Mitochondrial Structure Studied by High Voltage Electron Microscopy of Thick Sections of *Candida utilis*

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SUMMARY

Mitochondrial structure in yeast cells under various physiological conditions has been studied by high voltage electron microscopy of sections that are 0.5 to 2.0 μm thick. Such thick sections of the yeast *Candida utilis* had a small number of long, branched tubular mitochondria per cell. The mitochondria extended into cell buds and unseparated daughter cells. It was apparent from parallel studies with thin sections that most of the rounded mitochondrial profiles viewed in thin sections should not be interpreted as being numerous small individual mitochondria. Attempts to study thick sections of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were frustrated by poor contrast.

INTRODUCTION

Copper deficiency in mice results in the appearance of giant mitochondria in the liver (Suzuki, 1969; Dallman & Goodman, 1970). Electron microscopy of thin (about 75 nm) sections of *Candida utilis* apparently showed, in addition, that copper deficiency caused replacement of numerous rounded mitochondria by a smaller number of much enlarged mitochondria, or giant mitochondria (Davison, Downie & Garland, 1972; Keyhani, 1973). However, yeast mitochondria can be tubular (Yotsuyanagi, 1955; Kawakami, 1961), and tubular mitochondria repeatedly crossing the plane of a thin section would appear as numerous individual mitochondrial profiles. Furthermore, if these tubular mitochondria then swelled, their appearance in thin section would reveal fewer but much larger mitochondrial profiles. To settle whether the typical mitochondrial morphology usually deduced from thin sections is in error, two techniques are available. One is serial sectioning and reconstruction by model building, and was used to demonstrate mitochondrial morphology in yeasts by Keddie & Barajas (1969), who reported that there were only one to four large branched mitochondria per cell. The other technique is high voltage electron microscopy (h.v.e.m.) of thick sections. We have used this latter method for studying the mitochondrial morphology of the yeast *C. utilis*. It is concluded that inspection of single thin sections leads to quite erroneous interpretations of mitochondrial size and number. Examination of thick sections by h.v.e.m. provides a simpler and faster method of inspecting mitochondrial morphology than does serial sectioning, and permits many samples to be studied. We describe our experience with this technique. A preliminary report has been published (Davison & Garland, 1974). We also describe the effects of copper and other nutrient limitations on the electron microscopic appearance of *C. utilis*.

METHODS

Candida utilis strain 193, from the National Collection of Yeast Cultures, Nutfield, Surrey, was grown in continuous culture with glycerol limitation as described by Light & Garland (1971), or with sulphate limitation (Haddock & Garland, 1971), or with copper limitation (Downie & Garland, 1973). The dilution rate of the chemostat was 0.2 h^{-1} unless otherwise stated. For some experiments batch culture was used; the medium was 500 ml of the defined medium described by Light & Garland (1971) for glycerol-limited continuous culture. Cells were harvested by centrifugation and washed twice with distilled water. Sphaeroplasts, used for histochemical staining for cytochrome oxidase, were prepared by the use of snail gut enzyme (Light & Garland, 1971) and washed twice in 0.22 M -sucrose- 0.1 M -potassium phosphate buffer (phosphate-sucrose buffer), pH 7.4.

Fixation of sphaeroplasts for electron microscopy was effected by incubation for 2 h in 2.5% (v/v) glutaraldehyde in phosphate-sucrose buffer pH 7.4 at 4°C , followed by two washes in the phosphate-sucrose buffer to remove glutaraldehyde. Treatment of sphaeroplasts for visualization of cytochrome oxidase by the 3,3'-diaminobenzidine method of Seligman *et al.* (1968) was as follows. Sphaeroplasts from 0.4 g wet wt cells were incubated for 20 to 45 min at room temperature in 7.0 ml of phosphate-sucrose buffer containing 3,3'-diaminobenzidine (0.5 mg/ml) and H_2O_2 (30 mM). A control incubation was set up by adding the sphaeroplasts to 7.0 ml of phosphate-sucrose buffer containing 1 mM -KCN, adding H_2O_2 and diaminobenzidine after 10 min, and continuing the incubation for a further 20 to 45 min. The sphaeroplasts from both test and control incubations were then washed three times in phosphate-sucrose buffer at 4°C , followed by fixation in 1% (w/v) osmium tetroxide in phosphate-sucrose buffer at 4°C . The fixed sphaeroplasts were then washed twice in phosphate-sucrose buffer, dehydrated with an ethanol/water series, and embedded in Spurr's (1969) resin.

Preparation of yeast cells for lead-citrate staining was carried out as follows. Cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M -sodium cacodylate buffer pH 7.0 at 4°C for 2 h. The cells were then placed in freshly made 2% (w/v) potassium permanganate solution at 4°C for 2 h, followed by a further 2 h in a fresh solution at room temperature. Next, the cells were washed at least five times with distilled water and placed in a solution containing 1% (w/v) potassium dichromate and 1% (w/v) uranyl acetate for 2 h at 4°C . The cells were then washed several times with distilled water, embedded in agar, cut into small blocks, dehydrated with ethanol and embedded in Spurr's resin.

Sections 0.5 to $2.0 \mu\text{m}$ thick (i.e. thick sections) were cut with glass knives using a Reichert OM U3 ultramicrotome. To avoid lateral tears (Favard & Carasso, 1973) which obscure detail when the sections are viewed, two or three sections about $70 \mu\text{m}$ thick (i.e. thin sections) were interposed between thick sections. Sections were stretched by heat, using a modified battery-operated gas lighter in preference to chloroform vapour which caused the thicker sections to curl. To prevent the thick sections from falling off the grids during staining and washing procedures, and during viewing, it was necessary first to flame the grids until clean and dip them in a solution of Sellotape adhesive in chloroform; alternatively, sections could be mounted on collodion-coated copper grids. Sections were stained by immersion in freshly prepared lead citrate solution (Reynolds, 1963) diluted approximately 30-fold in freshly prepared 0.01 M -NaOH, and afterwards washed by dipping in 0.05% (v/v) nitric acid and then in several changes of distilled water.

Thin sections were viewed with an AEI 801 electron microscope. Thick sections were viewed with an AEI EM7 high voltage electron microscope at the Department of Metallurgy and Materials Science, Imperial College, London.

RESULTS

Electron microscopy of thin sections of C. utilis grown in continuous culture under a variety of growth limiting conditions

Figures 1 to 5 show electron micrographs of thin sections of *C. utilis* cells grown in continuous culture where the growth-limiting nutrient was glycerol (Fig. 1), iron (Fig. 2), sulphate (Figs. 3 and 4) and copper (Fig. 5). These Figures may be used as points of reference for comparison with thick sections. They illustrate the cell and mitochondrial morphologies resulting from growth conditions that produce cells with well-documented alterations in the mitochondrial respiratory chain and associated energy conservation mechanisms (Garland, 1970; Light & Garland, 1971; Haddock & Garland, 1971; Light, 1972; Keyhani & Chance, 1971; Downie & Garland, 1973).

The structure of glycerol-limited *C. utilis* shown by electron microscopy of thin sections stained with lead citrate (Fig. 1) shows a familiar picture for aerobically grown yeasts, there being easily-discernible mitochondrial profiles that are mainly round or oval and about 0.1 to 0.3 μm in diameter. Usually there are 6 to 10 such mitochondrial profiles per section. Neither iron limitation (Fig. 2) nor sulphate limitation (Fig. 3) caused any noticeable change in mitochondrial structure. Iron-limited cells showed vacuoles and thick walls, presumably due to excess carbon being available. Sulphate-limited cells showed similar changes, although thickening of the cell wall was less marked than in iron-limited cells. Aeration of sulphate-limited cells for several hours, a treatment that restores site 1 energy conservation (Haddock & Garland, 1971), did not significantly alter cellular or mitochondrial structure as shown by electron microscopy of thin sections. However, if sulphate-limited cells were aerated in 1 mM-sulphate, a treatment that results in the appearance of a cyanide-insensitive respiratory chain (Haddock & Garland, 1971), the cells also exhibited altered mitochondrial morphology. This is shown in Fig. 4, where it is seen that the mitochondrial profiles, which are larger but fewer per cell, are well-filled with cristae in contrast to the relative scarcity of cristae in the mitochondria of glycerol-limited cells (Fig. 1).

Growth of either wild-type *C. utilis* or the copper-independent variant in copper-deficient media resulted in cells where mitochondrial profiles became irregular in outline, enlarged and fewer per cell (Fig. 5), as described by Davison *et al.* (1972) and Keyhani (1973).

Electron microscopy of thick sections of C. utilis stained with lead citrate

For Figs. 6, 7 and 8, photograph pairs were taken at $\pm 4^\circ$ tilt and should be examined with the aid of a stereoscopic viewer. An oval-shaped *C. utilis* cell is about 3 to 4 μm long and 1 to 2 μm in diameter, and thus except for cell buds even the thickest sections (2 μm) are unlikely to include a whole unsectioned cell. Nevertheless, there is sufficient depth in the sections to permit considerable overlap of subcellular organelles. Contrast in thick sections is affected not only by the amount and nature of overlaying material, but also in a more specific manner by cylindrical or tubular objects (see below) such as mitochondria. A tubular mitochondrion running vertically through the section would be expected to show enhanced contrast of its membranes, presenting a well-marked profile resembling those seen in thin sections, whereas a tubular mitochondrion lying with its long axis parallel to the plane of the section would be relatively less well contrasted.

Figure 6 shows stereo-pair photographs of a 1 μm -thick section of chemostat-grown, glycerol-limited *C. utilis* stained with lead citrate. The cell is budding, and a tubular mitochondrion about 4 to 5 μm long is seen extending from the parent cell through a ring-shaped

Fig. 1. Thin section of chemostat-cultured glycerol-limited *C. utilis* cells, stained with lead citrate, showing several mitochondrial profiles (M) per cell.

Fig. 2. Thin section of chemostat-cultured iron-limited *C. utilis* cells, stained with lead citrate, showing mitochondrial (M) and thickened walls (W).

exit in the cell wall into the daughter cell. There is insufficient detail in the structure in the lower part of the cell (i.e. nearer the bottom of the photograph) to decide whether they are mitochondria or not. However, it seems likely that more than one tubular mitochondrial structure is extruding into the cell bud, and one of these terminates in a distinct mitochondrial profile, suggesting that it is turned out of the plane of the section. Long tubular mitochondria were present in all cells examined, and all cells had some branched tubes. Figure 7 shows a 1 μm -thick section of a *C. utilis* cell from the exponential growth phase of a batch culture in glycerol medium. The cell is budding, and not more than two long mitochondrial tubes can be identified in the parent cell; one tube runs around the top-right periphery of the cell and turns out of the section at the top, whereas the other is branched, and two of the branches appear to join up again to make a ring.

The identification of mitochondria in lead citrate-stained thick sections was made on the expectation that there would be cytoplasmic structures of suitable dimensions to account for the structures seen in the better-documented thin sections. Further evidence that these tubular structures are indeed mitochondria came from the presence of what appeared to be cristae seen running in the long axis of the tubular mitochondria, and showing themselves in a more familiar view when the mitochondrial tube turned to leave the section and gave a typical mitochondrial profile as seen in thin section. We were unable to obtain satisfactory contrast with thick sections of iron- or sulphate-limited cells, because of interference by vacuoles.

*Electron microscopy of thick sections of sphaeoplasts of C. utilis
stained by the diaminobenzidine method*

3,3'-Diaminobenzidine is oxidized by cytochrome *c* and cytochrome *c* oxidase (EC. 1.9.3.1) to yield an osmiophilic deposit on the outer surface of the inner mitochondrial membrane (Seligman *et al.* 1968). Although diaminobenzidine can be used for a histochemical localization of other haemoproteins, such as peroxidase (EC. 1.11.1.7) and catalase (EC. 1.11.1.6), Keyhani (1972) has shown by the use of normal and copper-deficient *C. utilis* that cytochrome *c* oxidase is responsible for the staining of the inner mitochondrial membrane. We have therefore used this histochemical technique because of the advantages offered by its specificity and, particularly important for thick sections, its high contrast. Figure 8 shows a stereo-pair of photographs of a 2 μm -thick section of a sphaeroplast of *C. utilis* taken from the stationary phase of a batch culture in glycerol medium. A single long tubular mitochondrion, looped back on to itself, is clearly indentifiable. The cristae are preferentially stained, and run along the length of the tube. There are also several uniformly stained round structures which show no evidence of cristal structure; they are presumably microbodies or peroxysomes (Osumi *et al.* 1974). Figure 9 shows a thick section of a *C. utilis* sphaeroplast from a glycerol-limited chemostat culture. There were probably three or four separate stretches of mitochondrial tube in this section, though it is possible that they joined with each other outside the section.

Electron microscopy of thick sections of copper-deficient C. utilis cells

Figure 10 shows the structure of the copper-independent variant of *C. utilis* grown in copper-deficient glycerol-limited continuous culture. Despite the poor contrast, the mitochondrial structures have highly irregular outlines.

Figs. 3-5. For legend see opposite page.

The effect of growth conditions on mitochondrial morphology

Examination of thick sections showed that branched tubular mitochondria were characteristic of *C. utilis* grown in glycerol-limited continuous culture at dilution rates varying from 0.2 to 0.6 h⁻¹, or grown in batch culture in glycerol medium and sampled during exponential growth or stationary phases. Variations of morphology related to growth rate or growth phase (Yotsuyanagi, 1955) were not seen.

The number of mitochondrial profiles per cell in thick section

If the several rounded mitochondrial profiles seen in thin section were indeed separate mitochondria, then the number of separate mitochondrial structures observed in thick section should increase over that in thin section. Conversely, if the several profiles seen in thin section were parts of one or two longer branched mitochondria, then the number of separate mitochondrial structures in thick section should decrease. The second of these two possibilities is favoured by the thick sections shown in Figs. 6 to 9, and it is not difficult to envisage how a thin section across such cells could show mitochondrial profiles that are sections across the branched tubular mitochondria. Occasionally, a thin section might by chance run down the long axis of a tubular mitochondrion, and an example of this is shown in Fig. 11. However, we have also seen in thick sections the opposite situation, of numerous small separate mitochondrial structures (Fig. 12). It is conceivable that these mitochondrial structures (Fig. 12) communicated in the cell outside the section. Alternatively they may indeed represent separate mitochondria, perhaps related to a specific stage of the cell cycle. We are exploring this latter possibility by serial section of *C. utilis* at different cell cycle stages.

Scanning transmission electron microscopy of thick sections of C. utilis

We have made a limited study of the use of scanning transmission electron microscopy of thick sections of *C. utilis*. Figure 13 shows the result with a 0.5 µm thick section of a *C. utilis* sphaeroplast from the same culture as that shown in Fig. 8, and stained by the diamino-benzidine procedure. A single long tubular mitochondrion is seen, about 6 µm long. Clearly a thin section in a different plane would show three round mitochondrial profiles that in fact were all sections through the same tubular structure. The contrast in this 0.5 µm-thick section taken at an accelerating voltage of 100 kV was very similar to that obtained by high voltage electron microscopy at 400 to 500 kV with a 2 µm-thick section (Fig. 8). The scanning transmission technique did not give as satisfactory stereoscopic viewing as did the high voltage technique. This may be due in part to the 'top-bottom effect' described by Gentsch, Gilde & Reimer (1974).

DISCUSSION

Although mitochondrial size, shape and number in microbes used often to be estimated from studying random thin sections of cells, this practice has now been thoroughly discredited by the use of serial sections and reconstruction (e.g. Keddie & Barajas, 1969; Arnold *et al.* 1972;

Fig. 3. Thin section of chemostat cultured sulphate-limited *C. utilis* cell stained with lead citrate. M, mitochondria.

Fig. 4. Lead citrate-stained thin section of chemostat-cultured sulphate-limited *C. utilis* cells after an overnight sulphate-recovery treatment with 1 mM sulphate (see Haddock & Garland, 1971). Numerous cristae occur in the mitochondria (M) and a typical large vacuole (V) is shown in one cell.

Fig. 5. Thin section of copper-deficient variant cells of *C. utilis* stained with lead citrate, showing irregular shaped mitochondrial profiles (M).

Fig. 6. Stereo-pair of a lead citrate stained thick section ($1\ \mu\text{m}$) of a chemostat-cultured glycerol-limited *C. utilis* cell, cultured at a dilution rate of $0.6\ \text{h}^{-1}$. The accelerating voltage was 1000 kV. Two tubular mitochondria (M) are seen, one 4 to $5\ \mu\text{m}$ long, and a peroxysome (P).

Fig. 7. Stereo-pair of a lead citrate-stained thick section ($1\ \mu\text{m}$) of a batch-cultured *C. utilis* cell harvested from a glycerol-defined medium in exponential growth phase. The accelerating voltage was 1000 kV, tilt $\pm 6^\circ$. The section shows the cut end of a branched tubular mitochondrion (M) with cristae in lumen and a branch point (arrow).

Fig. 8. Stereo-pair of a thick section ($2\ \mu\text{m}$) of a batch cultured *C. utilis* sphaeroplast harvested in stationary phase from a glycerol-defined medium and stained with 3,3'-diaminobenzidine and osmium tetroxide. The accelerating voltage was 500 kV. The section shows a tubular mitochondrion with two cut ends (M) and cristae which run along the length of the mitochondria. The darkly

Hoffmann & Avers, 1973; Osafune, 1973; Atkinson, John & Gunning, 1974; Burton & Moore, 1974). One cannot predict from random thin sections whether serial sections and reconstruction will show numerous individual mitochondria or a single large branched mitochondrion per cell, or situations intermediate between these extremes. For instance, Keddie & Barajas (1969) demonstrated that the numerous rounded mitochondrial profiles seen in single thin sections of the yeast *Pityosporum orbiculare* were part of one to four mitochondria per cell, whereas in *Trichoderma viride* conidia Rosen *et al.* (1974) showed that the numerous mitochondrial profiles of a thin section mostly corresponded to individual small mitochondria. These latter authors also emphasized that the constrictions of mitochondrial profiles often seen in thin sections should not be interpreted, as they often are, as indicating mitochondrial division.

High voltage electron microscopy of thick sections offers a convenient alternative to serial sectioning and reconstruction for studying mitochondrial morphology. There are however, limitations and a critical account has been given by Glauert (1974). First, high voltage instruments are not commonplace. Secondly, there must be sufficient contrast, as stressed by Weibull (1974) in his study of the yeast *Trichosporon cutaneum*. Although we have achieved good resolution and contrast with *C. utilis*, we have so far failed to do so with thick sections of *S. cerevisiae* or *Schizosaccharomyces pombe*. Thirdly, the use of thick sections loses some of its attraction if the cell under study is so much larger than the thickness of the section that only a small slice of the cell is studied. Fourthly, the interpretation of overlapping structures is difficult. However, our results show that high voltage electron microscopy has some advantages for studying mitochondrial morphology. Given a small yeast such as *C. utilis*, with good contrast from the staining procedures, then many cells from a variety of culture conditions can be studied fairly rapidly; although the information on mitochondrial morphology given by this method may lack some of the stereological precision of serial sectioning and reconstruction, it is nevertheless a considerable advance over that obtained from random thin sections. The study of the relationship between cell division and mitochondrial division in the cell cycle in yeasts, and the effects of mutations and inhibitors affecting one or the other, may well be valuable applications of the high voltage technique to thick sections.

Reconstruction may be possible from serial thick sections. Provided that the requirement (Favard & Carosso, 1973) for cutting, say, two thin sections between each thick section did not prevent alignment and continuity, then not more than five sections each 1.0 μm thick would encompass virtually all yeast cells, and give a resolution that might be adequate for answering some of the questions concerning mitochondrial division.

Our studies of the way mitochondrial structure varies with growth conditions illustrate only too well the pitfalls of interpreting from random thin sections. For instance, it has been concluded that copper deficiency in *C. utilis* reduces the number of mitochondria per cell (Davison *et al.* 1972; Keyhani, 1973). Yet it is clear from our studies of thick sections (e.g. Figs. 6 to 8) that swelling of the branched tubular mitochondrial structure would of necessity reduce the number of separate mitochondrial profiles per thin section but increase their area, as observed in Fig. 6.

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Figs. 9-13. For legend see opposite page.

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Fig. 9. Thick section (1 μ m) of chemostat-cultured glycerol-limited *C. utilis* sphaeroplast, stained with 3,3'-diaminobenzidine and osmium tetroxide. The accelerating voltage was 400 kV. A branched tubular mitochondrion is seen.

Fig. 10. Thick section (1 μ m) of chemostat-cultured copper-deficient variant *C. utilis* cells, stained with lead citrate. The accelerating voltage was 1000 kV. The section shows a wider and more irregular mitochondrial structure than in Figs. 6 to 9.

Fig. 11. Lead citrate-stained thin section of a chemostat-grown sulphate-limited *C. utilis* cell, after an overnight recovery procedure without added sulphate (see Haddock & Garland, 1971). The section shows a single branched mitochondrion running along the length of the cell.

Fig. 12. Thick section (1 μ m) of a chemostat-cultured glycerol limited *C. utilis* cell stained with lead citrate. The accelerating voltage was 1000 kV. Numerous small mitochondrial structures are seen.

Fig. 13. Scanning transmission electron micrograph of a thick section (0.5 μ m) of a batch-cultured *C. utilis* sphaeroplast harvested in stationary phase from a glycerol-defined medium, sphaeroplasted and stained with 3,3'-diaminobenzidine and osmium tetroxide. The accelerating voltage was 100 kV. A single tubular mitochondrial structure can be discerned.

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