

Monitoring growth phase-related changes in phosphatidylcholine-specific phospholipase C production, adhesion properties and physiology of *Bacillus cereus* vegetative cells

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Abstract The physiological status and metabolic heterogeneity of *Bacillus cereus* cells within a culture during an 8-h batch fermentation process was measured using flow cytometry (FCM). Concurrently, production of the toxin, PC-PLC, and the extent of cell adhesion of live and dead cells were monitored using novel fluorescent assays. Flow cytometry analysis detected growth phase-related changes in the physiological profiles of cells over the course of the fermentation, with variation in the percentage of cells displaying membrane damage and intracellular esterase and redox activities. As the exponential phase proceeded, populations became more uniform in terms of protein content as measured using FCM in tandem with a cell tracking dye, with the majority of cells becoming membrane intact, esterase positive and redox active. PC-PLC activity appeared strongly related to cell density. Permeabilisation of cells was accompanied by a loss in adherent properties, while 25–100% of cells with intracellular esterase activity possessed adhesion properties. Cells in late exponential phase appeared to have reduced adherence properties compared to cells in early exponential or lag phase. As well as demonstrating the utility of FCM for measuring heterogeneity in terms of cell physiological status throughout the course of batch cultures, the methods utilised in this study could be used to relate processes such as toxin production or cell adhesion to cell physiological state.

Keywords Adhesion · *Bacillus cereus* · Flow cytometry · Growth phase · Phospholipase C

Introduction

While the growth of *Bacillus cereus* as a batch culture in artificial media has been well characterised [1], the physiological status of individual *B. cereus* cells in batch cultures over the course of a fermentation process has not. In this study, as well as monitoring growth using traditional methods, flow cytometry (FCM) together with three staining regimes was utilised to assess the physiological status of individual cells during an 8-h batch culture. To date, FCM has been applied to study lag times, numbers of cell divisions and the extent of injury of *Lactobacillus plantarum* [2], the physiology of *Escherichia coli*, *Rhodococcus* spp. and *Sacharomyces cerevisiae* in batch and fed-batch fermentations [3, 4], the population dynamics of *B. licheniformis* in batch and continuous cultures [5, 6], forward scattered light (FSC) properties [7] glucose uptake [8], DNA content [9] and the appearance of “ghost cells” of *E.coli* during batch culture [10]. FCM has been applied to measure DNA and polyhydroxybutyrate content of *Variovorax paradoxus* [11] and *Ralstonia eutropha* during continuous culture [12] and the protein and nucleic acid content of *B. subtilis* [13].

Concurrent with flow cytometric evaluation of physiological status, phosphatidylcholine-specific phospholipase C (PC-PLC) production and cell adhesion were measured and related to growth phase and cell physiological status. PC-PLC is a zinc-metallophospholipase C which hydrolyses phosphocholine-containing phospholipids such as lecithin and is responsible, along with other enzymes, for producing off-flavours in contaminated foodstuffs [14, 15]. However, its role in causing human illness is not clear [16, 17]. It has been suggested that PC-PLC may play a major role in *B. cereus*-mediated endophthalmitis [18, 19]. Despite the emerging importance of PC-PLC, it has not been studied to nearly the same extent as the various diarrhoeal toxins and the emetic

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toxin secreted by *B. cereus* and little published information exists on when the toxin is expressed during the growth of *B. cereus* and the conditions favouring its production [20–22].

The adhesion property of *B. cereus* is an important factor in the initiation and persistence of contamination of process equipment and the central role of S-layers in the adhesion process has been established [23, 24]. However, little is known about the adhesion properties of *B. cereus* vegetative cells and whether these properties vary with growth phase and culture conditions. Hence, the objective of this study was to study cell physiological status and relate this to PC-PLC production and cell adhesion properties of *B. cereus* over the course of an 8 h batch fermentation process.

Materials and methods

Bacterial strain used, preparation of pure suspensions of vegetative cells and enumeration of vegetative cells and endospores using plate counts.

Bacillus cereus type strain, *B. cereus* NCTC 7464 (National Collection of Type Cultures, London, UK) was the test organism in this study. Endospore-free suspensions of vegetative cells were achieved by using a double-pass method and harvesting cells prior to the appearance of endospores [25, 26]. Plate counting and phase contrast microscopy confirmed that such suspensions did not contain detectable levels of endospores. Plate counts for vegetative cells or endospores were performed using nutrient agar (NA; Oxoid, Basingstoke, UK) or NA supplemented with 10 mmol l⁻¹ L-alanine respectively. 0.1% peptone water or sterile distilled water were the diluents used for vegetative cell or endospore counts respectively. For the enumeration of endospores, dilutions were heated to 80 °C for 10 min prior to plating in order to kill vegetative cells and activate any endospores present.

Experimental design

The experimental design for this study is outlined in Fig. 1. Batch cultures were initiated by transferring 500 µl of endospore-free culture of OD₆₀₀ 0.15 to a one conical flask containing 49.5 ml sterile nutrient broth (NB; Oxoid), which was then incubated at 30 °C without shaking until the experiment was terminated after 8 h (0 h). Five batch cultures of each cell type (stained and unstained) were tested using each of the analysis methods below.

Measurement of OD₆₀₀, A₂₆₀, A₃₁₀, A₃₄₀, pH and conductivity

One ml aliquots were aseptically removed from batch cultures at sampling times and the OD₆₀₀ of the raw suspensions and absorbencies at 260 nm (A₂₆₀), 310 nm (A₃₁₀),

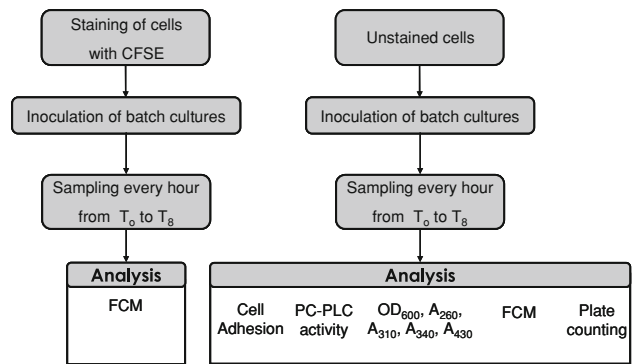


Fig. 1 The design of experiments enquiring into phosphatidylcholine-specific phospholipase C production, adhesion properties and physiological status of *Bacillus cereus* vegetative cells during 8 h batch culture. A₂₆₀ the absorbance at 260 nm etc.; CFSE carboxyfluorescein diacetate succinimidyl ester; FCM flow cytometry; PC-PLC phosphatidylcholine-specific phospholipase C

340 nm (A₃₄₀) and 430 nm (A₄₃₀) of cell-free supernatants (achieved by centrifuging at 8,000g for 10 min) were read using an Ultrascop 2000 spectrophotometer (GE Healthcare, Buckinghamshire, UK). OD₆₀₀ was measured in order to estimate cell density, A₂₆₀ the extent of released nucleic acids, A₃₁₀ the extent of released calcium dipicolinic acid, A₃₄₀ the extent of released reduced pyridine nucleotides, such as NADH, and A₄₃₀ the extent of released flavins [27, 28]. The pH and conductivity values of suspensions were then measured using a calibrated Jenway 3310 pH meter (Barloworld Scientific, Essex, UK). For each batch culture, duplicate samples were tested.

Fluorescent staining of cells and flow cytometric analysis of physiological status

Three FCM staining regimes were used to monitor the physiological status of cells during batch culture; SYTO 9 (Invitrogen, Paisley, UK) together with PI (Sigma-Aldrich, Dublin, Ireland), carboxyfluorescein diacetate (5[and 6]-CFDA “mixed isomers”; Invitrogen) and Hoechst 33342 (Sigma-Aldrich) and C₁₂-resazurin and SYTOX Green (constituents of the “Live/Dead® Cell Vitality Assay Kit”, Invitrogen). PI and SYTOX Green are nucleic acid dyes which only enter cells with permeabilised membranes, non-fluorescent CFDA is hydrolysed by non-specific intracellular esterases to form the fluorescent product, carboxyfluorescein (CF), non-fluorescent C₁₂-resazurin is reduced by cells with redox activity to fluorescent C₁₂-resorufin, while the permeant nucleic acid dyes, SYTO 9 and Hoechst 33342, are effectively counterstains. All dyes are excited 488 nm light, bar Hoechst 33342, which is excited at 325 nm. Emission maxima for the dyes are as follows; SYTO 9: 520 nm (green); PI: 617 nm (red); CF: 515 ± 5 nm (green); Hoechst 33342: 464 nm (violet);

C₁₂-resorufin: 575 nm (orange); SYTOX Green: 530 nm (green). Staining conditions, dye concentrations used and flow cytometer instrument set-up for the three FCM staining regimes are described in detail in a previous publication [26]. For these staining regimes, 270 µl aliquots removed from batch cultures at sampling times comprised the samples for staining and FCM analysis.

Fluorescent staining of cells for flow cytometric tracking of cell division

Cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) which forms stable conjugates with aliphatic amines, yielding carboxamides resistant to hydrolysis, and can be used to track cell division based on the principle that when a cell divides, its daughter cells will emit half the fluorescence of the mother cell [2, 29]. CFSE is excited at 488 nm and has an emission maximum at 518 nm (green). Unlike the previous staining regimes, cells were stained at the outset of batch cultures. At sampling times, 300 µl aliquots of suspensions were removed and analysed directly using FCM. Cells were stained as follows: 475 µl of the suspension normally used to begin experimental batch cultures was mixed with 25 µl of 1 mM CFSE, to give a final concentration of CFSE of 50 µM. This mixture was held at 30 °C in the dark for 30 min, before being centrifuged at 8,000g for 10 min and the pellet resuspended in 500 µl NB. This volume was then used to initiate a batch culture in the same manner as for unstained cells.

Measurement of phosphatidylcholine-specific phospholipase C activity

Measurement of PC-PLC activity was carried out using the Amplex[®] Red Assay Kit (Invitrogen). This enzyme-coupled assay works on the basis of detecting the fluorescent product of the Amplex[®] Red reagent (10-acetyl-3,7-dihydrophenoxazine), resorufin (absorption maximum: 571 nm; emission maximum: 585 nm). Measurement was performed in a Synergy[™] HT absorbance/fluorescence/bioluminescence microplate reader (BIO-TEK[®] Instruments Inc., Vermont, USA) together with Costar 96-well sterile, black, clear- and flat-bottomed polystyrene plates (Corning, New York, USA). Assay volume was 200 µl per well. Instrument settings included: 530/25 nm excitation filter, emission filter of 590/20 nm, top reading, photomultiplier tube sensitivity of 57, shaking of plates prior to reading at intensity “3” for 10 s, taking of 10 sample readings per well, pre-heating of plates to 37 °C prior to reading, top probe vertical offset of one mm, delay before sampling of 350 ms and a delay between sampling of 1.0 ms. On each plate a standard curve consisting of known activities of pure PC-PLC

from *B. cereus* (0.1, 0.2, 0.4, 0.8, 1.6 U ml⁻¹, where one unit is defined as the amount of enzyme that will liberate 1.0 µmol of water-soluble organic phosphorous from L- α -phosphatidylcholine per minute at pH 7.3 at 37 °C), which was supplied with the kit, was included. Using the above settings, good agreement was obtained between actual and measured concentrations (typical $R^2 \approx 0.883$). For the analysis of samples from batch cultures, one ml aliquots were removed at sampling times and cell-free extracts immediately frozen and stored at -80 °C. At a later date, samples were rapidly thawed and analysed. Freezing, storage at -80 °C and thawing of samples did not have any detectable effects on activity (data not shown). Triplicate samples were analysed per batch culture using this assay, with positive controls provided by dilutions for constructing the standard curve and negative controls by sterile NB.

Measurement of cell adhesion

For the measurement of cell adhesion, a modification of the Vybrant[™] Cell Adhesion Assay Kit (Invitrogen) was utilised, whereby, for measuring cell permeability, PI was used instead of the supplied SYTOX Green dye. This allowed the simultaneous measurement of cell permeability, using PI, and esterase activity, using the kit's other component, calcein acetoxymethyl ester (calcein AM), which is similar to CFDA. The cleavage product, calcein, has an absorption maximum at 494 nm and an emission maximum at 517 nm (green). The assay was performed in a microplate format, with final concentrations of calcein AM and PI of five and 10 µM respectively. At 0 h, 197 µl of the suspension used to begin experimental batch cultures was placed in each well of the microplate, which was incubated at 30 °C in between dye loading or washing and reading. 30 min prior to each sampling time, 2 µl of PI and 1 µl of calcein AM stock solutions were added to wells randomly designated as to be assayed during that sampling time. Blanks consisted of unstained suspensions. The microplate was then held at 30 °C in the dark for 30 min. Following this, the liquid in assay wells was gently removed and the wells carefully washed four times using a swirling motion with sterile pre-warmed NB. Finally, 200 µl of NB was added to each washed well and the green and red fluorescence of the remaining adherent cells was read using the Synergy[™] HT microplate reader. (A number of wells per sampling time did not have liquid removed and were not washed in order to be able to compare the fluorescence from all cells (adherent and non-adherent) with that of adherent cells). At each sampling time six wells were washed and tested and two wells tested without washing.

For this assay Costar 96-well plates (see above) were employed. For calcein, 485/40 nm excitation and 528/20 nm emission filters were used and for PI, 530/25 excitation

and 645/40 emission filters. No crosstalk between fluorophores and little autofluorescence from cells was recorded. For both fluorophores, the following settings were chosen as optimal; top reading, photomultiplier tube sensitivities of 50, shaking of plates prior to reading at intensity “1” for 1 s, taking of ten sample readings per well, pre-heating of plates to 37 °C prior to reading, a top probe vertical offset of 1 mm, a delay before sampling of 350 ms and a delay between sampling of 1.0 ms.

Statistical analysis and presentation of data

Data were analysed using Microsoft® Excel 2000 9.0.2720 (Microsoft Corporation, Redmond, WA, USA) and SPSS 13 (SPSS Corporation, Chicago, USA). Figures and graphs were also prepared using these software packages. Unless otherwise stated, means are presented \pm their 0.95 confidence interval.

Results

Over the course of 8 h, mean OD₆₀₀ of batch cultures increased from \sim 0.001 at 0 h to 0.507 ± 0.003 at 8 h, with a simultaneous increase in \log_{10} CFU ml⁻¹ from 5.32 ± 0.27 at 0 h to 7.55 ± 0.44 at 8 h. pH decreased somewhat from 7.0 to 6.84, conductivity increased from -25.0 to -9.66 mV while slight increases in the A_{260} , A_{310} , A_{340} and A_{430} of suspension supernatants were noted. Endospores were not detected in cultures until 8 h at which time they accounted for $0.22 \log_{10}$ CFU ml⁻¹ of the total count. According to a graph of time versus \log_{10} CFU ml⁻¹, lag phase lasted until approximately 3 h (see below).

Analysis of SYTO 9- and PI-stained cells using scatter plots of green versus red fluorescence allowed the identification of four discrete sub-populations, namely cells displaying very high red fluorescence only (Region I; denoted as highly permeabilised, possibly dead), cells displaying medium red fluorescence only (Region II; denoted as mildly permeabilised, possibly live), cells displaying high green fluorescence only (Region III; intact live cells) and cells displaying both high red and green fluorescence (Region IV; dual permeabilised and possibly dead). These populations were enumerated by plotting of regions around population borders (Fig. 2). Population profiles at each sampling time were different. For the first hour of batch culture, the most populous region was Region I (\sim 40–50% of cells), followed by Region II (\sim 30% of cells; Fig. 3). By 2 h, Region II-type cells dominated (\sim 43%), followed by Region III-type cells (\sim 33%). Over the period 3–7 h, Region III was the most populated region (\sim 46–75%), with Region II accounting for the majority of the remaining cells (\sim 30–20%). At 8 h, equal numbers of cells were located in

both these regions (\sim 45%). From 4 h onwards, the percentage of cells located in Region I was $<4\%$.

Cells showing intracellular esterase activity were identified and enumerated using scatter plots of green versus violet fluorescence. A discrete region of highly green fluorescent events corresponded to cells with high esterase activity, a second region of green fluorescent events corresponded to cells with moderate amounts of esterase activity and a third region of non-fluorescent events corresponded to esterase-negative cells (Fig. 2). At 0 and 1 h, esterase negative cells comprised the majority of cells (\sim 88 and \sim 72% of cells, respectively; Fig. 3). At 2 h, the combined percentage of highly and moderately esterase-positive cells equalled the percentage of esterase negative events. From 3 h onwards, the majority of cells were highly esterase-positive, with moderately esterase-positive cells comprising the vast majority of the remaining proportion of cells from 4 h onwards. Esterase negative cells made up $<4\%$ of cells from 4 h onwards.

C₁₂-resazurin- and SYTOX Green-stained cells analysed using scatter plots of green versus orange fluorescence, were classified into three groups: cells reducing C₁₂-resazurin to C₁₂-resorufin (redox active cells; red but not green fluorescent), cells permeable to SYTOX Green (green but not red fluorescent) and cells designated as intact but not metabolically active (neither green nor red fluorescence displayed; Fig. 2). Batch cultures sampled at 0 and 1 h comprised approximately equal percentages of redox active, intact and permeabilised cells (Fig. 3). Beginning at 2 h, cultures were predominantly composed of redox active cells, such that from 4 h onwards, $>96\%$ of cells were redox active.

CFSE-stained cells were analysed using single parameter histograms of green fluorescence. Mean fluorescence values of suspensions decreased over time, with the relationships between time and mean fluorescence ($R^2 = 0.899$; $P < 0.001$; $y = -77.045x + 703.29$) and mean fluorescence and \log_{10} CFU ml⁻¹ for the same sampling time ($R^2 = 0.845$; $P < 0.001$; $y = -234.09x + 1819.5$) being statistically significant and strong. Variation in the protein content amongst cells in batch cultures also decreased during culture, evident from the narrowing in distributions of histograms of green fluorescence and reflected statistically by a decrease in standard deviations of mean green fluorescence over time (Fig. 4). This relationship was also statistically significant and strong ($R^2 = 0.945$; $P < 0.001$; $y = -113.24x + 914.66$).

The PC-PLC assay revealed the presence of this toxin at the outset of culture (possibly due to carry over from the inoculum). Activity gradually increased over the first 2 h of culture (Fig. 5). Between 3 and 6 h, levels of toxin increased in a linear fashion, reaching 0.041 ± 0.006 U ml⁻¹ at 6 h. During the final 2 h of culture, the rate

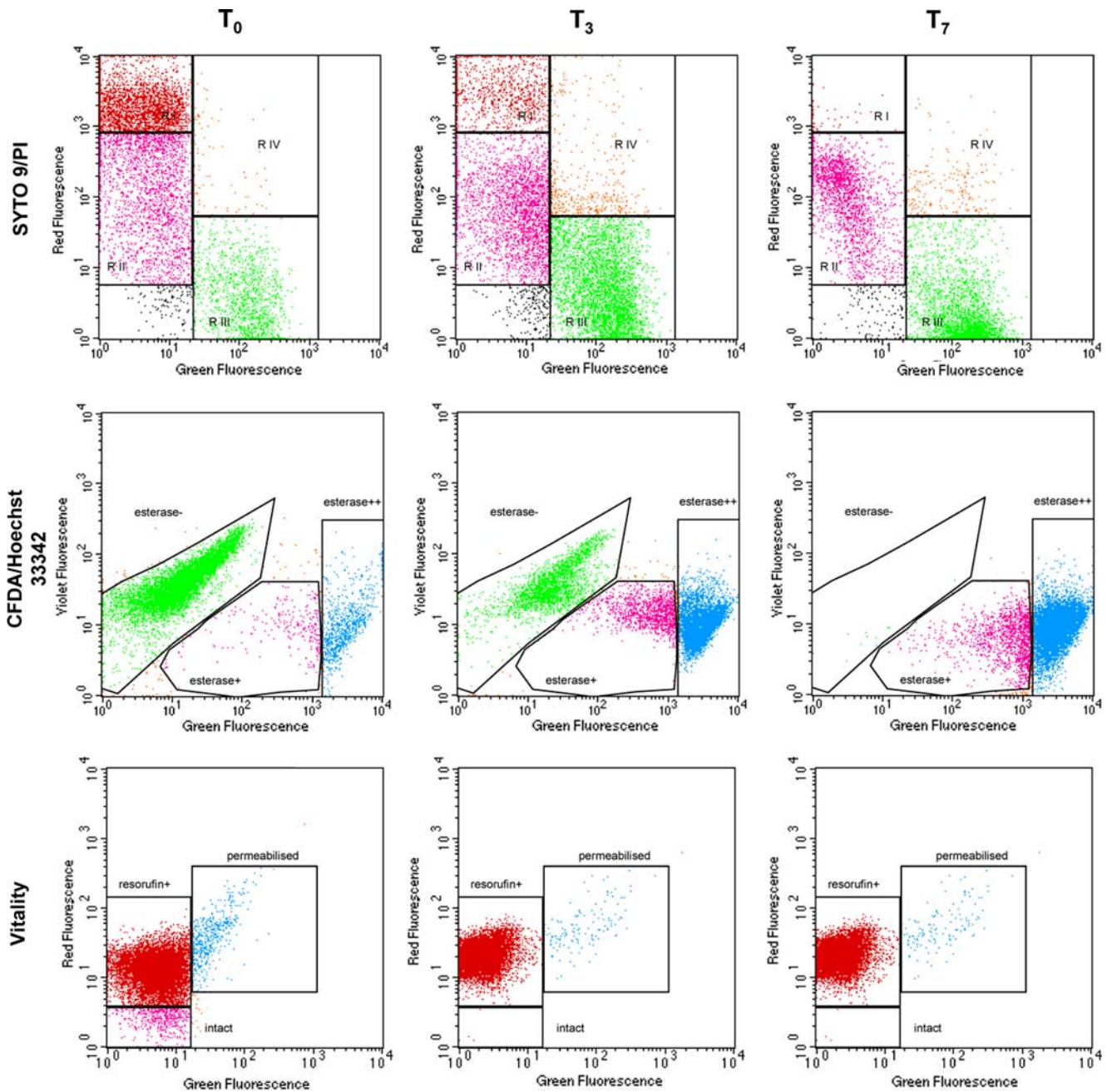


Fig. 2 Scatter plots of *Bacillus cereus* vegetative cells growing as batch cultures in NB at 30 °C over 8 h and analysed using flow cytometry. The *first column* shows cells taken from batch cultures at 0 h, the *second column* at 3 h and the *third column* at 7 h. (*SYTO 9/PI*) row shows the green versus red fluorescence profiles of SYTO 9 and PI-stained cells. R I is populated by highly PI-permeable cells, R II by moderately PI-permeable cells, R III by intact SYTO 9-only stained cells and R IV by moderately PI- and SYTO 9-permeable cells. (CFDA/Hoechst 33342) Green versus violet fluorescence profiles of

CFDA and Hoechst 33342-stained cells. Highly green and moderately green fluorescent cells, the esterase activity of which has cleaved CFDA to the fluorescent CF, are indicated. (vitality) Green versus red fluorescence of C_{12} -resazurin- and SYTOX Green-stained cells. Indicated are: red-fluorescent cells the metabolic activity of which have converted C_{12} -resazurin to the fluorescent C_{12} -resorufin, cells permeable to SYTOX Green, and intact cells without reductive capacity. NB nutrient broth; CF carboxyfluorescein; CFDA carboxyfluorescein diacetate; PI propidium iodide

of increase of activity tailed off. A statistically significant and strong relationship was found between PC-PLC activity and \log_{10} CFU ml⁻¹ over time ($R^2 = 0.849$; $P < 0.001$; $y = 52.916x + 4.7732$).

Cell adhesion properties of the culture varied over time with ~25–100% of viable cells (esterase-positive green fluorescent) adherent (Fig. 6). Generally, the only clear trend found for viable cells during batch culture was a

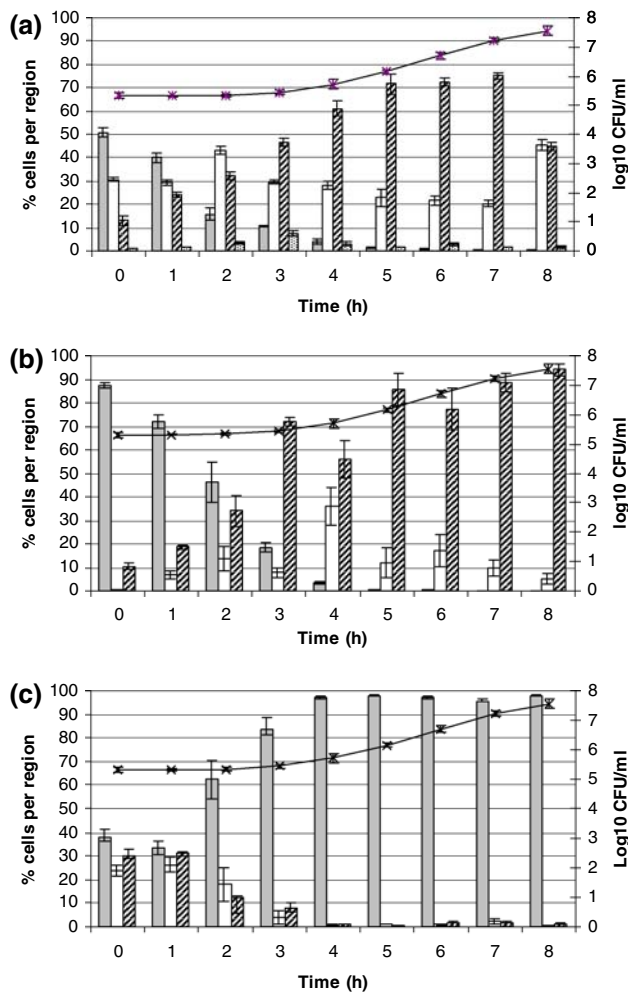


Fig. 3 The physiological profiles of suspensions of *Bacillus cereus* vegetative cells growing as batch cultures in NB at 30°C over 8 h and analysed using flow cytometry. **a** The mean percentage of cells stained with SYTO 9 and PI classified as being highly PI permeable (black bars), moderately PI permeable (clear bars), SYTO 9-only permeable (grey bars) and moderately permeable to both PI and SYTO 9 (bars with squares). **b** The mean percentage of CFDA and Hoechst 33342 cells displaying high amounts of esterase activity (grey bars), moderate amounts of esterase activity (clear bars) and no esterase activity (black bars). **c** The mean percentage of C₁₂-resazurin- and SYTOX Green-stained cells analysed using flow cytometry and designated as being metabolically active (grey bars), intact with no metabolic activity (clear bars) or permeable to SYTOX Green (black bars). Included in each panel is a plot of the mean log₁₀ CFU ml⁻¹ of *B. cereus* vegetative cells growing as batch cultures in nutrient broth at 30 °C over 8 h ($n = 5$). Lag phase ends approximately 3 h after inoculation. NB nutrient broth; CF carboxyfluorescein; CFDA carboxyfluorescein diacetate; PI propidium iodide

linear reduction in the percentage of adherent cells (i.e. a reduction in the percentage of green fluorescence post- and pre-washing) between 6 and 8 h. For damaged (PI-permeable red-fluorescent) cells, a ~97–99% reduction in red fluorescence was recorded at every sampling time, indicating that only between ~1 and 3% of membrane-compromised cells were adherent.

Discussion

In this study, distinct growth phase-related changes in the physiological profiles of *B. cereus* cells over time during batch culturing were detected using FCM. During lag phase, the majority of cells showed membrane damage, lacked intracellular esterase activity and had low levels of redox activity. This could either demonstrate the poor physiological condition of cells that form the inoculum or the extent of stress experienced by cells upon removal from their original medium and after transfer to another medium. As the lag phase continued, cells began adjusting to and altering their new environment [30], reflected by FCM profiles displaying a lesser degree of membrane damage, a greater percentage of cells with intracellular esterase activity and higher percentages of redox-active cells. These results agree with a previous FCM-based study, where *E. coli* cells were shown to adjust to their new environment through alterations in cell structure reflected by increases in FSC throughout the lag phase [7]. Upon entry into the exponential phase at 3 h, population profiles were largely unaltered until 8 h. Additionally, populations became more uniform, with the vast majority of cells being membrane intact, esterase positive and redox active. This increase in uniformity of cells within batch cultures as the exponential phase proceeded, previously reported for FCM analysis of *E. coli* [9] and *B. subtilis* [13], was reflected in this study by the decreasing variation of cellular protein content as measured by CFSE-staining of cells comprising the inoculum.

In addition to measuring population variability of cellular protein content, CFSE-staining was useful for tracking *B. cereus* cell division. Indeed, strong correlations were found between the reduction in fluorescence of stained cells and both time and log₁₀ CFU ml⁻¹. Staining of inoculum cells with CFSE has previously been shown to produce useful results as regards monitoring bacterial cell division [31]. This method has possible applications in the rapid screening of antimicrobial compounds and treatments, where the rate of decrease in green fluorescence due to cell division of treated cells and controls can be compared [29].

PC-PLC activity in batch cultures of *B. cereus* appeared strongly related to cell density and as cultures entered the exponential phase activity increased in a linear fashion over time. This was also found for the production of *B. cereus*' emetic and diarrhoeal toxins [14, 32, 33] and supports the possibility that PC-PLC expression is likely under the control of a quorum sensing system [34, 35]. PC-PLC production also coincided with the acquisition by the majority of cells in culture of both intracellular esterase and redox activity and may indicate that only cells possessing such activities are capable of toxin production. However, further research is necessary to separate the effects of cell density and cell physiological state. The levels of PC-PLC activity

Fig. 4 Histograms of the green fluorescence as measured using flow cytometry of CFSE-stained *Bacillus cereus* vegetative cells grown as batch cultures in NB at 30°C. Sample times, mean green fluorescence and mean standard deviations ($n = 5$) are indicated in each panel. CFSE carboxy-fluorescein diacetate succinimidyl ester; NB nutrient broth; SD standard deviation

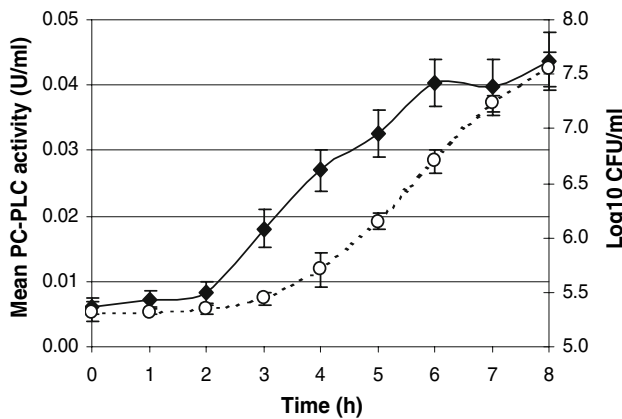
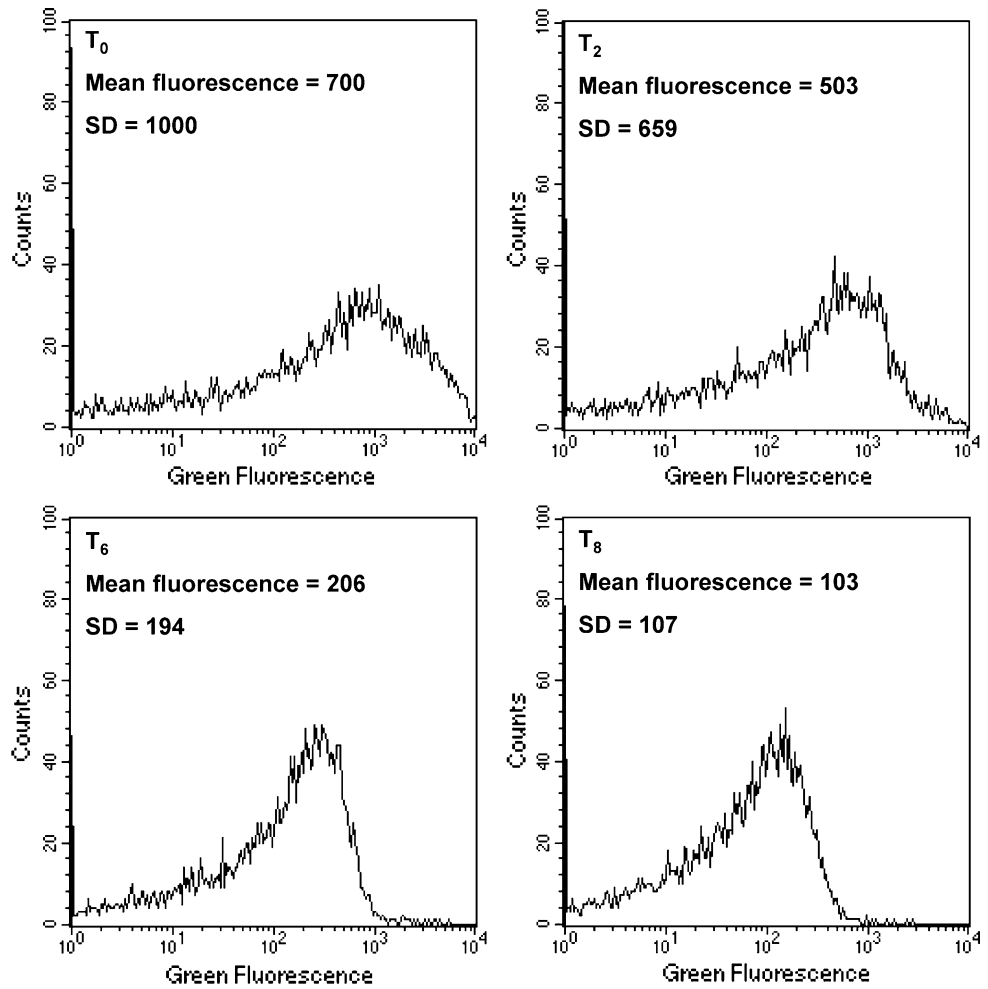


Fig. 5 PC-PLC activity over time in U ml⁻¹ in batch cultures of *Bacillus cereus* vegetative cells growing in NB at 30 °C (filled diamond). Included is a plot of the mean log₁₀ CFU ml⁻¹ during the same batch cultures (open circle; $n = 5$). NB nutrient broth

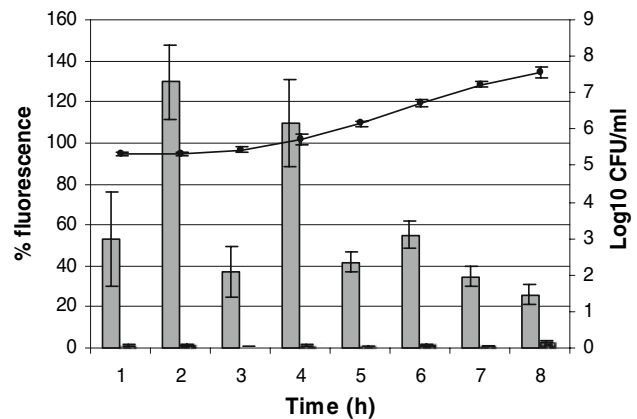


Fig. 6 The percentage of green (grey bars) and red fluorescence (black bars) remaining following four washes in sterile NB compared to starting fluorescence arising from *Bacillus cereus* vegetative cells stained with calcein AM and PI and grown in polypropylene microtitre plates at 30 °C over 8 h. Calcein AM calcein acetoxyethyl ester; NB nutrient broth; PI propidium iodide

recorded in this study (0.01–0.04 U ml⁻¹) are similar to those in food contaminated with *B. cereus* suspected as the cause of a food-poisoning outbreak (0.04–0.05 U ml⁻¹;

[21]), implying that toxin levels equivalent to those measured in heavily contaminated food can be reached in batch culture after 6 h of incubation. In contrast to other toxins,

special factors in the medium or particular environmental conditions do not seem to be required in order to stimulate PC-PLC expression or activity e.g. aeration has been found necessary for the production of emetic toxin [36].

Data from the cell adhesion assay indicated that permeabilised (and very possibly dead) cells do not adhere to the polypropylene surface of microtitre plate wells, whereas ~25–100% of cells possessing esterase activity (and very possibly viable) also possessed adhesion properties. This suggests that either adhesion is primarily a property of live cells or that damage to the cell membrane retards adhesion. In agreement with [37], up to ~50% of live cells are capable of adhesion 1 h into batch culture, indicating that *B. cereus* contamination of surfaces, aided by adhesive cells, can quite quickly become problematic from a sanitation viewpoint. Given the fact that a linear reduction in the percentage of adherent live cells was observed between 6 and 8 h, it may be that, as the exponential phase proceeds, live cells begin to lose their ability to adhere.

In agreement with [10], this report demonstrates the utility of FCM as a tool for studying the behaviour of individual bacterial cells in batch cultures. FCM is the only rapid method capable of yielding multiple measurements from large numbers of individual cells and is an excellent tool for studying heterogeneity in bacterial populations [38]. Using FCM in combination with three staining protocols, the various physiological changes over the course of a batch fermentation could be separated into distinct events: stress responses of cells at the outset of a batch culture, recovery from stress with the onset of exponential growth and the development of cell homogeneity within exponential phase populations. The richness of the data yielded by FCM is in contrast with absorbance data; A_{260} , A_{310} , A_{340} and A_{430} altered very little over the course of the culture. The FCM methods utilised in this study may potentially be applied to evaluating culturing methods to reduce the stress on cells during the lag phase in order to increase the efficiency of industrial fermentations. Conversely, FCM could evaluate methods to increase stress during food production processes in order to enhance food preservation. The methods described in this report may also be used to enhance existing models of *B. cereus* growth during batch culture and in contaminated foodstuffs.

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