In *Saccharomyces cerevisiae* deletion of phosphoglucone isomerase can be suppressed by increased activities of enzymes of the hexose monophosphate pathway

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*Saccharomyces cerevisiae* mutants defective in the structural gene *PGI1* lack phosphoglucone isomerase and hence cannot grow on glucose. Spontaneous mutants were isolated by selecting for the regained ability to grow on YEPD (yeast extract/peptone/glucose). Three complementation groups called *spg29-31* (suppressor of *pgi1A*) were identified. The metabolism of [2-13C]glucose was studied by 13C NMR spectroscopy. This led to the conclusion that in a *spg29* mutant suppression of the glycolytic defect was achieved by increased carbon flux through the hexose monophosphate pathway. The specific activities of enzymes of the hexose monophosphate pathway (except glucose-6-phosphate dehydrogenase) and NAD- and NADP-dependent glutamate dehydrogenase were increased in the bypass mutant.

Keywords: *Saccharomyces cerevisiae*, *pgi1A* suppressor mutations, hexose monophosphate pathway, 13C NMR

INTRODUCTION

Phosphoglucone isomerase is the enzyme which catalyses the interconversion of glucose 6-phosphate and fructose 6-phosphate. Thus, it is both the second step of the glycolytic sequence and the last step of gluconeogenesis. Mutants of *Saccharomyces cerevisiae* defective in the structural gene (*PGI1*) for phosphoglucone isomerase have been described by many authors (Maitra, 1971; Herrera & Pascual, 1978; Clifton et al., 1978; Ciriacy & Breitenbach, 1979; Aguilera, 1986). All such *pgi1* mutants cannot grow on glucose, the assumption being that in *S. cerevisiae* flux through the hexose monophosphate pathway is inadequate to support growth. It is reckoned that the hexose monophosphate pathway is only capable of 8% of the carbon flux that occurs during growth on glucose (Bruinenberg et al., 1986). In this respect *S. cerevisiae* is different from both *Escherichia coli* and *Kluyveromyces lactis*, in which mutants lacking phosphoglucone isomerase can still grow on glucose (Vinopal et al., 1975; Goffrini et al., 1991).

One way of trying to understand all aspects of the phenotype of *pgi1* mutants has been the isolation of extragenic suppressor mutations which confer upon *pgi1* mutants the ability to grow on glucose. Aguilera (1987) described *spg1* (suppressor of *pgi1A*) mutations which restored growth on glucose and resulted in obligately high levels of mitochondrial respiration and no ethanol formation. Gamo et al. (1993) described *rgl1* and *rgl2* (resistance to glucose) mutations from which it was also presumed that glucose may be channeled through the hexose monophosphate pathway to respiration. Recently, Boles et al. (1993) showed that overexpression of *GDH2*, which encodes the NAD-dependent glutamate dehydrogenase, can suppress the growth defect on glucose caused by *pgi1A* mutations. The explanation for this is that overexpression of NAD-dependent glutamate dehydrogenase causes a cycle of metabolic interconversion between 2-oxoglutarate and glutamate in which the anabolic reaction results in the conversion of 2-oxoglutarate to glutamate with concomitant conversion of NADPH to NADP, whilst the catabolic reaction results in the formation of 2-oxoglutarate from glutamate with conversion of NAD to NADH. Hence, the rapid depletion of NADP which would otherwise result from overuse of glucose-6-phosphate dehydrogenase (if flux via the hexose monophosphate pathway was increased in *pgi1A* bypass mutants) is avoided and the extra NADH which is formed can be oxidized by the electron transport chain. This also explains the high obligatory respiration observed in *pgi1* bypass mutants.

Boles et al. (1993) also showed that deletion of the *ZWF1*
Fig. 1. The pathways of catabolism of glucose in *S. cerevisiae*. The figure is redrawn from a version by Fraenkel (1982). The structures of all of the intermediates and products discussed in the text are given. Only the β anomer of the substrate D-glucose is drawn. The 13C label at C-2 of glucose is marked by (●). Enzymes which are specifically mentioned are identified by three-letter acronyms as follows: ZWF, glucose-6-phosphate dehydrogenase; PGI, phosphoglucose isomerase; GND, 6-phosphogluconate dehydrogenase; RPE, ribulose-5-phosphate 3-epimerase; TAL, transaldolase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; PDH, pyruvate dehydrogenase; GDH, glutamate dehydrogenase. Irreversible reactions are denoted by single-headed arrows. The presence of two arrows indicates a separate enzyme for each direction of a reaction. Reversible reactions are represented by bi-directional arrows (except, for simplicity, the four reversible reactions ‘below’ glyceraldehyde-3-phosphate).
gene (which encodes glucose-6-phosphate dehydrogenase) blocks suppression of a pgilA mutation. This is also highly indicative of the importance of the hexose monophosphate pathway, but it must be stressed that, to date, none of the studies have proved that the suppression of a pgil mutation actually involves a metabolic bypass in which glucose is catabolized by means of increased flux via the hexose monophosphate pathway; i.e. the flow of carbon from substrate to product(s) has not been demonstrated. One could postulate three possible metabolic routes. Firstly, increased flux via the hexose monophosphate pathway. Secondly, use of the glycolytic sequence involving an isoenzyme of phosphogluconic isomerase encoded by a gene which is normally cryptic. This is not without precedent in S. cerevisiae: for example ADH4 (encoding an isoenzyme of ethanol dehydrogenase) was discovered in adbl adh2 adh3 triple mutants (Paquin & Williamson, 1986). Thirdly, the use of a hitherto-unknown pathway of glucose catabolism. This concept has also been suggested previously to explain the bypass of pfk1 pfk2 double mutants which lack phosphofructokinase (Breitenbach-Schmitt et al., 1984).

13C nuclear magnetic resonance (NMR) spectroscopy is a very good technique for metabolic studies (London, 1988; Davies & Brindle, 1992); hence it was decided to use 13C-labelled glucose to analyse the patterns of metabolism by S. cerevisiae: for example ADH4 (encoding an isoenzyme of ethanol dehydrogenase) was discovered in adbl adh2 adh3 triple mutants (Paquin & Williamson, 1986). Thirdly, the use of a hitherto-unknown pathway of glucose catabolism. This concept has also been suggested previously to explain the bypass of pfk1 pfk2 double mutants which lack phosphofructokinase (Breitenbach-Schmitt et al., 1984).

METHODS

Media, strains and genetic methods. Cultural conditions and complex growth media have been described previously (Dickinson, 1991). Complex media contained, per litre: yeast extract (10 g), bacteriological peptone (20 g), adenine (0.1 g), uracil (0.1 g) and the specified carbon source (YPEP, 20 g d-fructose). Minimal medium with fructose (2%, w/v) as major carbon source was supplemented with 0.1% (w/v) glucose. Standard genetic techniques were used for mating, sporulation and dissection (Sherman, 1975; Mortimer & Hawthorne, 1975). S. cerevisiae strain AAG2 (MATa ura3 his4 can pgil125::LEU2) was obtained from A. Aguiera (University of Seville, Spain). Strain S9 (MATa ura3 his4 can pgil125::LEU2 spg29-1) was derived by crossing E9 (obtained as a spontaneous revertant of AAG2 as described below) to strain 329 (MATa ade8) and repeated backcrossing of appropriate MATa segregants to AAG2. Strain OH1 (MATa ura3 his4 can) is a wild-type (i.e. PG11) haploid derived from a cross between 329 and AAG2. Strains 26.1 A (MATa ade8 ade2 his7 pgil-1) and 26.6B (MATa ade2 his7 pgil-1) were constructed from mutant 9520b/TIC (MATa pgil-1) (Maitra, 1971), which was provided by P. K. Maitra (Tata Institute of Fundamental Research, Bombay, India).

Isolation of mutants carrying suppressors of pgil1A mutations. A 100 ml batch of strain AAG2 was grown overnight in YEPF to OD$_{600}$ 1.00. The cells were then harvested by
centrifugation and resuspended in 100 ml YEPD. The culture was divided into 10 separate aliquots, each of which was incubated in a 100 ml conical flask at 30 °C for 6 d. After this time 0·1 ml samples were removed from each small culture and plated directly onto YEPD. The YEPD plates were inspected after 3, 5 and 7 d. Colonies which had appeared at each time and were large enough to handle were transferred to YEPF, incubated for 4–5 d and then replica-plated to YEPF and YEPD. Clones which were able to grow well on YEPD when re-tested were retained for further analysis.

NMR analyses. For in vivo NMR analysis cells of S9 were grown in YEPD to OD_600_4·0 (late exponential phase for this strain), harvested by centrifugation, resuspended in 2 ml 50 mM potassium phosphate buffer pH 6·0 containing 30% (v/v) D_2O and transferred to a 10 mm NMR tube with a gassing system which provides both aeration and mixing (described by Lloyd et al., 1993). Then 70 mg [2,13C]glucose (99 atom % enrichment) was added and 13C data were collected in blocks of 500 accumulations (each of 8K data points over 22000 Hz) with an acquisition time of 0·15 s and a 1 s delay between pulses using a Bruker WM360 spectrometer operating at 90·5 MHz. High-power 1H-decoupling was used during acquisition, with low-power decoupling during delays; preliminary experiments had determined the correct settings to effect proton decoupling with sufficient dielectric heating to maintain a temperature of 30 °C ± 1 °C.

Metabolic analyses were also done on cells which had been grown to OD_600_4·0 in YEPD, harvested by centrifugation and resuspended in YEP containing [2-13C]glucose. After 150 min incubation at 30 °C cells were harvested and perchloric acid extracts prepared for 13C NMR analysis as described previously (Dickinson & Hewlins, 1988, 1991). The spectrum was recorded for the solution in a 5 mm NMR tube, using 32K data points over 22000 Hz, with broad-band 1H-decoupling, and also by the DEPT method to determine the number of protons attached to each carbon signal. A two-dimensional 13C–1H chemical shift correlation spectrum was carried out using the XHCOORD routine in the standard Bruker NMR software. All chemical shifts are reported in p.p.m. relative to the signals (1H and 13C as appropriate) from sodium 3-(trimethylsilyl)propane-1-sulphonate (assigned δ = 0 for both nuclei) measured in D_2O solution as the external standard.

Enzyme assays. Cells were harvested by centrifugation, resuspended in 50 mM potassium phosphate buffer pH 7·4 containing 2 mM EDTA and 2 mM 2-mercaptoethanol and disrupted using a Braun homogenizer as described by Dickinson & Williams (1986). Aliquots of this homogenate were used immediately as the source of enzyme. Phosphoglucone isomerase (EC 5.3.1.9), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glucuronate-6-phosphate dehydrogenase (EC 1.1.1.43) were assayed as described by Maitra & Lobo (1971). Ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) was assayed according to Williamson & Wood (1966). Transaldolase (EC 2.2.1.2) was assayed by the method of Tchola & Horecker (1966). NAD- and NADP-dependent glutamate dehydrogenases (EC 1.4.1.2 and 1.4.1.4) were both assayed in 0·1 M imidazole buffer pH 7·9 in a total volume of 1·8 ml. NADP-dependent glutamate dehydrogenase was assayed by following NADPH disappearance when 2-oxoglutarate (22·8 μmol) was converted to glutamate in the presence of ammonium acetate (614 μmol) using 0·03 μmol NADPH. NAD-dependent glutamate dehydrogenase was monitored by following the increase in absorbance at 340 nm due to NADH formation when glutamate (25 μmol) was converted to 2-oxoglutarate using 0·375 μmol NAD.

RESULTS

Isolation and genetic characterization of mutants carrying suppressors of pgi1Δ mutations

The spontaneous mutants which arose on YEPD after 3 d were called 'early mutants' and numbered E1–E33. The 'mid mutants' collected after 5 d were numbered M1–M14 and the 'late mutants' were numbered L1–L29. There was no correlation with the original time of appearance of an individual mutant and its rate of proliferation when examined on YEPD plates or in YEPD liquid medium: i.e. there was a range of generation times amongst all three sets of mutants. However, all of the 'mid mutants' subsequently died over the next 4 weeks (irrespective of whether they were stored on YEPD or YEPF) before they had been characterized genetically. Nevertheless, the frequency of suppressor mutations would seem to be 10⁻⁵–10⁻⁸. Sixteen of the remaining mutants which grew most rapidly on YEPD were mated on YEPF to strain 26.1A. The diploids which formed were selected on fructose minimal medium supplemented with 0·1% glucose. These diploids were then replicated onto YEPD: none of them grew on YEPD, showing that the suppressor mutations were all recessive. A series of outcrosses to suitable wild-type haploids allowed a subsequent complementation analysis, which indicated the existence of at least three distinct spg mutations. It seemed prudent to retain the terminology of Aguilera (1987) for such mutants rather than add a further acronym to an already crowded literature of phenotypes. However, to avoid the possibility of a future overlap of mutants from our different laboratories it was decided to call these three mutations spg29 to spg31. It was not possible to

Fig. 2. Typical growth curves for S9 (pgi1Δ spg29-1; •) and wild-type haploid OH1 (PG11; ○) in YEPD liquid medium. Both strains were inoculated into fresh pre-warmed medium from starter cultures grown to exponential phase in YEPD.
definitely assign all of the mutants, mainly due to their different rates of proliferation on YEPD prior to mating. The spg29 mutation was represented by three alleles all resulting in very similar phenotypes, the most prominent of which was the most rapid growth (generation time of 335 min in exponential phase) in glucose (Fig. 2).

**Fig. 3.** Time course of utilization of [2-13C]glucose by strain S9. In vivo time-elapsed proton decoupled NMR spectra are shown. α, C-2 of α-D-glucose; β, C-2 of β-D-glucose; T, C-2 of trehalose; G, C-1,3 glycerol.

**Fig. 4.** 13C NMR spectrum of a perchloric acid extract of strain S9 made after growth in YEP-[2-13C]glucose. E, C-2 of ethanol; 4, C-4 of glutamate; 3, C-3 of glutamate; 2, C-2 of glutamate; N, C-4 of glutamine; other resonances marked are as in Fig. 3.

13C NMR analysis of metabolism in a pgi1Δ spg29 mutant

In vivo NMR analysis of strain S9 showed that the major metabolic product derived from [2-13C]glucose was glycerol labelled at C-1 and C-3 (Fig. 3). This signal corresponding to glycerol (65.3 p.p.m.) increased with time as did the resonance corresponding to C-2 of the storage compound trehalose (75.7 p.p.m.). Three other resonances can be seen in Fig. 3 (78.6 p.p.m., 72.4 p.p.m. and 63.5 p.p.m.); they are explained below. The in vivo experiment was not run for longer because although it could be seen (from comparison of the peak heights of the signals due to C-2 of glucose) that very little glucose had been metabolized, the experiment had served its purpose, i.e. identification of the metabolic pathway by which the glucose was catabolized. Detailed isotopomer analysis is always better done on perchloric acid extracts because field inhomogeneity within the sample of in vivo experiments leads to signal broadness.

One possible criticism of the in vivo NMR study above is that the cells are in a non-growing situation because no nitrogen source was provided, although other workers actually strive to devise conditions of rigorous non-growth for metabolic analysis (e.g. Benevolensky et al., 1994). However, in this case the criticism would seem to be irrelevant because analysis of an extract made from cells grown in YEP-[2-13C]glucose gave a similar result (Fig. 4). In addition, the expanded spectrum allows the identification of resonances at 18.9 p.p.m. (ethanol C-2), 29.4 p.p.m. (glutamate C-3), 35.6 p.p.m. (glutamate C-4) and 57.3 p.p.m. (glutamate C-2). Although small, the signal due to ethanol C-2 is highly significant because it confirms that 13C in this molecule has been derived via the hexose monophosphate pathway (as explained in the Introduction). The fact that the intensity of the signal for glutamate C-4 is significantly greater than that for C-3, which in turn is much greater than the intensity of the glutamate C-2 resonance, is also important because these data indicate that glutamate is being labelled as predicted for metabolism of [2-13C]glucose around the hexose monophosphate pathway to yield pyruvate labelled at C-3 and C-1. This is decarboxylated to [2-13C]acetate, which then enters the tricarboxylic acid cycle. Indeed, it is just possible to observe the appearance of label in the C-4 position of glutamate first, followed by label at C-3 and C-2, in the time-elapsed spectra in Fig. 3. The resonance at 34.6 p.p.m. is due to C-4 of glutamine formed directly from glutamate. The multiplets at 98.6 p.p.m. and 94.8 p.p.m. are respectively C-1 of β-D-glucose and α-D-glucose which are visible mainly due to natural abundance of 13C at this position. The singlet (centre line) resonance in each case corresponds to glucose labelled only at C-1; the doublets are due to interaction with the heavily labelled C-2. Similarly, the resonance at 63.5 p.p.m. seen here and noted in Fig. 3 along with the resonances at 78.6 p.p.m. and 72.4 p.p.m. are due to glucose C-6, C-3 and C-4 respectively; all were observed due to natural abundance.

Initial signal assignment was made by comparison of 13C chemical shifts with those of standard compounds recorded under comparable conditions. Additional information was obtained from further NMR experiments carried out on the perchloric acid extract. The DEPT method was used to establish the number of protons attached to each 13C nucleus and this confirmed CH or CH₂ for each signal in accordance with the assignments.
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The largest increase recorded for a single enzyme was for
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of the depletion of an important intermediate of the
tricarboxylic acid cycle.

In a study of carbon metabolism during sporulation in
acetate it was noted that at a distinct time in the process
metabolic flux around the hexose monophosphate path-
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Table 1. Specific activities of various enzymes in strains AAG2 (pgi1A) and S9 (pgi1A spg29)

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>Specific activity [mU (mg protein)]</th>
<th>Activity in suppressed strain relative to parent (%)</th>
</tr>
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<tbody>
<tr>
<td>G6PDH</td>
<td>2065</td>
<td>94:1</td>
</tr>
<tr>
<td>GND</td>
<td>819</td>
<td>169:1</td>
</tr>
<tr>
<td>RPE</td>
<td>10</td>
<td>2:1</td>
</tr>
<tr>
<td>TAL</td>
<td>744</td>
<td>117:6</td>
</tr>
<tr>
<td>NAD-GDH</td>
<td>244</td>
<td>57:2</td>
</tr>
<tr>
<td>NADP-GDH</td>
<td>427</td>
<td>86:3</td>
</tr>
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</table>

*G6PDH, glucose-6-phosphate dehydrogenase; GND, 6-phosphogluconate dehydrogenase; RPE, ribulose-5-phosphate 3-epi-
merase; TAL, transaldolase; NAD-GDH, NAD-dependent glutamate dehydrogenase; NADP-GDH, NADP-dependent glutamate
dehydrogenase.

given above. Further information was obtained from the
13C-1H correlation spectrum. In particular this confirmed
that the 13C at 65.3 is attached to protons at δH 3.47
and 3.55, as expected for glycerol. The carbon signals attributed
to ethanol C-2, glutamate C-4, glutamine C-4 and
β-D-glucose C-1 were shown to be connected to protons
having chemical shifts of 1-3, 2-33, 2-45 and 4-6
respectively in accord with standard values.

Enzyme assays on the pgilA parent and the
pgilA spg29 suppressed double mutant

Phosphoglucone isomerase was undetectable in the
parental strain AAG2 and in strain S9, confirming that both
strains carry the pgilA mutation and that suppression in S9
was not due to the regaining of this activity. The specific
activity of glucose-6-phosphate dehydrogenase was virtu-
ally identical in both strains, but the specific activities of
all of the other enzymes assayed were all increased in the
bypass mutant to 150-235% of the levels present in the
parental strain (Table 1). The greatest increase in specific
activity occurred in the case of NAD-dependent glutamate
dehydrogenase.

DISCUSSION

Spontaneous mutants were isolated carrying mutations
which suppress the pgilA mutation, thus allowing growth
on glucose. In many respects these newly isolated spg
mutants seem similar to those described by Aguilera
(1987) and also the regl mutants of Gamo et al. (1993). The
reason for isolating these mutants was to establish the
metabolic pathway used which enables a pgilA suppressed
mutant to utilize glucose. 13C NMR studies clearly showed
that the hexose monophosphate pathway was used to
suppress the defect in phosphoglucone isomerase. Despite
the fact that previous studies all suggested the hexose
monophosphate pathway was used in suppressed pgil
strains (Aguilera, 1987; Gamo et al., 1993; Boles et al.,
1993), this is the first time that the flux of carbon has
actually been proved.

The specific activities of enzymes of the hexose mono-
phosphate pathway were increased in the suppressed
strain compared with the parental pgilA strain with the
exception of glucose-6-phosphate dehydrogenase. These
results serve to confirm the NMR study and lead to the
conclusion that glucose-6-phosphate dehydrogenase is
not the limiting step of the metabolic bypass. Aguilera
(1987) reported no significant differences in specific
activities between pgilA and pgilA spg1 double mutants.
He also reported the specific activity of 6-phospho-
gluconate dehydrogenase as lower in the suppressed
strains than in thepgilA parents whereas we observed that
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haps this up-regulation in sporulating diploids involves a gene which is mutated in spg mutants. Cloning of SPG genes will allow an examination whether the same genes are involved in controlling expression of hexose monophosphate pathway functions in sporulation and glycolytic bypass mutants.

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