

## FULL PAPER

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## A possible role of the key enzymes of the glyoxylate and gluconeogenesis pathways for fruit-body formation of the wood-rotting basidiomycete *Flammulina velutipes*

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**Abstract** Biochemical roles of the representative enzymes involved in carbon metabolism of glucose were investigated in relation to the fruit-body formation of the basidiomycete *Flammulina velutipes*. Changes in specific activities of the enzymes of the tricarboxylic acid (TCA) cycle and glyoxylate (GLOX) and gluconeogenesis pathways were measured at different stages of development of the fungus. The enzyme activities of malate synthase (MS) and fructose bisphosphatase (FBP) as the key enzymes for the GLOX-gluconeogenesis pathways increased in mycelia during the fruit-body formation. The activities of isocitrate dehydrogenase (IDH) for the TCA cycle and NADP-linked glutamate dehydrogenase (GLTDH (NADP)) for glutamate synthesis increased more markedly. Moreover, the mycelial mat of the cultures producing fruit bodies yielded greater enzyme activities of isocitrate lyase (ICL), MS, FBP, and IDH than that of the cultures that did not produce fruit bodies. These results suggest that the GLOX-gluconeogenesis pathways as well as the glutamate synthesis have a strong correlation with the fruit-body formation of *F. velutipes*.

**Key words** *Flammulina velutipes* · Gluconeogenesis · Glyoxylate cycle · TCA cycle

### Introduction

The glyoxylate (GLOX) cycle, which was discovered from acetate-grown bacteria by Kornberg and Krebs (1957), has been known as an important biochemical pathway for an

anaplerotic shunt to support the tricarboxylic acid (TCA) cycle in coordination with gluconeogenesis for synthesis of glucose. The anaplerotic shunt involving the two key enzymes, isocitrate lyase (ICL) and malate synthase (MS), permits the net conversion of 2 mol acetyl-CoA (derived from the two carbon substrates or from fatty acid) to C<sub>4</sub> acids, yielding oxaloacetate (OAA). Then, OAA is converted to phosphoenolpyruvate (PEP) by PEP carboxykinase, yielding eventually a simple sugar by mediation of the key enzyme fructose bisphosphatase (FBP), which catalyzes the irreversible conversion of fructose-1,6-bisphosphate to fructose-6-phosphate to direct the gluconeogenesis pathway. Thus, a metabolic role of the GLOX cycle has received much attention in morphogenesis of microorganisms or spore formation of filamentous fungi such as *Neurospora crassa* Shear et Dodge (Turian and Combépine 1963), *Aspergillus niger* van Tiegh. (Galbraith and Smith 1969), *Saccharomyces cerevisiae* Meyen ex Hansen (Duntze et al. 1969), and *Sclerotium rolfsii* (Curzi) Tu and Kimbrough (Maxwell et al. 1975). Alternatively, a role of the GLOX cycle was also investigated for fruit-body formation of *Coprinus lagopus* (Fr.) Fries (Casselton et al. 1969), *Schizophyllum commune* Fries (Cotter et al. 1970), and *C. cinereus* (Schaeffer. ex Fr.) S.F. Gray (Moore and Ewaze 1976). However, no positive participation of the key enzymes of the GLOX and gluconeogenesis pathways in fruit-body formation has been reported, probably because they failed to detect any higher activity of the key enzymes due to focusing on the enzymes in pilei but not in mycelia of those cultures, although MS activity was detected from basidiospores of a wide variety of basidiomycetes (Ruch et al. 1991).

Nevertheless, quite recently, we have explored a new physiological role of the GLOX cycle enzymes for biosynthesis of oxalic acid in mycelia of the wood-rotting basidiomycetes (Munir et al. 2001a,b). Thus, we were motivated to investigate a biochemical role of the GLOX cycle and gluconeogenesis for the fruit-body formation of the wood-rotting basidiomycete *Flammulina velutipes* (Curt.: Fr.) Sing., focusing on the enzymes occurring in mycelia. This investigation reports for the first time a possible role of the

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GLOX and gluconeogenesis pathways for the fruit-body formation of the basidiomycete. The results are discussed in relation to the role of other representative enzymes involved in the related carbon metabolic pathways.

## Materials and methods

### Chemicals

All chemical and biochemical reagents were of reagent grade. NAD, NADP, acetyl coenzyme A, DL-isocitric acid, 4-aminobutyric acid, fructose-1,6-bisphosphate, and 2-oxoglutaric acid were obtained from Nacalai Tesque (Kyoto, Japan). Coenzyme A and  $\beta$ -NAD<sup>+</sup>, thiamine pyrophosphate, and glyoxylic acid were obtained from Oriental Yeast (Tokyo, Japan), Wako (Osaka, Japan), and Sigma (St. Louis, MO, USA), respectively. The protein assay kit and glucose assay kit, succinic semialdehyde, glucose-6-phosphate isomerase, and glucose-6-phosphate dehydrogenase were purchased from Bio-Rad Laboratories (Hercules, CA, USA) and Boehringer (Mannheim, Germany), respectively.

### Microorganism

*Flammulina velutipes* (Curt.: Fr.) Sing. IFO 7777 strain was used as a model fungus in this study as developed by one of the authors (Terashita 1992).

### Culture conditions

Mycelia were grown on potato dextrose agar plates. Each single plug of mycelium prepared from a fully grown mycelial colony by using a cork borer (5 mm diameter) was inoculated to a 250-ml culture bottle (polycarbonate; Iwaki, Tokyo, Japan) containing 50 ml potato glucose liquid (PGL) medium (pH 5.5). The medium was composed of the extract of 200 g potato and 20 g glucose in 1000 ml distilled water. To cultivate mature fruit bodies, the fungus was first grown at 25°C for 14 days in the dark, and then transferred into the incubator at 16°C in the light (100–200 lx) until fruit bodies

appeared. To repress fruit-body formation, the fungus was grown at 25°C for 28 days in the dark. The developmental stages were divided into five, as indicated in Table 1.

### Preparation of cell-free extracts and enzyme assays

In principle, of 50 cultures, 5 or 7 cultures were harvested at each stage to get cell-free extracts. Cell-free extracts were prepared by homogenizing the *F. velutipes* mycelia (2–3 g fresh weight) with a pestle in an ice-chilled mortar with 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and a small amount of sea sand. When necessary, stipes and pilei were separated from each other and used for assay of the enzyme activity. The homogenate was centrifuged at 14000 g at 4°C for 30 min. This manipulation was performed twice, and the combined supernatant was used as the crude enzyme solution. All enzyme activities were determined spectrophotometrically by using a double-beam spectrophotometer equipped with a temperature controller. Activities of ICL and MS were assayed by the methods of Dixon and Kornberg (1959). Both malate dehydrogenase (MDH) and 2-oxoglutarate dehydrogenase (ODH), and isocitrate dehydrogenase (IDH) activities were determined by measuring the increase in absorbance at 340 nm resulting from the reduction of NAD and NADP, respectively, on the basis of reported methods (Moore and Ewaze 1976). The FBP activity was measured by the modified method described by Pontremoli and Melloni (1974). The activities of glutamate dehydrogenase (GLTDH) requiring for NAD or NADP were assayed by measuring the decrease in absorbance at 340 nm from the oxidation of NADH or NADPH, respectively, on the basis of reported methods (Moore and Ewaze 1976).  $\gamma$ -Aminobutyrate transaminase (GABAT) and succinic semialdehyde dehydrogenase (SSADH) were assayed by reported methods (Moore and Ewaze 1976; Jakoby 1962). One unit (U) of enzyme activity is defined as the amount of an enzyme that catalyzes the formation of 1  $\mu$ mol product per minute or the consumption of 1  $\mu$ mol substrate per minute under the conditions described; all enzyme activities are given in terms of specific activity and expressed as U/mg protein.

**Table 1.** Developmental stages in fruit-body formation of *Flammulina velutipes*

Stage number and name	Term in cultivation <sup>a</sup> (days after inoculation)	Description
1. Stage of the vegetative mycelium	0–14	Growth of the vegetative mycelium
2. Stage of the primordia	14–17	Formation of the primordium of fruit bodies
3. Stage of the young fruit bodies	17–21	Differentiation of the pileus
4. Stage of the immature fruit bodies	21–28	Formation of basidiospores; gills and slightly concave gill surface were visible
5. Stage of the mature fruit bodies <sup>b</sup>	28–30	Maturation of basidiospores; gill surface were flat and/or curving upward

<sup>a</sup>The temperature of the incubator was shifted down to 16°C on day 14

<sup>b</sup>The spores matured were observed at this stage by use of a light microscope

Determinations of glucose consumption, protein, and lipid contents

Glucose remaining in the culture fluid was determined enzymatically by using the glucose assay kit (Boehringer). Protein concentrations were determined by the Bradford method (1976) with bovine serum albumin as a standard. Lipid content in the cultures was measured by the method reported by Hiroi (1982).

## Results

Changes in dry weights of the mycelium and fruit bodies during the development of *F. velutipes*

The vegetative mycelium that had been grown in the dark began to produce a cluster of primordia 2–3 days after being transferred into an incubator at 16°C under light illumination (100–200lx). Figure 1A shows the changes in dry weights of vegetative mycelium and fruit bodies harvested at different stages during cultivation. The dry weight of the vegetative mycelium increased rapidly from day 7 to day 21, reaching the maximum (190 mg/culture), and decreased thereafter. Around day 17, numerous primordia appeared at one time and the dry weight of primordia increased, reaching the maximum on day 21, and decreased to one-third of the maximal value on day 25. Along with decreases in dry weight of mycelium and primordia, the stipes and pilei of fruit bodies began to increase significantly. This finding clearly indicates that nutrients in both mycelium and primordia were utilized for the growth of fruit bodies.

Figure 1B shows the pattern of glucose consumption in the medium and an increase in the lipid content in mycelia during the cultivation of the fungus. The rapid glucose consumption coordinated with the increase in total weight of biomass produced (Fig. 1A), including mycelium, primordia, stipes, and pilei of the growing *F. velutipes*. About 50% of glucose in the medium had been consumed before the primordial fruit-body appeared, which is fairly consistent

with the finding that the fruit-body formation of *F. velutipes* was initiated at the stage of about 50% glucose consumption (Kitamoto and Gruen 1976). Figure 1B further indicates that most of the glucose in the medium was rapidly utilized during primordium formation. The lipid content also increased rapidly at the early stage of development and increased moderately along with rapid consumption of glucose during colony development.

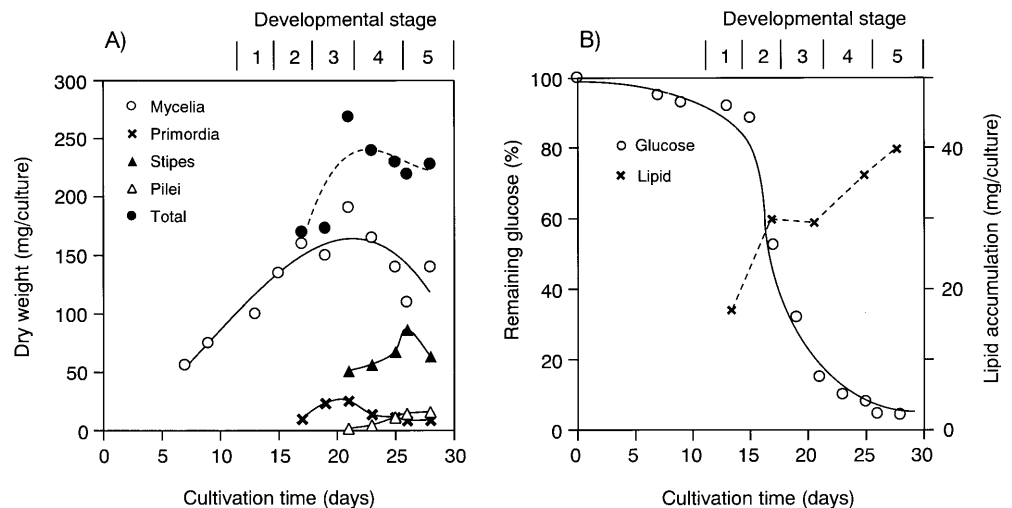
Changes in activities of the key enzymes of the GLOX and gluconeogenesis pathways and the TCA cycle during fruit-body development

Figure 2 shows the changes in the specific activities of ICL and MS for the GLOX pathway, FBP for gluconeogenesis, IDH and ODH for the TCA cycle, and GLTDH (NADP) for glutamate synthesis in mycelia during the development of *F. velutipes*. The results show that the enzyme activity of GLTDH (NADP) increased strikingly, accompanied by IDH, a key enzyme of the TCA cycle at stage 3–5 (during fruit-body development). In contrast, ODH, another key enzyme of the TCA cycle, was not detected throughout all stages. However, both FBP and MS activities began to increase moderately but significantly at stages of the fruit-body development (stage 3–4), reaching the highest level at maturation of fruit bodies (stage 5). However, ICL activity was only slightly elevated at stages 4 and 5. As a rule, however, the observed increases in both MS and FBP activities suggest that these enzymes support gluconeogenesis by use of C<sub>4</sub> acid intermediates derived from the lipid accumulating in the mycelia (Fig. 1B).

Comparison of the enzyme activities of the GLOX and gluconeogenesis pathways, TCA cycle, and the glutamate-GABA route between mycelium, stipes, and pilei

Table 2 shows the activities of the enzymes responsible for the GLOX and gluconeogenesis pathways, the TCA cycle, and glutamate synthesis in mycelium and fruit bodies of *F.*

**Fig. 1.** **A** Changes in dry weights of the vegetative mycelium, primordia, stipes, and pilei of *Flammulina velutipes*. **B** The consumption of glucose in the culture medium and accumulation of lipid in the mycelium during development of *F. velutipes*. The residual glucose contents are given as percentage of the initial concentration (20.0 g/l). The amounts of lipid accumulated in the mycelium were determined at each developmental stage



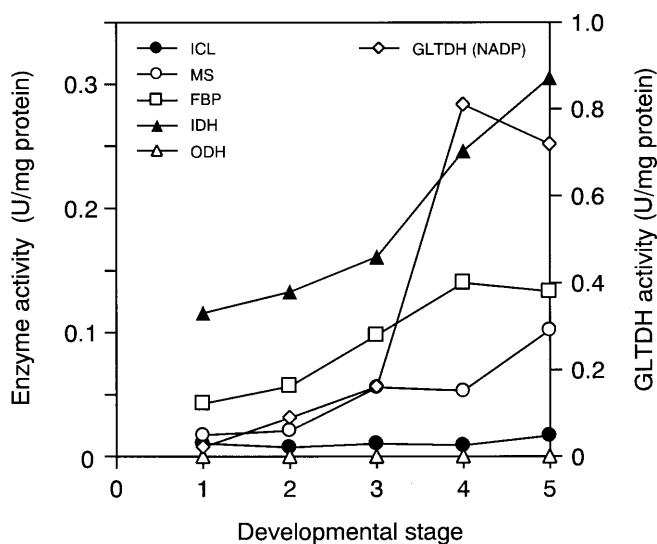
*velutipes*. The results are compared between these different tissues at stage 4. It is noteworthy that the activities of both GLTDH (NADP) and GLTDH (NAD) for glutamate synthesis and IDH for the TCA cycle in mycelium are 2- to 8-fold as compared with those in stipes and pilei; the FBP activity especially is 8- to 18-fold, whereas the GABAT activities in stipes and pilei are significantly greater than those in mycelium, although there are no significant differences in the SSADH activities between mycelium, stipes, and pilei. However, ODH activity was detected neither

from mycelium nor from stipes and pilei. In mycelium and fruit body, it is noteworthy that the metabolic flow toward glutamate synthesis is favored by greater activities of IDH and GLTDH (NADP), which couple by using a common NADP/NADPH electron carrier. However, glutamate may not be efficiently utilized by the GABA route in mycelium, because the activity of GABAT is much smaller in mycelium than that in stipes and pilei. On the other hand, the activities of the two GLOX pathway key enzymes are greater in pilei than those in mycelium and stipes, although there are no significant differences in MS activity between mycelium and pilei.

Nevertheless, it is noteworthy that FBP activity coordinating with MS activity is much greater in mycelium than that in other tissues. However, the extremely high activity of MDH obtained suggests that the enzyme plays an important role not only for gluconeogenesis but also for other pathways of metabolism, including NADH production in mitochondria and cytoplasmic sites.

Comparison of the enzyme activities of ICL, MS, FBP, and IDH in mycelium between the cultures with and without fruit-body formation

Because we found that the fungal cultures that had not been treated with the temperature downshift or with light illumination did not produce fruit bodies, we compared the enzyme activities of ICL, MS, FBP, and IDH in mycelium between the two different cultures with and without fruit bodies at stage 5. Both cultures yielded almost equal amounts of mycelia (about 180 mg dry weight each). However, dry weight of fruit bodies produced in one culture was about 140 mg. Table 3 shows that these marker enzymes from the fruiting cultures were two to three times those for the nonfruiting cultures, suggesting that these enzymes significantly contribute to fruit-body formation.



**Fig. 2.** Changes in activities of the key enzymes of glyoxylate [isocitrate lyase (*ICL*) and malate synthase (*MS*)] and gluconeogenesis [fructose biphosphatase (*FBP*)] pathways and tricarboxylic acid (*TCA*) [isocitrate dehydrogenase (*IDH*) and 2-oxoglutarate dehydrogenase (*ODH*)] cycles in the mycelium during the development of fruit bodies. The developmental stages (1–5) are defined in Table 1. The mycelium of stage 1 was sampled on day 13–14 after inoculation

**Table 2.** Specific activities of the enzymes of the glyoxylate (GLOX) and tricarboxylic acid (TCA) cycles, gluconeogenesis pathway, and  $\gamma$ -aminobutyric acid (GABA) route in mycelium and fruit bodies of *F. velutipes*

Carbon metabolism	Representative enzymes	Enzyme activity <sup>a</sup> (mU/mg protein)		
		Mycelium	Stipes	Pilei
GLOX cycle	ICL	10.0	3.8	21.0
	MS	52.0	23.0	55.0
	MDH	11 000.0	NT	NT
Gluconeogenesis	FBP	158.0	9.0	20.0
TCA cycle	IDH	305.0	128.0	153.0
	ODH	0	0	0
Glutamate synthesis	GLTDH (NAD)	389.6	45.0	95.0
	GLTDH (NADP)	809.0	345.0	328.0
GABA route	GABAT	1.7	14.0	9.8
	SSADH	5.1	3.5	4.3

ICL, isocitrate lyase; MS, malate synthase; MDH, malate dehydrogenase; FBP, fructose biphosphatase; IDH, isocitrate dehydrogenase; ODH, 2-oxoglutarate dehydrogenase; GLTDH, glutamate dehydrogenase; GABAT,  $\gamma$ -aminobutyrate transaminase; SSADH, succinic semialdehyde dehydrogenase

NT, not tested

<sup>a</sup> Enzyme activities are defined in the text for fungus harvested at stage 4

**Table 3.** Comparison of specific activities of the enzymes related to gluconeogenesis in mycelia of the two different cultures

Enzyme	Enzyme activity <sup>a</sup> (mU/mg protein)		A/B
	A <sup>b</sup>	B <sup>c</sup>	
ICL	17.2	10.0	1.7
MS	75.3	25.6	3.0
FBP	171.4	56.4	3.0
IDH	203.0	120.5	1.7

ICL, isocitrate lyase; MS, malate synthase; FBP, fructose biphosphatase; IDH, isocitrate dehydrogenase

<sup>a</sup> Enzyme activities were determined for the fungus grown for 28 days

<sup>b</sup> A, mycelium of the culture with fruit-body formation

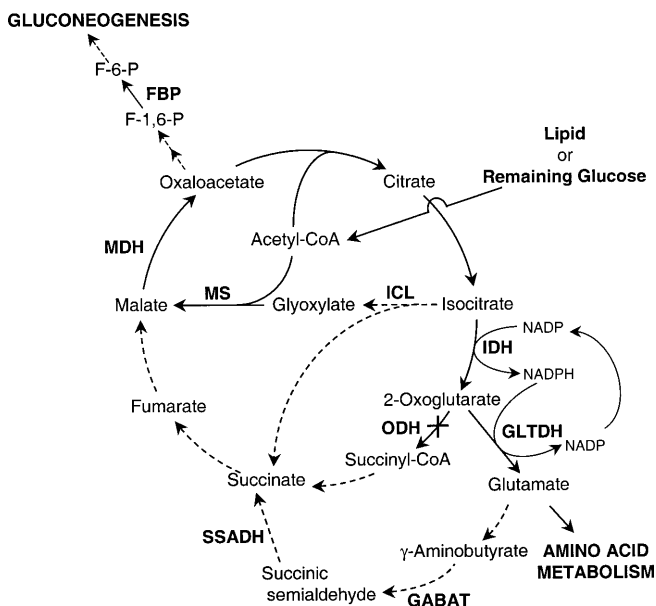
<sup>c</sup> B, mycelium of the culture without fruit-body formation

## Discussion

This investigation has revealed that the enzyme activities of FBP and MS for the GLOX-gluconeogenesis pathways increased in mycelium during the fruit-body development of *F. velutipes*, although both IDH and GLTDH (NADP) were more predominant in both mycelium and pilei (Fig. 2, Table 2). It is noteworthy that the FBP activity is about eightfold in mycelium. Moreover, the mycelial mat of cultures with the fruit bodies yielded greater activities of the enzymes of the GLOX-gluconeogenesis pathways than that of the cultures not producing fruit bodies (Table 3).

However, no marked increase in ICL activity was recognized during the development of the fungus (Fig. 2), and the ICL activities were much less than we had assumed. Moreover, the ODH activity was not detected from the fungus at any developmental stage. These results suggest that the metabolic flow was controlled or blocked at the GLOX or TCA cycle, respectively. Thus, this investigation led us to propose that there are mainly two metabolic routes that are linked with the TCA cycle to a different degree in mycelium and pilei as proposed in Fig. 3; the one is a route channeled to gluconeogenesis via a GLOX shunt involving MS, MDH, and FBP and the other is to glutamate synthesis involving IDH and GLTDH (NADP). For the two metabolic routes, acetyl-CoA is a common intermediate derived from the remaining glucose or the lipid accumulating in mycelium (see Fig. 1B). In both mycelium and fruit body, it is noteworthy that the glutamate synthesis is a major route, which is consistent with the finding reported by Moore and Ewaze (1976). Although glutamate synthesis is well known to play a central role in reshuffling a variety of amino acids and urea formation during fruit-body formation, it is intriguing to speculate that glutamate is a carbon source of glyoxylate to support the GLOX pathway as the alternate anaplerotic shunt in *Streptomyces collinus* Lindenbein (Han and Reynolds 1997), whereas the glycine-metabolizing systems (Goldman and Wagner 1962; Sanders et al. 1972) liberate glyoxylate and  $\text{NH}_4^+$ , which are required for malate and glutamate synthesis, respectively.

Furthermore, our finding that the enzymes (MS and FBP) in the GLOX-gluconeogenesis pathways are more significant in mycelia than those in pilei during fruit-



**Fig. 3.** Proposed carbon metabolic mechanism in the mycelium of the wood-rotting basidiomycete *F. velutipes* during fruit-body development

body formation is in good agreement with the findings reported by Kitamoto and Gruen (1976) that polyol and trehalose are synthesized in mycelia and translocated into the fruit bodies, as reported in a number of studies on the polyol metabolism in relation to fruit-body formation of mushrooms such as *Agaricus campestris* Linnaeus (Edmundowicz and Wriston 1963), *Schizophyllum commune* (Niederpruem et al. 1965), *Agaricus bisporus* (Ruffner et al. 1978), *Favolus arcularius* (Fr.) Ames, 69B/ATCC 24461 (Kitamoto et al. 1978), *Coprinus phlyctidiosporus* Romagnesi (Kitamoto et al. 1999), and *F. velutipes* (Kitamoto et al. 2000).

Taken together, this investigation provides new enzymatic evidence for the occurrence of GLOX-gluconeogenesis pathway in the mycelium, which might be supported by glutamate synthesis during the fruit-body formation of *F. velutipes*. However, further research is needed to elucidate the regulatory mechanisms at a molecular level for the fruit-body formation of basidiomycetes.

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