

## Isolation and Characterization of a Cycloheximide-Resistant Mutant of *Acanthamoeba castellanii* Neff

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A cycloheximide-resistant mutant was isolated from the amoeba *Acanthamoeba castellanii* Neff. Drug resistance was found to be due to a ribosomal modification.

*Acanthamoeba castellanii* Neff is a small free-living soil amoeba isolated in 1957 by R. J. Neff (5). It has been reported to be sensitive to a number of drugs including cycloheximide. Cycloheximide-resistant mutants have been isolated in several other organisms (3, 4, 7, 8) and have been used to study the genetics of these organisms. Since there are no reported genetic analyses of *A. castellanii*, cycloheximide resistance was selected as a possible starting point for the investigation of the genetics of this organism. A positive growth selection was employed after mutagenesis, and a resulting resistant mutant was then characterized by both in vivo and in vitro studies.

Cultures of *A. castellanii* were grown in 250-ml Falcon plastic tissue culture flasks with Neff growth medium (6). Stock cultures (20 ml) were transferred by thousand-fold dilutions at 5- to 7-day intervals; cells for experimentation were grown in 10-ml cultures with an initial density of  $2 \times 10^4$  to  $4 \times 10^4$  cells/ml and were harvested at  $9 \times 10^5$  to  $10 \times 10^5$  cells/ml. All cultures were grown at room temperature (22 to 25°C).

Cells were mutagenized in 50-ml tubes containing the following:  $2 \times 10^7$  cloned wild-type cells, 30 ml of 6.65 mM phosphate buffer (pH 6.0) with 0.85% NaCl (phosphate-buffered saline), 10 ml of glass-distilled water, and 2 mg of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, for 30 min at room temperature. To obtain cycloheximide resistance mutations, 50 µg of mutagen per ml was used, which gave a survival of 8 to 10% of the cells. The mutagenized cells were collected by centrifugation (3 min at  $400 \times g$ ) and suspended in 140 ml of Neff growth medium. The resulting cell suspension was dispensed into seven 250-ml culture flasks and incubated for 6 days at room temperature (seven to eight generations) before applying selective pressure to the culture. Cells were collected by centrifugation (3 min at  $400 \times g$ ), resuspended at a concentration of  $5 \times 10^5$  cells/ml, and dispensed

into 25-ml culture flasks (4 ml per flask). Cycloheximide was added at a final concentration of 100 µg/ml, and the flasks were incubated at room temperature. When exposed to 100 µg of cycloheximide per ml, the growth of wild-type cells is completely inhibited. After 24 h of exposure to this drug dosage, about 50 to 70% of the cells had undergone a morphological change from the normal amoeboid shape to a rounded form that often detached from the flask surface. By 48 h, all the cells had rounded up, and all but a few were detached from the flask. Cytoplasmic protrusions in the rounded cells appeared to become lysis sites, and over a period of 2 to 3 weeks cell destruction ensued. After 2 weeks, removal of the cycloheximide and resuspension in fresh Neff growth medium did not rescue any of the cells.

These effects of cycloheximide on wild-type cells provided the basis of a selection method for resistance mutants. After 17 days of exposure to 100 µg of cycloheximide per ml, only resistant cells were present as growing amoeboid cells. Due to the nature of the selection procedure, cultures of resistant cells could not be assumed to be independent. Thus, pure cultures of the isolated resistant cells were obtained by cloning. Cloning was done by harvesting 2 to 3 ml of a culture in a log phase ( $10^4$  to  $100 \times 10^4$  amoebas per ml) and diluting it to a titer of 1 cell/ml, with cycloheximide present at 7.5 µg/ml. Samples (100 µl) of the suspension were placed in microtiter wells (Falcon plate no. 3040). The plate was then sealed and incubated at room temperature. Five to 10 days later, individual clones were removed and transferred to 250-ml culture flasks. This procedure resulted in clones being recovered from 95 to 100% of the original cells. One clone (Cyc R-1) was selected for further characterization. Before characterization the mutant was cultured for at least 12 generations without cycloheximide present. Upon reexposure of the cells to the drug at 100 µg/ml, few

if any of the cells failed to grow.

The first step of characterization was to assay the effect of cycloheximide on *in vivo* protein synthesis of cloned Cyc R-1 cells compared to cloned F 11 wild-type cells. Cells were collected from 10-ml cultures with cell densities of  $0.75 \times 10^6$  to  $1.0 \times 10^6$ /ml. After centrifugation (3 min at  $400 \times g$ ), the cells were suspended in defined medium DM 3 of Adams (1, 2) with 0.1 mM L-methionine, at  $2.3 \times 10^6$  cells/ml, and incubated for 1.5 h at room temperature. After incubation, 0.9-ml samples of cell suspension were added to tubes containing cycloheximide (final concentration ranging from 0 to 400  $\mu\text{g}/\text{ml}$ ) in 0.1 ml of water, plus 50  $\mu\text{l}$  of L- $^{35}\text{S}$ ]methionine (1,245 Ci/mmol, 23.5  $\mu\text{Ci}/\text{ml}$ ). The reaction mixture was incubated at 24°C for 30 min, and incorporation was then terminated by chilling to 4°C in an ice-water bath. After centrifugation at 4°C, the cell pellets were dissolved by suspension in 1 ml of 0.9 M NaOH and incubated for 15 min at 37°C. A 2-ml volume of ice-cold 20% trichloroacetic acid was added, followed by incubation for 10 min at 0°C. Precipitates were collected on GF/A filters and washed three times with cold 5% trichloroacetic acid (Fig. 1). Fifty percent inhibition of L- $^{35}\text{S}$ ]methionine incorporation was found at 11  $\mu\text{g}$  of cycloheximide per ml for the F 11 wild type and 51  $\mu\text{g}/\text{ml}$  for the Cyc R-1 mutant.

*In vitro* protein synthesis was assayed for the two cell types, to distinguish whether the cycloheximide resistance was due to a defect in the protein synthetic apparatus or a change in membrane permeability. Approximately  $7 \times 10^7$  cells were harvested from cultures with densities between  $0.8 \times 10^6$  and  $1.0 \times 10^6$  cells/ml and were washed three times with 30 ml of ice-cold phosphate-buffered saline (pH 6.9). The pellet was then suspended in 2 ml of ice-cold TKM buffer plus  $\beta$ -mercaptoethanol (0.01 M KCl, 0.01 M Tris [pH 7.6], 0.0015 M  $\text{MgCl}_2$ , and 2.8 mM  $\beta$ -mercaptoethanol) and allowed to swell for 10 min before being homogenized with 20 strokes of a Dounce homogenizer. After homogenization, 0.2 ml of ice-cold buffer P (1.2 M KCl, 50 mM magnesium acetate, 200 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid [pH 7.2], 50 mM  $\beta$ -mercaptoethanol) was added, and the crude homogenate was centrifuged for 10 min at  $10,000 \times g$ . The supernatant was decanted from the pellet and stored in an ice-water bath until assayed. The assay mixture (kept at 0°C) consisted of: 1.8 ml of supernatant; 0.21 ml of ATP-GTP solution (15 mM ATP, 2.8 mM GTP); 0.19 ml of energy regenerating solution (25.4 mg of creatine phosphate and 2 mg of creatine kinase per ml in TKM); 0.18 ml of amino acid solution

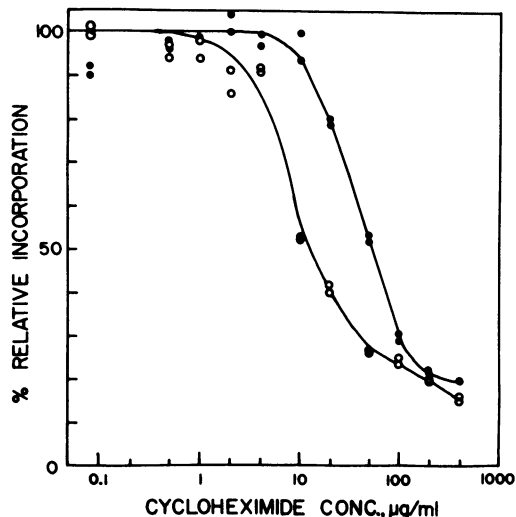


FIG. 1. *In vivo* cycloheximide inhibition of L- $^{35}\text{S}$ ]methionine incorporation into acid-precipitable material. Details of the experiment are in the text. Incubation time with the label was 30 min; incorporation was linear over this time interval and was insignificant without incubation. The results are plotted as percent of incorporation relative to control samples without cycloheximide present. The control sample had  $1.2 \times 10^4$  to  $1.5 \times 10^4$  precipitable cpm. Symbols:  $\circ$ , F 11 wild type;  $\bullet$ , Cyc R-1 resistant mutant.

(1.08 mM alanine, 0.26 mM arginine, 1.08 mM asparagine, 1.52 mM aspartate, 0.36 mM cysteine, 2.85 mM glycine, 0.97 mM glutamate, 1.03 mM glutamine, 2.50 mM histidine, 4.38 mM isoleucine, 0.95 mM lysine, 0.18 mM methionine, 0.84 mM phenylalanine, 0.74 mM proline, 0.91 mM serine, 0.90 mM threonine, 0.16 mM tryptophan, 0.44 mM tyrosine, and 1.65 mM valine); and 65  $\mu\text{l}$  of L- $^{14}\text{C}$ ]leucine (324 mCi/mmol, 50  $\mu\text{Ci}/\text{ml}$ ). Samples (80  $\mu\text{l}$ ) of the assay mixture were placed into tubes containing cycloheximide (final concentration ranging from 0.02 to 20  $\mu\text{g}/\text{ml}$ ) in 20  $\mu\text{l}$  of distilled water. Tubes were incubated for 30 min at 24°C; the reaction was terminated by addition of 50  $\mu\text{l}$  of 0.9 M NaOH containing nonradioactive leucine at 1 mg/ml. After incubation for 10 min at 37°C, to discharge all tRNA molecules, precipitation with trichloroacetic acid was performed as described above. Figure 2 shows that the concentration of cycloheximide giving 50% inhibition of L- $^{14}\text{C}$ ]leucine incorporation differs for the two cell types; 0.12  $\mu\text{g}/\text{ml}$  for the F 11 cells and 2.1  $\mu\text{g}/\text{ml}$  for the Cyc R-1 cells.

These results indicate that the modification in the resistant mutant is cytoplasmic, affecting either a soluble factor involved in protein syn-

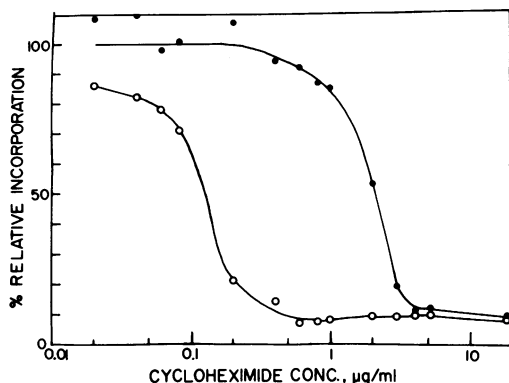


FIG. 2. *In vitro* cycloheximide inhibition of L-[<sup>14</sup>C]leucine incorporation into acid-precipitable material. Details of the experiment are in the text. Incubation time with the label was 30 min; incorporation was linear over this time interval and was insignificant without incubation. The results are plotted as percent of incorporation relative to control samples without cycloheximide present. The control samples had  $8 \times 10^3$  to  $10 \times 10^3$  precipitable cpm. Each point represents the average of duplicate samples which agreed with  $\pm 10\%$ . Symbols:  $\circ$ , F 11 wild type;  $\bullet$ , Cyc R-1 resistant mutant.

thesis or the ribosomes. To distinguish between these two possibilities, the cell-free extract was fractionated, reconstituted, and assayed for L-[<sup>14</sup>C]leucine incorporation. Approximately  $7.5 \times 10^7$  cells were collected from cultures with densities between  $0.80 \times 10^6$  and  $0.90 \times 10^6$  cells/ml, and cytoplasmic extracts were prepared as described above. This extract was then centrifuged for 90 min at  $300,000 \times g$ . The top two-thirds of the supernatant was removed and designated as high-speed cytoplasmic fraction; the last third was discarded. The high-speed pellet was washed with 0.3 ml of cold TKM buffer and suspended in 1.5 ml of the same buffer (10 ml of TKM buffer plus 2.8 mM  $\beta$ -mercaptoethanol), using a small Dounce homogenizer. Separate assay mixtures were made up with the four different possible combinations of high-speed supernatants and pellet fractions from the mutant and wild-type cells. Composition of the assay mixtures were (solutions as before): 0.5 ml of high-speed supernatant fraction; 0.5 ml of pellets (22.4 absorbance units at 260 nm per mixture); 0.12 ml of ATP-GTP solution; 0.11 ml of energy regenerating solution; 0.1 ml of amino acid solution; and 36  $\mu$ l of L-[<sup>14</sup>C]leucine. Eighty-microliter volumes of the assay mixtures were placed into tubes containing cycloheximide (final concentration, 1.0  $\mu$ g/ml) in 20  $\mu$ l of water. This concentration of cycloheximide (Fig. 2) allows over 80% *in vitro* incorporation for the

unfractionated Cyc R-1 extract but less than 10% incorporation for the unfractionated F 11 extract. The samples were incubated for 30 min at 24°C before incorporation was terminated by addition of 50  $\mu$ l of 0.9 M NaOH containing nonradioactive leucine at 1 mg/ml (Table 1). F 11-F 11 and Cyc R-1-F 11 cytoplasmic supernatant-pellet combinations gave approximately the same incorporation, which is much less than that for F 11-Cyc R-1 and Cyc R-1-Cyc R-1 combinations. These results show the modification of Cyc R-1 is associated with the washed pellet fraction, indicating that the modified element is most likely a ribosomal component.

This *A. castellanii* mutant joins a number of other characterized cycloheximide-resistant mutants of other organisms, including *Saccharomyces* (3), *Physarum* (4), *Dictyostelium* (8), and Chinese hamster ovary cells (7). At least one mutant of each of these organisms has been shown to have modified ribosomes, with the exception of *Dictyostelium* mutants which have not been biochemically characterized. Cycloheximide resistance mutations modifying ribosomes in *Saccharomyces* (3) and *Physarum* (4) have been shown to be recessive. In yeast, this modification is located in the 60S ribosomal subunit (3). The Cyc R-1 *A. castellanii* cells were four-fold more resistant to cycloheximide than the wild-type amoeba. This increase in resistance has been shown by our data to be due most likely to a ribosomal modification, although the exact site of the modification is not known. The significant difference in sensitivity to the drug we observed in *in vivo* versus *in vitro* translation assays may indicate that Cyc R-1 actually is a double mutant, with the other mutation affecting permeability to the drug. However, differences of this type are commonly observed with other eucaryotic translational systems and may simply reflect intrinsic differences between the

TABLE 1. L-[<sup>14</sup>C]leucine incorporation into acid-precipitable material by recombined polyribosome and high-speed supernatant fractions

High-speed supernatant source <sup>a</sup>	Pellet source	Incorporation <sup>b</sup>		Inhibition (%)
		- Cycloheximide (cpm)	+ Cycloheximide (cpm)	
WT	WT	713	281	61
WT	Cyc R-1	620	404	35
Cyc R-1	WT	870	263	70
Cyc R-1	Cyc R-1	841	482	43

<sup>a</sup> WT, Wild type F 11; Cyc R-1, cycloheximide-resistant cells.

<sup>b</sup> Averages from duplicate incorporation assays. Cycloheximide, when present, was at 1  $\mu$ g/ml.

conditions in which ribosomes are functioning in the two instances.

The Cyc R-1 mutant, which is the first reported for *A. castellanii*, opens the prospect of further mutants being isolated. This organism has already been used for a number of biochemical studies and is easy to culture and manipulate. The development of a genetic analysis would enhance its utility for further research.

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