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The effect of coenzyme Q10 included by γ-cyclodextrin on the growth of fission yeast studied by microscope Raman spectroscopy

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HIGHLIGHTS

- Addition of CoQ10-CD complex recovered the growth of a fission yeast mutant strain.
- Oxygen consumption rate of this strain was not restored by the addition of the CoQ10-CD complex.
- Raman measurements suggested the recovery of the growth of this strain was brought about not by the restoration of respiration function.
- The anti-oxidative property of CoQ10 resulted in the decrease in the oxidative stress to grow the strain.

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ABSTRACT

The inclusion complex of coenzyme Q10 (CoQ10) by γ-cyclodextrin (γ-CD), CoQ10-CD complex, was recently developed. The addition of the CoQ10-CD complex recovered the growth of a fission yeast mutant strain, *Δdps1*, which otherwise cannot grow well due to the lack of coenzyme Q producing ability. However, the oxygen consumption rate of this strain was not restored by the addition of the CoQ10-CD complex. The addition of two other anti-oxidative reagents, glutathione and ascorbic acid, also recovered the growth of the *Δdps1* strain as well. These results indicated that the recovery of the growth of *Δdps1* was brought about by the anti-oxidative property of CoQ10. The intensity of Raman spectra of *Δdps1* at 1602 cm^{-1}, which is prominently observed for the wild type of the fission yeast, was compared between before and after addition of the CoQ10-CD complex. The signal was very weakly observed for *Δdps1* and did not increase in intensity by the addition of the CoQ10-CD complex. These results suggested the recovery of the growth of *Δdps1* was brought about not by the restoration of respiration function of *Δdps1* but by the anti-oxidative property of CoQ10 to result in the decrease in the oxidative stress.

1. Introduction

Coenzyme Q10 (CoQ10) is a well known coenzyme which functions as an electron transfer substance in eukaryotes to produce ATP in mitochondria. CoQ10 has a benzoquinone moiety attached with a long isoprenyl chain [1–3]. One more important role of CoQ10 is an anti-oxidative property to diminish the oxidative stress from reactive oxygen species. CoQ10 is bio-synthesized in human body, but the production of CoQ10 is most active at 1920s and gradually decreases with aging [4]. This fact promotes the industrial production of various supplements of CoQ10 which are supposed to make up for the decreased CoQ10 concentration in human body [5]. Some CoQ formula, such as just dissolved in oil or cast in tablet form, are orally uptaken but absorption rate by human body is not high.

Low absorption rate of CoQ10 as well as its instability against light or acid can be overcome using cyclodextrins (CDs) [6]. CDs are oligosaccharides composed of 6–8 glucopyranosyl units with a hydrophobic internal cavity and are well known as molecular capsules, which include hydrophobic compounds or ions into their internal cavities to form so called inclusion complexes [7]. The good points of using CDs as a molecular capsule are as follows; stabilizing unstable guests, but not toxic, and easily digested in our body, etc. [8]. Recently an inclusion complex of CoQ10 stabilized by γ-CD (CoQ10-CD complex) has been developed [9–11]. A chemical analysis and molecular modeling study

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revealed that this inclusion complex is formed by five CD molecules with two staggered CoQ10 molecules into their internal cavities to build up an inclusion complex [12]. Using this CoQ10-CD complex, it was shown that CoQ10 concentration in human or beagle dog blood got elevated soon after oral uptake of the complex and gradually dropped again with proceeding time [9,13].

It is very important to study whether the CoQ10 molecules stabilized by γ-CD will be brought into living cells and whether they function as coenzyme for respiration or not. We have selected a fission yeast, *Schizosaccharomyces pombe*, as a model living organism. It produces a CoQ molecule with a side chain composed of 10 units of isoprenyl chains just as the same as that of human CoQ [1]. We used two different strains of the fission yeast, the wild type (W.T.) and a mutant (*Δdps1*) strains. The *Δdps1* lacks in a gene of decaprenyl diphosphate synthase which function at the first step of a CoQ10 biosynthetic route [1,14]. The *Δdps1* strain consequently lacks in the respiration activity, but it can live by fermentation. However the strain cannot grow well alone in the minimal medium due to the lack of CoQ10 producing ability. The delay in growth of *Δdps1* is thought to be relevant to the lack in the anti-oxidative ability by the fact that the addition of anti-oxidative reagents to the cultivation medium recovers the growth of the same strain [14–16]. In this study, we first have tried to make it clear that if the addition of CoQ10-CD complex in the minimal medium recovers the growth of *Δdps1* or not, as well as if it restores the respiration activity to the same strain or not.

The metabolic activity of fission yeast strains can be estimated by microscopic Raman spectroscopy. A Raman signal observed at 1602 cm^{-1} named "the Raman spectroscopic signature of life" was discovered by Hamaguchi et al. [17]. The signal is thought as a spectroscopic measure for metabolic activity of fission yeast cells because it gives a strong intensity only when the cells have metabolic activity. The result of Raman mapping of the signal showed that the signal is strong at the area overlapping to that of mitochondria which was visualized by GFP [17]. The signal quickly lost intensity when sodium cyanide, a strong inhibitory reagent for respiration, was added to the cultivation medium [17]. These results strongly suggested that the signal has relationship with the respiration activity of fission yeast. One potent candidate for the origin of this signal is ergosterol, a cholesterol equivalent for higher animals [18], however another possible candidate for this signal is intermediate compound of CoQ10 being considered that the distribution of the signal overlaps to that of mitochondria. The corresponding Raman signal is also observed for budding yeast just at the same Raman shift at 1602 cm^{-1}. Raman mapping results showed that it disappeared at the time of death of the cells [19]. The photo-bleaching of the Band of budding yeast cells by strong laser radiation seems to suggest that the Raman band at 1602 cm^{-1} is relevant to mitochondrial metabolic activity [20]. Another report suggests the signal is related to haem function of budding yeast [21]. Other researchers reported on the observation of the band for human cells and that it is related to mitochondrial activity as well [22]. The assignment of this band is thus still puzzling, but it is very probably that the band is related to some mitochondrial metabolic activity of yeasts.

One of the aims of this study is to investigate whether the CoQ10-CD complex will be brought into living cells and whether they function as coenzyme for respiration or not. Another aim of this study is to investigate the intensity change of the Raman signal at 1602 cm^{-1} for the measure of metabolic activity of two strains of fission yeast, W.T. and *Δdps1*, in the presence and absence of CoQ10-CD complex, which may influence the metabolic activity of the fission yeast cells.

2. Methods

2.1. Reagents for growth of fission yeasts

Standard yeast culture media were used as described [23]. Fission yeast (*Schizosaccharomyces pombe*) strains were grown in complete YES medium (0.5% yeast extract, 3% glucose, 225 mg/l each of adenine, leucine, uracil, histidine, and lysine hydrochloride) or in minimal PM medium (0.3% potassium hydrogen phthalate, 0.56% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts). The appropriate auxotrophic supplements were added as necessary (75 mg/l of leucine, or uracil).

2.2. Used fission yeast strains

Two strains of fission yeast were used for this study. One strain is PR110 (wild type, W.T.) and another is a mutant strain LJ1030 (*Δdps1*), which cannot synthesize CoQ10. The genotypes of these strains were *h^+, leu1-32, ura4-D18* for W.T. and *h^+, leu1-32, ura4-D18, dps1::kanMx6* for *Δdps1*, respectively.

2.3. Culture of fission yeast and determination of growth curves

Fission yeast was cultured by two steps, pre-culture and main culture as follows.

2.3.1. Pre-culture

In order to control the cell numbers of fission yeast to an equal order, a pre-cultivation was performed prior to the main cultivation. Cells of fission yeast were plated on complete medium and were incubated at 30 °C for 2 or 3 days. Then a single colony was taken and inoculated in a 10 ml of YES medium and was incubated with shaking at 30 °C for 14 to 16 h to reach stationary phase. The cell numbers was about 1×10^7 cells/ml.

2.3.2. Main culture

Cell numbers were measured by a cell counter (Sysmex, Kobe, Japan). The cells were diluted into PMLU medium at 1×10^5 - cells/ml. CoQ10-CD complex, glutathione and ascorbic acid at various concentrations were added in the medium, followed by the adjustment of the volume of the PMLU medium at 10 ml. Then, the yeast strains were cultured in an incubator for 3 or 4 days until the fission yeast cells reached to the stationary phase. The cell numbers of fission yeast were measured within the culture time to obtain a growth curve by plotting culture time for the horizontal axis and the logarithm of the cell numbers for the vertical axis.

2.4. Measurements of the oxygen consumption

A W.T. strain was pre-cultured in YES medium for 15 h (1.0×10^5 cells/ml) and was cultured in PMLU medium for 72 h. The *Δdps1* strain was similarly pre-cultured in YES medium. The cells of *Δdps1* strain were cultured for 72 h in PMLU medium containing 1.63 mmol/ml of glutathione or 1 mmol/ml of the inclusion complex of CoQ10, respectively. The net molar concentration of CoQ10 corresponds to 13.6 μmol dm^{-3}. Each of the final cell numbers in mediums was measured by a cell counter. All fission yeast cells were collected by centrifugation with 2000 rpm for 2 min. Excess medium for each sample was removed to adjust the cellular concentration at 1.5×10^8 cell number/ml. 500 μl of thus prepared samples were taken and moved to Eppendorf tubes after thorough shaking. The amount of oxygen consumption was measured with a Biological Oxygen monitor (Yellow Springs Instrument YSI model 53).

2.5. Measurements of Raman spectra

The Raman spectra of fission yeast strains were obtained either in sugar solution (diluted glucose solution at about 0.1%) or PMLU medium. Sugar solution is transparent in visible light region to give good Raman spectra. However it lacks other necessary components than sugar to keep fission yeast cells for a long time. On the other hand, PMLU medium includes minimal necessary nutritious components. We used sugar solution for short time measurements and PMLU medium for long time measurements.

2.5.1. Preparation of bottom dish samples for fission yeast cells in sugar solution

Fission yeast cells were pre-cultured in YES medium for 15 h. 1 ml of the medium was taken and centrifuged at 2000 rpm for 2 min. The supernatant was discarded and 10 ml of sugar solution at appropriate concentration was added to the pellet followed by dispersing it with a vortex mixer. Again 1 ml of the sugar solution of cells was taken and another 10 ml of sugar solution was added. The cell concentration at this step is about 1.0×10^5–1.0×10^6 cells/ml. 50 μl of this solution was taken on a bottom dish. The diameter of the bottom dish is 35 mm with a glass hole of 14 mm diameter at the center. On the surface of the center hole, concanavalin A (0.1 w/v aqueous solution) was preliminary pasted for the purpose of fixing the fission yeast cells still. Thus prepared bottom dishes were left at least 10 min to ensure the fix of the cells.

2.5.2. Preparation of bottom dish samples for fission yeast cells in PMLU medium

Fission yeast cells were pre-cultured in YES medium for 15 h. The cultured cells were moved in PMLU medium after adjusting the cell numbers at about 1.0×10^6 cells/ml. 50 μl of this medium was taken on a bottom dish similar to those in sugar solution as described.

2.5.3. Procedure of Raman spectral measurements

The con-focal microscope Raman spectra of fission yeast cells were measured with bottom dishes either in sugar solution or PMLU medium as described. The apparatus for Raman spectral measurements was a home made system with a Cromex single monochromator equipped with a Princeton Instruments SPEC-10 CCD system combined with an Olympus inverted microscope IX71. The excitation line was 632.8 nm with an average power of 2.1 mW at the sample point. The normal exposure time was 1 s/point with intervals of 0.6 μm/point for Raman mapping measurements. The system was controlled with a LabVIEW software (National Instruments) and analyzed with a IGOR Pro software (HULINKS).

3. Results and discussion

The W.T. fission yeast in PMLU medium gradually grew with proceeding time for several hours followed by the log growth phase for about 20 h and became stationary after 24 h (Fig. 1, ●). The *Δdps1* stopped growing due to the lack of CoQ10 producing ability (■). The addition of γ-CD did not exhibit any effects on the growth curves of W.T. or *Δdps1* (data not shown). The addition of CoQ10-CD complex (0.1 mg/ml) also did not exhibit any effects on the growth curve of W.T. In contrast, the addition of CoQ10-CD complex (0.1 mg/ml) dramatically recovered the growth of *Δdps1* as plotted by ▲ in Fig. 1. The addition of CoQ10-CD complex was effective at more diluted concentration. It was effective at higher concentration than 0.001 mg/ml.

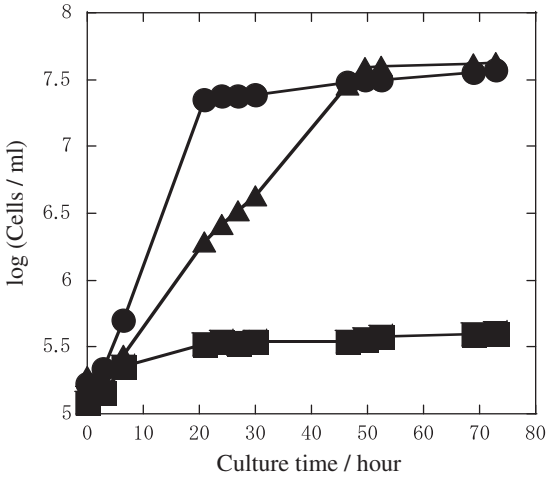

Fig. 1. The growth curves of W.T. (●) and *Δdps1* (■) in the PMLU medium and *Δdps1* (▲) in the presence of 1 mg of CoQ10-CD complex in the same medium (10 ml).

We next examined a possibility of the respiration recovery in the *Δdps1* strain by CoQ10-CD complex. The oxygen consumption rates for the W.T. and *Δdps1* in PMLU medium without the CoQ10-CD complex were 9.17 and 0.97%/min, respectively as shown in Fig. 2. Although the oxygen consumption rate of *Δdps1* was not zero, very small amounts of oxygen consumption, compared to W.T., indicated the lack in respiration activity of the *Δdps1* mutant strain. It has been known that there is a small amount of oxygen consumption by the non-respiratory reactions in the cells, so that oxygen consumption never becomes zero [24,25]. The addition of CoQ10-CD complex did not increase the oxygen consumption rate for either W.T. or for *Δdps1* at all as shown Fig. 2. This result strongly suggests that the recovery of the growth of *Δdps1* is not brought about by the recovery of the respiration activity of the strain.

Then the effects of other anti-oxidative reagents, glutathione and ascorbic acids, on *Δdps1* were studied after confirming of they did not affect the growth of W.T. (figures not shown). The *Δdps1* did not grow well alone in PMLU medium as shown in Fig. 3 (■) while W.T. (●) grew normally. When glutathione was added to the medium at 1 mg to the culture medium of 10 ml, the growth of *Δdps1* was dramatically recovered as (▲). The effect of the addi-

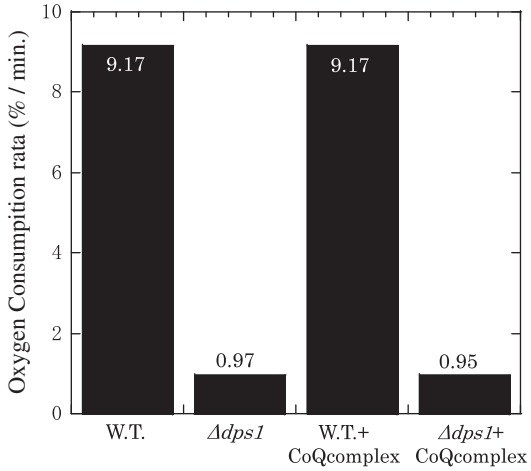

Fig. 2. The oxygen consumption rates for W.T., *Δdps1*, W.T. in the presence of CoQ10-CD complex and *Δdps1* in the presence of CoQ10-CD complex.

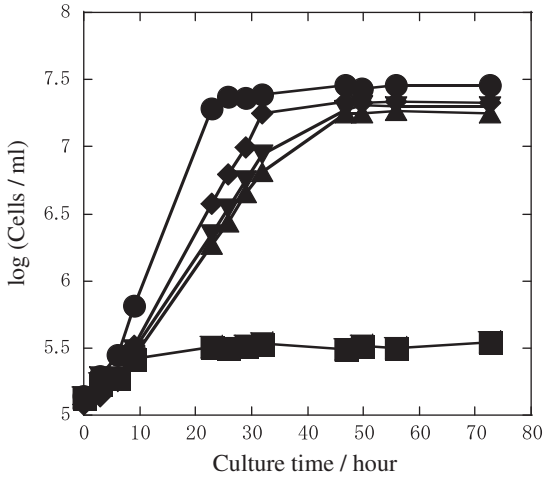

Fig. 3. The growth curves of W.T. (●), *Δdps1* (■) in PMLU medium and *Δdps1* in the presence of 1 mg (▲), 5 mg (▼) and 10 mg (♦) of glutathione in the same medium (10 ml).

tion of glutathione was more effective at higher concentrations, 5 mg (▼) and 10 mg (♦), as shown in Fig. 3. This result suggests that recovery of the growth of *Δdps1* by the addition of CoQ10-CD complex was not due to the recovery of the respiration activity of the mutant strain but by the anti-oxidative property of CoQ10.

The effect of another anti-oxidative reagent, ascorbic acid, was checked its capability of growth recovery in *Δdps1* similarly as shown in Fig. 4. By the addition of ascorbic acid at lower concentration than 5 mg, 1 mg (▲) or 5 mg (▼), the growth of *Δdps1* was not recovered. The addition of 10 mg (♦) ascorbic acid recovered the growth of *Δdps1* although the effect was not as strong as that of glutathione, but apparently it restored the growth of *Δdps1*. Clear amount of ascorbic acid as a threshold was difficult to determine, probably due to the greater instability of the acid than glutathione. These results indicated that addition of antioxidatants restored the growth capability in the *Δdps1* strain.

The typical one-dimensional Raman spectra of W.T. and *Δdps1* in sugar solution just after the pre-culture, at the area where mitochondria supposed to be distributed, are shown in Fig. 5 with Raman shift values for major bands. The band at 1650 cm^{-1} is an overlapped band of the amide I of proteins and C=C stretching

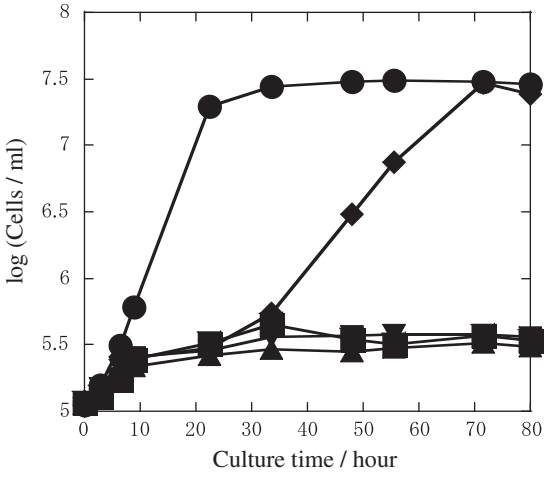

Fig. 4. The growth curves of W.T. (●), *Δdps1* (■) in PMLU medium and *Δdps1* in the presence of 1 mg (▲), 5 mg (▼) and 10 mg (♦) of ascorbic acid in the same medium (10 ml).

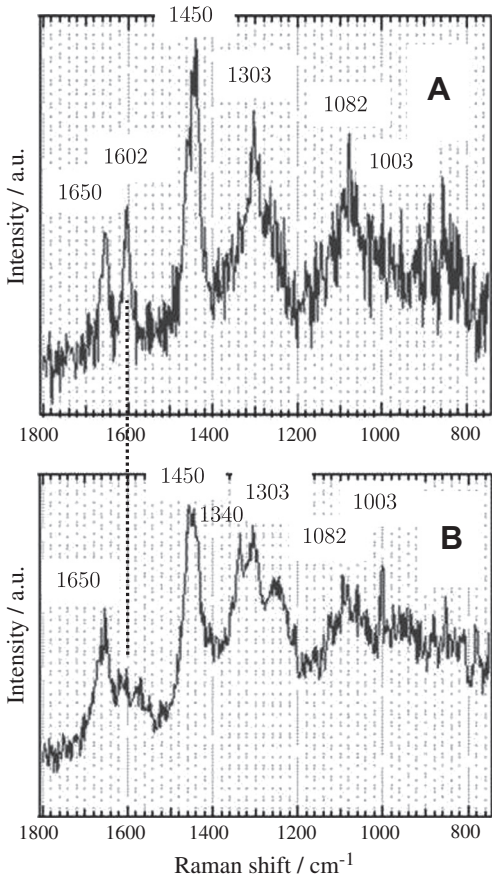

Fig. 5. The typical Raman spectra of W.T. (a) and *Δdps1* (b) in sugar solution for at the area where the Raman signal at 1450 cm^{-1} was strong.

bands [17,26]. Other Raman bands due to proteins are found between 1300 and 1250 cm^{-1} (amide III) and at 1003 cm^{-1} due to the breathing mode of phenylalanine [26]. The band at 1450 cm^{-1} was used for the measure of distribution of mitochondria, because the vibrational mode of the band is CH bending and is reported to be observed strongly for phospholipid membrane of mitochondria [17]. Although the overall features of two Raman spectra were similar, the distinct signal at 1602 cm^{-1} clearly observed for W.T. (A) could be observed only very weakly for *Δdps1* (B). The lack of this signal, "the Raman spectroscopic signature of life", suggests the weaker metabolic activity of *Δdps1* than W.T. A relatively distinct signal only observed for *Δdps1* (B) at 1340 cm^{-1}, assignable to CH_2 deformation might be due to the change in concentration of lipid, but not clear at present. Another change found at about 1260 cm^{-1} might be due to change in the conformational change of proteins, but similarly not clear at present.

In order to check the metabolic activity of W.T. by the intensity of the signal at 1602 cm^{-1} as a measure, we followed the Raman spectral change with proceeding time where the signal at 1450 cm^{-1} was observed prominently. The results are summarized to give Fig. 6. The left column (A) of this figure shows the optical images of a W.T. cell (60, 120, 180 and 360 min after the start of culture). The corresponding Raman mapping images (B) obtained by the intensity distribution of the Raman signal at 1450 cm^{-1} with typical one dimensional Raman spectra at the right column (C), where the signal at 1450 cm^{-1} is prominently observed. The stronger intensity in the Raman mapping images is indicated by more red color and weaker intensity by more blue color in this kind of figures. The optical image of W.T. did not change within the Ra-

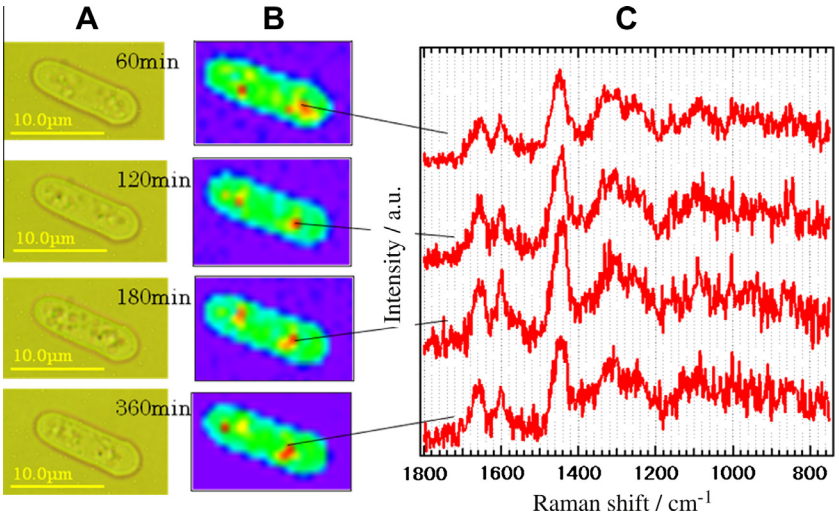

Fig. 6. The optical images of a W.T. fission yeast cell (a) kept in PMLU medium, the corresponding Raman mapping image, (b) given by the intensity distribution by the Raman signal at 1450 cm^{-1}, and the one dimensional Raman spectra and (c) at the position pointed by black arrows.

man measurements for 6 h. Raman spectroscopic changes accompanying the cell division cycle of fission yeast showed intensity changes of some Raman bands within 20 h has been reported [27]. Considering that an ordinary W.T. cell divides within two or 3 h, it is possible that the division of this cell was inhibited by the fixation with concanavalin A. Even though this may have occurred, we can suppose that the metabolic activity of this cell is maintained, as judged by the strong intensity at 1602 cm^{-1} in this figure. As a whole, Fig. 6 shows the metabolic activity of W.T. could be observed fairly by this method. The corresponding Raman mapping images for W.T. in the presence of CoQ10-CD complex were similarly observed. The images (not shown) essentially corresponded to those observed for W.T. in the absence of CoQ10-CD complex. This fact suggests that little change was given by the CoQ10-CD complex on the metabolic activity of W.T. The result probably reflects the fact that oxygen consumption rate was not changed by the addition of the complex to the culture medium as shown in Fig. 2.

The similar measurements were performed for *Δdps1* to give Fig. 7. As can be readily noticed, the signal at 1602 cm^{-1} was observed very weakly or not detectable in Fig. 7C. Within Raman measurements for 6 h, the optical image and Raman mapping image for the signal at 1450 cm^{-1} gave little difference. Although some intensity changes in one dimensional spectrum (C) were observed, for example in the region between 1350 and 1250 cm^{-1}, the metabolic activity of this cell can be regarded as very weak within the time for Raman measurements. This result probably reflects the incapability of growth of *Δdps1* in PMLU medium.

The effect of the addition of CoQ10-CD complex was studied by Raman measurements to be summarized as shown in Fig. 8. The signal at 1602 cm^{-1} did not increase with proceeding time within 6 h suggesting that the metabolic activity of *Δdps1* was not improved by the addition of CoQ10-CD complex. As a result, we concluded that the addition of CoQ10-CD complex recovered growth of *Δdps1*, but the respiratory activity of the mutant strain was not recovered. The recovery of the growth of the *Δdps1* is supposed to be brought about by the anti-oxidative property of CoQ10.

In order to summarize the Raman spectroscopic estimate of the metabolic activity of fission yeasts, the intensity ratio of the Raman signal at 1602 cm^{-1} against that at 1450 cm^{-1} was plotted for incubation time to give Fig. 9. The ratio for W.T. (●) was between 0.65 and 0.75 within the incubation time. If the ratio can be treated

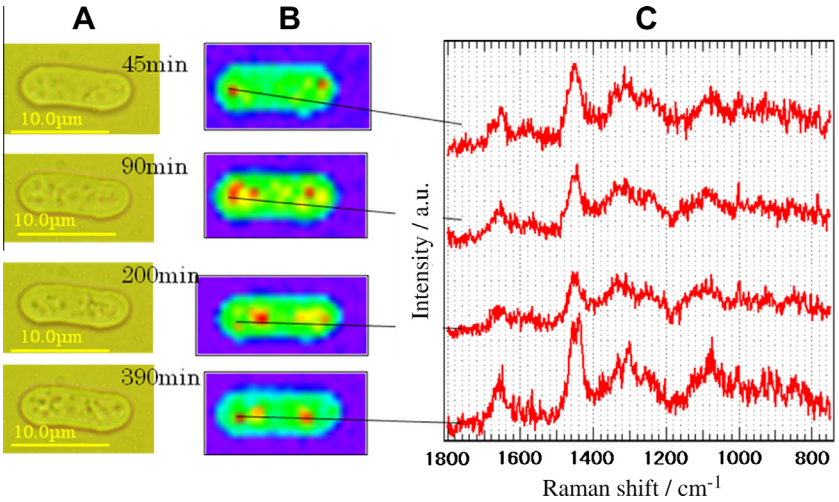

Fig. 7. The optical images of a *Δdps1* fission yeast cell (a) kept in PMLU medium, the corresponding Raman mapping image, (b) given by the intensity distribution by the Raman signal at 1450 cm^{-1}, and the one dimensional Raman spectra and (c) at the position pointed by black arrows.

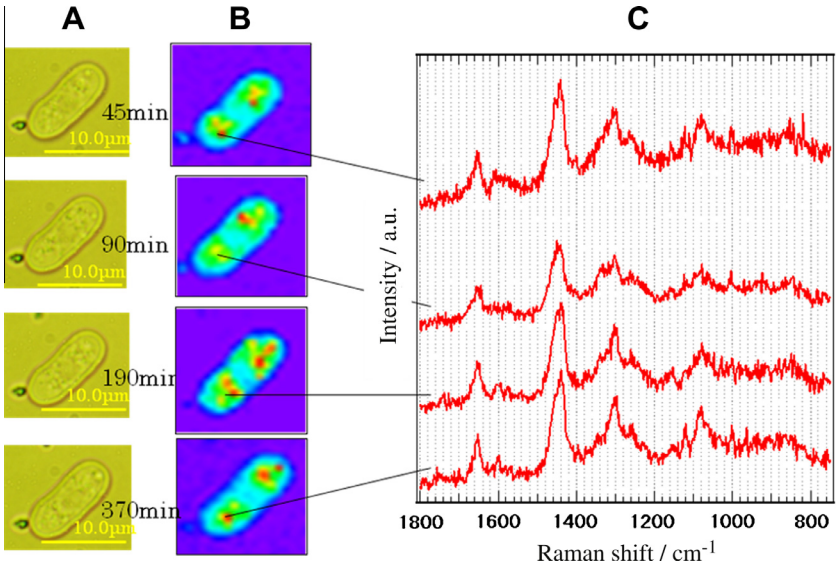

Fig. 8. The optical images of a *Δdps1* fission yeast cell (a) kept in PMLU medium in the presence of 1 mg of CoQ10-CD complex, the corresponding Raman mapping image, (b) given by the intensity distribution by the Raman signal at 1450 cm^{-1}, and the one dimensional Raman spectra and (c) at the position pointed by black arrows.

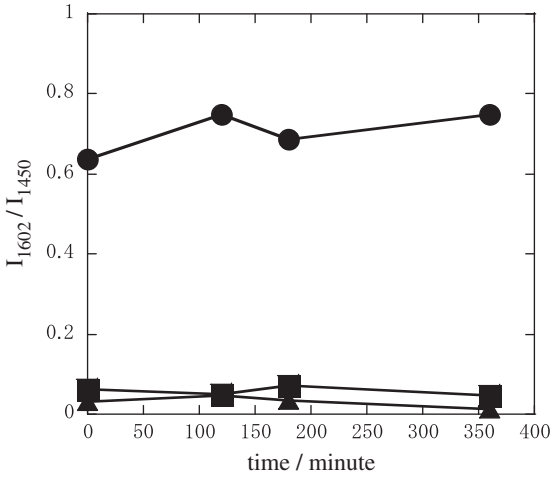

Fig. 9. The intensity ratio of the Raman signal at 1602 cm^{-1} against that at 1450 cm^{-1} for W.T. (●), *Δdps1*(■) and *Δdps1* (▲) in the presence of CoQ10-CD complex.

as a metabolic activity of fission, W.T. keeps its metabolic activity within the incubation time. The ratio was not changed by the addition of CoQ10-CD complex (data not shown). The ratio for *Δdps1* (■) was less than 0.1, almost zero, within the incubation time and did not change by the addition of CoQ10-CD complex (▲). These results and the oxygen consumption rate comprehensively suggest that the addition of COQ10-CD complex did not affect the metabolic activity of W.T. and *Δdps1* strains, and did not recover the respiratory activity of *Δdps1* either. For the purpose of estimating more detailed metabolic activity by Raman spectroscopy, the assignment and thorough investigation in the change of intensity of so called "the Raman spectroscopic signature of life" is needed.

4. Conclusion

The growth of a mutant fission yeast strain, *Δdps1*, in PMLU medium, was recovered by the addition of CoQ10-CD complex. The results that oxygen consumption rate of the mutant strain was not recovered by the addition of CoQ10-CD complex and that other anti-oxidative reagents, glutathione and ascorbic acid, restored the growth of the strain, indicate that the anti-oxidative property of CoQ10 was supported by the CoQ10-CD complex. The Raman spectroscopic measurements supported this conclusion as judged by the intensity change at 1602 cm^{-1} as a measure of metabolic activity.

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