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Screening for and characterization of phospholipase A_1 hypersecretory mutants of *Tetrahymena thermophila*

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Abstract We have described a procedure for the isolation of mutants of *Tetrahymena thermophila* with hyperscretion of phospholipase A_1 (PLA_1). Using random chemical mutagenesis, uniparental cytogamy, genetic crossing and a new, fast and effective screening procedure, four PLA_1-hypersecretory mutants were isolated. The screening procedure is based on the formation of a halo appearing around cylindrical holes in a lecithin-containing agar plate filled with cell-free supernatants. About 3,940 clones were tested with this procedure in primary screening for hypersecretory features, of which 60 putative hypersecretory mutants were isolated, subcloned and tested in a secondary screening. Of these, four selected mutants showed 1.8–2.2 more PLA_1 activity in the cell-free supernatants compared to the wild-type strain CU 438.1. Hypersecretion was only observable for PLA_1; no increased activity for two other lysosomal enzymes could be detected. These hypersecretory mutants of *T. thermophila* can be very useful for increasing the yield of PLA_1 in fermentation processes. This is particularly relevant because, in contrast to other phospholipases, PLA_1 is not available on the commercial market for fine chemicals and little is known about the role of PLA_1 in cell signaling and metabolism.

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Introduction

Unusual microorganisms still constitute a largely untapped source of interesting metabolites and enzymes (Vandamme 1994). These organisms include the ciliated protozoa, e.g. species of *Tetrahymena*, which exhibit a large biotechnological potential (Munro 1985). Gosslin et al. (1989) developed a fed-batch mass culture for the production of γ-linolenic acid by *Tetrahymena rostrata*. Another species, *Tetrahymena thermophila*, is known to release large amounts of acid hydrolases into the extracellular medium (Müller 1972; Rothstein and Blum 1974) and is now a focus for biotechnology of protozoa. Kiy and Tiedtke (1991) developed a technique for mass cultivation of immobilized cells, allowing easy harvesting of secreted acid hydrolases for this species. Until now seven glycosidases, two phospholipases and six other hydrolases have been either detected or partially purified from spent culture medium of *T. thermophila* (Tiedtke 1993). Finally, the set-up of a perfused bioreactor for continuous high-cell-density fermentation together with the formulation of inexpensive culture media provides an economical and effective fermentation technology (Kiy and Tiedtke 1992a, b). The production of commercially interesting enzymes and other metabolites is on the way (Kiy and Tiedtke 1993) and the suitability of glycosidases of *Tetrahymena* in bioconversion reactions for synthesis of specific carbohydrates has recently been shown (Kiy et al. 1995).

Aside from fermentation techniques, strain improvement by genetic manipulation is a method for increasing the yield of products. Random chemical mutagenesis followed by a suitable screening procedure represents the classic route for this purpose. Chemical mutagenesis, e.g. of *Penicillium occitanis* (Jain et al. 1990) or *Dictyostelium discoideum* (Ebert et al. 1990), followed by screening led to strains which were hypersecretory for antibiotics or extracellular enzymes. A screening procedure following random mutagenesis is easier with target

organisms having a haploid life cycle (bacteria or some fungi), in which also recessive mutations are expressed immediately. Ciliated protozoa contain a diploid germline micronucleus and a highly polyploid somatic macronucleus governing the vegetative life of these cells. To get a mutation expressed in the diploid germline micronucleus, a sexual process must follow mutagenization. Cytogamy, autogamy in paired cells, is one such process which eventually results in the formation of whole-genome-homozygous zygotic nuclei in both pairing partners. Two postzygotic mitotic divisions and gene amplification in two of the division products produce new diploid micronuclei and allow the development of new polyploid macronuclei expressing the mutated phenotype in high numbers.

In this study we used uniparental cytogamy (UPC), a genetic crossing protocol developed by Cole and Bruns (1992) to bring chemically induced mutations to expression. After cloning the mutagenized UPC-progeny, the clones were screened for hypersecretion of phospholipase A (PLA). We chose the phospholipase A as targets for our screening for the following two reasons: (1) In contrast to PLA_2, little is known about the role of PLA_1 in cell signaling and metabolism, and (2) *Tetrahymena* normally secretes a phospholipase A_1 (Florin-Christensen et al. 1985, 1986; Arai et al. 1986) into the medium. This type of enzyme is not commercially available and can be used for the preparation of *sn*-2-acyl-lysophospholipids as precursors for phospholipid synthesis. Presently, the only phospholipases A industrially produced are those of type A_2. As a consequence, only *sn*-1-acyl-lysophospholipids are prepared in large quantities by commercial companies. This implies a serious limitation in organic biochemistry with regard to the starting reagents for phospholipid synthesis, especially in view of the fact that position *sn*-2 is particularly difficult to acylate, due to steric hindrances (Gupta et al. 1977). This difficulty can be overcome by using *sn*-2-acyl-lysophospholipids with an unhindered primary alcohol. In addition, phospholipase A_1 can also be used for the isolation of the fatty acids esterifying position *sn*-1 of phospholipids, thus providing a valuable tool in the analysis of phospholipids.

Materials and methods

Microorganisms

The screening procedures were carried out with the inbred B strain CU 438.1 (mating type IV) of *T. thermophila*. This functional heterokaryon carries the drug marker for paromomycin resistance homozygously in the micronucleus (Pmr). Due to the macronuclear wild-type alleles, it is phenotypically sensitive to this drug. The mating partner was the star strain B*VI (mating type VI) which has a defective micronucleus. Like CU 438.1, strain B*VI is paromomycin-sensitive (Pmr^+), because it carries no paromomycin resistence alleles in its micronucleus, nor in its macronucleus. Both strains were obtained from Dr. P. Bruns (Cornell University, Ithaca, N.Y., USA).

Chemicals

Skimmed milk powder was obtained from Milchwerke Lippstadt (Lippstadt, Germany), yeast extract and proteose peptone from Difco (Detroit, Mich., USA) and Sequestrene from Ciba-Geigy (Greensboro, N.C., USA). *N*-Methyl-*N*-nitro-*N*-nitroso-guanidine (MNNG) for mutagenesis and 4,6-diamidino-2-phenylindoledihydrochloride (DAPI) for staining nuclei and paromomycin were purchased from Sigma (Deisenhofen, Germany). *p*-Nitrophenyl-phosphate and *p*-nitrophenyl-α-D-glucopyranoside as substrates for glycosidases were purchased from Sigma (Deisenhofen, Germany). Phosphatidylcholine (96%) and taurocholic acid were obtained from Calbiochem (Bad Soden, Germany); 1-palmitoyl-2-(1-^{14}C)-linoeoyl-phosphatidylcholine (P-^{14}cLPC, 110 μCi/μmol) as substrate for phospholipase A_1 from Amersham (Braunschweig, Germany), silica gel G on glass plates from Merck (Darmstadt, Germany), bovine serum albumin from Sigma (Deisenhofen, Germany) and Ficoll from Pharmacia (Freiburg, Germany).

Medium

For precultures and mutagenesis PPYS medium was used, containing 1% proteose peptone, 0.1% yeast extract and 0.003% Sequestrene. Mating reactivity was induced by starving the cells in 0.01 M Tris-HCl, pH 7.5. The growth medium for cloning and culture of mutagenized cells, first described by Kiy and Tiedtke (1992a), contained 2% skimmed milk, 0.5% yeast extract, 0.003% Sequestrene and 1% glucose. All experiments were conducted at 30 °C.

General experimental procedure

The sequential steps of the screening protocol are outlined in a schematic diagram shown in Fig. 1.

Mutagenesis and induction of mating

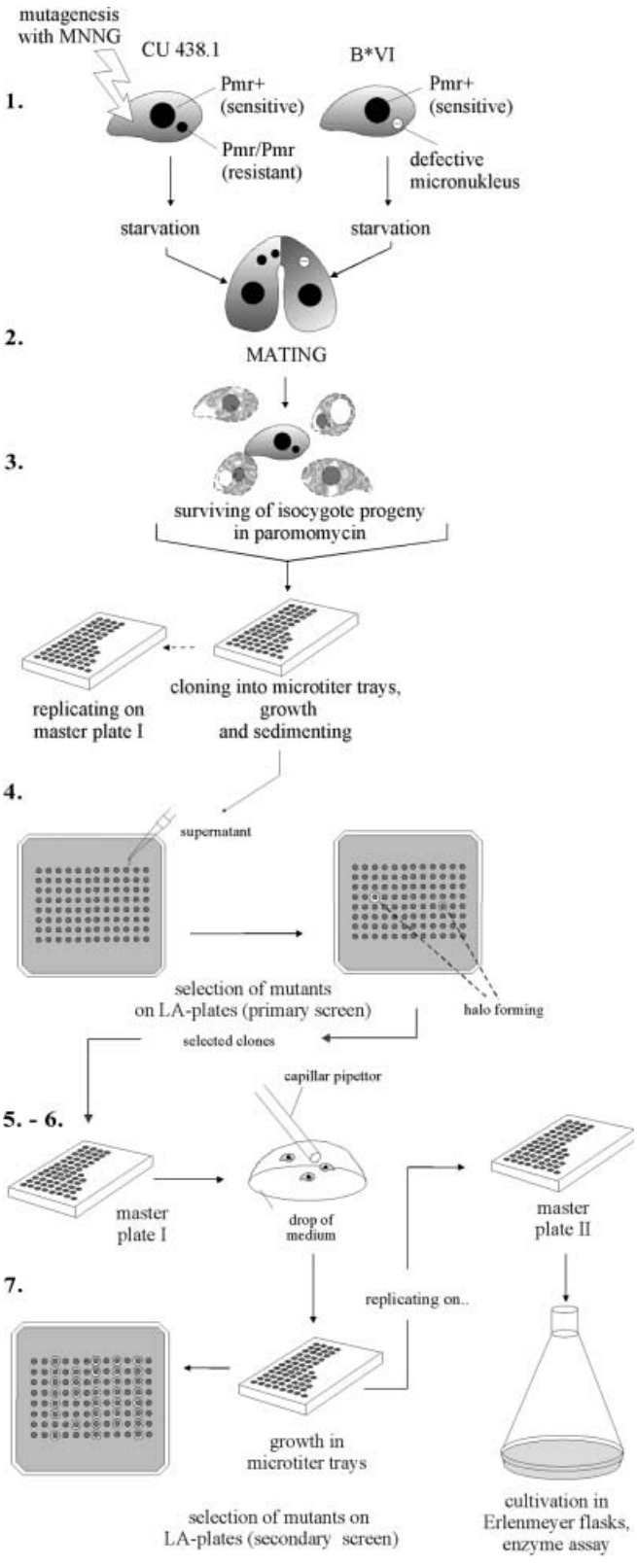
All steps of mutagenesis, UPC and drug selection were carried out in general as described by Cole and Bruns (1992) with a few modifications: cultures of the strains CU 438.1 and B*VI were grown to $2–3 \times 10^5$ cells/ml. To 5 ml of cultures of CU 438.1 MNNG was added at a final concentration of 10 μg/ml. After 4 h incubation without agitation at 30 °C, the mutagenized CU 438.1 cultures and the B*VI cultures were washed three times in starvation medium and allowed to starve overnight.

Cytogamy and environmental shock

After 18 h starvation, 5-ml samples of each strain (adjusted to the same cell number) were mixed in a petri dish. The mating process was controlled by monitoring the mating rate: 3 h and 6 h after mixing, small samples were taken, in order to count the number of pairs and unpaired cells, to calculate the pairing rate and to stain their nuclei using the fluorochrome DAPI. Cells were fixed in methanol–acetic acid (3:1) and stained by adding an equal volume of 100 μg DAPI/ml. A Zeiss fluorescence microscope was used to examine the nuclear configurations of paired cells in order to determine the time at which the majority of the pairs were ready to exchange their haploid migratory nuclei. At this time (~6.5 h after mixing the starved pairing partners) a hyperosmotic shock was delivered to the mating cells by adding 1 ml of 10% glucose to 8 ml of mating cells (final concentration: 1.4%). After 40 min, the cytogamy mixture was diluted first 1:10 in distilled water; and subsequently, the same volume of double-concentrated PPYS medium was added to allow growth of UPC progeny.

Selection of excytogamonts and cloning

Progeny of UPC were exposed to 100 μg paromomycin/ml for 4 days in a 1-l Fernbach flask at room temperature. The selection



process was controlled by light microscopy. Surviving drug-resistant excytogamonts and offspring of short-circuit genomic exclusion were easily discriminated from dying non- and exconjugants.

Cells were distributed using a 8-channel pipette to deliver volumes of 150 µl of skimmed milk, containing on average only a single cell into the v-shaped wells of microtiter plates. Under these conditions, about 37% (1/e) of the wells were expected to have received a single cell, according to the Poisson distribution. This procedure was previously described by Hünseler et al. (1987). Two wells of each plate were inoculated with parent strain (wild-type CU 438.1) as an internal control. After incubation at 30 °C for 4 days, plates were united and replica-plated.

◀

Fig. 1 Schematic diagram of the screening steps: *1* mutagenesis with *N*-methyl-*N*-nitro-*N*-nitroso-guanidine (MNNG) and induction of mating, *2* cytogamy and environmental shock by adding glucose (1.4%), *3* selection of drug resistant isozygote cytogamonts, *4* primary screen with lethicin-agarose screening (LA) plates, *5* cloning of selected mutants by single cell isolation, *6* secondary screen and *7* characterization of hypersecretory mutants with phospholipase A (PLA_1) enzyme assays

Screening procedure

To assay hypersecretory phospholipase A mutants, lecithin-agarose screening plates (LA plates: square dishes, 14 × 14 cm, containing screening medium, see below) were used as described by Kim and Rhee (1994) with the following variations: the screening medium was composed of 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \times 7H_2O$, 1 g of $(NH_4)_2SO_4$, 15 g of agarose, 5 g of 97% phosphatidylcholine, 2.5 g of taurocholic acid/l dissolved in 10 mM acetate buffer, pH 4.5, containing 100 mM $CaCl_2$. After solidification of the agarose, holes were punched using a 96-well microtiter plate raster. The punched agar cylinders, 2.5 mm in diameter, were aspirated and the plates were stored at 4 °C.

The cells grown for 7 days in the wells of the microtiter plates were then underlayed with 20 µl of Ficoll (100 g/l) and the plates were centrifuged at 1000 *g* for 5 min at 10 °C. A 20-µl sample of the supernatant was removed from each well for enzyme assays and stored at 4 °C. Samples (8 µl) of the supernatants were filled into the cylindrical holes in LA plates using an eight-channel pipettor and were incubated at 30 °C for 24 h in a moist chamber. The evaluation of the plates was done by comparing the beginning of formation of a transparent halo around the holes with that of controls (parental strain CU 438.1). Clones that had formed clear halos significantly earlier than the controls were selected for single cell isolation and a secondary screen.

Single cell isolation

Ten single cells from each of the wells containing putative PLA-hypersecretory "clones" (primary screen) were separated with a capillary pipettor under binocular control into a drop of medium on a Petri dish and incubated for 3 days at 30 °C. The resulting clones were replicated for the second screen into holes of LA plates to prove their hypersecretory performance.

Photometric enzyme assays

The enzyme activities of acid phosphatase (EC 3.1.3.2) and *N*-acetyl-β-hexosaminidase (EC 3.2.1.52) were assayed at 30 °C using *p*-nitrophenyl phosphate and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrates. Both enzymes were assayed as described previously (Kiy et al. 1996) with the following changes. A sample of 20 µl was added to 50 µl of 10 mM *p*-nitrophenyl substrate dissolved in 0.1 M citrate puffer, pH 4.6, supplemented with bovine serum albumin (0.2%). After incubation for the preselected time, 200 µl of 0.4 M glycine/NaOH buffer (pH 10.4) were added to stop the reaction. The amount of liberated *p*-nitrophenol was determined photometrically at 405 nm. One unit was defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol/min. All assays were done in 96-F-well microtiter plates using an Anthos HT III microtiter plate reader for photometric measurements.

Radiochemical enzyme assay

A radiochemical assay was used for the determination of phospholipase A_1 activities (EC 3.1.1.32) with a natural substrate. This assay was carried out in a total volume of 200 µl, containing 160 µl of cell-free supernatant or water (in the case of the substrate blank), 20 µl of substrate and 20 µl of 1 M sodium acetate buffer, pH 4.75. The substrate was prepared by mixing chloroform solutions of labeled and cold phosphatidylcholine (PC), evaporation of the chloroform and resuspension in distilled water, followed by vigorous vortexing and sonication in a water sonicator. The substrate contained 20,000 cpm of radioactively labeled and 0.6 mg cold PC/sample. The labeled PC was: 1-palmitoyl-2-(1-^{14}C)linoleoyl-PC and the cold PC was crude soy bean lecithin. Water-saturated diethylether (200 µl) was added to the samples to inhibit lysophospholipase A activity. After incubating the reaction mixtures at 37 °C for 1 h, the reactions were stopped and the lipids extracted according to Bligh and Dyer (1959). The organic (lower) phases were separated and evaporated under nitrogen. Then the lipids were spotted onto silica gel G plates and chromatographed in two sequential steps: the first solvent mixture was: chloroform/methanol/water (65:35:5, v/v) to separate polar lipids. This mixture was allowed to run up to the middle of the plates. After removing the plates from the chamber and letting the solvents dry, they were introduced into a second thin layer chromatography (TLC) chamber with the following solvent mixture: petroleum ether/diethyl ether/acetic acid/methanol (90:20:2:3, v/v) to separate neutral lipids. This mixture was allowed to run to the top of the plates. The TLC plates were then subjected to autoradiography on Kodak X-ray film for about 5 days. The autoradiograms were developed using Kodak GBX developer and fixer. Then, the silica gel containing the different lipid spots was scraped off the plates into scintillation vials. The radioactivity was quantified in a scintillation counter after addition of scintillation fluid (0.5% PPO, 0.01% POPOP, 33% Triton X-100, 67% toluene). As the substrate is only labeled in position *sn*-2, the increase in radioactivity in the lysophosphatidylcholine spot is indicative of phospholipase A_1 activity. The number of micromoles of lysophosphatidylcholine has been calculated from the specific activity (110 µCi/µmol).

Characterization of the mutants

For further characterization, the isolated mutant clones were cultivated in 100-ml Erlenmeyer flasks containing 5 ml of skimmed milk medium for 72 h at 30 °C. The flasks were inoculated with cells from the stationary phase at a concentration of 5×10^4 cells/ml. Samples for cell counts and enzyme assays were taken after 72 h. Cells were counted in fixed samples using a Fuchs-Rosenthal counting chamber. Cell samples for enzyme assays were sedimented at 1,000 ×*g* for 4 min into a cushion of 10% (w/v) Ficoll. The supernatants were aspirated and stored at 4 °C.

For secretion kinetics of mutant "12 1 C", the isolated mutant clone was cultivated for 48 h in a 2-l Fernbach flask containing 100 ml of skimmed milk medium. The flask was inoculated with cells from the stationary phase at a concentration of 5×10^4 cells/ml. Samples for cell countings and enzyme assays were taken after 3, 6, 12, ±18, 24, 30, 36, 42 and 48 h. Cell counts and enzyme assays were made as described above.

Results

The screening procedure used in the primary screen is outlined in Fig. 1. Hypersecretory clones as detected with the LA plate screen quickly form a distinct halo around the hole. Figure 2 shows the halo formed by clone "20 2 D" after 12 h incubation with cell-free supernatants. Altogether 6,286 wells were screened on LA plates. According to the Poisson lottery, 3,940 were considered to be potential clones. Out of these, a number of 60 wells contained putative phospholipase A-hypersecretory mutants. These formed larger halos in a shorter time than (the spent media from) the wild-type parent (CU 438.1), whose spent media were incubated at the same temperature (30 °C) for 12, 18 and 24 h. The 60 putative phospholipase A-hypersecretory mutants were subcloned by single-cell isolation and screened on LA plates a second time. Four of these clones proved hypersecretory for phospholipase A_1 when assayed as described (see Fig. 2). A minimum frequency of 2.2% PLA-hypersecretory mutants (four mutants in 3,960 viable clones) may be calculated. This is probably not a correct estimate because, on the one hand, some clones may have been lost during both the primary and secondary screenings; and on the other hand, several mutants with hypersecretory characteristics could be 'clone sisters'.

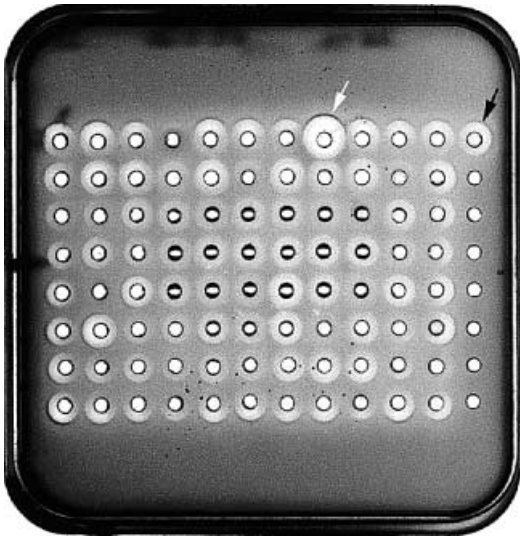


Fig. 2 Photographs of a LA plate of the primary screen. The strong, clear halo around the hole (*white arrow*) containing cell-free supernatant of the (PLA-hypersecretory) clone "20 2 D" is formed in 12 h incubation at 30 °C. At this time, holes containing cell-free supernatants of the other clones (other holes) and the wild-type parent (CU 438.1, *black arrow*) showed only a weak halo formation

Table 1 summarizes the results of the primary and secondary screen. The mutants "12 1 C", "20 2 D", "11 4 F" and "22 8 H", respectively, released about 2.1, 2.2, 1.8 and 1.9 times more phospholipase A_1 activity than wild-type CU 438.1, as measured with the radiolabeled substrate assay. These mutants were selected for further experiments, because they turned out to be best with regard to stability and fertility. We regard strains that release more phospholipase A_1 to the extracellular medium than the parent strain as hypersecretory. In this case, all isolated mutants are hypersecretory mutants. The hypersecretion of phospholipase A_1 was also confirmed in the secretion kinetics of mutant "12 1 C" (Fig. 3A). A remarkable hypersecretion could be observed in the early logarithmic growth phase, but the secretion of the enzyme was more pronounced in the late

Table 1 Comparison of primary and secondary screens. From each putative hypersecretory clone detected in the primary screen, ten single cells were isolated and grown-on. Two clones proved hypersecretory in the secondary screen. They originated from different clones of the primary screen

Screening step	Number of wells
Primary screen	
Screened wells	6,286
Clonal wells	3,940
Hypersecretory clones	60
Secondary screen	
Single cell isolations	600
Hypersecretory clones	113
Selected mutants	4

Table 2 Extracellular phospholipase A_1 activity of wild-type (parent strain CU 438.1) and hypersecretory mutants (samples of cell-free supernatants were taken at t_{72h} from 100-ml Erlenmeyer flasks containing 5 ml of cell suspension grown in skimmed milk medium at 30 °C without shaking). The enzyme substrate was 1-palmitoyl-2-(1-^{14}C)linoleoyl-phosphatidylcholine (P-^{14}cLPC)

Strain	Cell concentration (cell/ml × 10^6)	Extracellular enzyme activity (mU/ml)	Extracellular enzyme activity (mU/10^6 cells)
CU 438.1	2.10	17.2 ± 1.98	7.24
"12 1 C"	1.54	34.9 ± 0.83	20.91
"20 2 D"	1.72	35.2 ± 2.60	19.53
"11 4 F"	1.95	30.3 ± 2.11	15.54
"22 8 H"	1.92	33.4 ± 2.75	17.40

stationary phase (t_{48h}). In contrast to this, no hypersecretion could be measured for two other lysosomal enzymes. The enzyme activity for acid phosphatase and for β-hexosaminidase had nearly the same value as the wild-type after 48 h cultivation (Figs. 3B, C).

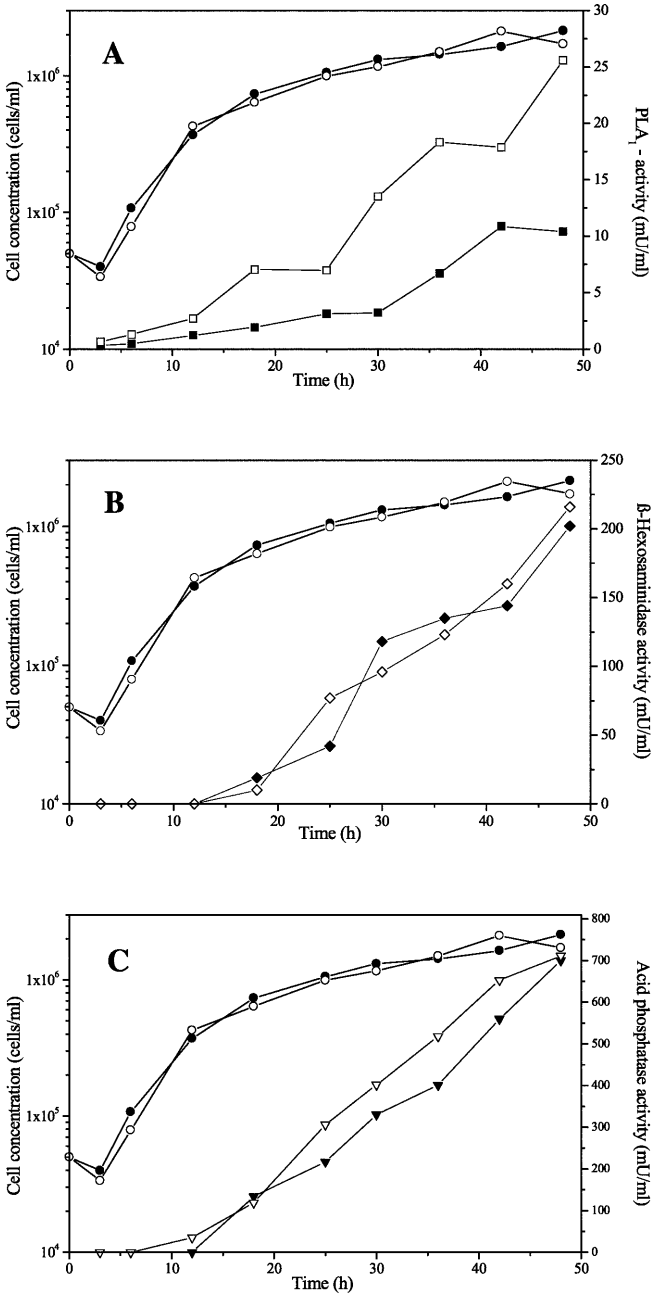


Fig. 3A–C Secretion kinetics of three lysosomal enzymes of mutant "12 1 C" and wild-type. Illustrated are the cell densities of the wild-type (-●-) and the mutant "12 1 C" (-○-), the extracellular activity of (**A**) phospholipase A_1 for the wild-type (-■-) and the mutant "12 1 C" (-□-), the extracellular activity of (**B**) β-hexosaminidase for the wild-type (-◆-) and the mutant "12 1 C" (-◇-) and the extracellular activity of (**C**) acid phosphatase for the wild-type (-▼-) and the mutant "12 1 C" (-▽-). Cells were cultivated in 2-l Fernbach flasks as described in Materials and methods

Discussion

Dimond et al. (1983) first described mutants of *Dictyostelium discoideum* defective in the regulation of secretions. Hünseler et al. (1987) later isolated and characterized a mutant blocked in the secretion of lysosomal enzymes in *T. thermophila*. In this study, short-circuit genomic exclusion (Bruns et al. 1976) was used as a crossing technique to bring micronuclear mutations into macronuclear expression; and a screening method was designed for identifying the mutants blocked in the secretion of acid phosphatase and β-hexosaminidase. One of the resulting mutants, MS-1, secreted less than 5% of the enzymatic activities into the medium. In comparison with the present study, there were no data about the number of clones that were screened to obtain this mutant, but with reference to the findings of Cole and Bruns (1992), only a small fraction of mating pairs (less then 5%) proceeded through postzygotic development via short-circuit genomic exclusion. As a consequence, a large part of the induced mutations were not expressed. Additionally, this progeny have a tendency toward infertility, which is probably a result of the possession of aneuploid micronuclei (Cole and Bruns 1992). In contrast, UPC produces a large number of whole-genome homozygous (= isozygote) and highly fertile progeny, which could easily be selected from

heterozygote non- or exconjugants by using dominant drug-resistance markers. From this it can be concluded that a large fraction of mutations are brought into expression. The only limiting step in the production of desired mutants of *T. thermophila* with the mutant described here is the screening procedure itself. In the present work a lecithin-agarose plate assay was improved for semi-quantitative detection of phospholipase A_1 activities in cell-free supernatants. This screening procedure allows the checking of a large number of clones for PLA-hypersecretory features in a fast, efficient and effective way. About 3,940 clones were tested in one week by one person; and 77 or about 2.2% of all clones showed hypersecretion of phospholipase A. Four selected hypersecretory mutants showed about 1.8–2.2 times more phospholipase A_1 activity than the wild-type CU 438.1, measured with the radioactive phospholipase A_1 assay system. In no case did we observe reversion of the mutant phenotypes. Since the macronucleus of the UPC progeny contains exclusively copies (45) of the mutant allele, reversion of the mutant phenotype is not expected to occur often. The secretory ability for lysosomal enzymes has been investigated with the mutant "12 1 C". The hypersecretion appears in the early logarithmic growth phase and gets stronger in the late stationary phase. A hypersecretion was only observable for phospholipase A_1, because no increased activities of two other lysosomal enzymes could be detected, compared with the wild-type.

This screening system should be compared with other improvement procedures to evaluate their effectiveness, aimed at improving strains. Unfortunately, there is very little information about screening for enzyme hypersecretory mutants in other protozoans. Using *D. discoideum*, Ebert et al. (1990) carried out chemical mutagenesis with subsequent screening for mutants. More than 11,700 clones of *D. discoideum* were screened for secretory mutants for several lysosomal enzymes. After a secondary screen, eight mutants were identified, showing oversecretion of acid phosphatase. The relationship between extracellular and total enzyme activities have been calculated in the final characterization of the mutants. Thus, in this work there were no data about the actual enzyme activities in the supernatant. These workers defined oversecretion as a displacement of the relationship between intra- and extracellular enzyme activities and not as an increase of the enzyme activity in the supernatant of the mutant in comparison with wild-type supernatant. For example, the mutant HMW 570 secreted more than 80% of the total enzyme activity of acid phosphatase into the extracellular medium (wild-type Ax3: 40%) and showed a decrease in total enzyme activities in comparison with the wild-type.

A random mutagenesis procedure, followed by a screening technique based on commonly used enzyme plate assays, is often used for the improvement of strains of enzyme-secreting fungi. Jain et al. (1990) isolated a cellulase–pectinase hypersecretory mutant of *Penicillium occitanis*. Their procedure included seven rounds of mutagenesis using UV light, ethylmethansulfonate and MNNG. The isolated mutant, Pol 6, showed a 5–7-fold cellulase activity in cell-free supernatant, compared with the wild-type. Brown et al. (1987) gave more data about the experimental procedures. They screened approximately 2,500 colonies of *P. pinophilum* on agar plates to identify strains that overproduced cellulase and β-glucosidase. After two rounds of mutagenesis their best mutant, JAB/3C/1C, showed 3.7-fold higher β-glucosidase activity and more than 7-fold higher cellulase activity than the original strain C22c. Chelius and Wodzinski (1994) used two rounds of mutagenesis for improving a strain of *Aspergillus niger* for phytase production. Their isolated mutant, 2DE, produced 3–6-fold more phytase activity into mycelium-free cultivation broth than the wild-type. In summary, the average improvement of secretory abilities in fungal systems is about a factor of 1.5–3 for each round of mutagenesis, which is comparable with data for *Tetrahymena* in this work.

Similar to fungal hypersecretory mutants, it should be possible to combine several rounds of mutagenesis in order to construct hyper-yielding strains of *T. thermophila* for enzyme production. However, the screening method allows a fast and effective improvement of the strain and we could also apply this method to identify hypersecretory mutants for other lysosomal enzymes (unpublished results). To our knowledge, the present study reports for the first time on the successful screening for and the isolation of hypersecretory mutants of ciliated protozoa. Future studies will characterize the mutants in order to reveal the primary cause of their hypersecretory activity. Once such mutants are available, we will cross hypersecretory mutants from different screening cycles in order to combine their hypersecretory genes for further improvement of the strain.

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