormant) have the same characteristic.

It is apparent that a major factor currently limiting the application of SdFFF to the rapid determination of bacterial biomass is uncertainty as to the variation in specific fluorescence of intact, DAPI-stained bacteria. In this paper, we report on measurements of the specific fluorescence of four genera of bacteria under different growth phases, including starvation and subsequent recovery, to quantify the variation in specific fluorescence of intact bacterial cells.

MATERIALS AND METHODS

Bacteria

The four strains of bacteria were: *Escherichia coli* (from Microbiology Department, Monash University, Clayton, Australia), *Pseudomonas aeruginosa* (isolated from the Ovens River, north-east Victoria; Boon 1991); *Proteus mirabilis* and *Staphylococcus epidermidis* (both from Department of Environmental Management, Victoria University of Technology, St Albans, Australia). *Escherichia coli* has a G+C content of 51–53% and a genome size of $2{\cdot}4$–$2{\cdot}7 \times 10^9$ Da (Gillis *et al.* 1970). We have no comparable data for the *Ps. aeruginosa* strain, but other *Pseudomonas* species have G+C contents of 58–70% and genome sizes of $2{\cdot}5$–$2{\cdot}7 \times 10^9$ Da (Gillis *et al.* 1970; Prescott *et al.* 1993). *Proteus* spp. have G+C contents of 38–41% and genome sizes of $2{\cdot}0$–$2{\cdot}1 \times 10^9$ Da (Wallace and Morowitz 1973; Prescott *et al.* 1993). Various *Staphlococcus* species have G+C contents of 30–38% and genome sizes of $1{\cdot}1$–$1{\cdot}4 \times 10^9$ Da (Wallace and Morowitz 1973; Prescott *et al.* 1993).

Culture conditions

An inoculum culture of each strain was obtained by growing bacteria for 18 or 48 h (depending on the growth rates of the various taxa) in 10 ml of LB medium (Sambrook *et al.* 1989) at 37°C, with constant shaking. Fresh LB medium (80 ml) was inoculated with each culture (80 μl), incubated with shaking, and growth measured spectrophotometrically (600 nm) at frequent intervals. Subsamples (10 ml) were taken at various stages of growth, fixed with formalin solution (4% v/v, final concentration) and stored at 4°C.

To examine the effect of starvation and subsequent metabolic recovery on specific fluorescence, 15 ml of bacterial culture were removed when cells were in log phase, centrifuged at 12 000 *g* for 5 min, the supernatant fluid taken off, and the pellet resuspended in 15 ml of M9 minimal medium lacking a carbon source (Sambrook *et al.* 1989). The centrifugation-washing step was performed twice on each culture, to maximize the removal of residual nutrient solution. The harvested bacteria were starved, constantly shaken, in M9 minimal media at 22°C for 7 d. At the end of the starvation period, cells were revived by adding 1·6 ml of fresh 10 × LB medium to 15 ml of each bacterial starvation culture. Subsamples were taken 0, 2, 8 and 24 h after adding the new medium for processing as described below.

Specific fluorescence

Cells were stained with DAPI (0·95 mg l^{-1}, final concentration) for 20 min. To remove unincorporated DAPI and minimize background fluorescence, cell suspensions were washed and centrifuged twice (29 000 *g*) with 0·1 mmol l^{-1} aqueous tetrasodium pyrophosphate. Total fluorescence (excitation = 350 nm; emission = 460 nm; 18 nm bandwidth) was measured immediately by pumping the DAPI-stained cell suspensions through a Waters 470 scanning fluorescence detector (flow cell volume = 16 μl) at 0·28 ml min^{-1}. Cell numbers were determined with u.v.-excitation epifluorescence microscopy, using an Olympus BH-2 microscope. Two or three irgalan-black stained filters were prepared per sample, and 10–40 fields of view examined per filter. Specific fluorescence was calculated by dividing the fluorescence signal by the number of cells in the flow cell.

RESULTS

Figure 1 shows specific fluorescence of the four bacterial strains at various stages of growth, after starvation, and after being relieved from carbon limitation. Data are comparable across species and times because a single fluorimeter was used for all measurements. Specific fluorescence varied from 0·22 arbitrary fluorescence units (AFU) per cell (*E. coli* in late stationary phase) to 103 AFU $cell^{-1}$ (*Staph. epidermidis* 8 h after recovery from starvation). Specific fluorescence was always markedly greater for cells in early log phase than for those in late log or stationary phase. The week-long starvation in minimal medium caused a marked decrease in specific fluorescence compared with log-phase cells: specific fluorescence for *E. coli* decreased from 4·0 AFU $cell^{-1}$ at log phase to 1·5 AFU $cell^{-1}$ after a week of starvation; *Pr. mirabilis* from 13·1 to 4·5 AFU $cell^{-1}$; *Ps. aeruginosa* from 16·3 to 1·7

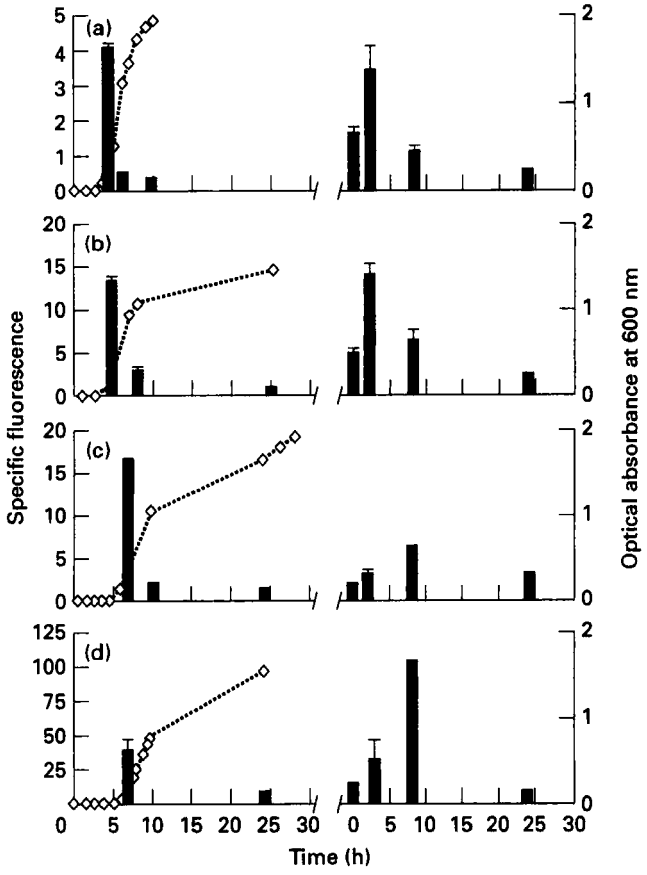

Fig. 1 Variations in the cell-specific fluorescence of four bacterial taxa harvested at various growth phases and after 7 d starvation and subsequent recovery. The left-hand axis shows cell-specific fluorescence (bar graph) and the right-hand axis shows optical absorbance at 600 nm (line graph). Means and standard deviations are shown ($n = 2$); in some cases the standard deviation is too small to be shown on the figure. (a) *Escherichia coli*; (b) *Proteus mirabilis*; (c) *Pseudomonas aeruginosa*; (d) *Staphylococcus epidermidis*

AFU cell^{-1}, and *Staph. epidermidis* from 37·3 to 5·3 AFU cell^{-1}. Specific fluorescence increased within 2 h of relieving cells of their carbon limitation. After 24 h from adding the fresh medium, specific fluorescences returned to values not dissimilar to those measured for stationary-phase cells in the earlier experiments.

DISCUSSION

Bacteria in samples from complex or natural environments will come from a diversity of taxa and be in a wide range of growth conditions. Our finding that the specific fluorescence of intact bacterial cells stained with DAPI varied by over 500-fold means it is impossible to determine total bacterial biomass of complex or environmental samples directly using SdFFF with fluorescence detection. Subsamples from SdFFF runs will need to be taken at various points along the fractogram, and the number of bacteria in each of these fractions determined with traditional methods of enumeration, such as epifluorescence microscopy. These values combined with data on biomass per cell generated directly by SdFFF will allow the calculation of total bacterial biomass (Sharma 1994). Note that the SdFFF procedure yields biomass per cell directly, despite the variation in specific fluorescence. The calibration is used only to generate information on bacterial numbers. This use of epifluorescence microscopy for enumeration will not be subject to the wider range of errors that occur when microscopy is used for determining bacterial biomass.

The magnitude of the variation we have established also has significant implications for other techniques used in microbial ecology. Great care must be taken when using automated image analysis of fluorescently-stained preparations, lest poorly-fluorescing cells be missed. Starved cells, which are common in natural environments, fluoresce only weakly and so could be easily overlooked if threshold levels were set too high. Variation in specific fluorescence also affects the accuracy of sizing fluorescently-labelled bacteria. The extent of the halo around the cell is influenced markedly by the intensity with which the cells fluoresce (Fry 1988; Krambeck *et al.* 1990). This halo effect is especially troublesome with photographic preparations, and thus for the manual estimation of bacterial biovolume via epifluorescence microscopy (Fry 1988).

The variations have significance for indirect estimates of bacterial abundance using DNA contents (e.g. Walser and Güde 1994). The specific fluorescence of DAPI-stained intact bacteria is determined in part by the DNA content of the cells, and indeed this phenomenon has been widely used for the quantification of cellular DNA contents via flow cytometry (Crissman and Steinkamp 1990). The magnitude of variation in specific fluorescence that we detected suggests that the bacterial DNA content varies at least 10-fold, according to taxa and nutritional status of the cells. Bacterial DNA content is thus sufficiently variable that indirect approaches for measuring bacterial biomass, that require a reasonably constant cellular DNA content, must be viewed with scepticism.

The results presented here indicate clearly the significance of growth phase, and thus number of copies of the genome per cell, in affecting specific fluorescence. Our finding that specific fluorescence was greatest in cells harvested in early log phase is consistent with the fact that rapidly-dividing cells have multiple copies of their genomic material (Christensen *et al.* 1993).

The situation with starved bacteria is more complex. Early in starvation, especially if onset is rapid, cellular DNA content may continue to increase as bacteria are committed to com-

plete already initiated rounds of DNA replication. With the slow onset of starvation or upon extended starvation, starved cells probably have only a single copy of the genome. The results presented here, indicating very low specific fluorescence of starved bacteria, are consistent with the observation of others that starved cells typically have a single copy of their genome e.g. *E. coli* (Christensen *et al.* 1993). As an additional effect, the DNA content of starved bacteria may decrease by as much as 46% over 6 weeks, presumably due to the breakdown of extrachromosomal DNA or partially replicated copies of the chromosome (Dawes 1989). Once starved bacteria are released from their carbon or nutrient limitation, the number of genomes per cell increases rapidly. For example, in *Salm. typhimurium* cellular genome content increased from 1·5 to 3 within 40 min of nutrient upshift (Kjeldgaard *et al.* 1958). The results presented here are also consistent with this early finding, since specific fluorescence increased soon after bacteria were relieved from starvation.

The combined effects of genome size and number of copies of the genome can probably account for about a 10-fold variation in the DNA content of intact bacterial cells. We detected variations over 500-fold in specific fluorescence of DAPI-stained cells, so other factors must be operative. The bacterial genome is a circular supercoil, and access of the fluorochrome to binding sites may alter among bacterial strains if the DNA is packaged differently in different cells. The topological state of bacterial DNA is affected by a range of stress factors (Oliver 1993; van Elsas and van Overbeek 1993), but the effect on specific fluorescence is unclear.

Taxonomic position appears to affect the specific fluorescence of intact bacteria, as the specific fluorescence of *Staph. epidermidis* was higher than for the other species at similar stages of the growth curve. The factors responsible are difficult to elucidate. Some of the variation may be due to interactions between DAPI and cell surfaces of formalin-fixed bacteria (Zweifel and Hangström 1995).

DAPI binds preferentially to the A-T base residues of DNA, giving less fluorescence with G-C base pairs (Lin *et al.* 1977; Larsen *et al.* 1989), leading to differences in specific fluorescence of bacterial taxa with different base-pair compositions, even if their genomes are the same size. Although the low G+C ratio of *Staph. epidermidis* would give greater specific fluorescence than taxa having higher G+C ratios, this effect is unlikely to be sufficient to account for the 5–10-fold difference shown in Fig. 1. The empirical significance of variations in base-pair composition is not clear, since the G+C ratios of *Ps. aeruginosa* and *E. coli* are not greatly dissimilar yet the specific fluorescence of log-phase cells of these two taxa varied by a factor of 4. In spite of also having a low G+C ratio and, presumably, a larger genome, *Pr. mirabilis* did not yield such high specific fluorescence as did *Staph. epidermidis.*

Variations in specific fluorescence may also be related to the choice of fluorogenic stain. The Hoechst family of benzimidazole derivatives offer potential for use with SdFFF, as they may give a lower background fluorescence and smaller coefficients of variation than DAPI (Paul and Myers 1982; Monger and Landry 1993). However, measurements obtained with the Hoechst dyes will suffer from all the other sources of variation discussed above, including selectivity towards A-T residues.

It may yet be possible to determine bacterial biomass directly from the SdFFF fractogram, by detecting and counting individual fluorescent cells. With this direct counting approach there is no requirement for a constant specific fluorescence of stained cells. Automated single particle counters utilizing the fluorescence signal of individual cells are currently available in the form of flow cytometers. Single purpose instruments with higher sensitivity and lower cost are required to make the application to SdFFF an attractive proposition. This possibility is currently being investigated.

REFERENCES

Beckett, R., Nicholson, G., Hart, B.T., Hansen, M. and Giddings, J.C. (1988) Separation and size characterization of colloidal particles in river water by sedimentation field-flow fractionation. *Water Research* **22**, 1535–1545.

Boon, P.I. (1991) Bacterial assemblages in rivers and billabongs of southeastern Australia. *Microbial Ecology* **22**, 27–52.

Christensen, H., Bakken, L.R. and Olsen, R.A. (1993) Soil bacterial DNA and biovolume profiles measured by flow cytometry. *FEMS Microbiology Ecology* **102**, 129–140.

Crissman, H.A. and Steinkamp, J.A. (1990) Cytochemical techniques for multivariate analysis of DNA and other cellular constituents. In *Flow Cytometry and Sorting*, 2nd edn, ed. Melamed, M.R., Lindmo, T. and Mendelsohn, M.L. pp. 227–247. New York: Wiley-Liss.

Dawes, E.A. (1989) Growth and survival of bacteria. In *Bacteria in Nature. Vol. 3. Structure, Physiology and Genetic Adaptability* ed. Poindexter, J.S. and Leadbetter, E.R. pp. 67–187. New York: Plenum.

Fry, C. (1988) Determination of biomass. In *Methods in Aquatic Bacteriology* ed. Austin, B. pp. 27–72. Chichester: John Wiley.

Giddings, J.C., Myers, M.N., Caldwell, K.D. and Fisher, S.R. (1980) Analysis of biological macromolecules and particles by field-flow fractionation. In *Methods of Biochemical Analysis. Vol. 26* ed. Glick, D. pp. 79–136. New York: John Wiley.

Gillis, M., de Ley, J. and de Cleene, M. (1970) The determination of molecular weight of bacterial genome DNA from renaturation rates. *European Journal of Biochemistry* **12**, 143–153.

Karl, D.M. (1986) Determination of in situ microbial biomass, viability, metabolism, and growth. In *Bacteria in Nature. Vol. 2. Methods and Special Applications in Bacterial Ecology* ed. Poindexter, J.S. and Leadbetter, E.R. pp. 85–176. New York: Plenum.

Kjeldgaard, N.O., Maaløe, O. and Schaechter, M. (1958) The transition between different physiological states during balanced growth of *Salmonella typhimurium*. *Journal of General Microbiology* **19**, 607–616.

Krambeck, C., Krambeck, H.J., Schroeder, D. and Newell, S.Y. (1990) Sizing bacterioplankton: a juxtaposition of bias due to shrinkage, halos, subjectivity in image interpretation and asymmetric distributions. *Binary* **2**, 5–14.

Larsen, T.A., Goodsell, D.S., Cascio, D., Grezeskowiak, K. and Dickerson, R.E. (1989) The structure of DAPI bound to DNA. *Journal of Biomolecular Structure and Dynamics* **7**, 477–491.

Lin, M.S., Comings, D.E. and Alfi, O.S. (1977) Optical studies of the interaction of 4′-6′-diamidino-2-phenylindole with DNA and metaphase chromosomes. *Chromosoma* **60**, 15–25.

Monger, B.C. and Landry, M.R. (1993) Flow cytometric analysis of marine bacteria with Hoechst 33342. *Applied and Environmental Microbiology* **59**, 905–911.

Oliver, J.D. (1993) Formation of viable but nonculturable cells. In *Starvation in Bacteria* ed. Kjelleberg, S. pp. 239–272. New York: Plenum.

Paul, J.H. and Myers, B. (1982) Fluorometric determination of DNA in aquatic microorganisms by use of Hoechst 33258. *Applied and Environmental Microbiology* **43**, 1393–1399.

Prescott, L.M., Harley, J.P. and Klein, D.A. (1993) *Microbiology*, 2nd edn. Dubuque: Wm C. Brown.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Sharma, R. (1994) Physical characterization, quantification and biomass determination of bacteria using sedimentation field flow fractionation. PhD Thesis, Water Studies Centre, Monash University, Australia.

Sharma, R.V., Edwards, R.T. and Beckett, R. (1993) Physical characterization and quantification of bacteria by sedimentation field-flow fractionation. *Applied and Environmental Microbiology* **59**, 1864–1875.

van Elsas, J.D. and van Overbeek, L.S. (1993) Bacterial responses to soil stimuli. In *Starvation in Bacteria* ed. Kjelleberg, S. pp. 55–79. New York: Plenum.

Wallace, D.C. and Morowitz, H.J. (1973) Genome size and evolution. *Chromosoma* **40**, 121–126.

Walser, R. and Güde, H. (1994) DNA and RNA contents of bacteria-sized particles from Lake Constance. *Archiv für Hydrobiologie* **131**, 257–276.

Zweifel, U.L. and Hagström, Å. (1995) Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts). *Applied and Environmental Microbiology* **61**, 2180–2185.