Characterization of acetate-requiring mutants, *ace-1* and *ace-5*, of *Neurospora crassa* with special reference to deficiency in glycerol utilization

BY Nobuo NAKAGIRI, Yasuhisa MATSUSHIRO, Toshihiro ASAHI and Homare KUWANA

Faculty of Science, Kwansei Gakuin University, Nishinomiya, Hyogo 662

(Received September 28, 1987)

ABSTRACT

Glycolytic enzyme activities of the two acetate-requiring mutant strains, *ace-1* and *ace-5*, were not so different from the wild type strain as to explain their acetate requirements. Accumulation of glycolytic intermediates coincided with these data. While the wild type strain grows well on glycerol, the growth of the *ace-1* and *ace-5* strains did not increase by adding glycerol over the level of that in a medium containing acetate. Coincidentally with the inability to use glycerol as a carbon source, these strains did not induce glycerol kinase even in the presence of glycerol. These strains, however, were able to induce this enzyme after incubation at 5°C for 10 h even in the absence of glycerol. This means that the glycerol kinase gene per se is normal, but expression of this enzyme in the presence of glycerol is impaired in these strains. Inability of the *ace-1* and *ace-5* mutants to utilize glycerol does not explain directly the acetate requirement of these strains, but there may be some relationship between them.

1. INTRODUCTION

Eight acetate-requiring mutant strains have been isolated and examined in *Neurospora crassa* (Kuwana and Okumura 1979, Kuwana and Kubota 1983). All the eight genes were mapped on eight scattered loci on all chromosomes except the sixth. Mutants of the three genes, *ace-2*, *ace-3*, and *ace-4*, were found to lack the activity of pyruvate dehydrogenase complex (Okumura and Kuwana 1979). Mutant suc (or *ace-6*) is deficient in pyruvate carboxylase activity (Beevers 1973, Kuwana and Okumura 1979), mutant *ace-7* is deficient in glucose-6-phosphate dehydrogenase activity (Nishikawa and Kuwana 1985), and mutant *ace-8* is deficient in pyruvate kinase activity (Kuwana and Kubota 1983). Enzyme lesions in *ace-1* and *ace-5* mutants have not yet been analyzed.

1) To whom correspondence should be addressed.

Following abbreviations were used for the enzyme names. HK, hexokinase; GPI, glucose-6-phosphate isomerase; PFK, phosphofructokinase; ALD, fructose-bisphosphate aldolase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; GK, glycerol kinase; G3PDH, glycerol-3-phosphate dehydrogenase.
In *Saccharomyces cerevisiae*, many mutants which do not grow on glucose or related sugars were isolated and analyzed (Lam and Marmur 1977, Clifton et al. 1978, Ciriacy and Breitenbach 1979, Lobo 1984). They are reported to lack one of the glycolytic enzymes, GPI, TPI, ALD, PGK, PGM, or PK. Multiple genes for HK, PFK, GAPDH, and ENO were also reported (Lobo and Maitra 1977, Holland et al. 1981, Clifton and Fraenkel 1982, Holland et al. 1983). In *Neurospora*, however, only GPI and PK mutants were hitherto isolated as glycolytic mutants (Murayama and Ishikawa 1975, Kuwana and Kubota 1983).

The mutants ace-i and ace-5 share some characteristics in common, although their genes are located in quite different positions on the *Neurospora* linkage map (Kuwana and Okumura 1979). They do not grow on a complex medium containing yeast extract and peptone, while other acetate mutants except ace-3 and ace-8 grow well on such a complex medium. Growth of the ace-1 and ace-5 strains on a medium containing glycerol plus acetate as carbon sources is very bad (Kuwana and Okumura 1979).

In this paper, we report some characteristics of the ace-1 and ace-5 mutants, with special emphasis on glycerol utilization.

### 2. MATERIALS AND METHODS

**Strains**

The wild type strain, KGa, is an isolate from a cross of the wild type strains Em5256A and Em5297a, which were obtained from the Fungal Genetics Stock Center, USA. The strains Y2492a (ace-1), 234A (gtp-1) and JC17-Y30539yA (gtp-2, yto-1) were also obtained from the Stock Center. The acetate-requiring strains 512a (ace-5) and 539a (ace-7) were obtained in our laboratory and have been described earlier (Kuwana and Okumura 1979). The strain gtp-2, yto-1 will be hereafter called as gtp-2 in this paper for simplicity.

**Determination of growth**

The extent of growth was expressed in mg of dry weight of mycelium obtained from 20 ml liquid medium in 100 ml Erlenmeyer flasks. Vogel's minimal salt mixture was used as the base of medium (Vogel 1964). Sugars were autoclaved separately from other ingredients. Each flask was inoculated with $2 \times 10^8$ conidia, and cultivated at 34°C for 3 days. An average of triple flasks was recorded.

**Preparation of enzyme solutions and determination of enzyme activities**

Preparation of enzyme solutions were as follows. Mycelium was obtained from aeration culture at 34°C unless otherwise stated, and was ground in a
chilled mortar with the same weight of acid-washed sand and 4 volumes of buffer. All preparations were carried out at 0-4°C.

Preparations for HK and GPI were supernatant fractions obtained after centrifugation at 180,000 × g for 25 min of the ground material prepared in 0.02M Tris-HCl buffer, pH 7.5. Preparations for PFK, ALD, TPI, GAPDH, PGK, PGM, ENO and PK were similar fractions prepared in 0.02M potassium phosphate buffer, pH 7.5, containing 0.25M sucrose and 0.15% bovine serum albumin. Preparations for GK and G3PDH were the same as described by Courtright (1975).

Enzyme activities were determined by the methods previously described by the following authors. HK, Joshi and Jagannathan (1966); GPI, Noltmann (1966); PFK, Uyeda and Kurooka (1970); GAPDH, Duggleby and Dennis (1974); PGK, Krietsch and Bucher (1970); PGM, Grisolia and Carreras (1975); ENO, Asaga and Konno (1975); and PK, Kapoor and Tronsgaard (1972). ALD and TPI were determined by following oxidation of NADH coupled with G3PDH reaction in 50 mM Tris-HCl buffer, pH 7.5. For ALD determination 2.5 mM fructose-bisphosphate, and for TPI determination 0.15 mM glyceraldehyde-3-phosphate were added to reaction mixtures as substrates. GK was determined as described by Hayashi and Lin (1967), and G3PDH by Courtright (1975).

Specific activities of enzymes were shown as μmol/min/mg protein. Protein was determined by the method of Lowry et al. (1951).

**Determination of intermedial accumulates**

Fresh mycelium was ground with chilled sand and four volumes of 5% perchloric acid, and the paste was allowed to stand for 10 minutes. Denatured proteins were removed by centrifugation, and the supernatant solution was adjusted at pH 7.0-7.5 with potassium carbonate. Metabolite concentrations in this solution were stable at least for 24 hours at 4°C. Determination of intermediates was carried out using the Bergmeyer's enzymatic method (Bergmeyer 1978). All the final determinations were coupled with absorbancy change at 340 nm due to formation or oxidation of NADH or NADPH.

**3. RESULTS**

**Growth and utilization of glycerol**

Relationships between growth and various concentrations of glucose, maltose, and glycerol were determined in the wild type, ace-1, and ace-5 strains (Fig. 1). The wild type used these carbon sources for growth well. The growth of the mutants ace-1 and ace-5, on the other hand, did not increase by adding increasing concentrations of glycerol over the basal level of the growth with acetate.
Activities of glycolytic enzymes

Glycolytic enzyme activities were determined in young mycelium (Table 1). Activities of some enzymes, e.g., PFK, GAPDH and ENO, of the ace-1 and ace-5 strains are rather weak as compared with those of the wild type. Similarly, the activities of PFK, GAPDH, ENO and PK of the ace-7 strain, which is known to be deficient in glucose-6-phosphate dehydrogenase activity, are also weaker than those of the wild type (Table 1).

Accumulation of glycolytic intermediates

Accumulations of glycolytic intermediates in the mycelia of the wild type,
ace-1, ace-5 and ace-7 strains cultivated in the medium containing 2% glucose plus 0.3% sodium acetate as carbon sources were determined. Concentrations of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate plus dihydroxyacetone phosphate, 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate in the mycelia of the wild type, ace-1 and ace-5 strains cultivated for 1 to 4 days did not differ significantly among strains. The ace-7 strain accumulated higher concentrations of 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate as compared with the wild type strain. The ace-1 and ace-5 strains also accumulated higher concentration of pyruvate in early stages of culture (Table 2). The accumulation of pyruvate in the mycelia of the wild type and ace-1 strains cultivated with the medium containing 1% sodium acetate as a sole carbon source also showed a similar result (Table 2).

Enzymes of glycerol metabolism

The activities of GK and G3PDH were determined and are shown in Fig. 2 and Table 3, respectively. The ace-1, ace-5 and glp-1 mutants showed weak GK activity, and this seems to be the cause of the inability of the ace-1 and ace-5 strains to grow with glycerol as in the glp-1 strain. The wild type and ace-7 strains showed an high activity peak of GK (Fig. 2). The possibility of existence of an activator of GK in the wild type cells and that of an inhibitor in the ace-1 and ace-5 cells was neglected, because the sum of the GK activities of both the wild type and ace-1 or ace-5 strains was not changed when both extracts were mixed. The G3PDH activities of the ace-1 and ace-5
strains were in between those of the wild type and glp-1 strains, while the glp-2 strain completely lacks the activity (Table 3).

While both ace-1 and ace-5 strains show little GK activity during the culture of several days (Fig. 2), they can induce the enzyme activity by the cold treatment, in which mycelium was cultivated for the first 2 days at 34°C and then transferred to 5°C (Fig. 3). The GK activity of the ace-1 and ace-5 strains, as well as the wild type, increased after 5 to 10 hours in the cold. The glp-1 strain, however, did not show the GK activity as long as 25 h after the temperature shift (Fig. 3).

Table 3. Activity of glycerol-3-phosphate dehydrogenase (flavin) in various strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific activity (μmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>0.47</td>
</tr>
<tr>
<td>ace-1</td>
<td>0.25</td>
</tr>
<tr>
<td>ace-5</td>
<td>0.27</td>
</tr>
<tr>
<td>ace-7</td>
<td>0.51</td>
</tr>
<tr>
<td>glp-1</td>
<td>0.16</td>
</tr>
<tr>
<td>glp-2</td>
<td>ND</td>
</tr>
</tbody>
</table>

Mycelia were cultivated in the medium containing 2% glycerol and 0.3% sodium acetate as carbon sources for 2 days. ND means that the activity was not detected.
The ace-1 and ace-5 mutants of Neurospora

Accumulation of sn-glycerol-3-phosphate by the ace-1 and ace-5 strains was comparable to that of the glp-i mutant, while that by glp-2 mutant was much more (Table 4), consistent with the enzyme activity data of Table 3.

4. DISCUSSION

Among eight acetate-requiring mutants of Neurospora crassa, ace-1 and ace-5 are the only two which have not yet been characterized in terms of enzymatic deficiency. In this study, growth characteristics, enzyme activities and intermedial accumulations of these two mutants were investigated. Although activities of some of the glycolytic enzymes of the ace-1 and ace-5

Fig. 3. Induction of glycerol kinase activity after the culture temperature was shifted from 34°C to 5°C. Medium contains 2% sucrose and 0.3% sodium acetate as carbon sources. ○, wild type; △, ace-1; □, ace-5; ●, glp-1.

Table 4. Accumulation of sn-glycerol-3-phosphate in mycelia of various strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration (μmol/g dry mycelium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>0.7</td>
</tr>
<tr>
<td>ace-1</td>
<td>1.2</td>
</tr>
<tr>
<td>ace-5</td>
<td>0.9</td>
</tr>
<tr>
<td>glp-1</td>
<td>2.0</td>
</tr>
<tr>
<td>glp-2</td>
<td>17.7</td>
</tr>
</tbody>
</table>

Mycelia were cultivated in the medium containing 2% glycerol and 0.3% sodium acetate as carbon sources for 3 days.

Accumulation of sn-glycerol-3-phosphate by the ace-1 and ace-5 strains was comparable to that of the glp-1 mutant, while that by glp-2 mutant was much more (Table 4), consistent with the enzyme activity data of Table 3.

4. DISCUSSION

Among eight acetate-requiring mutants of Neurospora crassa, ace-1 and ace-5 are the only two which have not yet been characterized in terms of enzymatic deficiency. In this study, growth characteristics, enzyme activities and intermedial accumulations of these two mutants were investigated.

Although activities of some of the glycolytic enzymes of the ace-1 and ace-5
strains are lower than those of the wild type strain, all the enzyme activities of the ace-1 and ace-5 strains are larger than one fourth of those of the wild type strain (Table 1). The fact that all the activities of glycolytic enzymes exist in vitro does not necessarily show that these enzymes are actually acting in vivo. Some enzyme or isozyme which has the physiological importance in vivo might not be operative in vivo condition. In this case, amounts of intermedial accumulates will reflect the abnormality of the metabolic flux. No significant difference in accumulation of glycolytic intermediates was observed among the wild type, ace-i and ace-5 strains, except accumulation of pyruvate in early period of cultivation (Table 2). The wild type shows the same tendency to accumulate pyruvate in a small quantity at a very early stage of culture period. The flux of glycolysis in the ace-i and ace-5 strains does not seem to differ greatly from the wild type.

Various glucose-nonutilizing mutant strains which lack glycolytic enzymes were isolated in Saccharomyces cerevisiae, and these strains accumulate corresponding intermediates before the impaired step (Ciriacy and Breitenbach 1979). It seems to be improbable from the present data of enzyme activities and intermedial accumulations that the acetate requirement of the strains ace-i and ace-5 was caused from the impaired glycolytic enzymes.

If one of the enzymes of the tricarboxylic acid cycle between isocitrate and malate is lacking, the cycle stops and sufficient ATP for growth is not produced. But when acetate was supplied, the glyoxylyte cycle enzymes would be induced (Flavell and Woodward 1970), and a new cycling could operate to produce ATP. Some acetate-requiring mutants of Neurospora might be this case. So, activities of the tricarboxylic acid cycle enzymes between isocitrate and malate, that is, NAD-linked and NADP-linked isocitrate dehydrogenases, oxoglutarate dehydrogenase complex, succinyl-CoA synthetase (ADP-forming), succinate dehydrogenase and fumarate hydratase, were determined, but no case of deficiency of activity was found (data not shown).

The growth of the ace-i and ace-5 strains in the medium containing glycerol in addition to acetate did not exceed the level of that in the medium containing only acetate as a carbon source (Fig. 1). The same was true of the medium containing 0.5% maltose and 0.3% acetate as a base. The growth of ace-i and ace-5 in this medium did not increase by adding glycerol, while that of the wild type increased to the same extent by the addition of glucose or glycerol.

In Neurospora, glycerol non-utilizing mutants are already known. The strain glp-i lacks inducible GK activity (Holm et al. 1976, Nilheden et al. 1975), and glp-2 lacks G3PDH activity (Denor and Courtright 1978). This means that the pathway through GK and G3PDH is the physiological one in which glycerol is metabolized in vivo. The GK activity of the ace-i and ace-5 strains was as weak as the glp-i strain (Fig. 2), and this weak activity seems to cause
The ace-1 and ace-5 mutants of Neurospora

The glycerol non-utilization in the ace-1 and ace-5 strains. G3PDH activity of the ace-1, ace-5 and glp-1 strains was a little weak when compared with that of the wild type and ace-7 strains (Table 3). Accumulation data for sn-glycerol-3-phosphate (Table 4), however, shows that the glp-2 strain is defective in G3PDH step, while glp-1, ace-1 and ace-5 strains are rather normal in this step. So, glycerol non-utilization in the ace-1, ace-5 and glp-1 strains may be caused by the defective GK step.

Two kinds of GK were reported in Neurospora, inducible and constitutive (North 1974, Holm et al. 1976). The low basic activity of GK in the ace-1, ace-5 and glp-1 strains may be due to constitutive type (Fig. 2). That is, these three strains cannot induce GK even in the presence of glycerol. The ace-1 and ace-5 strains and the glp-1 strain, however, are distinctly different, because the former two require acetate for growth, and all three gene locations are quite different.

In Neurospora, GK is induced even in the absence of glycerol, after cultivation of the wild type strain in sucrose minimal medium at around 5°C (North 1973, 1974). The two acetate mutants, ace-1 and ace-5, induced GK after transfer to 5°C, while the medium without glycerol was not changed (Fig. 3). The glp-1 strain, on the other hand, did not induce GK after transfer to 5°C (Fig. 3). The acetate mutants, ace-1 and ace-5, have the genetic capability to produce GK, but are defective in induction of the enzyme by glycerol. The relationship between this defect and the acetate requirement of these strains remains to be determined.

The authors wish to thank Mr. T. Kawamura for his technical assistance in some of the experiments.

REFERENCES


