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An Efficient Plating System for Rapid Isolation of Mutants from Plant Cell Suspensions

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Summary. A plating system for cell suspensions of soybean, SB-1, (*Glycine max* L. cv. 'Mandarin') and *Datura innoxia* D.I. (Mill) was developed using feeder cells. The characteristics of the system are: a) the efficiency of plating (EOP) is high (0.5-0.6), b) over a range of 10-300 plated clumps the EOP is constant, c) the growth rate of plated cells resembles that of suspension cultures (generation time 24 hr.). Clumps with few or with many cells have similar plating efficiencies.

Employing the plating system, a mutant resistant to 8 azaguanine (8AG) was isolated from SB-1 in 7 days and purified and tested within an additional 3 weeks. Feeder plates were used to selectively re-isolate 8 AG resistant and maltose utilizing mutants from a 1000-fold excess of wild type cells.

The plating technique also can be utilized to isolate auxotrophic mutants since free amino acids are not produced by the feeder suspension. Other applications of this plating technique are discussed.

Key words: Efficient plating system – Mutant isolation – Soybean (*Glycine max* L.) – Haploid *Datura innoxia* Mill. – Suspension cultures – HGPRT

Abbreviations

8AG	8 Azaguanine
6TG	6 Thioguanine
EMS	Ethyl methanesulfonate
EOP	Efficiency of plating
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8)

Introduction

Cells growing in suspension culture divide rapidly and can be grown in large quantity, increasing the probability of selecting mutants and decreasing the time necessary to purify mutant strains. The selection of mutant colonies on agar plates may be more difficult, due to slow growth or poor plating efficiency. Methods for plating described so far were unable to solve these problems satisfactorily (Raveh et al. 1973; Engvild 1974; Logemann and Bergmann 1974, Werry and Stoffelsen 1978).

The procedure to be described here allows rapid growth but avoids two problems which may be encountered during mutant selection in liquid cultures: toxic effects of media in which cells are dying and deprivation of beneficial effects (feeder effects) upon replacing media in which large numbers of cells have been growing. This paper reports a procedure for rapid colony growth and selection in which cells are grown on a solid support system (membrane filter) which can be transferred to different selection media. The growth of cells is supported by the presence of feeder cells below the membrane. The use of feeder cells is essential because cultured cells stop growing when cell concentrations drop below critical titers (which in the case of soybean (SB-1) is 5×10^4 cells/ml). On the other hand, when a large proportion of cells is killed by selection the survivors often also grow poorly or die, particularly if the cell concentration is high.

Materials and Methods

Cell Cultures and Media

The cell lines used are shown in Table 1. All wild type strains were obtained from Dr. O.L. Gamborg, Prairie National Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada.

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Table 1. Cell lines used in experiments

Cell line	Description	Derived from	Characteristics	Reference
SB-1	Wild type soybean	*Glycine max* L. cv. 'Mandarin'	2n – 3 = 37 (Kao et al., 1970) doubling time: Sucrose, 24 hr; Maltose, 200 hr. Sensitive to 1μg/ml 6TG, 10 ng/ml 8AG	Gamborg (1965)
SB-6TG	6 Thioguanine resistant soybean	SB-1	Resistant to 60μg/ml 6TG	this paper
SB-8AG	8 Azaguanine resistant soybean	SB-1	Resistant to 1μg/ml 8AG	this paper
M-24	Maltose utilizing soybean	SB-1	24 hr doubling time in Maltose medium	Limberg et al. (in press)
D.I.	Haploid wild type *Datura innoxia*	*Datura innoxia* Mill.	70% of cells n = 12	Gamborg et al. (in preparation)

The cultures were maintained as 50 ml batch suspensions in 250 ml Delong Flasks on a gyratory shaker (120 rpm) at 33°C in the dark (Chu and Lark 1976). B5 medium (Gamborg et al. 1968) containing 1 μg/ml 2,4 dichlorophenoxyacetic acid was used. Media for SB-1 and *D. innoxia* were additionally supplemented with 0.2% casein hydrolysate (casein hydrolysate, acid, vitamin and salt free, ICN Pharmaceutical Inc., Cleveland, Ohio). One line of *D. innoxia* was grown without amino acid supplements. The M-24 mutant of SB-1 was grown as described by Limberg et al., in press. All chemicals, unless indicated otherwise, were purchased from Fisher Scientific Company, Santa Clara, California.

Antimetabolites

1 mg/ml aqueous stock solutions of 6 Thioguanine (6 TG) (Sigma, St. Louis, Missouri) and 8 Azaguanine (8 AG) (Sigma, St. Louis, Missouri) were prepared. The pH of these solutions had to be adjusted to 11.5 to solubilize the compounds. When diluted and added to tissue culture media no change of pH was observed in the media.

Counting of Cells

Numbers of cells were determined by protoplasting cells from suspensions within 1 hr after adding the following enzyme mixture: 5% cellulase 'Onozuka' R-10 and 2% Macerozyme R-10 (Kinki Yakult Mfg. Co., Ltd., Nishinomiya, Japan) dissolved in 0.55 M sorbitol and 3.9 mM $Ca(H_2PO)_2 \times H_2O$. The number of protoplasts (usually the efficiency of protoplasting was greater than 90%) was counted in a Neubauer hemacytometer. The distribution of cells in clumps was determined by counting the number of cells per clump in cell suspension. Viability of cells was determined using the vital stain trypan blue (trypan blue 0.4% in normal saline, Grand Island Biological Company, Grand Island, N.Y.) which stains dead cells. Viability is expressed as the percent of live cells in the sample.

Plating

Feeder Plates

Feeder cultures (Fig. 1) were set up in polystyrene dishes (100 mm × 20 mm) (Falcon Plastic No. 3003, Division of Becton and Dickinson Co., Oxnard, California). Circular stainless steel screens (mesh width 1-2 mm) were placed in the bottom of the dishes. The edges of the screens were bent in such a manner as to raise the screens approximately 3 mm above the plastic. Polyurethane pads obtained through a local supplier (50 × 50 mm, 3-4 mm thick) were placed on top of the screen and covered by 50 × 50 mm squares of nitrocellulose membrane filters, pore size 0.45 μm (BA 85, Schleicher and Schuell, Keene, N.H., U.S.A. Similar plating efficiencies have been obtained using cellulose triacetate filters obtained from Gelman (GA6) (The latter are less expensive.)

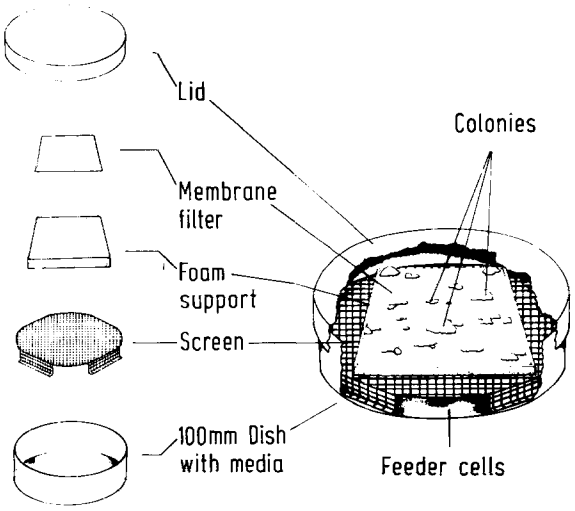

Fig. 1. Diagrammatic representation of the plating system used

Preparing Plates

For sterile routine experiments, the polyurethane foam pad was placed on the bottom of a petri dish and covered with an inverted stainless steel screen. 20 ml of sterile culture medium were then added. Care was taken to insure that the foam pads were thoroughly soaked with culture medium. The assembly then was inverted, so that the foam would rest on top of the screen. A sterile membrane filter was then placed on top of the foam pad.

2 ml of feeder cells from a logarithmically growing culture were pipetted into the bottom of the dish. The final concentration corresponded to the more dilute range of the logarithmic growth phase of the culture (1-3 X 10^5 cells/ml). Various concentrations of cells, suspended in up to 1 ml of medium, were plated on top of the membrane filters. (The liquid passes through the filter leaving the cells spread on top.)

Test for Amino Acid Secretion by Feeder Cells

Medium from 2, 4, or 8-day old feeder cultures as well as non-conditioned medium was mixed with an equal amount of bacterial minimal medium (Vogel and Bonner 1956) and solidified with agar (1.5%). Various auxotrophic strains of *Salmonella typhimurium* were plated on this medium. Strains auxotrophic for the following markers were kindly provided by Dr. J. Roth, Department of Biology, University of Utah, Salt Lake City: his$^-$, asp$^-$, pro$^-$, trp$^-$, ile$^-$, leu$^-$, phe$^-$, ser$^-$, ilv$^-$, arg$^-$, lys$^-$, gly$^-$, thr$^-$, tyr$^-$, guaB$^-$, bio$^-$, purB$^-$.

Mutagenesis

50 ml logarithmically growing SB-1 cells were diluted to a titer of 2 X 10^5 cells/ml (total of 1 X 10^7 cells), and 0.16% ethyl methanesulfonate (EMS) (Eastman Kodak Co., Rochester, N.Y.) was added. The cells were incubated under normal culture conditions for 1 generation (24 h.). Through dose response experiments it was determined earlier that 0.16% EMS yields 40-50% survivors.

After incubation the cells were washed 3 times in 10 ml of culture medium and cultured in fresh B5 medium. The recovery from mutagenic treatment was monitored by testing viability and measuring the increase in number. Within 8 days the mutagenized cells had recovered. They were then plated on selective media.

Results

Plating Efficiency

Dilutions of soybean and *Datura* suspension cultures were plated on membrane filters (see Materials and Methods) and incubated over soybean feeder cultures (*Datura* was plated on *Datura* feeders with similar success). Using this technique, colonies developed rapidly within 6 days. Figure 2 presents the combined results of several experiments. The frequency of colonies has been graphed against the number of cells plated. Since no attempt was made to disperse clumps of cells, a second value is given on the abscissa denoting the number of cell clumps plated. Two results are immediately evident from the data. The technique is reproducible yielding similar results from one experiment to another and a linear relationship is observed between the number of clumps plated and the number of colonies obtained. From this it can be concluded that no interaction between clumps is occurring such that more than one clump is required to form a

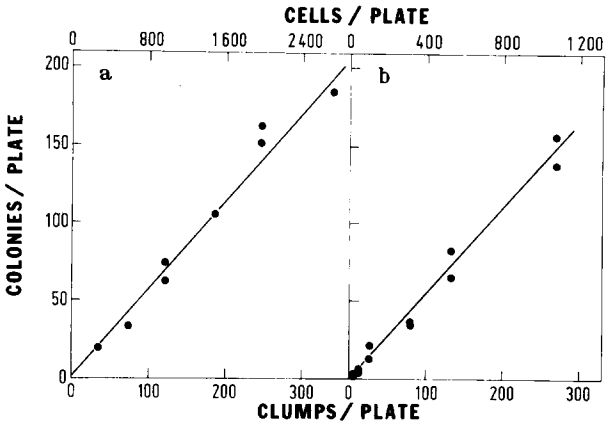

Fig. 2a and b. Plating of SB-1 and *D. innoxia* on feeder plates. Different concentrations of clumps of SB-1 (a) with a mean cell number of 8 cells per clump and *D. innoxia* (b) with a mean cell number of 4 cells per clump were plated on feeder cells. (The D.I. cells were prefiltered to remove very large clumps of material.) The lower abscissa shows the number of clumps plated estimated from the cell number (upper abscissa); the ordinate gives the number of colonies counted after 7 days

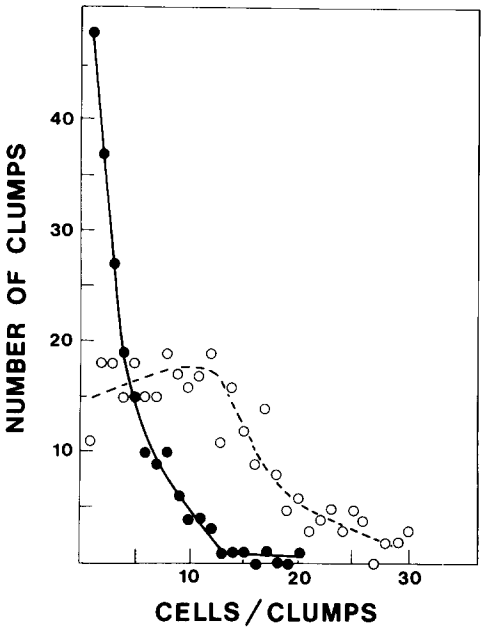

Fig. 3. Size distribution of clumps of *D. innoxia* after filtering through nylon screens. *D. innoxia* suspensions were fractionated by filtering through 215 μm nylon screens. The distributions of a fraction ≤ 215 μm, mean clump size 4, (●-●), and of one < 215 μm, mean clump size 12, (o-o), are shown. The abscissa presents the number of cells/clump, the ordinate, the total number of such clumps

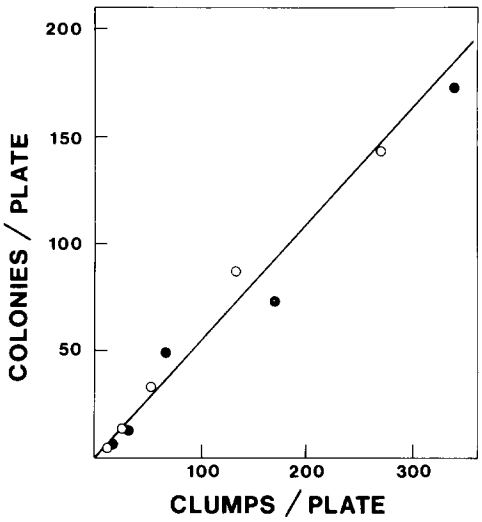

Fig. 4. Plating efficiencies of two populations of filtered *D. innoxia* cells with different sizes of cell clumps. The two filtered fractions of *D. innoxia* (described in Fig. 3) were plated on feeder plates. After seven days the number of clumps plated (abscissa) was compared with the number of colonies which had appeared (ordinate). Clumps with a mean size of 4 cells ($\leq$ 215 μm) (•-•) or clumps of 12 cells (< 215 μm) (o-o) were plated. The line was drawn from the data in Fig. 2 B and represents an efficiency of plating of .55 per clump

colony. The plating efficiency of clumps is quite high, varying between 50 and 60%. Because the majority of clumps are small (ca. 4 cells for *Datura* after filtering through a 215 μm nylon screen) we can conclude that these are plating with a high efficiency. On the other hand, the technique would not distinguish a low efficiency of plating for large clumps.

To determine the plating efficiency of clumps of different sizes, we obtained populations of large and small clumps by sieving *Datura* cells through graded screens of nylon mesh. The distribution of clump sizes of the population obtained are shown in Figure 3. The plating efficiencies of the two populations are shown in Figure 4 and compared to the plating efficiency of populations plated in routine experiments (Fig. 2b). No significant difference in plating efficiency was observed.

Excretion of Amino Acids by Feeder Cells

In order to utilize this plating technique for isolation and study of auxotrophs, it is important that feeder cells do not excrete required metabolites. We tested B5 medium (without added casamino acids), in which *D. innoxia* cells had been growing for 2, 4, or 8 days, for the presence of amino acids or purines. None of the following could be detected in quantities sufficient to allow growth of *Salmonella typhimurium* (< 0.1 μg/ml): histidine, aspartic acid, proline, tryptophan, isoleucine, leucine, phenylalanine, serine, valine, arginine, lysine, glycine, tyrosine, threonine, guanine or adenine. Thus, this plating system can be used for the isolation of auxotrophic mutants. (*D. innoxia* plates with equal efficiency on feeder cells growing in medium containing or not containing added casein hydrolysate.)

Isolation of 8 Azaguanine and 6 Thioguanine Resistant Mutations

8 azaguanine and 6 thioguanine are analogues of guanine and hypoxanthine which inhibit growth of soybean and *Datura* and, at high concentrations, kill them. Attempts to select 8 AG-resistant strains using 1 μg/ml 8 AG in liquid culture failed (all cultures died). However, a 6 TG resistant strain (SB-6 TG) was selected by growth in increasing concentrations of 6 TG over a period of 6 months. This strain, which can survive concentrations of 6 TG up to 60 μg/ml, was used in reconstruction experiments.

Wild type (SB-1) and 6 TG resistant (SB-6 TG) cells were plated on membrane filters and incubated over either SB-1 or SB-6 TG feeder cells suspended in 6 TG medium (Table 2).

When SB-1 feeder cells were used, no resistant colonies appeared, although some growth occurred prior to death. However, growth on SB-6 TG feeder cells resulted in colonies, comparable in number to the number of SB-6 TG clumps plated.

We used the membrane plating technique to select a cell line, resistant to 8 AG. A total of 2.4×10^6 clumps, from a mutagenized culture of SB-1, was plated on membranes and incubated over SB-1 feeder cells in 8 AG medium. To avoid the phenomenon of cooperative death, the membrane was transferred every two days to fresh media containing new feeder cells. After 8 days a few colonies (10) had appeared. These were picked and transferred to new membranes. One of these, SB-8 AG, re-

Table 2. Growth of SB-1 or SB-6TG cell clumps on 6TG media[a]

		Cells plated (125 clumps/plate)	
Plated cells		SB-1	SB-6TG
Feeder cells[b]	SB-1	0	0
	SB-6TG	0	85[c]

[a] Culture medium contained 60 μg/ml 6TG
[b] No exchange of feeder cells was per formed
[c] Colonies counted after 7 days incubation

mained resistant to 8 AG after passage through non-selective medium. The selection and testing of the 6 TG resistant cell line using liquid culture had taken 6 months. In contrast, the selection, isolation and testing of the 8 AG resistant line required 4 weeks. From experiments not shown here we have concluded that the SB-8 AG line does not lack the enzyme, HGPRT. However, it is possible that either the membrane or HGPRT enzyme have been altered to exclude incorporation of 8 AG.

Reconstruction Experiments for Mutant Selection

Mixtures of SB-1 and SB-8 AG were plated on SB-1 feeder cells in medium containing 8 AG (Table 3). Pure cultures of either SB-1 or SB-8 AG were also plated. Colonies resistant to 8 AG were selected from among an excess of wild type cells and the plating efficiency was similar to that of SB-8 AG plated in the absence of SB-1.

Another experiment was carried out using a maltose utilizing mutant of SB-1, SB-M 24. Again mixtures of SB-1 and SB-M 24 as well as pure cultures of each were plated on selective (Maltose) or non-selective media. SB-M 24 could be selected in spite of the presence of 1000-fold excess of wild type cells (Table 4).

Table 3. Selection of SB-8AG clumps on 8AG media

	Number of colonies/plate after 8 days Medium	
Cell lines and titers (clump/plate) plated	1B5	1B5 with 500ng 8AG/ml
10^5 SB-1	+	0
10^2 SB-8AG	16, 14	15, 25, 33
10^3 SB-8AG	100, 95, 135, 98	100, 122, 143
10^2 SB-8AG with 10^5 SB-1	N.E.	13, 7, 28, 36, 19[a]
10^3 SB-8AG with 10^5 SB-1	N.E.	39, 65, 105, 87, 115[a]

+ = Confluent lawn of growth, N.E. = No experiment
[a] When colonies were picked and tested they were found to be resistant to 8 azaguanine

Table 4. Selection of M-24 cells on maltose media

	Number of colonies/plate after 8 days	
Cell lines and titres (clump/plate) plated	MS-medium 2% sucrose	MS-medium 2% maltose
10^5 SB-1	+	0
10^3 M-24	N.E.	241, 259
10^3 M-24 with 10^5 SB-1	N.E.	220, 185, 215, 180, 165

+ = Confluent lawn of growth, N.E. = No experiment

Discussion

We have described a new plating technique which can be used to rapidly isolate mutants of cells growing in suspension culture. The method was developed recognizing that, if feeder cells grow well, cells on top of the membrane will also grow rapidly (provided a specific selection is not in progress). On the other hand, when feeder cells grow poorly (for any reason) the cells on top of the membrane will also be inhibited. Even the lag in growth, observed upon transferring feeder cells to fresh unconditioned medium, can retard the growth of colonies otherwise well established on the membrane surface.

The membrane support allows clones of growing cells to be in contact with medium shared by cells in suspension. By transferring cells, under selection, to fresh selection media, before death of feeder cells begins, contact with the products of dead or dying cells can be avoided. Essential nutrients are provided by the healthy feeder cells and growth rates on the membrane can be achieved which are comparable to the growth rates of the feeder cells in suspension.

Added advantages of the system are: (1) membranes can be transferred successively to different selection or enrichment conditions. Thus, cells on membranes can be passaged in the presence or absence of essential amino acids, purines or pyrimidines, or treated periodically with inhibitors. (2) Clumps of mutant cells are kept separate and therefore different clones of mutants can be isolated from the same suspension of mutagenized cells.

One disadvantage is that clumps cannot grow efficiently at densities above 50 clumps per cm^2. This limits the number of mutants which can be isolated on a single membrane to frequencies of $1/10^3$ and restricts use of the technique to mutagenized cells.

Another use of this plating technique may be to develop efficient assays for essential factors which promote or inhibit growth. By supplementing medium with appropriate extracts or laying chromatographs of extracts between the sponge and the membrane and monitoring plated cells, assays for factors influencing embryogenesis, senescence and differentiation may be developed.

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