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PURIFICATION AND CHARACTERIZATION OF PLASMA MEMBRANES FROM *PHYSARUM POLYCEPHALUM* AMOEBAE

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In this study we describe a method for preparing plasma membranes from *Physarum polycephalum* amoebae. The cells were swollen in hypotonic medium (1 mM $ZnCl_2$, 10 mM Tris-HCl, pH 8.0) and then broken in a Thomas tissue grinder fitted with a teflon pestle. The plasma membranes were collected by differential centrifugation and purified by centrifugation on a continuous 20–50% sucrose gradient. The membranes sedimented in a single band having a density of 1.16 g/cm^3. They were found by enzymatic assay and by electron microscopy to be free of lysosomes, mitochondria, and nuclei and minimally contaminated by endoplasmic reticulum. The membrane proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis; 10 major and 20 minor bands were seen. Periodic acid Schiff's staining of electrophoretically separated proteins revealed five major and six minor bands containing glycoproteins. Radioiodinated cell surface proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis into six bands with apparent weights greater than 68 000.

Introduction

The acellular slime mold *Physarum polycephalum* has been used as a model system for numerous studies on the mitotic cell cycle and cell differentiation [1–5]. In the sexual life cycle of this organism, compatible haploid amoebal cells fuse to form a diploid zygote, which then differentiates into the large multinucleate plasmodium. The differentiation of the zygote is controlled by the multiallelic locus *mt* [6–8].

The fusion of amoebal cells is also genetically regulated. Only amoebae carrying different alleles of the multiallelic *mat*B locus fuse efficiently [9–12]. In addition genetically compatible amoebae become competent to fuse at a critical cell density [13]. Shipley and Holt [14] presented evidence suggesting that fusion competence is induced by a filter-transmissible material that accumulates during amoebal growth. In another study Shinnick et al. [15] demonstrated that the genetic locus *imz* affected the pH at which amoebal cell fusions can occur. These genetic and physiological studies have not yet been complemented by biochemical analyses.

As a first step in the biochemical analysis of amoebal cell competence and fusion, we present in this paper a method for the purification of amoebal plasma membranes. These were found by enzymatic assays and by electron microscopy to be free of lysosomes, mitochondria, and nuclei, and minimally contaminated by endoplasmic reticulum. Total membrane proteins, glycoproteins and external membrane proteins were analysed by gel electrophoresis.

Materials and Methods

Amoebal cell culture

The amoebal strain Cld-Axe, which grows in

axenic medium [16], was supplied to us by Dr. Jennifer Dee. These amoebae were routinely grown in 500 ml baffled flasks containing 200 ml of Daniel and Baldwin's semidefined medium [17]. The cultures were constantly shaken at 26°C.

Purification of plasma membranes

Exponentially growing amoebae at a density of $(5-10) \cdot 10^6$ cells per ml were collected by a 2-min centrifugation at $400 \times g$ in an International clinical centrifuge. The cells were twice resuspended and centrifuged in cold 0.14 M NaCl. The pellet of washed cells was suspended in room temperature homogenization buffer consisting of 1 mM $ZnCl_2$, 10 mM Tris-HCl, pH 8.0 at a density of 10^9 cells per 15 ml. After a 15-min incubation at room temperature the cells were cooled on ice to 4°C. All of the subsequent operations were carried out at 4°C. The cells were broken with 30 strokes of a mechanically driven Thomas tissue grinder fitted with a tight teflon pestle. Cell breakage was monitored microscopically.

The cell homogenate was centrifuged in a Sorvall SS-34 rotor for 15 min at $20000 \times g$. The resulting pellet was suspended in 10% (w/w) sucrose at a volume equal to half of that of the cell homogenization buffer and vigorously homogenized with five mechanical strokes of the previously used Thomas tissue grinder. The suspension was loaded onto 20% to 50% (w/w) linear sucrose gradients in aliquots of 10 ml per gradient. Centrifugation was carried out at 4°C for 2 h at 26000 rpm in a Beckman SW27 rotor. The bottoms of the tubes were pierced, and 1.5-ml fractions were collected by upward displacement with a 65% (w/w) sucrose solution. An ISCO density gradient fractionator model 185 was used for this purpose. The membranes were located in the gradient by enzyme assay. The appropriate fractions were pooled, diluted with 5 volumes of distilled water, and collected by a 20-min centrifugation at $20000 \times g$ in a Sorvall SS-34 rotor.

Enzyme assays

Alkaline phosphatase activity was determined as the Mg^{2+}-dependent alkaline *p*-nitrophenylphosphatase activity as described by Perkarthy et al. [18]. Appropriate fractions to be assayed were incubated in 2 ml of a solution containing 10 mM Tris, 5 mM $MgCl_2$, and 10 mM *p*-nitrophenyl phosphate at pH 10. After 10 min the reaction was stopped by the addition of 0.2 vol. of 1 M K_2HPO_4, 0.25 M EDTA, pH 10. Absorbance was measured at 420 nm in a Bauch and Lomb model 710 spectrophotometer.

To assay alkaline phosphatase activity with intact cells the method of Parish and Pelli was used [19]. Cells were washed in 0.14 M NaCl and then placed into 5 ml of a solution containing 50 mM Tris-HCl, pH 8.5, 50 mM $MgCl_2$, and 1.25 mg of *p*-nitrophenyl phosphate. After incubation at 26°C for between 5 and 20 min a 0.9-ml aliquot was removed. The cells were sedimented in a clinical centrifuge, and 1.8 ml of 0.2 M Na_2CO_3 was added to the supernatant. The absorbance at 400 nm was measured.

The 5′-nucleotidase activity was determined as described by Evans [20]. The final concentration of the incubation mixture was 100 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, 10 mM potassium sodium tartrate, 5 mM AMP, and 5–50 μg of protein to be assayed. The volume of the incubation mixture was 0.5 ml. After 15 min at 26°C, the reaction was stopped with 50 μl of 20% (w/v) trichloroacetic acid. The inorganic phosphate released was measured by the method of Chen et al. [21].

The glucose-6-phosphatase activity was assayed as described by Swanson [22]. The enzyme activity was measured in 1 ml of a solution containing 100 μM sodium acetate, pH 6.2, 4 mM EDTA, 0.5 mM NaF, and 2 mM glucose 6-phosphate. After a 15-min incubation at room temperature the reaction was stopped by the addition of 250 μl of 50% (w/v) trichloroacetic acid. The inorganic phosphate released was assayed, according to the method of Chen et al. [21]. Since initial experiments showed that sucrose interfered with the enzyme assay, trace amounts of sucrose were removed by repeated suspension and centrifugation of the fraction in water.

Acid phosphatase was assayed as described by Barnett and Heath [23]. The reaction mixture contained 5 mM sodium acetate adjusted to pH 5.0 with acetic acid, 5 mM *p*-nitrophenyl phosphate. After 20 min at room temperature the reaction was stopped with 100 μl of a solution containing 1 M Tris, 1 M K_2HPO_4, and 0.25 M EDTA at pH

10.5. The absorbance was measured at 420 nm. Before assaying the fractions, they were washed with water to remove sucrose and twice frozen in liquid nitrogen and thawed to break the lysosomes.

In each of the above enzyme assays one unit of enzyme was defined as the amount that liberates 1 μM of product in 10 min under the conditions stated.

Cytochrome oxidase was assayed as described by Cooperstein and Lazrow [24]. In this reaction cytochrome *c* was reduced with a freshly prepared solution of sodium hydrosulfite. The final 1 ml reaction mixture contained 0.08 mM reduced cytochrome *c*, 60 mM phosphate buffer at pH 7.6 and the fraction to be assayed for cytochrome *c* activity. The rate of decrease in absorbance at 550 nm was determined. Enzyme activity was calculated as the decrease in the logarithm of the molar concentration of reduced cytochrome *c* per minute.

Radioiodination of cell surface proteins

The cell surface proteins were labelled using the lactoperoxidase-glucose oxidase method [25]. Exponentially growing cells were collected, washed twice in 0.14 M NaCl, 10 mM KNa_2PO_4 buffer at pH 7.2. About $3 \cdot 10^6$ cells were suspended in the above described phosphate-buffered saline containing 20 mM glucose. The cell surface proteins were labelled with carrier-free $Na^{125}I$. After a 2-min incubation at 0°C, the cells were washed twice with phosphate-buffered saline to remove enzyme and unreacted iodine.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide slab gels containing 15% acrylamide in the running gel and 4.5% acrylamide in the stacking gel were prepared according to the method of Laemmli [26]. The molecular weight markers used were bovine serum albumin (68 000), ovalbumin (45 000), trypsin soybean inhibitor (22 000), lysozyme (14 400) and cytochrome *c* (12 500). Total proteins were stained with Coomassie brilliant blue G-250. Glycoproteins were detected by the periodic acid Schiff method as described by Sergrest and Jackson [27]. For these experiments the gel was treated with 0.7% periodic acid in 5% acetic acid for 3 h. The gel was then transferred to a solution containing 0.2% sodium metabisulfite in 5% acetic acid for 3 h. Reaction with the Schiff's reagent was carried out overnight, in the dark at room temperature. The coloration was enhanced by washing the gel in 0.2% sodium metabisulfite in 5% acetic acid.

DNA, RNA, and protein determinations

DNA was measured by the diphenylamine method of Burton [28] and RNA by the orcinol method of Dische [29]. Proteins were estimated by either the method of Lowry et al. [30] or Bradford [31], using bovine serum albumin as a standard.

Electron microscopy

The membrane pellet was fixed for 90 min in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.2. The membranes were placed into a 20% (w/v) bovine serum albumin solution and fixed with the buffered glutaraldehyde for an additional 30 min. Post fixation was carried out in 1% osmic acid buffered with 0.1 M sodium cacodylate, pH 7.2. The pellet was rinsed in sodium cacodylate buffer, dehydrated in a graduated series of ethanol concentrations, and embedded in Spurr resin. The sections were cut with a diamond knife and double stained with lead citrate followed by uranyl acetate. The micrographs were a generous contribution of Dr. J.R. Colvin of the National Research Council of Canada.

Results and Discussion

Cell breakage

The purification procedure described in the preceding section involved the breakage of amoebae at 4°C in 10 mM Tris, 1 mM $ZnCl_2$ at pH 8.0, after an incubation period of 15 min at room temperature. The percent of cells broken increased with the number of homogenization strokes until a maximum of about 95% broken cells was obtained with 30 strokes of a mechanically driven Thomas homogenizer. We observed that the omission of the incubation period resulted in a substantially lower percentage of breakage (40%). We also noted that the cell breakage was highly pH dependent. At pH 7.2, for example, only 40% of the cells were broken (Fig. 1).

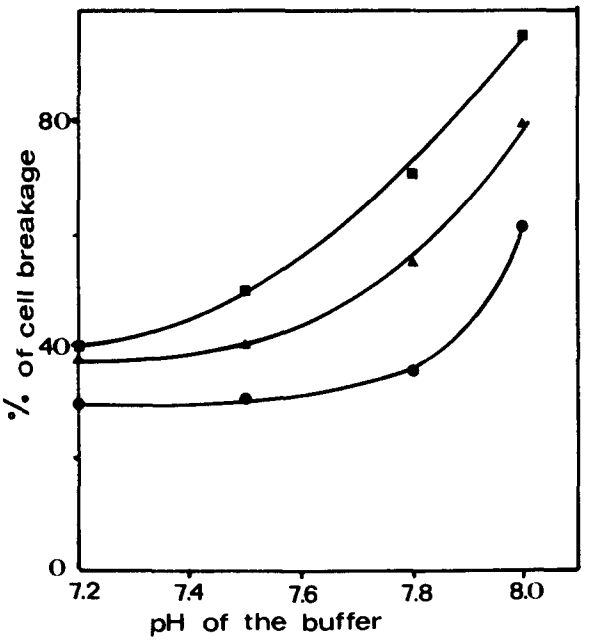

Fig. 1. Percent of amoebal cells broken as a function of the number of strokes of a mechanically driven Thomas tissue grinder and the pH of the homogenization medium. In four separate experiments $2.5 \cdot 10^7$ cell/ml were pre-incubated for 15 min at room temperature in 1 mM $ZnCl_2$, 10 mM Tris-HCl at either pH 7.2, 7.5, 7.8 or 8. In each experiment the cells were cooled to 4°C and then homogenized for three series of 10 strokes. After each series the percent of cell breakage was determined microscopically. ●———●, 10 strokes; ▲———▲, 20 strokes; ■———■, 30 strokes.

Plasma membrane enzymes

Before attempting to isolate the plasma membranes of *P. polycephalum* amoebae, we assayed cell homogenates for various enzyme markers generally used in plasma membrane studies with other organisms. The presence of $(Na^+ + K^+)$-dependent ATPase was tested, but the high background signal from the $(Na^+ + K^+)$-independent ATPase made the assay too imprecise to be useful. We next tested for the presence of alkaline phosphatase and 5′-nucleotidase and found that cell homogenates contained substantial activity for both enzymes. Recent histochemical data by Aldrich and Reiskind [32] showed that the 5′-nucleotidase is partly located at the cell surface of *P. polycephalum* amoebae and can therefore be used as a plasma membrane marker. To verify that alkaline phosphatase is also located at the cell surface, intact cells were assayed for this enzyme. The presence of significant activity with whole cells justified the use of alkaline phosphatase as a plasma membrane marker. In addition cells with iodine-labelled surface proteins were added to unlabelled cells and the mixture was used in a plasma membrane preparation. The maximum iodine labelling, alkaline phosphatase activity and 5′-nucleotidase activity fell in the same region of the sucrose gradient. This served as additional evidence that alkaline phosphatase and 5′-nucleotidase are useful plasma membrane marker enzymes in *P. polycephalum* amoebae.

Plasma membrane isolation

The two-phase system of Brunette and Till [33] has been used in several laboratories to isolate plasma membranes from the cellular slime mold *Dictyostelium discoideum* [34–37]. Our attempts to use this technique for the preparation of *P. polycephalum* amoebal plasma membranes were only partially successful. We did succeed in obtaining membranes that were enriched for alkaline phosphatase, but they were visibly contaminated with nuclei.

Since isopicnic sucrose gradient centrifugation has also been successfully used to prepare plasma membranes from *D. discoideum* [38–42] as well as from other cell types [20], we applied this technique to *P. polycephalum* amoebae. As a first step in the purification, the plasma membranes in the cell homogenate were collected by centrifugation at $20\,000 \times g$ for 15 min. The resulting pellet contained more than 95% of the total alkaline phosphatase activity and about half of the cell proteins. The specificity activity of the alkaline phosphatase was approximately 2-fold higher than that of the cell homogenate, indicating that the plasma membranes were partially purified at this step. The pellet of concentrated plasma membranes was then vigorously suspended in 10% sucrose with five strokes of a mechanically driven Thomas homogenizer. This step is important for the preparation of plasma membranes. Well suspended membranes fall in a narrow band with a average density of 1.16 g/cm^3 after sucrose gradient centrifugation (Fig. 2), while incompletely suspended membranes gave a broader distribution with an average density of 1.2 g/cm^3 (results not shown).

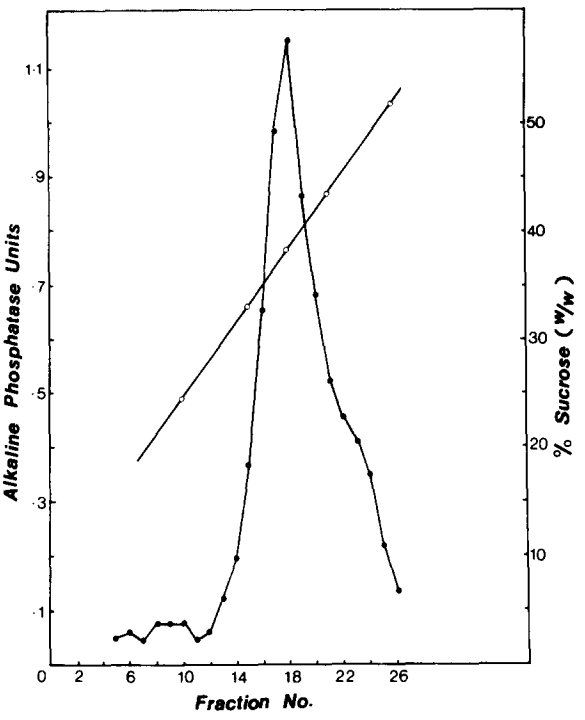

Fig. 2. Sucrose gradient profile of *P. polycephalum* amoebal plasma membranes. Centrifugation was carried out in a Beckman SW27 rotor during 2 h at 26000 rpm. Fractions of 1.5 ml were collected and assayed for alkaline phosphatase activity (●——●). The percent of sucrose in the fraction was also determined (○——○).

The distribution of the alkaline phosphatase activity across the sucrose gradient in a typical plasma membrane preparation is shown in Fig. 2. The peak activity is found at 38% (w/w) sucrose. The area indicated in Fig. 2 contained 20% of the total alkaline phosphatase activity and 2% of the total cell proteins. Very similar results were obtained with 5′-nucleotidase.

Plasma membrane purity

The sucrose gradient fractions containing the highest alkaline phosphatase activity were collected and analysed (see Fig. 2). The increase in the specific activity of alkaline phosphatase and 5′-nucleotidase in the membrane fraction indicated that the plasma membranes were purified about 13-fold (Table I). Contamination by mitochondria and lysosomes was minimal as estimated by measuring cytochrome oxidase and acid phosphatase activity, respectively. Very little DNA or RNA was detected, indicating low levels of contaminating nuclei and ribosomes. There was a positive enrichment for glucose-6-phosphatase, an enzyme typically associated with the endoplasmic reticulum (Table I). However, the amount of this enzyme associated with the plasma membranes was minimal as we found that only 6% of the total glucose-6-phosphatase activity in the plasma membrane fraction. A similar result was found for *D. discoideum* where 2.6% of the total glucose-6-phosphatase activity was recovered in the plasma membrane fraction [39].

TABLE I

PURITY OF AMOEBAL PLASMA MEMBRANE FRACTION PURIFIED BY SUCROSE GRADIENT CENTRIFUGATION

The specific activities were calculated as units assayed per mg of fraction protein. The enrichments were determined by dividing the specific activities of the fraction by that of the homogenate. The result for alkaline phosphatase is the average of 12 different experiments. All other experiments were performed at least twice.

Assay	Enrichment relative to homogenate
DNA (nucleus)	0.23
RNA	0.05
Cytochrome oxidase (mitochondria)	0.005
Acid phosphatase (lysosomes)	0.63
Glucose-6-phosphatase (endoplasmic reticulum)	3.9
Alkaline phosphatase (membranes)	12.6
5′-Nucleotidase (membranes)	12.5

Under the electron microscope the membranes appeared as vesicles. At high magnification the typical trilaminar plasma membrane structure was visible. There were no recognizable mitochondria, lysosomes, nuclei or rough endoplasmic reticulum (Fig. 3). The plasma membranes of *P. polycephalum*, as judged by enzyme assays and microscopic examination were comparable in purity to those of the cellular slime mold *D. discoideum* [34–42].

Gel electrophoresis

Total amoebal proteins and plasma membrane proteins were separated by electrophoresis on

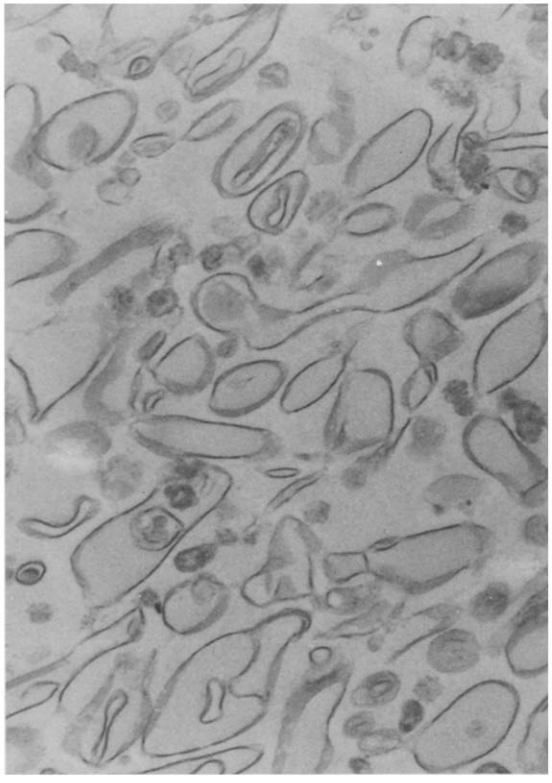

Fig. 3. Electron micrograph of sucrose gradient isolated amoebal plasma membranes (10000×). The micrograph is the courtesy of J.R. Colvin.

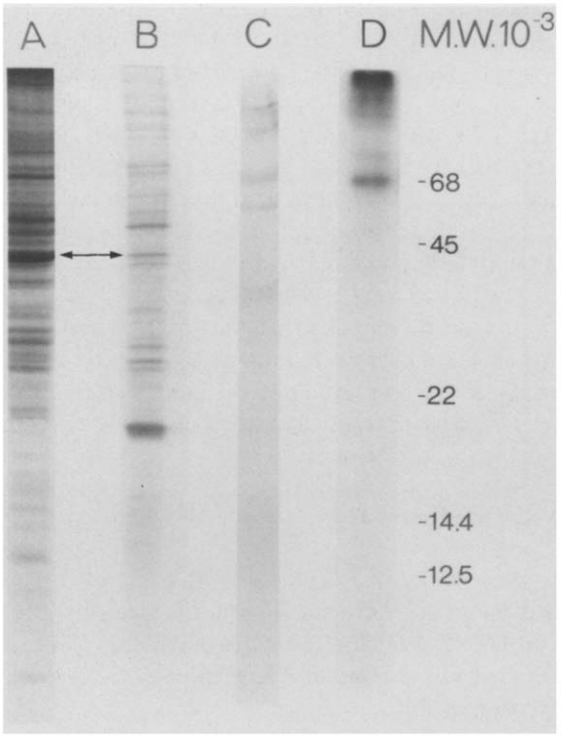

Fig. 4. Analysis of amoebal total cell and plasma membrane proteins by electrophoresis on 15% polyacrylamide SDS slab gels. (A) Total cell proteins. (B) Plasma membrane proteins. (C) Plasma membrane glycoproteins. (D) Autoradiogram of ^{125}I-labelled cell surface proteins. The (↔) indicates the bands that comigrate with rabbit actin.

sodium dodecyl sulphate acrylamide slab gels and were stained with Coomassie blue. The protein profiles of these two preparations were distinctly different, with each having its own characteristic major bands (Figs. 4A and 4B). Some prominent bands in the plasma membrane profile were only minor components of the total proteins, while some of the major total cell proteins were undetectable or present as minor components in membranes. As expected, the pattern from the total amoebal proteins was more complex than that of plasma membranes.

About 10 major and 20 minor bands were visible in the plasma membrane profile. The most prominent series of bands had an apparent molecular weight of about 20000. In electrophoresis on polyacrylamide gradients this complex was seen to contain three bands (results not shown). A band at 43000 comigrated with rabbit muscle and *P. polycephalum* actin. The presence of actin associated with plasma membranes has been reported for *D. discoideum* [39,40] for other types [43–51].

A typical SDS-polyacrylamide electrophoretic gel of plasma membrane proteins was stained with the periodic acid Schiff's technique to reveal the glycoproteins (Fig. 4C). With this technique five major and six minor bands, having apparent molecular weights greater than 33000 were seen. Nine of the eleven glycoproteins had apparent molecular weights greater than 72000. From these results it is apparent that the glycoproteins represent a subset of high molecular weight plasma membrane proteins.

Proteins at the surface of the plasma membrane can be selectively labelled by the lactoperoxidase-glucose oxidase radioiodination method. When the

surface proteins of exponentially growing amoebae were labelled and separated by SDS-polyacrylamide gel electrophoresis, six bands having molecular weights greater than 68 000 were seen (Fig. 4D). Like the glycoproteins, the cell surface proteins represent a minority class of apparent high molecular weight plasma membrane proteins.

These electrophoretic studies have permitted us to analyse the plasma membrane proteins of the genetically characterized Cld-Axe amoebal strain. A comparative analysis of the plasma membrane proteins of amoebal strains with different genetic backgrounds (for example, different *mat*B alleles) might give an insight to the biochemical basis for cell fusion in this organism.

Acknowledgements

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