Flow cytometric detection of specific genes in genetically modified bacteria using in situ polymerase chain reaction

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Abstract

Use of the polymerase chain reaction, coupled with flow cytometry, to detect a plasmid encoded $xy/E$ gene sequence in intact cells of Escherichia coli and Pseudomonas putida was investigated. Optimal incorporation of fluorescently labelled dUTP into a full length PCR product required substitution at a level of 2:3 dUTP:dTTP. Formaldehyde fixed cells of both species were counted before and after thermal cycling. Sufficient numbers of cells remained intact for subsequent detection using microscopy and flow cytometry but light scatter properties were altered. Intact cell suspensions of both species containing plasmid pLV1013 were subjected to thermal cycling with fluorescent dUTP in the reaction mix. Subsequent analysis by flow cytometry allowed detection of a fluorescent PCR product associated with cells. Control samples (without the plasmid) showed only background fluorescence. This method demonstrates the potential for applying DNA amplification methods for sensitive detection of specific sequences localized inside intact bacterial cells.

Keywords: Escherichia coli; Pseudomonas putida; Flow cytometry; In situ polymerase chain reaction

1. Introduction

Reports of the use of in situ polymerase chain reaction (isPCR) for the enzymatic amplification of nucleic acid sequences in intact cells was first described in fixed samples of mammalian tissue by Haase et al. [1], and has remained limited to eukaryotic studies of viral infection (for a review, see [2]). The technique may have great potential for prokaryotic studies, allowing sensitive detection of sequences without selective culture steps. PCR detection of prokaryotes is often limited to a presence/absence analysis, with quantitative measurements being problematic. Combining isPCR with flow cytometry or fluorescence microscopy extends the use of PCR to morphological studies of prokaryotes, allowing determination of the range and spatial distribution of cells carrying a target sequence. Its success depends upon the specificity of DNA amplification and retention of the PCR product within the cell during and after thermal cycling. A major problem is the compromise between allowing access of reagents into the cell while limiting product diffusion out of the cell.

Most published reports using isPCR have performed DNA amplification with samples attached to microscope slides with the slide being placed on the
heating block of the thermal cycler. The more sophisticated approach of a thermal cycler modified for this procedure is now available from several manufacturers. However, Haase et al. [1] performed is-PCR on cells in suspension without reporting significant cell lysis. Detection of amplified product has been achieved using either direct incorporation of labelled nucleotides [3-6], incorporation of fluorescent primers [7] or by subsequent in situ hybridization of the amplified DNA [5,8]. Additionally, in situ reverse-transcription PCR has been demonstrated [7].

In this study, we have combined for the first time the speed and automation of flow cytometry (FCM) with the sensitivity of in situ PCR to attempt flow cytometric detection of bacterial cells containing specific nucleic acid sequences. The target sequence selected was the xylE gene [9], using incorporation of fluorescent nucleotides for the detection of a specific gene inside intact bacterial cells.

2. Materials and methods

2.1. Bacterial strains, growth conditions and fixation

Escherichia coli and Pseudomonas putida with and without the plasmid pLV1013 were obtained from C. Winstanley [9,10]. Cultures were grown and maintained in nutrient broth (Lab M, Bury, UK), solidified with 1.5% agar where appropriate. Media for growth and maintenance of strains containing plasmid pLV1013 were supplemented with kanamycin A at 100 μg/ml. E. coli was grown at 37°C; P. putida at 30°C. Harvesting and fixation of cells was essentially as outlined previously [11]. Briefly, exponential phase cultures of the bacteria were harvested by centrifugation at 5000 × g for 20 min at 4°C, washed once in phosphate buffered saline (PBS; filtered three times through 0.22 mm pore size filters before use) and the colony forming units /ml determined in triplicate. The remaining cell suspension was mixed 1:3 vol/vol with freshly prepared (within 1 h) paraformaldehyde in PBS before fixation at 4°C for 16 h. Cells were then washed once in PBS and resuspended to 1.0 × 10^9 CFU/ml before mixing with an equal volume of filtered absolute ethanol at -20°C. Fixed cells were stored at -20°C for up to 4 weeks before being discarded.

2.2. In situ PCR: experimental procedures

In situ PCR experiments were repeated up to four times. Initially, fixed cell samples were washed and resuspended in TE buffer, and subjected to PCR cycling in Taq DNA polymerase buffer (Boehringer Mannheim). Samples were analysed by FCM before and after cycling, and counts were determined using both FCM and microscopy.

Aliquots of fixed cultures were lysed by the addition of 0.05 volumes 10% SDS and 0.05 volumes proteinase K (2 mg/ml). Samples were shaken at 37°C until clear before subjecting to phenol/chloroform extraction [12]. DNA was ethanol precipitated [12] and resuspended in 1 × TE. Cultures of E. coli ED8654 (+ pLV1013) were treated in this way, before amplifying target sequences using the polymerase chain reaction (PCR). Initially, it was confirmed that both the 16S rRNA and the xylE genes could be successfully amplified. PCR conditions were (per 100 μl reaction mix) -5 μl 1 mM dNTP solution in Hipersolv (BDH, UK), 1 μl each primer (10 pmol μl^-1 stock in Hipersolv), 10 μl 10 × Taq buffer with MgCl₂ (Boehringer Mannheim) and 0.5 μl Taq DNA polymerase. This was made up to 90 μl with Hipersolv, before adding 10 μl target DNA. Primers for the xylE gene, designated xyl1 and xyl2, were from Morgan et al. [13]. The reagents were then subjected to 30 cycles in a DNA Thermal Cycler 480 (Perkin Elmer, Beaconsfield, UK). Cycling conditions were 95°C, 1 min (initial denaturation) followed by 95°C, 40 s (denaturation), 55°C, 1 min (annealing) followed by 72°C, 2 min (extension) for 30 cycles. A final extension step was performed at 72°C for 3.5 min, after which samples were held at 4°C.

Products from these reactions were electrophoresed on 1% agarose gels, using 50% glycerol as a loading buffer and appropriate DNA weight markers (Marker VI, Boehringer Mannheim).

Conditions were optimised for the substitution of fluorescein-12-dUTP (Boehringer Mannheim) for dTTP in the PCR mixture, without loss of product length or yield, using the same reaction conditions as before, using varying ratios of labelled dUTP:dTTP. Amplified DNA was electrophoresed in ethidium bromide free apparatus (scrubbed and pre-electrophoresed for 1 h). Gels were examined under u.v.
illumination, incubated at room temperature in ethidium bromide solution [12] and re-examined. This allowed differentiation of fluorescence due to the labelled nucleotide, and fluorescence from EtBr stained DNA.

Whole fixed cells were washed and resuspended in an equal volume of TE before 3 μl of cell suspension in 7 μl Hipersolv were substituted for DNA in the PCR mixture containing fluorescein-12-dUTP. Samples were placed on ice and analysed immediately after cycling ceased, to limit product diffusion from the cells. Other samples were lysed as above before electrophoresis to confirm the presence and length of the PCR product.

2.3. Flow cytometry and microscopy

Flow cytometry was performed using a Skatron Argus flow cytometer set to trigger on forward angle light scatter. The instrument was set up and maintained as described previously [14]. All laboratory solutions were filtered three times (0.22 μm pore size) before passage through the cytometer. Forward angle light scatter (LS1) was measured at a photomultiplier tube voltage (PMT) of 590 V using linear amplification. Fluorescence from fluorescein labelled dUTP was measured through fluorescence detector 1 (FL1) using appropriate filter blocks, at a PMT of 650 V and logarithmic amplification.

Microscopy was performed using epifluorescence microscopy with ×1000 magnification. Cell samples were aspirated as a mixing step before removal from the PCR tubes, to reduce cell lysis due to vortexing. Samples were removed from beneath the mineral oil layer to avoid contamination, suspended in 2% formaldehyde and labelled with acridine orange [15]. Cells were then collected on 0.22 μm pore size black polycarbonate filters (Costar, High Wycombe, UK) before counting. Counts were made on duplicate filters [15].

3. Results and discussion

3.1. Optimization of fluorescent dUTP incorporation

Initial work confirmed that the xylE gene could be specifically amplified using the xylI and xyl2 primers [13], and that no non-specific amplification was observed. Incorporation of fluor-dUTP into the reaction mix was then optimised, where the labelled nucleotide was substituted for varying amounts of dTTP before amplification. Results are presented in Fig. 1, demonstrating that satisfactory detection was achieved with a substitution ratio of 2:3 fluor-dUTP:dTTP (Fig. 1a, lane 4). Using 100% fluor-dUTP gave no product (Fig. 1a,b, lane 7), indicating that incorporation of the modified nucleotide was not identical to that of dTTP.

3.2. Maintenance of cell integrity during thermal cycling

It was tested whether fixed cells were able to withstand the repeated heating (95°C) associated with
the PCR. Fixed samples of cells of both species were placed in normal *Thermus aquaticus* (*Taq*) buffer and put through 30 thermal cycles. Cells were enumerated before and after treatment, using total counts (LS1) from the cytometer, and from acridine orange direct counts. Changes in the light scatter were noticed for both species (Fig. 2a,b), but these changes did not prevent cell detection. Results from the two methods of counting are given in Table 1. It can be seen that although cell lysis did occur, and significantly fewer cells were present after PCR cycling, approximately half of the cells remained intact (Table 1).

### 3.3. Detection of the xylE gene after isPCR

Samples were prepared for *isPCR*, using both *E. coli* and *P. putida*. Subsequent FCM analysis showed that a fluorescent signal was generated in the presence of the plasmid for both species (Fig. 3a,b) and that the fluorescent signal was particle associated (or it could not have been detected by the cytometer). A clearer signal was observed from the *P. putida* samples than from the *E. coli* (Fig. 3). In addition, cells from samples which proved positive by flow cytometry were lysed, and electrophoresed in EtBr free apparatus. Fluorescent products of the correct size for the xylE gene were found only in those samples containing plasmid pLV1013 (data not shown).

It was also further investigated whether DNA could remain inside the cell during the fixing, washing and thermal cycling procedures. Cell samples subjected to thermal cycling were pelleted, and the supernatant was retained. The cell pellet was washed, resuspended and the DNA extracted. Both cell associated and supernatant DNA was then used as template for subsequent PCR amplification. Results presented in Fig. 4 show that for both species, sufficient DNA remained cell-associated after thermal cycling to act as template for PCR. Additionally, the approximately equal densities of the product bands generated by both cellular DNA and supernatant DNA suggest approximate equal starting levels of template. This would correspond with the estimation of approximately 50% cell lysis during the thermal

<table>
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<th>Table 1</th>
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<td><strong>Measurement of cell lysis after 30 thermal cycles</strong></td>
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<th>Flow cytometer count</th>
<th>Microscopic count</th>
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<tr>
<td></td>
<td>Before cycling</td>
<td>After cycling</td>
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<tr>
<td><em>E. coli</em></td>
<td>2.60 ± 0.44</td>
<td>1.09 ± 0.07</td>
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<tr>
<td><em>P. putida</em></td>
<td>3.14 ± 0.35</td>
<td>1.94 ± 0.2</td>
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* Both flow cytometric and microscopic counts are expressed as $\times 10^6$ cells ml$^{-1}$. Triplicate counts were made for each sample.
cycling procedure. Fig. 4 also demonstrates that the xylE fragment is only amplified from strains carrying the plasmid pLV1013 (Fig. 4, lanes 6 and 7). The maintenance of intracellular template DNA was confirmed for samples subjected to thermal cycling for 10, 20 and 30 cycles. Specific PCR products were found from each template DNA set, indicating that although extracellular DNA was present in the liquid phase, sufficient DNA for satisfactory amplification was retained intracellularly throughout thermal cycling.

3.4. Comments and conclusions

The approach taken in this work was to attempt amplification of a plasmid encoded, introduced gene. This allowed amplification of a 699 bp product [13]. Haase et al. [1] reported that product length was of critical importance, as a short product would leak from a cell, reducing specificity, while a long product would be more difficult to synthesize intracellularly. This product length appeared to be a satisfactory compromise between the two extremes. Specificity was easily confirmed by using identical reaction mixtures, but substituting the parent strain in place of the recombinant strain. Attempts to amplify the 16S rRNA gene intracellularly using primers pA and pH' [16] were unsuccessful, possibly indicating that the increased product length (approximately 1500 bp) or the complexity of the target template did not allow isPCR.

Sufficient numbers of cells remained intact for satisfactory detection by both FCM and microscopy, although cell lysis did occur. Alteration of the fixation conditions may decrease cell lysis considerably. However, the procedure described here demonstrates detection of specific gene sequences inside intact cells using isPCR and flow cytometry. The fluores-
cent PCR product must have been cell associated to have been detected by the flow cytometer. It was not, however, conclusively demonstrated that PCR product remained entirely inside the cell after thermal cycling, and was not merely cell associated.

The applications of combining the PCR with fluorescence dye technology for in situ measurements are wide ranging. Direct detection of bacteria using fluorescent in situ hybridisation is a rapidly expanding method for bacterial ecology [17], providing both spatial and phylogenetic information, with a measure of activity. Use of PCR will extend these measurements in terms of sensitivity, extending the choice of target nucleic acids sequences from that of ribosomal RNA to include chromosomal- or plasmid-located DNA target sequences. The exact copy number of the plasmid within the two species is not certain, but it is likely to be within the range of 10–30 copies [18]. This increases detection sensitivity below that currently required for fluorescent in situ hybridization. Although much work remains to be done before ecological studies may be undertaken, the approach may eventually allow far more detailed understanding of bacterial activity and functioning within natural populations.

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References


