Measurement of bioprocess containment by high flow rate air sampling and quantitative polymerase chain reaction

M. Noble^2, I. Lisi^1, J. M. Ward^2, A. D. Buss^3 & M. K. Turner^1*

^1* The Advanced Centre for Biochemical Engineering, Department of Chemical and Biochemical Engineering, University College London, Torrington Place, London, WC1E 7JE. Tel: +44 171 387 7050 ext.4415; email: michael.turner@ucl.ac.uk
^2. Department of Biochemistry and Molecular Biology, University College London, Gower Street, London, WC1E 6BT
^3. Bioprocessing Research Unit, Glaxo Wellcome Medicines Research Centre, Gannels Wood Road, Stevenage, Hertfordshire, SG1 2NY

*Corresponding author

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ABSTRACT

A competitive quantitative polymerase chain reaction (QPCR) method has been used to measure the concentration of cells of a genetically modified E. coli K-12 strain in aerosols released from bioprocess operations. The QPCR assay was shown to be specific for the process strain, and can measure the target organism concentration over a 6 decade range. Air sampling was achieved using an Aerodynamic General Cyclone which has a high sampling rate and is efficient in the capture of bacteria bearing aerosol particles. The release of process cells into fermenter exit-gas throughout a fermentation was monitored using the cyclone-QPCR methodology. It was found that over the course of a 5.5 hour period, 3 x 10^7 process cells were released. This number of cells is contained within less than 2 uL of the fermentation broth at harvest. Sampling of the air in the vicinity of a 50 L fermentation has shown that some cellular material is released during operation of the fermenter sampling valve. The extent of the releases recorded and the implications of the ability to measure bioprocess release are discussed with respect to the safe handling of recombinant microorganisms and the need to balance losses due to accidental and incidental release.

Keywords: bioprocess, containment, aerosol, air sampling, genetically modified micro-organism, E. coli K-12, polymerase chain reaction, fermentation, risk assessment.

INTRODUCTION

The design of bioprocesses to achieve effective containment of microorganisms, and the relationship between design and containment category, has received much consideration (Hambleton et al., 1991; Vranch, 1992). However, since
recommendations have generally been made in the absence of performance data, there is some question over the validity of design solutions (Titchener-Hooker et al., 1993). To evaluate the performance of a bioprocess design, in terms of containment, it is necessary to use a test method that will give a measure of the scale of any release of process microorganisms. Such methodology will also allow the contributions to overall release from routine operational (which we term 'incidental') and accidental failure to be assessed.

The release of microorganisms from bioprocesses may occur as a result of spillage or leakage of liquid or by the release into the air of bacteria-laden aerosols. Most attention has focused on release of microorganisms in aerosols since, in such a state, the released organisms may pose a threat to health and the environment and can not be easily contained or detected (Hambleton et al., 1992). Additionally, many bioprocess operations have the potential to release aerosols containing microorganisms (Dunnill, 1982; Hambleton et al., 1992).

Methods employed to detect the release of microorganisms from biotechnological processes include the placement of settle plates in the vicinity of the process (Tuijnenburg Muijs et al., 1987) and the active sampling of known volumes of air using an air sampling device (Cameron et al., 1987; Tuijnenburg Muijs et al., 1987; Tinnes and Hoare, 1992). Only active sampling techniques are likely to give quantitative results since the volume of air sampled can be measured. However, the method used to enumerate the collected microorganisms is often based on culture on an appropriate selective solid medium. The number of culturable cells recovered from aerosols may underestimate the total viable number present in the aerosol because of the effect of sampling stress (Alvarez et al., 1995). Additionally, it is now well accepted that a significant proportion of water borne viable cells may exist in a non-culturable state (Colwell et al., 1985), and that these viable but non-culturable cells can retain their pathogenicity (Colwell et al., 1990).

The polymerase chain reaction (PCR), which has been shown to detect non-viable bacterial pathogens (Josephson et al., 1993), has been extensively used for the detection of microorganisms in environmental samples such as soil (Picard et al., 1992) and water supplies (Bej et al., 1990, 1991a, b). Recently, researchers (Alvarez et al., 1994, 1995; Roll and Fujioka, 1995) have used PCR to detect airborne bacteria, and it has been shown that sampling stress does not affect detectability by PCR (Alvarez et al., 1995). Additionally, several workers (Mahon and Lax, 1993; Leser, 1995; Lee et al., 1996) have shown that quantitative PCR, using a competitive internal standard DNA approach (Becker-Andre and Hahlbrock, 1989; Gilliland et al., 1990), can be used to detect and measure bacteria in the environment.

In this study, we have developed a quantitative PCR method for the detection of a recombinant, plasmid bearing E. coli K-12 strain based on the competitive internal standard method. We show how this method can be applied to measurement of the microorganisms in aerosols collected by an Aerojet General glass cyclone impinger (Griffiths and DeCosemo, 1994; Upton et al., 1994). The cyclone has a high sampling efficiency for biological aerosols (Upton et al., 1994) and since the sample is collected into liquid, this can be used directly in the PCR. Additionally, the cyclone has a high volumetric sampling rate. Since the aim is to apply the developed methods in bioprocess suites where the source of a release might be removed from the sampling point and the room air is likely to be rapidly exchanged, then the high sampling rate of the cyclone is believed to offer a significant benefit.

In this paper we report the development of the methodology and demonstrate its application in monitoring the release of process microorganisms into unfiltered fermenter exit-gas and during the operation of a fermenter sampling valve.

MATERIALS AND METHODS

Microorganism strains and growth in shake flasks

The microorganism strains used in this study and their sources are listed in Table 1. E. coli JM107 pQR701 was the target strain for which the PCR was developed. All other strains were used to test the specificity of the PCR method. E. coli strains were grown at 37 C in 25 g L^-1 nutrient broth (Oxoid, Unipath Ltd., Basingstoke, UK) supplemented with 10 ug mL^-1 kanamycin (strains 1, 2, 3 and 5) or 100 ug mL^-1 ampicillin (strain 4). Cultures were grown overnight in 50 ml volumes in a 250 ml shake flask in an orbital shaker at 200 rpm. The yeast strains were grown up in 20 g L^-1 malt extract broth (Oxoid) at 28 C; otherwise the same procedure as for E. coli was used.

TABLE 1. Microorganism strains used in this study
<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Strain donated by:</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli JM107</td>
<td>Km^R, transketolase</td>
<td>Drs J. Ward^a</td>
<td>French and Ward, 1995)</td>
</tr>
<tr>
<td>pQR701</td>
<td></td>
<td>C. French^a</td>
<td>Yanisch-Perron et al., 1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli JM107</td>
<td>Km^R, alpha-amylase</td>
<td>Dr J. Ward^a</td>
<td>Bahri and Ward, 1990</td>
</tr>
<tr>
<td>pQR126</td>
<td></td>
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<td>E. coli JM83</td>
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<td>Drs J. Ward^a</td>
<td>Yanisch-Perron et al., 1985)</td>
</tr>
<tr>
<td>pQR187</td>
<td></td>
<td>C. French^a</td>
<td>et al., 1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli JM107</td>
<td>Ap^R, T4 lysozyme</td>
<td>Drs J. Ward^a</td>
<td>Yanisch-Perron et al., 1985)</td>
</tr>
<tr>
<td>pQR752</td>
<td></td>
<td>R. Sloane^a</td>
<td>et al., 1985)</td>
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<tr>
<td>E. coli JM107</td>
<td>Km^R, TOL meta-cleavage</td>
<td>Dr J. Ward^a</td>
<td>Yanisch-Perron et al., 1985)</td>
</tr>
<tr>
<td>pQR150</td>
<td></td>
<td></td>
<td>et al., 1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli JM107</td>
<td>unmodified</td>
<td></td>
<td>Yanisch-Perron et al., 1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae wt</td>
<td>unmodified</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>osmotically fragile</td>
<td>Prof. S.G. Oliver^b</td>
<td>Stateva et al., 1991</td>
</tr>
<tr>
<td>7d</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ap^R; ampicillin resistance: Km^R; kanamycin resistance: ^a; Department of Biochemistry and Molecular Biology, UCL, Gower Street, London, WC1E 6BT.
^b; Department of Biochemistry and Applied Molecular Biology, UMIST, Manchester, M60 1QD.
Strains 6 & 7 are available from the authors.

**Fermentation**

The medium used for fermentation studies was a modified version of T-broth (Tartof and Hobbs, 1987) comprising the following (concentrations in g L^-1 unless otherwise stated): KH2PO4, 2.3; K2HPO4, 3.8; bactotryptone (Oxoid), 8; yeast extract (Oxoid), 16; kanamycin (Sigma, Poole, Dorset), 0.02; glycerol, 3 ml L^-1; PPG (antifoam), 0.2 ml L^-1.

The 2 L fermenter was a glass vessel with a 6 blade Rushton turbine impeller (Inceltech (UK) Ltd., Reading, UK). The sparge rate was 1 v.v.m., (0.19 cm s^-1 superficial air flow rate). Inlet and outlet air lines were both 0.2 um filtered using Gelman Acro50 filters (Gelman Sciences, Ann Arbor, Michigan). The dissolved oxygen tension (DOT) was maintained above 20% by automatic variation of the stirrer speed between 300-1100 rpm. Temperature, pH and DOT were logged throughout the fermentation and exit gas was analysed by mass spectrometry when the exit line was not connected to the cyclone input. The fermenter was inoculated using an overnight shake flask culture; seed volume was 6%.

The 50 L fermenter (Chemap, BM Browne UK Ltd, Reading) was operated under the same conditions, using the same media. This fermenter was designed to provide complete primary containment; double mechanical rotary seals on the impeller are pressurised by sterile steam condensate. The sampling valves (shown schematically in Figure 1) are sterilised by steam.
**FIGURE 1.** Schematic representation of the contained sampling array fitted to the 50 L fermenter. The pre-sterilised sample bottle and piping are connected to the sampling line via the couplings A and B. The line is steamed through before and after taking the sample.

**Operation of the cyclone**

The air sampling device used in these studies was an Aerojet General Cyclone (Hampshire Glass Co., Southampton, Decker *et al.*, 1969; Upton *et al.*, 1994). The principle of operation is that airborne microorganisms drawn into the cyclone are deposited on the inner walls of the glass device due to the fast rotation of the air and are subsequently washed off by the circulating liquid. At the end of operation, all circulating liquid is collected, the volume is measured and the liquid sample is used in subsequent PCR assays. In these experiments, air was drawn into the cyclone by an air pump (Air Control Installations, Chard, Somerset, UK) at 360 L min^-1, and the collecting liquid, 80 mL thiosulphate Ringer's solution (Oxoid), was recirculated at 20 mL min^-1 using a peristaltic pump. Sampling was carried out in batch-mode for 10-25 minute periods. At the end of sampling, the volume of collection liquid was measured.

The cyclone was cleaned by immersion in 1% Tego solution (Th. Goldschmidt Ltd., Milton Keynes, UK) and soaking for a 15 minute period. During this time all tubing was wetted by squirting through the cleaning solution using a wash bottle. After the soaking stage, the cyclone and tubing were thoroughly rinsed with tap water and were allowed to dry by standing for 5-10 minutes. Fresh disposable containers were used for each sample. In early experiments, a 10% Chloros solution (Hays Chemical Distribution Ltd, Leeds, UK), which has been recommended for use in cleaning surfaces prior to PCR (Prince and Andrus, 1992), was found to be effective as a cleaning agent, but the glass of the cyclone became pitted.

**Microscopic cell counting**

The concentration of *E. coli* cells in suspension was determined by microscopic counting using a Helber Bacteria Counting Chamber with Thoma rulings (Weber Scientific International Ltd, Teddington, Middlesex, UK) at 400 magnification, using phase contrast. Yeast cells were counted using a haemocytometer with improved Neubauer rulings (Weber Scientific).

**Preparation of samples for PCR**

For the PCR of 'whole cells' during the development and validation of the PCR methods, a 0.8 mL sample of culture was taken 15-20 hours after inoculation. The A600 of this sample was measured and the cells in the sample were pelleted by centrifugation at 7000g for 2 minutes and resuspended in sterile thiosulphate Ringers solution to achieve a suspension of A600 of 2.5. Resuspended cells were diluted in sterile thiosulphate Ringers solution to achieve the desired concentration. The number of whole cells per ml of the samples was determined by microscopic counting of the 10^-2 dilution.

Purified plasmid DNA samples were prepared from an overnight culture of the relevant microorganism using a Wizard Miniprep Kit (Promega Corp, Madison, WI). Plasmid DNA was stored at -20 C and was used in the PCR without any further pretreatment. Dilutions of these DNA preparations were made using sterile reverse osmosis water (SROW) as the diluent. Samples derived from cyclone operation were used directly in the PCR without any pretreatment.

**Differentiation between total and extracellular plasmid concentration**

Extracellular plasmid (free in solution) was measured separately from total plasmid concentration according to the following method: a 0.5 mL sample was microfiltered by centrifugation for 5 minutes at 7000g in a Costar spin-X tube (0.2 um pore size, low DNA binding cellulose acetate membrane, Costar, Cambridge, MA). 10 uL of the filtrate was then used in the PCR and the results compared with those from an unfiltered sample.

**PCR**
A list of the primers used is given in Table 2.

TABLE 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'-3'</th>
<th>Anneals to;</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13R1</td>
<td>GGA CCA AGC TAT GAC CAT G</td>
<td>M13 sequence in mp18 derived vectors</td>
</tr>
<tr>
<td>CMTA1</td>
<td>CGT CAA AGA GTG TAT TGA GG</td>
<td>CmtA gene in transketolase insert in pQR701</td>
</tr>
<tr>
<td>TKL</td>
<td>GTG TAT TGA GGG ATC GAT</td>
<td>CmtA gene in transketolase insert in pQR701</td>
</tr>
</tbody>
</table>

The target strain used in these studies, *E. coli* JM107 pQR701, has a plasmid encoded transketolase gene which is derived from the chromosome of *E. coli*. Primers were therefore chosen such that the amplified section crosses the point of insertion of the *tkt* gene (Figure 2).

**FIGURE 2.** Schematic showing primer binding sites on pQR701

The size of the amplified product using the M13R1 and CMTA1 primers was 332 bp. The PCR was carried out in 25 µL reaction volume containing: 1.25 units *Taq* polymerase (Life Technologies, Uxbridge, UK); 20 mM Tris- HCl, pH 8.4; 50 mM KCl, 1.5 mM MgCl2; 0.2 mM each deoxynucleotide triphosphate (Pharmacia, Milton Keynes, UK); and 50 pmoles of each primer M13R1 and CMTA1. Sample volume was 10 µL. Each reaction mixture was overlaid with 25 µL light mineral oil (Sigma). PCR was carried out in a Hybaid OmniGene (Hybaid, Teddington, Middlesex, UK) temperature cycler, programmed for 94 C for 5 minutes followed by 30 cycles of 94 C for 30 sec, 55 C for 1 min, 72 C for 3 min and finally 10 min at 72 C.

The internal standard DNA (IS(T)) for quantitative DNA was prepared according to the method of Forster (1994) using primer TKL as the linker primer. The product was isolated from agarose by use of a Costar spin-X filter (Costar) and was purified using a QIAquick nucleotide removal kit (Qiagen, Chatsworth, CA) before quantitation by A260 measurement (using the approximation that ds DNA at 50 mg mL^-1 has an A260 of 1 (Sambrook *et al.*, 1989)). Three internal standard preparations were made by dilution in SROW: IS(T)1 at 1.3 x 10^4 molecules mL^-1; IS(T)2 at 1.3 x 10^6 molecules mL^-1; and IS(T)3 at 1.3 x 10^8 molecules mL^-1. In quantitative PCR assays (QPCR), IS(T) was added in a 1uL volume, the volume of SROW was reduced accordingly. Aliquots of IS(T) were stored at -70 C. The size of the IS(T) amplified product was 247 bp.

Analysis of PCR products

Agarose gel electrophoresis was performed as described in Sambrook *et al.* (1989). The running buffer used was 0.5 Tris borate EDTA with 0.25µg mL^-1 ethidium bromide. Aliquots of 7.5 uL of PCR product were mixed with 2.5 uL gel loading solution (Sigma) and were run on horizontal 2% agarose gels at 80V for 2-3 hours. The molecular weight marker used was phix-174 RF DNA Hinc II digest (Pharmacia). Gels were documented using a UV transilluminator and Gel Documentation System with ImageStore 5000 software and then analysed using Gelbase software (UVP Ltd., Cambridge, UK). Great care was taken to ensure that the detection system was not overloaded by the fluorescent intensity of the bands. This was controlled by adjusting the aperture setting of the camera. The precision of the measurement was found to be acceptable if the ratio of product bands was between 0.05 - 20, if the ratio was outside of this range the result was ruled invalid.

RESULTS
Selectivity of the PCR

Table 3 summarises the results of experiments where non-target *E. coli* or yeast strains were used in the PCR in the presence or absence of the target microorganism *E. coli* JM107 pQR701.

<table>
<thead>
<tr>
<th>Non Target Strain</th>
<th>Target strain (E. coli JM107 pQR701)</th>
<th>PCR Result^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>designation^a</td>
<td>Cells/PCR^b</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>-</td>
<td>5.0 x 10^4</td>
</tr>
<tr>
<td>JM83 pQR187</td>
<td>5.6 x 10^4</td>
<td>0</td>
</tr>
<tr>
<td>JM107 pQR150</td>
<td>6.1 x 10^4</td>
<td>0</td>
</tr>
<tr>
<td>JM107</td>
<td>4.7 x 10^4</td>
<td>0</td>
</tr>
<tr>
<td>S. cerevisiae wt</td>
<td>4.5 x 10^3</td>
<td>0</td>
</tr>
<tr>
<td>S. cerevisiae 7d</td>
<td>4 x 10^3</td>
<td>0</td>
</tr>
</tbody>
</table>

^a *E. coli* strains unless otherwise stated.

^b Cell counts by microscopy.

^c PCR result is positive if a band of the correct size (332 bp) is visible (by ethidium bromide staining) on agarose gel.

There are several points that can be made about these results: i) around 5 x 10^4 cells/PCR of all the *E. coli* strains tested (one 'wild type K-12' and 3 strains that are closely related to the target in terms of vector and host strain) do not produce a positive PCR result (a band of similar size to the target strain when subjected to PCR under the same conditions); ii) approximately 4 x 10^3 cells of a wild type and a genetically modified *Saccharomyces cerevisiae* strain do not produce a positive result; and iii) low numbers (25-50) of target cells will give a positive PCR even in the presence of a 1000 fold excess of other *E. coli* cells or a 100 fold excess of *S. cerevisiae* cells. In further experiments (data not shown) it was found that 4.6 x 10^6 cells of *E. coli* JM107 pQR150 in a PCR do not give rise to a positive PCR result and that 50 cells of *E. coli* JM107 pQR701 in a 'background' of 4.6 x 10^6 *E. coli* JM107 pQR150 cells can still be easily detected. The target strain can therefore be detected in the presence of a 1 x 10^5 fold excess of other *E. coli* cells.

Construction of calibration curves by QPCR

Competitive internal standard (IS(T)) was co-amplified with purified preparations of plasmid pQR701 to produce a series of standard curves for subsequent measurement of *E. coli* JM107 pQR701 cells. It was found that analysis of the two relative band intensities gave meaningful results only if the plasmid pQR701 and IS(T) were within a concentration range of +/- 1.4 logs relative to each other. Similarly, Chan *et al.* (1994) have found that the ratio of target fragment/internal standard is limited to a 3 log range. A calibration curve was therefore constructed using 3 fixed concentrations of IS(T) (denoted by IS(T)1, 2 and 3) which differ from each other by a factor of 100 (Figures 3 and 4).

**FIGURE 3.** Gel showing results of co-amplification of IS(T) and plasmid pQR701 at a range of concentrations.
FIGURE 4. Calibration curves for quantitation of pQR701 with linear regression lines. Error bars shown represent the standard deviation of the 3 replicate points used for each data point. The lines are drawn using a linear fit approximation.

This series of overlapping calibrations allows measurement of pQR701 concentrations over 6 orders of magnitude. The measurement of plasmid pQR701 concentrations within whole cell preparations using this calibration curve is shown in Figure 5. The standard deviation of measurement of pQR701 concentration within whole cells of *E. coli* JM107 pQR701 was found to be +/- 0.11 logs (n=5).

FIGURE 5. Measured pQR701 concentration in a dilution series of *E. coli* JM107 pQR701 cells. Figure shows measured number of pQR701 molecules per PCR assay versus number of cells/PCR (determined by microscopic cell counting).

Measurement of the number of plasmids per cell

Since the QPCR method measures the concentration of target plasmid in a sample and the aim of this research is to measure the quantity of process cells released, then it is necessary to convert plasmid to cellular concentration. The general approach to this is to determine the average number of copies of plasmid per cell within the bioprocess and to assume that any cells released from this process will have the same plasmid per cell average value. This is achieved by dividing the number of intracellular plasmids per ml of broth (i.e. total minus extracellular plasmid concentration) at each time point by the number of cells mL^-1 at the same time (see Figure 6).

FIGURE 6. Variation of the number of plasmids per cell throughout *E. coli*JM107 pQR701 fermentation. Error bars represent the SEM (data taken from 3 fermentation runs). Total and extracellular pQR701 concentrations were determined from samples taken from the fermentation at 1 hour intervals. Three fermentations were carried out under the same conditions to build up a reliable picture of the variation in total and extracellular pQR701 within the medium throughout a fermentation.

In the 2 L fermenter, the plasmid per cell values at the exit gas sampling times of 1.5, 3, 5 and 7 hours after inoculation were 146, 84, 64 and 64 respectively. In the 50 L fermenter the plasmid per cell value was found to be 139, all sampling was carried out after the onset of stationary phase in this instance. These figures were used to convert intracellular pQR701 concentrations collected in the exit gas to the number of cells collected.

Fermentation exit gas sampling

Release of cells into the exit gas stream was measured by disconnecting the exit gas filter and rapidly connecting the exit gas line to the cyclone air intake by means of an adapter (Figure 7).

FIGURE 7. Sampling fermenter exit-gas using the Aerojet-General Cyclone. Exit air flows at 1.5 x 10^-3 m^3 min^-1, whilst the air pump in the cyclone draws air in at 0.36 m^3 min^-1. Air flow through the cyclone is maintained by allowing unfiltered room air to enter. The distance travelled by exit gas from the fermenter to the cyclone is 1m.

Since the incoming room air was unfiltered, careful background measurements were made to determine whether the room air itself contained target *E. coli* JM107 pQR701 cells. For each measurement the cyclone was therefore run for 10 minutes before connection to the exit gas line. After this time, a 1 mL sample was aseptically taken from the sample pot. This sample was subjected to QPCR and the results were used to determine the cleanliness of the cyclone (i.e. how efficiently it had been cleaned since a previous sample) as well as any background level of *E. coli* JM107 pQR701 cells that may be present in the air. On connection to the exit gas supply, the cyclone was run for a further 15 minutes. On some occasions, the cyclone was run for the entire 25 minute sampling period without connection to the exit gas. This was to provide information about any background levels of *E. coli* JM107 pQR701 that might be present in the air. The rationale was that if the background was entirely due to poor cleaning, then the level after 25 minutes would be the same as that after 10 minutes and if the background was due to airborne *E. coli* JM107 pQR701 whole cells then the
level would be increased at the end of the complete sampling period. The sampling and cleaning times involved in using the cyclone meant that the turnaround time was 45 minutes; consecutive samples were therefore taken at least this time apart.

In all exit gas sampling experiments there was no detectable background of pQR701 either in the air around the fermenter (from where a large proportion of the total air is drawn when exit gas sampling is taking place) or from contamination by previous samples. This indicates that the cleaning procedure is adequate and that there is no detectable release of process organisms into the air surrounding the fermenter. Since background levels were not detected, it was not necessary to make a correction for the room air that was drawn into the cyclone. The number of cells detected at each sampling time in terms of cells released per minute is shown in Figure 8. The data are also expressed as the number of cells released per m³ of exit gas exiting the fermenter (Table 4). Notably, the proportion of total pQR701 that was found to be extracellular in samples collected from the exit gas was always less than 5%. In some experiments (data not shown) the exit gas was monitored downstream of an exit gas filter. In this instance, pQR701 was not detected in the exit gas stream.

![FIGURE 8](http://www.bioline.org.br/request?by97005)

**FIGURE 8.** Release of *E. coli* JM107 pQR701 cells into unfiltered exit-gas throughout fermentation. Error bars on mean release rate determinations (taken from 3 fermentation runs) represent the SEM. Integration of the curve describing the mean release rate between 1.5 and 7 hours after inoculation allows estimation of the total (cumulative) number of cells released into the exit gas during this period. This value is 3.0 x 10^7 cells.

### TABLE 4 Cell release rates expressed in terms of exit gas volume

<table>
<thead>
<tr>
<th>Sampling point (hours after inoculation)</th>
<th>Release rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells min⁻¹</td>
</tr>
<tr>
<td>1.5</td>
<td>8.7 x 10^3</td>
</tr>
<tr>
<td>3</td>
<td>9.5 x 10^4</td>
</tr>
<tr>
<td>5</td>
<td>7.8 x 10^4</td>
</tr>
<tr>
<td>7</td>
<td>1.7 x 10^5</td>
</tr>
</tbody>
</table>

^a Exit gas flow rate 1.5 x 10⁻³ m⁻³ min⁻¹

### Monitoring air during operation of fermenter sampling valve

Air sampling was carried out (for 15 minute periods) in the vicinity of the 50L fermenter during operation of the sampling valve, both before and after the fermenter contents had been steam sterilised. In addition, a series of background measurements were taken before inoculation of the fermenter and when the fermentation was proceeding, but when sample valve operation was not taking place. For all air sampling experiments, the cyclone inlet was positioned in the open room within 2 m of the sampling valve at 1.1m above ground level.

In these experiments, a consistent (background) quantity of the target process strain was found in the cyclone on air sampling both before inoculation of the fermenter and during fermentation when the sampling valve was not being operated. The actual quantity was 1.6 +/- 0.09 x 10^⁴ cells (n=6). However, on two occasions, when the air samples were taken during sampling valve operation, considerably higher concentration of process cells was detected: 4.7 x 10⁶ (n=2) when the sample valve was operated and the fermenter contents were alive and 2.7 x 10⁵ (n=2) when the sampling valve was operated after sterilisation of the fermenter contents.

### DISCUSSION

**Development of QPCR assay**

Before considering the implications of the results of this study, it is worth recalling that in control experiments, occurrence of background levels of target plasmid lysis may vary throughout the bacterial life cycle. Here, the assumption is made that lysis efficiency of cells collected relatively poor under these conditions. It is known that cell wall strength (Siegele and Kolter, 1992) and hence ease of plasmids per cell may reflect variations in lysis efficiency throughout the fermentation and that lysis efficiency is methods (Lewington and Day, 1986; Wrigley-Jones et al., 1992). We are aware that observed variations in the number plasmids per cell number, as determined by this technique, and plasmid copy number determined according to published methods (Spratt et al., 1986). Kuhnert et al. (1995) have recently described a PCR assay that selectively detects all E. coli K-12 strains, which is in contrast to the method we present here which is specific for a particular construct. Since our experiments were carried out in an environment where other E. coli strains were being used, it was necessary to use a technique that could identify the specific process organism being monitored. Although it is conceivable that detection of a particular strain based on the presence of a plasmid borne sequence may give false results due to loss or transfer of the plasmid, in the immediate environment of the bioprocess stream plasmid loss or transfer is highly unlikely. Microfiltration of the samples collected from the cyclone show that an insignificant proportion of measured plasmid concentration passes throughout the filter, whereas pure plasmid preparations show high transmission (data not shown). We regard this as evidence that cells, rather than cell-free plasmids, are being detected. S. cerevisiae strains were tested in the PCR since this is a widely used host organism in large scale processes employing genetically modified organisms.

Quantitation of target by PCR has been achieved by using a competitive internal standard fragment made according to the method of Forster (1994). The internal standard is a 247 bp fragment, which has the same primer binding sites as the target (332 bp) but has a section deleted. Some authors (Chan et al., 1994; McCulloch et al., 1995) have noted that despite sharing common primer binding sites, the amplification efficiency of internal standard and the target DNA fragment may not be equal. In this case it was found that 2 molecules of IS(T) were required for each molecule of pQR701 in order to produce bands on the agarose gel of equal intensity. However, the relationship between peak area ratio of the products and input molar ratio of target and IS(T) at the start of the PCR was linear over the +/- 1.4 log range used for each IS(T) concentration. The calibration curves used therefore corrected for this inequality in amplification efficiency of standard and target (Chan et al., 1994; Leser, 1995; McCulloch et al., 1995). The use of overlapping calibration curves (Chan et al., 1994) allows extension of the range of concentrations of target plasmid pQR701 that can be measured.

The minimum concentration of cells required for a positive response in the assay was 500 cells mL^-1 of cyclone collection fluid, equivalent to 5 10^3 cells m^-3 of sampled air. This was adequate for these experiments where either the total volume of exit gas was sampled or the sampling was carried out close to the point of release. Where a higher sensitivity is required, the large sample volume taken from the cyclone could be concentrated before PCR assay by filtration (Alvarez et al., 1994).

**Determination of the number of plasmids per cell**

Figure 5 shows that E. coli strain JM107 pQR701 can be accurately measured by the QPCR method, provided that the number of plasmids per whole cell are known. The initial calibration was set up using purified plasmid rather than whole cells because the number of plasmids per cell may not be constant between different experiments. Therefore, the approach that was used was to calibrate against known concentrations of pQR701 and to correct for the number of plasmids per cell in each experiment according to the method described. We intend to clarify the relationship between plasmid per cell number, as determined by this technique, and plasmid copy number determined according to published methods (Lewington and Day, 1986; Wrigley-Jones et al., 1992). We are aware that observed variations in the number of plasmids per cell may reflect variations in lysis efficiency throughout the fermentation and that lysis efficiency is relatively poor under these conditions. It is known that cell wall strength (Siegele and Kolter, 1992) and hence ease of lysis may vary throughout the bacterial life cycle. Here, the assumption is made that lysis efficiency of cells collected from the exit gas is the same as that for cells taken from the broth at the same time.

**Occurrence of background levels of target plasmid**

Before considering the implications of the results of this study, it is worth recalling that in control experiments,
background levels of the target process organism were occasionally observed. There are several potential sources of background when using this methodology in a bioprocess plant: poor cleaning of the cyclone between runs leaving residual whole or fragmented cells between one sample and the next; contamination of the cyclone and/or associated tubing by the operator; a periodic appearance of airborne target cells in the area used for taking background measurements; or cross contamination at the PCR stage. Under experimental conditions, it has been found that the cyclone cleaning method used consistently reduced the plasmid concentration within the cyclone by a more than 10^6 fold (data not shown). Whatever the cause of the background, the effect is to undermine confidence in the technique, since a positive result does not necessarily imply that a release of cells has been detected. With the results reported here the levels of cells detected during operation of the fermenter sampling valve are substantially higher than any recorded background levels, and this is construed to indicate genuine release. In early exit-gas monitoring experiments, background levels were occasionally observed, although in the experiments presented here all background readings were found to be nil. Clearly there is a need to understand the origins and nature of the background and to eliminate it, this is a major consideration in further development work.

**Measurement of cells released into the exit gas**

In these experiments the fermenter exit-gas flow rate is substantially lower than the cyclone air intake rate, necessitating the use of an adapter which allows 'make-up' air from the room in which the fermenter is situated to enter the cyclone. It was therefore necessary to make a series of room air control measurements. Since other air samplers are capable of efficient microbial capture from air flowing at lower rates than that required by the cyclone, then such devices might have been more suitable in this instance. However, the aim of this experiment was to provide a model system for cyclone air sampling where microbe-bearing aerosols were likely to be released.

**Figure 8** shows that, in general, there is an increase in the rate of release of cells into the exit gas as the fermentation proceeds. However, there is no simple relationship between cell density in the fermentation broth and the number of cells released into the exit gas. There are a series of factors that might be implicated in the variation of release rate as the fermentation proceeds. For instance, Pilancinski and co-workers (1990) have shown that aerosol formation from a fermentation broth is influenced by several factors such as air flow rate, agitation rate and the rheological properties of the liquid. Szewczyk et al. (1992) found that in an industrial pilot scale fermenter aerosol particle concentration decreased significantly with increasing cell density and that the change in particle concentration was more pronounced in the size range above 2 um. Huang et al. (1994) attempted to monitor aerosol generation and properties as a method for on-line biomass monitoring of an E. coli fermentation using T-broth as the growth medium. This group noted that effluent aerosol number concentration increased during the growth phase and subsequently decreased after bacterial cell concentration had reached a stable level. Metabolic changes in the composition of the fermentation medium caused the surface tension to decrease, leading to an increased foaming tendency which, in turn, causes greater aerosol release. However, as cell density increases, it is thought that clumping of cells will occur in the broth. Larger clumps of cells may not be lifted into an aerosol due to their size not being compatible with the particle size distribution of the aerosol. Additionally, the increase in settling velocity which occurs as the particle diameter increases may account for the fall in the number of cells detected. In the data presented here, the final sampling point shows a marked increase in the release rate that is unlikely to be caused by a reduction in clumping. Observation of the fermentation shows that foaming begins to occur at 5-6 hours after inoculation, at the end of the exponential growth phase. The increased rate of release of cells into the exit gas at this time, compared with the rate at the previous sampling point, is most likely to be caused by the first occurrence of foam in the intervening period.

Expression of the rate of release of cells in terms of the exit gas volume allows extrapolation of results to larger scale fermenters and the comparison of results with other workers who have used different methods to measure cell numbers in fermenter head space. Our data show that the release rate rises to 1.1 x 10^8 cells m^-3 during the stationary phase of the culture. Winkler (1987) has reported that in the fermenter head space there are about 10^6 contaminated particles per m^3 gas. Since each 'contaminated particle' contains at least one viable cell then this describes only the minimum number of culturable cells present. Other studies on aerosols produced by fermentation have concentrated on aerosol particle characteristics rather than cellular concentration in the aerosol. For instance, Pilancinski et al. (1990) measured over 10^8 particles m^-3 of which 30-40% exceeded 2 um in diameter above a complex broth stirred at 130 rpm. Szewczyk and co-workers (1992) measured the particle concentration at approximately 15 cm above the fermentation liquid and found that the level decreases from greater than 6 x 10^8 particles m^-3 at inoculation to 2.5 x 10^8 particles
Since we have used PCR, rather than a culture based method, to detect cells, it is not possible to directly compare findings with those of Winkler (1987). Additionally, in this study the exit gas has been sampled downstream of a condenser (see Figure 7), whereas other workers have sampled in the headspace of the fermenter (Winkler, 1987; Pilancinski et al., 1990; Szewczyk et al., 1992). The condenser may well have the effect of reducing the bacteria-bearing particle concentration in the exit gas stream.

**Sampling around the 50L fermenter during operation of the sampling valve**

During fermentation to a high cell density, there is no release (above background) of process organisms to the air surrounding the fermenter. This might be expected considering that the fermenter used is designed to operate to a high degree of containment and incorporates double mechanical seals with steam traces. However, there is evidence of a release of plasmid material during the use of the contained sampling array on two occasions. The amount of material captured equates to the equivalent of less than 0.5 uL of live cell broth in both instances. It was observed during both sampling events that after decoupling of the sampling array (Figure 1) a small amount of liquid leaks out. This liquid is likely to comprise mainly condensed steam, but there is a possibility that it also contains some material derived from the broth. Temperature mapping studies carried out as part of the commissioning program for the plant suggest that any cells that remained in the pipe work of the sampling array would be steam sterilised by the time of decoupling. Indeed, on the second occurrence of a release during sampling, the contents of the fermenter itself had been sterilised.

The use of a microfiltration step before PCR to distinguish plasmid that is contained within cells from plasmid that is extracellular showed that with both samples being discussed here, target plasmid was not detected in the filtrate. It was hoped that the ratio of intra- to extracellular pQR701 plasmid concentration measured in the samples might provide information on the condition of the cells which are released into the air and detected. For instance, steam sterilisation might have caused the intra/extracellular ratio to rise as lysis occurs in parallel to cell death. Since the pQR701 concentration in each sample was found to be entirely intracellular (or at least retained by a 0.45um filter), it can be concluded that this method is unable to distinguish steam killed cells from those that are viable. This is a failing of the currently available methodology as it is envisaged that there will be occasions when killed cells are present in the environment which could give rise to a false positive result for the presence of live cells. However, other workers have used a variety of additional steps to investigate the viability of cells detected by PCR (Mahbubani et al., 1991; McCarty and Atlas, 1993; Bej et al., 1996). It is possible that a similar step could be incorporated into our methodology. Since it is recognised that sampling from fermenters is an operation that might give rise to breach of containment, the design of sampling ports has received some consideration (Cameron et al., 1987; Hambleton et al., 1991; Leaver and Hambleton, 1992). Cameron et al. (1987) have in fact monitored the containment effectiveness of a sampling port with a B.subtilis spore suspension in a fermenter using a cyclone as the air sampling device. In this study, bacteria were detected (by culture) at times coincidental with the operation of the sample valve. Indeed, Hambleton and co-workers have suggested that where high containment is required, the use of secondary containment features may be necessary (Hambleton et al., 1991).

In summary, it seems most likely that non-viable cells were detected during fermenter sampling. However, at present, the methodology presented here is unable to distinguish between viable and non-viable cells. This is a refinement that will need to be addressed if sampling is likely to occur in areas where viable and non-viable cells may be released and the former present a hazard whilst the latter is, to a certain degree, acceptable.

**Efficiency of capture of microbial aerosols**

Assessment of the performance of bioprocess equipment, in terms of its effectiveness in containing process organisms, requires determination of the quantity of cells that are released during an operation. In order to provide such quantitative performance data, it is necessary to measure the number of cells collected by the sampling device and to determine what proportion of the cells released become entrained within the cyclone. Whilst the former has been achieved using QPCR, the latter is a more complex issue that we have not directly addressed in this paper. With the monitoring of fermenter exit-gas, the exit-gas stream was piped directly into the cyclone, so the proportion of airborne cells collected would be largely a function of the cyclone capture efficiency. Other researchers (Upton et al., 1994) have
shown that the cyclone is highly efficient in its capture of aerosol particles of greater than 2\textmu m diameter, and we (Ferris et al., 1995) have shown that approximately 40\% of bacteria released as an aerosol into a 0.36 m$^3$ cabinet can be recovered using this device under the operating condition used here. Therefore, in this instance, the number of cells captured and enumerated can be reasonably estimated as representing 50-100\% of those released. The situation when sampling the air at the time of operation of the fermenter sampling valve is more complex since we also need to take account of the proportion of cells that are released during the operation but do not reach the cyclone inlet, situated at a distance of 2m. There are two general approaches that could be used to determine this proportion: calibration or air flow modelling with particle tracking. The previously mentioned work where a capture efficiency of 40\% was observed in a 0.36 m$^3$ cabinet is one example of a calibration experiment; larger cabinets, which could enclose pilot scale equipment such as homogenisers, could be similarly calibrated (Ferris et al., 1995). However, at larger scale it will not be desirable to perform such calibrations. Modelling of air flow and particle tracking using Computational Fluid Dynamics is an approach that we have used to solve this problem and we report this in a forthcoming publication.

**Biosafety considerations**

Since there is a greater degree of certainty about the number of cells that were released into the exit-gas than into the air surrounding the fermenter during operation of a sampling valve, then we will use the former scenario in considering the impact that quantitative performance data might have on risk assessment in bioprocessing. The total number of cells released throughout the fermentation (between 1.5 and 7 hours) can be calculated by integration of the curve in Figure 8. This gives a value of 3.0 x 10$^7$ cells. Comparison of this figure with the total number of cells in the broth, 2.7 x 10$^13$ cells at stationary phase, reveals that the number of cells released is a very small proportion of the total. Indeed, the total number of cells released into the exit gas is the same as that contained within 1.7 \textmu L of the final broth.

Sampling of the exit gas downstream of a filter showed that no release was detectable. However, the long term value of installing a filter might be questioned if its only role were to reduce the number of cells released. One accident, say, the result of a 'foam-out', which released 1 mL of fermentation broth would allow approximately 10$^10$ cells to escape. This number of released cells will only be surpassed by the cumulative release from unfiltered exit gas of over 300 fermentations. Since it is presently unclear whether the long-term cumulative release of a small number of organisms exposes the operator and the environment to a greater hazard than the occasional release of a large number of organisms, the potential for accidents should be a key feature of risk assessment in industrial bioprocesses. Notably, Hesselink et al. (1990) have used a model to estimate the failure probability during an industrial centrifugation operation due to operator error. This model (Bello and Colombari, 1980) involves assessment of the type of operation, the time available for completion and the environmental ergonomic characteristics. In short, the complex engineering now installed to reduce the release to a minimum may overlook a more pressing need to simplify engineering practice to reduce the likelihood of accidents (Kletz, 1996). We believe that the development of quantitative methods of the kind described here will allow a more balanced approach to risk assessment in bioprocessing.

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FIGURE 1. Schematic representation of the contained sampling array fitted to the 50 L fermenter. The pre-sterilised sample bottle and piping are connected to the sampling line via the couplings A and B. The line is steamed through before and after taking the sample.
FIGURE 2. Schematic showing primer binding sites on pQR701
FIGURE 3. Gel showing results of co-amplification of IS(T) and plasmid pQR701 at a range of concentrations. Figure shows three sets of [pQR701]/[IS(T)] pairs. A: range of [pQR701] + IS(T)2; B: range of [pQR701] + IS(T)1; C: range of [pQR701] + IS(T)3. In all sets [pQR701] (top band of the doublet) increases from left to right. Analysis of peak areas allows construction of calibration curve (Figure 4).
FIGURE 4. Calibration curves for quantitation of pQR701 with linear regression lines. Error bars shown represent the standard deviation of the 3 replicate points used for each data point. The lines are drawn using a linear fit approximation.
FIGURE 5. Measured pQR701 concentration in a dilution series of *E. coli* JM107 pQR701 cells. Figure shows measured number of pQR701 molecules per PCR assay versus number of cells/PCR (determined by microscopic cell counting).
FIGURE 6. Variation of the number of plasmids per cell throughout *E. coli* JM107 pQR701 fermentation. Error bars represent the SEM (data taken from 3 fermentation runs). Total and extracellular pQR701 concentrations were determined from samples taken from the fermentation at 1 hour intervals. Three fermentations were carried out under the same conditions to build up a reliable picture of the variation in total and extracellular pQR701 within the medium throughout a fermentation.
FIGURE 7. Sampling fermenter exit-gas using the Acrojet-General Cyclone. Exit air flows at $1.5 \times 10^{-3}$ m$^3$ min$^{-1}$ whilst the air pump in the cyclone draws air in at 0.36 m$^3$ min$^{-1}$. Air flow through the cyclone is maintained by allowing unfiltered room air to enter. The distance travelled by exit gas from the fermenter to the cyclone is 1 m.
FIGURE 8. Release of *E. coli* JM107 pQR701 cells into unfiltered exit-gas throughout fermentation. Error bars on mean release rate determinations (taken from 3 fermentation runs) represent the SEM. Integration of the curve describing the mean release rate between 1.5 and 7 hours after inoculation allows estimation of the total (cumulative) number of cells released into the exit gas during this period. This value is $3.0 \times 10^7$ cells.