Characterisation of tetraploid and diploid clones of *Spodoptera frugiperda* cell line

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Abstract

We have isolated and characterised diploid and tetraploid clones from the normally heterologous *Spodoptera frugiperda* (Sf-9) cell line by dilution cloning technique. Tetraploid clones were found to have cell sizes in excess of 35% larger than that of the diploid clones. In contrast, the maximum cell numbers achieved in batch cultures of diploid clones were on average 185% higher than the tetraploid cell numbers. Growth rates and metabolic quotients during the exponential phase were similar for both clones. Tetraploid cells infected with wild-type and recombinant green fluorescent protein (GFP) baculovirus, resulted in more polyhedra or GFP product per cell. Importantly, the difference between the clones either completely diminished or reduced to 50% when the yield was assessed in terms of the amount of polyhedra or GFP per mL of medium, respectively. These results indicate that the existing heterogeneity in insect cell populations with respect to ploidy level, are correlated to cell growth and product yield.

Introduction

The baculovirus expression system is often the method of choice for the production of recombinant proteins as the system has many advantages, including short time-scales, safety, relative culture simplicity of the insect cell culture systems, and high levels of production that are frequently achieved. The system is based on the insect nuclear polyhedrosis viruses (NPVs); a group of large rod-shaped DNA viruses, into which the gene or genes of interest are cloned. The recombinant virus can then be used to infect insect cells in culture, with subsequent expression of recombinant proteins (Miller 1988; Vlak 1992; O'Reilly et al. 1994; Kioukia et al. 1995). The virus *Autographa californica* (AcNPV), which infects a limited number of insect species including *Spodoptera*

frugiperda (Sf-9) cells, is the most extensively used baculovirus (Francki et al. 1991; Jones and Morikawa 1996).

Clone Sf-9 originates from the ovarian tissue of the fall armyworm, *S. frugiperda* (Sf-21) (American Type Culture Collection 1988). Although much interest has been engendered in Sf-9 research, there is distinct lack of cytological data and the subsequent effects that cellular, nuclear or chromosomal attributes have on productivity, stability and longevity.

Homogenous cell lines are essential in industry and research if an optimised production system that provides reliable and reproducible data is to be obtained. Yet, DNA analysis of the Sf-9 cell line from different international laboratories has revealed that the ploidy levels of this insect cell line vary depending on its culture history and

origin (Jarman-Smith et al. 2002). Doverskog et al. (2000) reported the appearance of an octaploid subpopulation during the G2/M arrest, which was explained as due to the latent possibility of transition of G2/M cells into an endoreplicative state. Other observations (Vaughn et al. 1977; Disney and McCarthy 1985) suggested that the chromosomes seem to spontaneously fragment during growth and expansion which raises the question. Do the tetraploid cells arise from diploid cells during cell cultivation or from a cross-contamination of different cell populations or do the cells display an altered cell cycle prior to cell death?

What evidence is there that the Sf-9 cells are spontaneously fragmenting?

This genus is often described as having a higher polyploid state (Vaughn et al. 1977; Disney and McCarthy 1985; Lery et al. 1999) yet the polyploidy state of the Sf-9 cells has largely been ignored even though a third peak on the cell cycle obtained through flow cytometry has long been recorded, e.g. see Fertig et al. (1990). Chromosomal fragmentation has previously been reported in two *S. frugiperda* cell lines IPLB-Sf-21 and IPLB-Sf-1254 (Vaughn et al. 1977) as well as in TN358 cell lines (Disney and McCarthy 1985). Positive identification of tetraploid cells has also been made in the TN358 cell line (Disney and McCarthy 1985).

The polyploidism is believed to occur in Lepidoptera because the chromosomes are holokinetic, which means that during cell division the spindle fibre can attach to the chromosomes at any point on the chromosome (White 1973; Swanson 1981; Disney and McCarthy 1985). Consequently, if the chromosome splits into two, both pieces of the chromosome remain viable and fragments of chromosomes rapidly begin to accumulate in the cell. Whether these are random chromosomal breakages or programmed response to culture/environmental conditions has yet to be determined.

Having experimentally confirmed that a polyploid state in Sf-9 exists (Jarman-Smith et al. 2002), the significance of this finding needed to be explored in order to determine how ploidy arises in insect cell culture. What is the consequence of changes in chromosome number and what is the impact on productivity? Thus, a comprehensive

investigation into the properties of the individual ploidy states in the Sf-9 cell line was undertaken. The Sf-9 cell line used in this work is heterogeneous, comprised of two subpopulations. The low chromosome number subpopulation has been termed as "diploid" and the secondary karyotype as "tetraploid" (as the latter appears to have twice the DNA content of the diploid cell line) with these two subpopulations being able to coexist or exist independently (Jarman-Smith et al. 2002).

The growth characteristics of diploid and tetraploid subpopulations isolated from Sf-9 heterogeneous parental cells were determined. The cloned populations significantly reduced genetic variation, producing a pool of cells with closely related karyotypes and morphology.

Materials and methods

Cell lines, maintenance and samples for analysis

The Sf-9 parental line was obtained from GlaxoSmithKline (Harlow, UK) at passage number 104, and maintained at 27 °C in TC100 medium (Gibco, Paisley, UK) supplemented with 5% foetal bovine serum (FBS; PAA laboratories Ltd, Yeovil, UK), 10 units mL⁻¹ penicillin and 10 µg mL⁻¹ streptomycin. Sf-9 clones were obtained from the mid-exponential phase of heterogeneous parental Sf-9 cultures. Samples for viable cell number, viability, cell size, glucose utilisation, lactate production and product yield were obtained from the clonal cultures in the mid-exponential phase. Cell concentration and viability was estimated using a haemocytometer and trypan blue exclusion (Sigma, Poole, UK). A Multisizer II and Accucomp software (Coulter Electronics, Luton, UK) were used to measure absolute cell size.

Viruses

The wild-type AcMNPV baculovirus was kindly supplied by Dr B. Rooney (CeNes Ltd, Cambridge, UK) and the recombinant green fluorescent protein (GFP) AcMNPV virus from GlaxoSmithKline. The GFP gene in GFP AcMNPV had been artificially inserted in place of the very late expression

polyhedra gene, and was under the control of the strong polyhedrin promoter.

Recombinant protein or polyhedra production

Cells were resuspended at 8×10^5 cells mL⁻¹ and infected with either wild-type AcMNPV (multiplicity of infection (MOI) 5) or GFP AcMNPV (MOI 1).

Dilution cloning

Heterogeneous parental Sf-9 cell line in mid-exponential phase was extrapolated and serially diluted to 10^1 cells mL⁻¹. Suspension (100 μ L) was aliquoted into each well of a 96-well flat-bottomed micro titre plate (NUNC) precoated with 1% agarose to capture 1 cell per well. Plates were then placed in sterile air-tight bags containing autoclaved moist towels and incubated until clones were visualized (approximately 10–20 days). Clones were then subsequently expanded and ultimately transferred to 250 mL Belco flasks, agitated with a magnetic pendulum.

Preparation and analysis of cells using flow cytometry

Cells infected with GFP virus were washed in 1% cold (4 °C) paraformaldehyde solution. All samples were suspended at 1×10^6 cells mL⁻¹, centrifuged (1000 rpm for 5 min), the pellets resuspended in 20 mL of 70% cold (–18 °C) ethanol and the samples stored at –18 °C. Prior to use, GFP samples were centrifuged at 1000 rpm for 5 min to remove ethanol. Cell pellets were washed in $1 \times$ (calcium- and magnesium-free) phosphate buffered saline (PBS) at pH 6.4. Samples were recentrifuged and resuspended in 1 mL RNase solution (250 μ g mL⁻¹; Sigma) for 20 min at 37 °C. To each sample, 50 μ L of propidium iodide (PI; 50 μ g mL⁻¹, Sigma) was added, mixed and incubated at room temperature for a minimum of 5 min.

Flow cytometric analysis was performed using Coulter EPICS Elite Analyser (Coulter Electronics) equipped with an argon laser emitting 15 mW at 488 nm, as described by Al-Rubeai et al. (1992). Passing the cells individually through the argon laser beam activated the relative cellular DNA content of 10^6 stained cells. Forward-scattered (FS)

laser light was collected using a neutral density filter and the standard cross beam mask and 90° side scatter (SS) laser light was collected using a 488 nm band pass filter. PI and EGFP fluorescence was collected using a 488 nm long pass followed by a 635 nm band filter.

Intracellular pH (pHi)

Cells at 6×10^6 cells mL⁻¹ were taken from mid-exponential phase, centrifuged and resuspended in 2.5 mL of medium. Carboxy-seminaphtharhodafleur-1-acetoxymethyl ester (carboxy-SNARF-1-AM) (1 mM in dimethylsulphoxide (DMSO); Molecular probes, Leiden, The Netherlands) was added to give a final concentration of 10 μ M and cells incubated for 30 min at 37 °C. Cells were centrifuged (1000 rpm for 3 min) and resuspended in 3 mL of fresh medium for flow cytometry analysis. The pHi values were obtained by determining the fluorescence ratio emissions at 575 and 635 nm. A standard curve was generated by calibration of the fluorescence ratio in buffers of different ionic strength containing the proton ionophore nigericin (Molecular probes) to convert this fluorescence ratio in to intracellular pH (Ishaque and Al-Rubeai 1998).

Determination of the level of polyhedrin expression

Samples of cells infected with wild-type AcMNPV were repeatedly freeze-thawed to lyse cells. The number of polyhedra per mL was then determined by haemocytometer.

Measurement of glucose and lactate concentration in culture medium

Glucose and lactate concentrations were obtained using the Biolyser Rapid Analysis System (Eastman Kodak Co., USA) as per manufacturer's instruction.

Measuring oxygen uptake rates (OUR)

A dissolved oxygen probe (Digital Oxygen System Model 10, Rank Brothers Ltd) was used to measure the OUR of the diploid and tetraploid Sf-9 clones. Around 2.5 mL of cell suspension (5×10^5

cells mL^{-1}) was continually stirred in the probe chamber and the decrease in the level of dissolved oxygen was monitored on a chart recorder.

Flow cytometric identification of the Sf-9 cloned subpopulations

Serial dilution cloning generated single cell colonies, which were selected and propagated in increasing volumes until cultivation in T-flasks (Jarman-Smith et al. 2002). The DNA content of all 26 clonal isolates was screened using the flow cytometer to assess their ploidy state. About 16 of the clones were true diploid clones and two were tetraploid clones. Flow cytometry analysis of the relative DNA content and cell cycle phases was performed in the mid-exponential phase for Sf-9 heterogeneous parental cells and Sf-9 cloned cells.

Results

The growth characteristics of the Sf-9 parent, diploid and tetraploid clones in batch culture

The flow cytometry profiles illustrated in our previous paper (Jarman-Smith et al. 2002) clearly showed that the heterogeneous parental cell line (diploid G₁, S and G₂, combined with tetraploid G₁, S and G₂) was successfully separated into two distinct clones by dilution cloning technique. Figure 1 illustrates the DNA distribution of Sf-9 parental cells. Batch cultures of the Sf-9 heterogeneous parental cell line and Sf-9 clones are illustrated in Figure 2.

The cellular growth rate of each cell line was similar for the first 60 h of exponentially growing culture. The tetraploid cell cultures peaked 24 h before the diploid and parental cell lines reached their optimum cell density. Tetraploid clones 13 and 11 reached 11.1×10^5 cells mL^{-1} and 12.1×10^5 cells mL^{-1} respectively 60 h post seeding. Whereas 84 h post inoculation, the Sf-9 parental cells attained a cell concentration of 26.1×10^5 cells mL^{-1} more than double the tetraploid cell number and the diploid clones grew to in excess of 30×10^5 cells mL^{-1} roughly triple the tetraploid cell lines. The growth curve of the tetraploid clonal isolates (Figure 2) contradicts the opinion of Fertig et al. (1990) who suggested that the tetraploid cells

Figure 1. Flow cytometry profile of Sf-9 heterogeneous parental cell line illustrating that it contains a Diploid G₁, S and G₂ subpopulations combined with tetraploid G₁, S and G₂ subpopulations. The profile illustrates the number of cellular events versus relative DNA content.

synchronised in the G₂+ M phase were non-cycling as cell division was absent. However, it is now believed that tetraploid cells are capable of independent growth and the cells are cycling but their maximum potential cell density is limited.

Cellular viability started to decrease approximately 60 h post seeding, as the cell density began to decline, in a comparable trend in both tetraploid cell lines (Figure 3). The heterogeneous parental cell line and the diploid clones exhibited a similar decrease in cell viability as the batch culture progressed. The latter retained a high viability, in excess of 80% viable, until approximately 108 h post seeding, reflecting their extended exponential growth phase. Cell survival and maximum attainable cell densities were far superior in the diploid clones and heterogeneous parental cell lines when compared with the tetraploid cell lines. At 84 h the cell density of the parental cell line was more than double, and the diploid cell line reached triple the cell number attained with the tetraploid cell line. The trypan blue counts revealed that the tetraploid cells lose their viability at a greater rate in batch culture than its counterparts. Perhaps tetraploid cells are more susceptible to cell death caused by shear due to its enlarged cellular volume, or they are more sensitive to environmental influences such as nutrient deprivation.

They may have consumed higher quantities of an essential amino acid or toxic metabolite accumulation, though the cells seem to reutilise

Figure 2. Growth curve of batch cultures of Sf-9 heterogenous parental cell line and two cloned Sf-9 diploid cell lines and two cloned Sf-9 tetraploid cell lines. The insect cell density was assessed by trypan blue assessment.

Figure 3. The viability of Sf-9 parental, diploid and tetraploid cells in batch culture. The cellular viability of the insect cells was assessed by trypan blue assessment. Cells that excluded trypan blue were viable and cells stained blue were non-viable.

the lactate they produce so it does not collect in the cells. The onset of cell death coincided with a drop in viability to 74% which is in agreement with Cowger et al. (1999), who obtained flow cytometric evidence that *S. frugiperda* cells grown in rotating-wall vessel or shaker flask became apoptotic at 70% cell viability.

Both the parental and the diploid cell lines survived 24 h longer than the tetraploid cells in batch cultures. The presence of a tetraploid subpopulation in the parental cell line did not have a negative

impact on cell viability, may be because the second karyotype only represents a small amount of the whole population. No significant differences were observed between the mode and timing of cell demise between the heterogeneous parental and the diploid clones.

The growth characteristics derived from the batch cultivation of Sf-9 variants are summarised in Table 1. Using a Coulter Multisizer, it was shown that the parental and the diploid cells are quite similar in size but the tetraploid cells were

Table 1. The growth characteristics of the parent, diploid and tetraploid clones propagated in batch culture.

Sf-9 cell line	Initial cell density ($\times 10^5$ cells mL^{-1})	Max. cell density ($\times 10^5$ cells mL^{-1})	Time taken to reach max. cell density (h)	Duration of batch (h)	Mean specific growth rate (h^{-1})	QG ($\text{mmol L}^{-1} \text{h}^{-1}$ cells $^{-1}$)	QL ($\text{mmol L}^{-1} \text{h}^{-1}$ cells $^{-1}$)	pHi	Oxygen uptake rate (10^{-17} moles $\text{cell}^{-1} \text{s}^{-1}$)	Mean cell size (μm)
Sf-9 parent	2.5 Std = 0.19	26.1 Std = 0.55	84 $\pm$ 1	156 $\pm$ 0.5	0.024 Std = 0.01	0.004	0.0010	–	–	15.6 Std = 0.65
Diploid clone 4	2.5 Std = 0.23	30.9 Std = 1.09	84 $\pm$ 1	156 $\pm$ 0.5	0.024 Std = 0.01	0.004	0.0012	–	11.46 Std = 0.19	14.4 Std = 1.29
Diploid clone 9	2.5 Std = 0.26	34.9 Std = 1.35	84 $\pm$ 1	156 $\pm$ 0.5	0.028 Std = 0.01	0.003	0.0012	6.9	12.22 Std = 0.31	14.4 Std = 0.83
Tetraploid clone 11	2.5 Std = 0.21	12.1 Std = 0.80	60 $\pm$ 1	132 $\pm$ 0.5	0.024 Std = 0.01	0.006	0.0010	6.4	14.48 Std = 1.23	20.3 Std = 1.80
Tetraploid clone 13	2.5 Std = 0.27	11.1 Std = 0.59	60 $\pm$ 1	132 $\pm$ 0.5	0.025 Std = 0.01	0.006	0.0011	–	14.40 Std = 0.36	18.5 Std = 0.78

QG: specific glucose consumption rate; QL: specific lactate production rate; Std: standard error.

approximately 30% larger than the diploid cells. No significant difference was observed in the production rate of lactate over the entire culture time in each cell line. The mean specific growth rate was similar in all cell lines, but diploid clone 9 had a slightly higher value roughly 16% greater than its counterparts.

A dissolved oxygen probe was used to compare the oxygen uptake rates of exponentially growing diploid and tetraploid clonal isolates. OUR results shown in Table 1 agrees with data previously attained for insect cells ($\text{OUR} = 3.4 - 1.55 \times 10^{-17} \text{ moles cell}^{-1} \text{ s}^{-1}$ depending on the culture phase sampled; (Kioukia 1994). Only a very narrow margin of difference registered between the two tetraploid cell lines, but the latter had a greater oxygen consumption demand of about $2.9 \times 10^{-17} \text{ moles cell}^{-1} \text{ s}^{-1}$, approximately 18% higher than the diploid cells. Caution must be taken here though as large standard error was generated for tetraploid clone 11, but even taking this into account the diploid cell lines still have a lower oxygen requirement. This may be due to the smaller cell size of the diploid clones (Kioukia 1994). Similar results were obtained with CHO cells, where cells in the S/G₂ cell cycle phase have higher O₂ consumption rates than smaller cells in G₁ phase (Leelavatcharamas 1997).

Carboxy-seminaphtharhodafuor-1-acetoxymethylester, a pH sensitive fluorophore revealed a small and probably insignificant difference in the pH_i of the Sf-9 clones independent of cell volume, suggesting that these clonal isolates are physiologically similar. The intracellular environment of tetraploid clone 11 cells was slightly more acidic than diploid clone 9 cells, with a 0.5 pH difference. The pH_i of the Sf-9 cells was 6.4 for the tetraploid clone, which is the same as the external pH of the media, and 6.9 for the diploid clone 9 (Table 1). The slightly more acidic pH_i of the tetraploid clones may be due to their greater surface area and the more cytoplasmic material present.

The growth characteristics of the Sf-9 diploid and tetraploid cell lines infected with wild-type baculovirus at an MOI of 5

To prevent either cell ploidy type being exposed to suboptimal environments and subsequently

exhibiting sign of stress, all the cell lines were resuspended in fresh media prior to virus infection. All cell cultures were inoculated at $7.7 \times 10^5 \text{ cells mL}^{-1}$. Cell viability remained high until the cultures were inoculated with wild-type AcMNPV virus. The growth and infection characteristics of this experiment are summarised in Table 2. The mean cell size of the tetraploid cell lines again was found to be around 30% larger than the diploid cell lines but the glucose concentration in all the supernatants was similar. The difference in lactate production between the diploid and tetraploid cell lines was insignificant.

The tetraploid clones had produced an average polyhedra per cell approximately 30% greater than the diploid clones at 24 h. However, when the number of polyhedra produced is assessed in terms of polyhedra mL^{-1} instead of per cell, the difference in product yields between the tetraploid and diploid clones is not so large. The tetraploid clones generally fabricated 20% more polyhedra mL^{-1} in comparison with the diploid cells 24 h post infection (Table 2).

The growth characteristics of the Sf-9 diploid and tetraploid cell lines infected with GFP baculovirus at an MOI of 5

The tetraploid cell lines were more sensitive than the diploid cells to GFP baculovirus infection and their viability fell by 60% when measured by trypan blue assessment within 24 h post infection (results not shown). All cultures showed a similar trend, a drop in viable cell concentration in response to cellular infection. At the end of the experiment at 108 h both the tetraploid cell lines averaged 98% and the diploid cell lines around 88% non-viable cells. The infection characteristics of Sf-9 clones are summarised in Table 3.

The tetraploid cells, which were still approximately one-third larger in size than the diploid cells, consumed twice as much glucose but released roughly the same amount of lactate into the media. The mean channel GFP expression was analysed after 24 h post infection as shown in Table 3. Around 24 h post infection, the tetraploid cell lines synthesised superior yields of greater GFP production when compared with the diploid cells. A similar trend was found when product yields

Table 2. Growth characteristics derived from the batch cultures of Sf-9 heterogeneous parental cell line, two cloned Sf-9 diploid cell lines and two cloned Sf-9 tetraploid cell lines infected with MOI 5 wild-type AcMNPV.

Cell line	Initial cell density ($\times 10^5$ cells mL $^{-1}$)	Cell density at time of infection ($\times 10^5$ cells mL $^{-1}$)	Duration of batch culture (h)	Mean specific growth rate prior to virus infection (h $^{-1}$)	Max. product yield at 24 h pi (polyhedra cell $^{-1}$)	Max. product yield at 48 h pi (polyhedra mL $^{-1}$)	Max. product yield at 48 pi (polyhedra mL $^{-1}$)	QG (mmol L $^{-1}$ 10 $^{-6}$ cells $^{-1}$ h $^{-1}$)	QL (mmol L $^{-1}$ 10 $^{-6}$ cells $^{-1}$ h $^{-1}$)	Mean cell size (μ m)
Sf-9 diploid clone 4	2.5 Std = 0.26	7.9 Std = 0.21	143 $\pm$ 1.25	0.0236	11.5 Std = 1.87	7.0 Std = 0.17	22.2 Std = 1.87	7.16 Std = 0.17	0.0012	14.6 Std = 0.82
Sf-9 diploid clone 9	2.5 Std = 0.34	8.0 Std = 0.35	143 $\pm$ 1.25	0.0196	10.8 Std = 2.64	7.1 Std = 0.28	21.8 Std = 2.64	6.36 Std = 0.30	0.0014	14.5 Std = 0.86
Sf-9 tetraploid clone 11	2.4 Std = 0.28	7.3 Std = 0.16	116.5 $\pm$ 1	0.0175	16.2 Std = 2.23	8.7 Std = 0.17	20.7 Std = 2.23	5.78 Std = 0.16	0.0010	19.0 Std = 1.01
Sf-9 tetraploid clone 13	2.5 Std = 0.12	7.3 Std = 0.32	143 $\pm$ 1.25	0.0223	15.3 Std = 2.73	7.8 Std = 0.30	24.2 Std = 2.73	6.72 Std = 0.28	0.0011	18.9 Std = 1.07

QG: specific glucose consumption rate; QL: specific lactate production rate; Std: standard error.

Table 3. Growth characteristics derived from the batch cultures of Sf-9 heterogeneous parental cell line, two cloned Sf-9 diploid cell lines and two cloned Sf-9 tetraploid cell lines infected with MOI 1 recombinant GFP AcMNPV.

Cell line	Initial cell density ($\times 10^5$ cells mL ⁻¹)	Maximum cell density at the time of infection ($\times 10^5$ cells mL ⁻¹)	Mean specific growth rate		QG (mmol L ⁻¹ 10 ⁻⁶ cells ⁻¹ h ⁻¹)	QL (mmol L ⁻¹ 10 ⁻⁶ cells ⁻¹ h ⁻¹)	Mean cell size (μ m)	Max.	Max.	Maximum product yield (mean GFP channel units)		
			Duration of batch culture (h)	inoculation prior to virus				product yield at 24 h pi (GFP mL ⁻¹) ^a	product yield at 48 pi (GFP mL ⁻¹) ^a			
Sf-9 diploid clone 4	2.52	Std = 0.237.08	Std = 0.29	108 $\pm$ 1.5	0.0264	0.004	0.0012	14.5	Std = 0.95	975.5	838.9	96.3 (Std = 10.24)
Sf-9 diploid clone 9	2.53	Std = 0.277.33	Std = 0.34	132 $\pm$ 2	0.0271	0.003	0.0012	14.8	Std = 0.78	1401.8	1201.6	117.8 (Std = 6.37)
Sf-9 tetraploid clone 11	2.5	Std = 0.17	7.43	Std = 0.25	108 $\pm$ 1.5	0.0276	0.006	19.0	Std = 0.80	2571.6	450.7	288.3 (Std = 8.74)
Sf-9 tetraploid clone 13	2.53	Std = 0.357.36	Std = 0.22	108 $\pm$ 1.5	0.0271	0.006	0.0011	20.6	Std = 0.86	2299.9	735.7	207.2 (Std = 7.38)

QG: specific glucose consumption rate; QL: specific lactate production rate; Std: standard error.

^a The maximum product yield of GFP mL^{-1} was calculated by taking a flow cytometer reading of GFP production in a cell population $\times$ cell density.

Figure 4. The production of GFP in Sf-9 diploid and tetraploid clones infected with GFP baculovirus (MOI 1). These amounts were calculated by multiplying the mean GFP production in Sf-9 diploid and tetraploid clones infected with GFP baculovirus (MOI 1) 24 hpi by the cell density of each culture at that time point.

were assessed in terms of total cellular GFP (Figure 4). The tetraploid cells surpassed the GFP yields produced per cell in the diploid cell lines by 35–40%, and fabricated roughly 150% more GFP at 24 h post infection when the total GFP in culture was calculated.

Why does the tetraploid clone have a superior ability when compared with diploid cells to produce recombinant GFP protein, but can only match diploid productivity when producing polyhedra? When a three-dimensional structure, such as a polyhedra sphere, is packaged into a cell approximately 40% of the cell potential space is lost through voidage as detailed by Kozeny's rule (Coulson and Richardson 1999). So even though the tetraploid cell is larger than the diploid cell, polyhedra production in the tetraploids is not significantly greater. Also there comes a point when the non-secreted polyhedra spherical structures accumulating within the cell inflict too much stress upon the cell membrane, which only has a restricted elasticity, consequently the insect cell will burst open, thus again limiting the amount of polyhedra the cell can hold. These theories combined with the limited growth density means that the tetraploid cell is not capable of manufacturing more polyhedra than the diploid cell. Whereas in the case of GFP fabrication, the tetraploid cells are able to generate larger quantities of protein due to its nano-size and the continuous secretion of GFP out of the cell.

Discussion

Once *S. frugiperda* was identified as heterogeneous, containing a mixture of diploid and tetraploid cells (Jarman-Smith et al. 2002), it was essential to characterise these subpopulations. The data presented here has provided clear evidence that the diploid and tetraploid cell lines are physiologically similar despite the size difference between them. However, these cell lines have distinct growth profiles with different cell survival and maximum cell density.

Sf-9 parental cells were propagated to more than double the maximum tetraploid cell concentration number and the diploid clones grew to roughly triple the tetraploid cell density. In Sf-9 tetraploid cells infected with wild-type baculovirus infection, the early descent to death, the lower maximum cell density but with only a slight increase in volumetric productivity suggests that tetraploid cell lines are unsuitable for wild-type polyhedra production. In contrast tetraploid cells infected with GFP baculovirus despite their limited cell number consistently surpassed the GFP yields produced by the diploid cell lines. In this respect, the tetraploid cell line may confer an advantage when used as a host cell line for recombinant BEVS.

The polyploidy levels of *S. frugiperda* cells currently used for protein production are variable with the proportion of diploid to tetraploid varies according to origin and age (Jarman-Smith et al. 2002). This may explain the discrepancies often

found between different laboratories in product yields of insect cell–baculovirus systems. According to our results this variability is due to the polyploid state of *S. frugiperda*. Thus, polyploidy level should be determined when comparing production yields or other associated traits in insect cell lines. One way to guarantee reproducibility between two data sets from different laboratories is to set a criterion that *S. frugiperda* cells must only be a certain generation number old.

Conclusion

There appears to be two main Sf-9 chromosomal subpopulations (diploid and tetraploid) and some additional fragmentation. The genetic aberrations in Sf-9 were shown to have an effect on culture longevity, cell size, oxygen uptake rate, cell death, glucose consumption rate, intracellular pH and product yield. These results stress the need to fully characterise all cell lines used for commercial and research purposes and for cytogenetic monitoring to be routinely undertaken. Population studies into insect cell lines will be essential for selecting robust clones that thrive in serum and protein-free media and produce high concentration of recombinant proteins. This research may help to understand the implications of changes in chromosome number and morphology on cell death and product yields.

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