Growth, metabolism and baculovirus production in suspension cultures of an *Anticarsia gemmatalis* cell line

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Abstract The UFL-AG-286 cell line, established from embryonic tissue of the lepidopteran insect *Anticarsia gemmatalis*, has been identified as a good candidate to be used as a cellular substrate in the development of a process for in vitro production of the *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus, a baculovirus widely used as bioinsecticide. In order to characterize the technological properties of this cell line and evaluate its feasibility to use it for the large-scale production of *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus, UFL-AG-286 cells were adapted to grow as agitated suspension cultures in spinner-flasks. Batch suspension cultures of adapted cells in serum-supplemented TC-100 medium grew with a doubling time of about 29 h and reached a maximum cell density higher than 3.5×10^6 viable cells ml^{-1} . At the end of the growth period glucose was completely depleted from the culture medium, but L-lactate was not produced. Amino acids, with the exception of

glutamine, were only negligibly consumed or produced. In contrast to other insect cell lines, UFL-AG-286 cells appeared to be unable to synthesize alanine as a metabolic way to dispose the by-product ammonia. The synchronous infection of suspension cultures with *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus in the early to medium exponential growth phase yielded high amounts of both viral progenies per cell and reduced the specific demands of UFL-AG-286 cells for the main nutrients.

Keywords *Anticarsia gemmatalis* · Baculovirus · Cell suspension culture · Lepidopteran insect cell line · UFL-AG-286 · AgMNPV

Abbreviations

AgMNPV	<i>Anticarsia gemmatalis</i> multicapsid nucleopolyhedrovirus
NOV	non-occluded virus
PIB	polyhedral inclusion body
TCID _{50%}	tissue culture infectious dose 50%

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Introduction

Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) constitutes one of the main defoliating plagues in soybean crops in Latin America (Moscardi 1993). This insect is efficiently controlled with a baculovirus, *Anticarsia gemmatalis*

multicapsid nucleopolyhedrovirus (AgMNPV) (Moscardi et al. 1981; Moscardi 1999). Currently, the commercial production of AgMNPV is based on the in vivo propagation in infected *Anticarsia gemmatilis* larvae. This technology has been shown to be robust and economically competitive to satisfy current requirements. However, a growing demand for this bioinsecticide has spurred an interest in developing alternative processes based on the in vitro viral propagation in insect cell cultures (Rodas et al. 2005). The economic performance of in vitro processes for baculovirus production improves as the production scale increases, making them more attractive for large-scale production than in vivo processes. Moreover, better process control and product purity are additional advantages of in vitro production processes.

The selection of a cell line susceptible to virus infection and able to produce high volumetric yields of polyhedral inclusion bodies (PIBs) is the first requirement for developing a feasible process to produce an insecticidal baculovirus in insect cell cultures. Several cell lines have been evaluated for their ability to support AgMNPV replication (Castro et al. 1997; Claus et al. 1993; Grasela and McIntosh 1998; Lynn 2003; Rodas et al. 2005; Sieburth and Maruniak 1988b). These reports show that the UFL-AG-286 homologous cell line (Sieburth and Maruniak 1988a) exhibits a higher susceptibility to AgMNPV infection, as well as a larger capability to replicate the virus, than other insect cell lines. Thus, the UFL-AG-286 cell line could represent a promising substrate to develop a process for the in vitro production of AgMNPV.

The development of a large scale in vitro production process for an insecticidal baculovirus also requires the ability of insect cells to grow in suspension culture (Murhammer 1996). The best characterized insect cell lines differ in their ability to grow in suspension. The *Spodoptera frugiperda* cell lines IPLB-Sf-21 and Sf9 can grow either attached or in suspension cultures. On the other hand, the *Trichoplusia ni* cell line Tn-5 and its commercial derivative High-Five require either treatment with heparin (Dee et al. 1997) or the selection of adaptable cellular subpopulations (Rhiel et al. 1997) to grow in single cell suspen-

sion culture. The UFL-AG-286 cell line was originally established as static cultures of adherent embryonic cells of *Anticarsia gemmatilis* (Sieburth and Maruniak 1988a), and to the best of our knowledge there is no published data of its adaptability to grow in free suspension. Additionally, the kinetic parameters of UFL-AG-286 cell suspension cultures are also unknown.

The rational development of a production process using an animal cell line requires a detailed knowledge of its nutritional requirements and its metabolic behavior. Elucidation of the quantitative demands of key nutrients would permit to anticipate, and eventually also avoid, the nutritional limitations that can affect the production process. Moreover, the metabolic characteristics of a cell line can determine the selection of a particular cultivation strategy, such as batch, fed-batch or perfusion. As an example, the lack of production of toxic by-products in well-oxygenated cultures of the lepidopteran insect cell lines IPLB-Sf-21 and Sf9 makes it possible to use nutrient feeding strategies in order to keep cultures in production at very high cell densities (Nguyen et al. 1993; Bédard et al. 1997; Elias et al. 2000). On the contrary, Tn-5 and High-Five cell lines display a metabolic behavior characterized by the accumulation of lactate and ammonia (Rhiel et al. 1997) that could limit the applicability of nutrient feeding strategies in high density cultures of these cell lines (Ikonomou et al. 2003). These differences illustrate the diversity of metabolic profiles that can be found in different lepidopteran insect cell lines; they also emphasize the importance of characterizing the metabolic properties of any uncharacterized cell line to be used in production processes. Since the nutritional requirements of the UFL-AG-286 cell line have not been previously investigated, its specific metabolic profile remained unknown.

In this work it was demonstrated that UFL-AG-286 cells can readily adapt to grow as agitated suspension cultures in spinner flasks. The kinetic parameters of suspension cultures were determined, and the nutritional and metabolic profiles of both uninfected and AgMNPV-infected cultures were characterized. The viral production in cultures of UFL-AG-286 cells adapted to grow in suspension was also evaluated.

Materials and methods

Cell cultures

UFL-AG-286 cells, kindly provided by Dr. Víctor Romanowski (Instituto de Bioquímica y Biología Molecular, Universidad Nacional de La Plata, República Argentina), were cultivated in TC-100 medium (Invitrogen, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (Bioser, Buenos Aires, Argentina). Cultures, which were routinely maintained as attached cultures at 27 °C, were split 1:10 every 5 days. Suspension cultures were performed in 500 ml spinner flasks (Techne, Cambridge, UK), with 50 ml culture volume and agitated at 80 rpm.

Samples taken from suspension cultures were processed as follows: cellular suspensions were stained with 0.4% Trypan blue and then both total and viable cells were counted in duplicate using a Neubauer haemocytometer. In order to differentiate cells that grow in isolation from cells associated to clumps, cellular counts were made before and after a vigorous disruption of cellular aggregates. In addition, culture supernatants were separated by low-speed centrifugation at 4 °C, and then frozen (−20 °C) until they were analyzed. Glucose and lactate concentrations were determined using enzymatic reactions in a YSI 2700 analyzer (YSI, Yellow Springs, OH, USA). The concentrations of 18 different amino acids were determined by reversed-phase high-performance liquid chromatography (HPLC) (Beckmann, USA) using precolumn derivatization with *O*-phthalaldehyde (Ryll et al. 1990). The ammonia concentration was measured using a commercial kit (Wiener Lab, Rosario, Argentina).

Rates

The specific growth rate was calculated from the linear region of the semilog plot of cell density versus time. For cells in the exponential growth phase, specific consumption rates were determined by first evaluating the cell yield obtained from a plot of the component concentration of interest versus the viable cell density and then multiplying this value by the specific growth rate. For infected cells, the specific consumption rates

were determined by evaluating the rate of change of the component of interest either from 0 to 24 h or from 24 to 48 h post-infection, and then dividing by the average viable cell density during the respective time period.

Virus and virus quantification

The strain of AgMNPV was originally isolated in Oliveros, Provincia de Santa Fe, Argentina (Claus et al. 1993). The virus stock used for these experiments was prepared by infection of static cultures of UFL-AG-286 cells (2×10^5 cells ml^{−1}) at a multiplicity of infection of 0.1 TCID_{50%} cell^{−1} using the second passage of the original isolate. Samples obtained from infected suspension cultures were processed as previously described for non-infected cultures. Additional samples were taken from culture supernatants for NOVs titration and from cellular pellets for PIBs quantification. NOVs were quantified by end-point dilution analysis. Briefly, to determine the TCID_{50%}, cell suspensions (3×10^5 cells ml^{−1}) of UFL-AG-286 cells were seeded into 96-well plates (50 µl per well) and then an equal volume of each viral supernatant dilution was added (five replicates per supernatant). After incubation at 27 °C for 1 week, plates were scored for infection and then the entire supernatant from each well was added to freshly seeded wells containing 50 µl cell cultures. After 7 days, the second plate was scored and the TCID_{50%} was calculated (Reed and Muench 1938). After extraction of cellular pellets with 1% SDS, the number of PIBs was determined with a Neubauer haemocytometer by taking the average count after analyzing three separate aliquots.

Results

Culture growth

Starting from attached cultures, UFL-AG-286 cells were adapted to grow in agitated suspension cultures. Cells from static cultures in T-flasks were mechanically detached, suspended in culture medium at 3.5×10^5 cells ml^{−1}, and agitated in a spinner flask. Most cells initially aggregated in

clumps of different size. To gradually enrich suspension cultures in cellular subpopulations that tend to grow either as isolated cells or in small clumps, larger clumps were allowed to settle and were discarded before each subculture was made. Following this strategy, nearly 90% of the cells were growing as single cells after 30 passages in suspension culture, while cultures that were not subjected to settlement of cell clumps prior to each subculture did not significantly alter their original behavior.

The growth curve of a UFL-AG-286 cell culture adapted to suspension (saUFL-AG-286) is shown in Fig. 1. Cultures started at a high initial density of 3.7×10^5 cells ml^{-1} began to grow immediately after seeding with a constant specific growth rate ($\mu = 0.024 \text{ h}^{-1}$). A short lag phase could be observed in cultures started at a lower initial cell density (not shown), but the value of μ was independent of the initial cell density. saUFL-AG-286 cultures in TC-100 + 10% FBS medium usually reached maximum cell densities

Fig. 1 Kinetics of saUFL-AG-286 cell growth in TC-100 medium supplemented with 10% FBS. Cells were cultivated in 500-ml spinner flasks with 50 ml working volume at 28 °C and 80 rpm. Samples were taken daily and both total and viable cell concentrations were determined. Results represent the average and standard deviation of three experiments

higher than 3.5×10^6 viable cells ml^{-1} . Culture viability remained higher than 98% up to 96 h in culture. After that time, viability decreased rapidly, and no stationary growth phase could be observed.

Nutrients consumption and metabolism

Figure 2 shows the time course of change in glucose and L-lactate concentrations. Glucose levels gradually decreased with a constant cell specific consumption rate of 3.2×10^{-14} mol cell $^{-1}$ h $^{-1}$ until it became exhausted from culture medium after 120 h in culture, while the L-lactate concentration remained almost unchanged throughout the entire cultivation. The L-glutamine concentration in the culture supernatant increased during the first 21 h in culture, a behavior also observed with the remaining amino acids (Fig. 2). Thereafter, the L-glutamine concentration decreased gradually, with a variable cell specific consumption rate that

Fig. 2 Time course profiles of glucose, L-lactate, L-glutamine, L-alanine and ammonia during suspension cultures of saUFL-AG-286 cells in TC-100 medium supplemented with 10% FBS. Cells were cultivated in 500-ml spinner flasks with 50 ml working volume at 28 °C and 80 rpm. Samples were taken daily and concentrations were determined as described in 'Materials and methods'. Results represent the average and standard deviation of three experiments

peaked at 48 h (5.87×10^{-14} mol cell $^{-1}$ h $^{-1}$) and then decreased, until this amino acid was almost exhausted after 120 h in culture. The L-alanine concentration did not increase throughout the culture period. Conversely, the ammonia concentration increased sharply after the first 72 h reaching values higher than 15 mM at the end of the culture (Fig. 2).

Most amino acids were not consumed during saUFLAg-286 cell suspension cultures (Table 1). Only glutamine and methionine were consumed in percentages higher than 20% of their initial concentrations. On the other hand, arginine, aspartic acid, glycine, glutamic acid, histidine, isoleucine, lysine, phenylalanine, threonine and valine accumulated in culture supernatants, while alanine, asparagine, leucine, serine and tyrosine were only slightly consumed. The amount of glutamine consumed represented 77% of the total consumption of amino acids in saUFL-AG-286 cell suspension cultures.

AgMNPV infection

The behavior of saUFL-AG-286 suspension cultures infected with AgMNPV was also investi-

Table 1 Amino acid concentrations in a batch suspension culture of saUFL-AG-286 cells at the beginning and the end of growth

Amino acid	Concentration (mM)		% consumption
	0 h	96 h	
Alanine	2.78	2.64	5
Arginine	3.52	3.62	-3*
Asparagine	3.47	3.23	7
Aspartic acid	3.25	3.80	-17
Glycine	8.81	9.03	-2
Glutamine	4.11	1.30	68
Glutamic Acid	5.24	5.72	-9
Histidine	16.56	19.13	-16
Isoleucine	0.90	0.97	-8
Leucine	1.27	1.18	7
Lysine	4.12	4.19	-2
Methionine	0.76	0.60	21
Phenylalanine	1.34	1.39	-4
Serine	5.22	5.02	4
Tyrosine	0.49	0.48	2
Threonine	1.64	1.73	-5
Tryptophan	0.61	0.61	0
Valine	1.38	1.51	-9

* Negative sign indicates production

gated. Spinner flask cultures were inoculated with 3.5×10^5 cells ml $^{-1}$ and infected 36 h later, after they reached a density of 8.4×10^5 viable cells ml $^{-1}$, at a multiplicity of infection of 3 TCID $_{50\%}$ cell $^{-1}$. Samples were taken from infected cultures every 24 h. The evolution of the infected cultures is shown in Fig. 3. The first day post-infection was characterized by a slight increase of total cell density, while viability remained above 90%. Thereafter, the number of viable cells decreased slowly up to 48 h post-infection. Finally, viability decreased exponentially, reaching a percentage lower than 30% at 96 h post-infection. The cellular lysis was not very significant during this period, as the number of total cells remained nearly unchanged. Even though more than 90% of the cells showed clear cytopathic effects 24 h post-infection, PIBs-containing cells were first observed 48 h post-infection, when more than 50% of the cells showed signals of accumulation of occlusion bodies. The number of cells containing PIBs did not change afterwards.

Fig. 3 Time course profiles of total cells, viable cells and PIBs-containing cells during AgMNPV infection of saUFL-AG-286 cell suspension cultures in TC-100 medium supplemented with 10% FBS. Cells, cultivated in 500-ml spinner flasks with 50 ml working volume at 28 °C and 80 rpm, were infected at a multiplicity of infection of 3 TCID $_{50\%}$ cell $^{-1}$ and cells analyzed at specific time points post-infection. Results represent the average and standard deviation of three experiments

The production of both viral progenies with the characteristic biphasic profile of the baculovirus replication cycle is shown in Fig. 4. The production of NOVVs increased rapidly during the first 24 hpi, and then more slowly, to reach a maximum average titer of 6.3×10^8 TCID_{50%} ml⁻¹ at 96 hpi (cell-specific yield of 692 TCID_{50%} cell⁻¹). PIBs were first detected at 48 hpi, coincidentally with both the microscopic observation of PIBs-containing cells and the initial reduction of culture viability. The concentration of cell-associated PIBs increased rapidly up to 72 hpi, when it reached a maximum value of 5.8×10^7 PIBs ml⁻¹ (cell specific yield of 64 PIBs cell⁻¹).

Glucose and glutamine were the most consumed nutrients in saUFL-AG-286 cultures infected with AgMNPV (Fig. 5). However, their rates of consumption were reduced after infection in comparison to non-infected cultures. The

Fig. 4 Production kinetics of NOVVs and PIBs in saUFL-AG-286 suspension cultures infected with AgMNPV in TC-100 medium supplemented with 10% FBS. Cells, cultivated in 500-ml spinner flasks with 50 ml working volume at 28 °C and 80 rpm, were infected at a multiplicity of infection of 3 TCID_{50%} cell⁻¹ and NOV and PIB production analyzed at specific time points post-infection. Results represent the average and standard deviation of three experiments

Fig. 5 Time course profiles of glucose and glutamine concentrations during infection of suspension cultures of saUFL-AG-286 cells in TC-100 medium supplemented with 10% FBS. Cells, cultivated in 500-ml spinner flasks with 50 ml working volume at 28 °C and 80 rpm, were infected with AgMNPV at a multiplicity of infection of 3 TCID_{50%} cell⁻¹ and supernatants analyzed for glucose and glutamine concentration at specific time points post-infection. Results represent the average and standard deviation of three experiments

specific consumption rate of glucose was reduced from 3.39×10^{-14} to 1.23×10^{-14} mol cell⁻¹ h⁻¹ during the first 24 hpi, but subsequently increased to the same value as non-infected cultures for the period from 24 to 48 hpi. The specific glutamine consumption rate was also reduced immediately after infection from 2.56×10^{-14} to 1.43×10^{-14} mol cell⁻¹ h⁻¹. This rate did not change significantly during the 24–48 hpi period. Only five more amino acids were consumed in infected cultures: alanine, asparagine, lysine, methionine and tryptophan, while the other amino acid concentrations increased in the supernatant of infected cultures (Table 2). The amount of glutamine consumed represented 48% of the total consumption of amino acids in infected saUFL-AG-286 cell suspension cultures. There was neither consumption nor production of ammonia

Table 2 Amino acid concentrations in an AgMNPV-infected suspension culture of saUFL-AG-286 cells at the beginning and the end of the infection period

Amino acid	Concentration (mM)		% Consumption
	0 hpi	96 hpi	
Alanine	2.75	2.40	13
Arginine	3.24	3.38	−4*
Asparagine	3.26	3.04	7
Aspartic acid	3.23	3.54	−9
Phenylalanine	1.28	1.31	−2
Glycine	8.40	8.97	−7
Glutamine	3.22	2.38	26
Glutamic Acid	4.87	5.61	−15
Histidine	15.92	17.64	−11
Isoleucine	0.96	1.03	−7
Leucine	1.19	1.19	0
Lysine	3.84	3.72	3
Methionine	0.76	0.63	17
Serine	4.88	4.91	0
Tyrosine	0.47	0.49	−4
Threonine	1.62	1.75	−8
Tryptophan	0.67	0.57	15
Valine	1.48	1.53	−3

* Negative sign represents production

and lactate during the infection period, but ammonia concentration oscillated between 7.5 and 9.5 mM (not shown).

Discussion

The adaptation of the UFL-AG-286 cell line to agitated suspension cultures was the first aim of this work. Most cells transferred from static to agitated cultures immediately aggregated in clumps of different size, showing the same tendency as the *Trichoplusia ni* cell line Tn-5 (Wickham and Nemerow 1993). Using the procedure described by Rhiel et al. (1997), which separated cellular aggregates by settlement before each subculture, UFL-AG-286 cells that did not tend to aggregate were quickly obtained. On the contrary, the cell line did not significantly modify its tendency to aggregate when clumps were not separated before each subculture, indicating that a non-aggregating cellular subpopulation was segregated in cultures subjected to the selection procedure. This fact could be considered as evidence of the population complexity of the UFL-AG-286 cell line. The coexistence of diverse

cellular subpopulations with different properties in an established cell line was also demonstrated in other lepidopteran insect lines (Pasumarthu and Murhammer 1994).

The growth kinetics of saUFL-AG-286 batch suspension cultures in TC-100 + 10% FBS shows a very short or non-detectable lag phase followed by a long exponential growth phase with a constant specific growth rate whose value is well within the range reported for suspension cultures of other insect cell lines (Schmid 1996). The growth rate determined in this work was significantly higher than the rate measured by Sieburth and Maruniak (1988a) when the UFL-AG-286 cell line was established. As the UFL-AG-286 cell line was subjected to an undetermined number of passages since 1986, that difference could be explained by the growth rate improvement phenomenon previously described by Donaldson and Shuler (1998) as an effect of the long-term passaging of the Tn-5 insect cell line.

A remarkable characteristic of saUFL-AG-286 batch cultures is the stability of the specific growth rate during the exponential growth phase, as opposed to the instability observed in batch suspension cultures of *Spodoptera frugiperda* cell lines (Drews et al. 1995; Doverskorg et al. 1997; Ferrance et al. 1993). Specific growth rate instability could be considered as an expression of sensitivity of the regulatory mechanisms that govern the cellular proliferation to the changes, both environmental and cellular, that occur as a consequence of the cellular activities in the culture. That variability has been alternatively explained either by oxygen limitation (Ferrance et al. 1993), nutrient limitation/by-product accumulation (Bédard et al. 1994) or by dependence of the activity of autocrine growth factors (Doverskorg et al. 1997). Nevertheless, the regulation of saUFL-AG-286 cell proliferation mechanisms appears to be relatively unsusceptible to the cellular and environmental modifications that determine the variation of the specific growth rate in batch cultures of other lepidopteran insect cell lines.

The end of the exponential growth phase in saUFL-AG-286 suspension cultures was immediately followed by a rapid decline of culture viability. The beginning of the death phase was

accompanied by both the exhaustion of glucose and glutamine and the accumulation of ammonia. Several reports have shown that the viability of batch cultures of insect cells decayed after the exhaustion of carbohydrates (Drews et al. 1995; Palomares and Ramírez 1996; Mendonça et al. 1999; Meneses-Acosta et al. 2001). On the contrary, the viability of some insect cell lines is not affected by glutamine absence (Öhmann et al. 1996). The accumulation of by-products could also be the cause of viability reduction. Ammonia is a toxic metabolite in animal cell cultures, but the concentration reached in saUFL-AG-286 cultures should not be considered detrimental for culture viability, according to results obtained with other insect cell lines (Bédard et al. 1993; Öhman et al. 1996). Lactate, another by-product commonly produced in animal cell cultures, did not accumulate in saUFL-AG-286 culture supernatants. Therefore, the rapid increase of cellular mortality in saUFL-AG-286 batch cultures is more probably caused by nutrient exhaustion, either glucose alone or in combination with glutamine or other undefined nutrient(s), than by toxic by-product accumulation.

Carbohydrates constitute a main carbon and energy source in animal cell cultures. Glucose, the unique carbohydrate present in TC-100 medium, was the most widely consumed nutrient in saUFL-AG-286 cultures. The cell-specific glucose consumption rate in saUFL-AG-286 cultures was slightly lower than the maximum rates reported in cultures of other insect cell lines under similar culture conditions (Bédard et al. 1993; Drews et al. 1995; Mendonça et al. 1999; Rhiel et al. 1997). This low rate of glucose consumption could be due to the low concentration of glucose in TC-100 medium. Bédard et al. (1993) reported that both Sf9 and BTI-EAA cultures consumed glucose at a lower rate in TNM-FH medium than in other culture media that contained a higher glucose concentration. It should also be noted that glucose was consumed in saUFL-AG-286 cultures at a nearly constant rate along the culture, while in other insect cell lines the cell specific glucose consumption rate decays after reaching a maximum value at the initial to mid-term exponential growth phase (Drews et al. 1995).

Differently from glucose consumption rate, the cell specific glutamine consumption rate was not constant in batch cultures of saUFL-AG-286 cells. The maximum value of $5.87 \times 10^{-14} \text{ mol cell}^{-1} \text{ h}^{-1}$, reached after 48 h in culture, was higher than that measured in other lepidopteran insect cell lines (Bédard et al. 1993; Rhiel et al. 1997; Mendonça et al. 1999). This high consumption rate of glutamine could also be related to the low glucose concentration in the TC-100 medium, taking into account the role of glutamine as an alternative energy source to carbohydrates in insect cell cultures. Additional experiments should be performed to establish whether this behavior is either just an adaptive response of saUFL-AG-286 cells to the low concentration of carbohydrates in the TC-100 medium or whether they constitutively express a higher preference to use glutamine as an energetic source than other insect cell lines.

Methionine was the other amino acid consumed in a significant proportion by saUFL-AG-286 cell cultures, but its concentration did not reduce enough to constitute a potential nutritional limitation. The oversupply of amino acids by insect cell culture media with respect to the cellular requirements is also a fact reported in cultures of *Spodoptera frugiperda* cell lines (Bédard et al. 1993; Ferrance et al. 1993). It should be noted that the concentration of cysteine, an amino acid highly consumed by Sf9 cells and whose concentration determines the uptake rate of other amino acids (Doverskog et al. 1998), has not been analyzed in this work. For this reason it is not possible to speculate about its potential nutritional importance in saUFL-AG-286 cultures.

The saUFL-AG-286 cell line shows a production pattern of metabolic by-products characterized by the production of ammonia and the lack of production of both lactate and alanine. This pattern, that is different from those described in other well-characterized insect cell lines (Rhiel et al. 1997), could be the result of the expression of a specific arrangement of metabolic pathways. Although it will be necessary to verify this hypothesis through measurements of enzymatic activities and follow-ups of molecular tracers, a comparison with the information obtained with another insect cell lines would allow us to

speculate about some metabolic characteristics of saUFL-AG-286 cells. The lack of accumulation of lactate, a product of the glucose metabolism in mammalian cells and some insect cells, could indicate that saUFL-AG-286 cells metabolize glucose as efficiently as *Spodoptera frugiperda* cells (Neermann and Wagner 1996; Benslimane et al. 2005). Glycerol and ethanol, identified as by-products of glucose metabolism in Sf9 cells (Drews et al. 2000), were not investigated in this work. On the other hand, the active accumulation of ammonia and the lack of production of alanine would indicate that saUFL-AG-286 cells do not express the capability of other animal cells to synthesize alanine as a non-toxic alternative to eliminate the amounts of ammonia generated by catabolic degradation of amino acids. This particular behavior of saUFL-AG-286 cells resembles the glutamine metabolism under glucose limitation in Sf9 cells, where ammonia is generated by the activity of both glutaminase and glutamate dehydrogenase (Drews et al. 2000). However, in saUFL-AG-286 cells the peak of ammonia production started when glucose was still available. An alternative mechanism to detoxify ammonia in saUFL-AG-286 cells could explain its sharp increase at the end of the growth phase. The synthesis of uric acid is the main mechanism of detoxification of ammonia in most terrestrial insects, and Stavroulakis et al. (1991) showed that a *Bombyx mori* cell line produced uric acid as a main nitrogenous by-product. The uricotelic pathway to synthesize uric acid is complex and requires an energetic contribution. On the other hand, uric acid can be degraded through the uricolytic pathway, which leads to the production of ammonia. Thus, it could be postulated the existence of two different stages of ammonia elimination in saUFL-AG-286 cells: an initial stage characterized by uric acid synthesis during the exponential growth phase, in the presence of enough energetic resources, then followed by a second stage characterized by degradation of uric acid and the consequent production of ammonia, when energy is no longer available. This mechanism could explain the sharp increase of ammonia at the end of the growth period, but specific experiments should be made to either confirm or refuse this hypothesis.

No obvious nutritional limitation to AgMNPV replication could be observed because all measured nutrients were oversupplied under the infection conditions employed in these experiments (synchronous infection in the early to medium exponential growth phase). Moreover, the results obtained here show that the overall nutritional demand of saUFL-AG-286 cells was reduced after infection with AgMNPV, mainly during the first 24 h post-infection, when infected cells were synthesizing NOV. The effects of baculovirus infection on the nutritional demands of insect cells were previously studied under different experimental conditions, with contradictory results. Several papers have reported that baculovirus-infected insect cells consume the main nutrients at higher rates than uninfected cells (Sugiura and Amman 1996; Radford et al. 1997; Rodas et al. 2005). However, other papers have shown that the consumption rates of the main nutrients either do not change (Wong et al. 1994; Kamen et al. 1996; Palomares et al. 2004) or decrease (Rhiel and Murhammer 1995) after baculovirus infection in insect cells. The explanation to these contradictory results could be based on the different experimental conditions (cell line, culture medium, culture strategy, time and multiplicity of infection) that were employed in the cited studies. Rhiel et al. (1997) showed that the changes of the nutritional demands after infection depend on the cell line and the culture medium. On the other hand, the change of the nutritional demands after infection should not be exclusively determined by the specific needs of virus replication but should also be dependent on both the metabolic state of the cells at the time of infection and the effects of infection on the cell metabolism. The markedly reduced consumption of the main nutrients verified in the first hours post-infection could be explained, in these experiments, by the growth arrest that occurred immediately after infection of the cells that were actively dividing. In this context of plentiful intracellular availability of both carbon and energetic resources, the reduced uptake of glucose and glutamine after infection could indicate that the nutritional requirements to support the viral replication would be lower than that for biomass multiplication. Nevertheless, the rela-

tively enhanced uptake rates of some amino acids (alanine, lysine and asparagine) in infected cultures suggest that there is no overall reduction of nutrient consumption. Instead, infected cells preserve their capacity to incorporate the nutrients specifically needed to face the viral replication process.

AgMNPV infection in saUFL-AG-286 cells evolved with the same characteristic pattern as observed in static cultures of UFL-AG-286 cells (Castro et al. 1997; Sieburth and Maruniak 1988b). Therefore, the selected subpopulation of saUFL-AG-286 cells appears to exhibit a susceptibility to AgMNPV infection comparable to that observed in the whole population of UFL-AG-286 cells. High yields of both viral progenies were obtained in saUFL-AG-286 cells, showing that they also preserved their high capability to replicate AgMNPV. The cell-specific yield of AgMNPV PIBs obtained in this work (64 PIBs per cell) is significantly higher than that obtained in cultures of other insect cell lines. Rodas et al. (2005) reported to achieve a specific yield of 27.60 AgMNPV PIBs per cell in suspension cultures of the Sf9 cell line in Sf900 II serum-free medium, while Batista et al. (2005) obtained a volumetric yield of 1.60×10^7 AgMNPV PIBs ml^{-1} in suspension cultures of the same cell line infected in a serum-supplemented medium at a cell density of 1×10^6 cells ml^{-1} (maximum specific yield of 16 AgMNPV PIBs per cell). Grasela and McIntosh (1998) assayed the production of AgMNPV PIBs in static cultures of the homologous cell line BCIRL-AG-AM1 in serum-supplemented TC-100 medium, obtaining a maximum specific yield of 14.20 AgMNPV PIBs per cell. Zhang et al. (2005), infecting suspension cultures of the IPLB-Sf-21 cell line in TC-100 medium supplemented with 10% fetal calf serum, obtained a maximum volumetric yield of 1.10×10^6 PIBs ml^{-1} in cultures infected at an initial cell density of 4×10^5 cells ml^{-1} (maximum specific yield of 2.75 AgMNPV PIBs per cell). Although the results obtained in this work show the superior potential of saUFL-AG-286 as a producer cell line for AgMNPV PIBs when compared to other insect cell lines, the comparison with the in vitro production of other insecticide baculoviruses suggests possibilities for yield improvement.

Chakraborty et al. (1999) assayed the production of PIBs of the *Helicoverpa armigera* singly enveloped nucleopolyhedrovirus in serum-free cultures of the insect cell line BCIRL-HZ-AM1, obtaining specific yields varying from 27 PIBs per cell in a serum-supplemented medium to 112 PIBs per cell in a serum-free medium. Higher specific yields of PIBs of *Autographa californica* multicapsid nucleopolyhedrovirus were obtained by McKenna et al. (1997) in suspension cultures of Tn-5B1-4 cells in several serum-free media, where they attained up to 185 PIBs per cell.

In summary, the cell line UFL-AG-286 could be adapted to grow in suspension through the selection of a subpopulation of cells capable to proliferate free of aggregates (saUFL-AG-286). The adapted cultures grew with kinetics similar to that of other characterized lepidopteran insect cell lines, but exhibited specific nutritional and metabolic behaviors. The high yields of AgMNPV in saUFL-AG-286 suspension cultures sustain the feasibility of using this cell line in the development of an industrial process to produce AgMNPV polyhedra in vitro. The data obtained in this work could aid to design both a specific medium for cultivating saUFL-AG-286 cells and a specific strategy to produce AgMNPV PIBs in this cell line.

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