Enzymatic and Metabolic Studies on Retrograde Regulation Mutants of Yeast†

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ABSTRACT: Two nuclear genes, *RTG1* and *RTG2*, which sense the functional state of yeast mitochondria, have been described recently. Yeast strains with null alleles of either of these two genes (Δ*rtg1*, Δ*rtg2*) cannot grow on acetate as the sole carbon source and are auxotrophic for glutamate and aspartate. We report here a series of metabolic experiments and enzyme activity measurements that were made in an attempt to determine the reason for the acetate$^-$ phenotype and the glutamate/aspartate auxotrophy. Decreases in the activities (~50%) in mitochondrial citrate synthase (CS1), acetyl-CoA synthetase, NAD isocitrate dehydrogenase, and pyruvate carboxylase were noted. When CS1 was overexpressed in the Δ*rtg1* and Δ*rtg2* mutants, these strains could grow on acetate but were still auxotrophic for glutamate/aspartate. We propose that, in the mutant strain, CS1 activity becomes limiting for efficient acetate utilization, but that other complex metabolic interactions are affected, limiting production of intermediates that would allow synthesis of glutamic and aspartic acids.

Liao and Butow (1993) have recently described two nuclear genes, *RTG1* and *RTG2*, the expression of which is influenced by the functional state of mitochondria. This mechanism has been called retrograde communication. It had previously been shown that in *Saccharomyces cerevesiae* ρ° petite mutants (which lack mitochondrial DNA), in cells with no CS1,[1] or in wild-type (ρ$^+$) cells grown in the presence of the respiratory cleavage inhibitor antimycin A there is a large increase in the expression of the *CIT2* gene product, CS2 (Liao & Butow, 1993). CS2 functions as part of the glyoxylate cycle in yeast peroxisomes. Liao and Butow (1993) identified an activating sequence within a 76-bp region upstream of the *CIT2* gene which is critical for transcriptional control of *CIT2* retrograde expression. *RTG1* and *RTG2* were cloned and characterized. The deduced product of the *RTG1* gene has been shown to be a member of the basic helix−loop−helix family of transcription factors. The function of the protein encoded by the *RTG2* gene is not currently known. However, a recent report shows that the *RTG2*-encoded protein has an ATP binding domain with similarity to bacterial phosphatases that hydrolyze the transcriptional regulators ppGpp and pppGpp (Koonin, 1994).

While *RTG1* and *RTG2* are required for both basal and retrograde expression of *CIT2* expression, two unexpected collateral phenotypes of the Δ*rtg1* and Δ*rtg2* strains were observed: an inability to grow in acetate and auxotrophy for glutamate/aspartate. The inability to grow on acetate is a common phenotype of cells which lack a Krebs TCA cycle enzyme. In an effort to locate the metabolic lesion underlying the growth phenotype of Δ*rtg1* and Δ*rtg2* cells, we have assayed the enzymes of the Krebs TCA cycle, the glyoxylate cycle, acetate activation, mitochondrial metabolite transport, mitochondrial respiration, and ^{13}C-labeled substrate utilization and the growth patterns of the mutant cells on a variety of carbon sources. Among the enzyme activities tested, the only missing enzyme was CS2, confirming the requirement of *RTG1* and *RTG2* for *CIT2* expression (Liao & Butow, 1993). However, we have shown previously that CS2$^-$ yeast cells have a growth phenotype that is indistinguishable from that of the parental cell (Kispal *et al.*, 1994). Thus, the lack of growth on acetate cannot be attributed to a lack of CS2 alone. We show here that 50% reductions in CS1, acetyl-CoA synthetase, NAD isocitrate dehydrogenase, and pyruvate carboxylase activities are the only major abnormalities in the mutant cells. We also show that overexpression of CS1 (>100-fold) in Δ*rtg1* and Δ*rtg2* results in a change in the acetate$^-$ phenotype with a very slow rate of growth on acetate, but with no change in glutamate/aspartate auxotrophy. We discuss the possibility that these phenotypes for the Δ*rtg* mutant cells result from the cumulative effect of many small changes in the enzyme composition of the cell.

MATERIALS AND METHODS

The parental yeast strain COP161 U7 (*MAT a, ade1, lys1, ura3*) and its three derivatives containing disruption mutations in *RTG1* (Δ*rtg1*), *RTG2* (Δ*rtg2*), or both (Δ*rtg1*Δ*rtg2*) have been described (Liao & Butow, 1993). Subcellular fractionation of yeast cells was based on the procedure of Daum *et al.* (1982) as modified by Rosenkrantz *et al.* (1986).

Enzyme and Transport Assays. References for the enzyme assays are as follows: CS, Srere (1969); malate dehydrogenase, Kitto (1969); fumarase, Hill and Bradshaw (1969); succinate dehydrogenase, Veeger *et al.* (1969); αKGDC,

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[1] Abbreviations: CS1, mitochondrial citrate synthase; CS2, peroxisomal citrate synthase; PDC, pyruvate dehydrogenase complex; αKGDC, α-ketoglutarate dehydrogenase complex; RCR, respiratory control ratio.

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Porpaczy *et al.* (1983); aconitase, Plank and Howard (1988); isocitrate lyase, Daron and Gunsalus (1962); malate synthase, Dixon and Kornberg (1962); and carnitine acetyltransferase, Fritz *et al.* (1963). Isocitrate dehydrogenase was assayed by a modification of the procedure of Cook and Sanwal (1969). PDC was assayed largely as described previously (Sumegi & Alkonyi, 1983) except cysteine was used as a reducing agent, and BSA (5 mg/mL) was added to stabilize the protein. Acetyl-CoA synthetase was assayed by a modification of the coupled enzyme method described by Bergmeyer and Möllering (1974) for measurement of acetate. Succinate thiokinase was assayed by measuring the formation of succinyl-CoA from succinate and CoA in the presence of ATP and magnesium (W. A. Bridger, personal communication). Citrate transport was measured by the procedure of Grigorenko *et al.* (1990) except that only 1,2,3-benzenetricarboxylic acid (BTC), a specific inhibitor of citrate transporter, was used to stop the reaction. Transport was calculated as BTC-insensitive uptake of [^{14}C]citrate by intact mitochondria.

Respiration Measurements. Oxygen consumption was determined using the polarographic assay as described by Estabrook (1967). Briefly, a Clark electrode coupled to a biological oxygen monitor (YSI Scientific, Yellow Springs, OH) was immersed in a 0.6-mL chamber containing 0.6 M mannitol and 1 mg/mL bovine serum albumin and buffered with 20 mM KPO_4 (pH 6.8). Substrates including NADH, α-ketoglutarate, citrate, pyruvate/malate, succinate, and isocitrate were used to measure the respiratory activity of intact isolated mitochondria. State 4 versus state 3 respiration was monitored by observing the rate of oxygen consumption before and after the introduction of ADP into the reaction vessel.

[2-^{14}C]Acetate Oxidation. Nondividing or nonmitotic conditions in phosphate buffer were used with a constant number of cells. The precultures of cells were grown on selective media with 2% glucose for 12 h and transferred into 500 mL of YP with 2% galactose for 24 h. Cells (25 mg wet weight) were incubated in 50 mM potassium phosphate buffer (pH 6.5) for 5 min at 30 °C in rubber-stoppered 50-mL vessels. The reaction was started by adding 5 mM [2-^{14}C]acetate with 40 000 cpm/μmol specific activity and stopped by adding perchloric acid (0.2 N final concentration), and the $^{14}CO_2$ produced during 2 h more shaking at room temperature was trapped in small plastic vials containing 100 μL of 1 N KOH. Values are averages of triplicate measurements.

Growth Method. For all experiments start cultures (10 mL) contained 1% YP and 2.5% raffinose in 50-mL Erlenmeyer flasks. Raffinose is a carbon source which does not repress tricarboxylic acid cycle enzyme expression. Synthetic medium contained 0.50% ammonium sulfate; 0.17% YNB without amino acids and ammonium sulfate; 20 mg/L each of adenine sulfate, L-arginine HCl, L-methionine, and L-tryptophan; 30 mg/L each of L-isoleucine, L-lysine HCl, and L-tyrosine; 50 mg/L of L-phenylalanine; 150 mg/L of L-valine; and 2% glucose. For growth of the parental cells, synthetic medium contained 60 mg/L L-leucine and 20 mg/L uracil; for mutant strains (*Δrtg1*, *Δrtg2*, *Δrtg1Δrtg2*) only leucine was added to the synthetic medium. Start cultures were grown for 24 h. Three milliliters of these cells was added to the growth media containing different carbon sources, either 2% raffinose, 2% of an EtOH–glycerol mixture (20% EtOH, 30% glycerol), or 1% sodium acetate or potassium acetate (pH 5.5). Start cultures for the acetate experiment contained 1% potassium acetate and 2% glucose. Cells were grown in 125-mL side-arm flasks in 25 or 50 mL of medium, and growth was measured at 540 nm with a Klett colorimeter.

^{13}C NMR Spectroscopy. Packed, washed yeast cells (2 g) were suspended in 10 mL of minimal medium (Difco yeast nitrogen base containing 0.5% ammonium sulfate), containing 5 mg of [3-^{13}C]pyruvate (sodium salt) (4.5 mM). The cells were incubated at 30 °C for 30 min with vigorous shaking to supply oxygen. The reaction was terminated by adding perchloric acid to the incubation mixture to a final concentration of 4%. The mixture was centrifuged, and the supernatant fraction was neutralized with KOH, centrifuged to remove salt, and lyophilized. The resulting powder was suspended in 0.65 mL of D_2O and microfuged to remove any residual particulates.

High-resolution ^{13}C NMR spectra were recorded on a GN-500 spectrometer at 11.75 T. The number of scans used for each sample varied between 2000 and 8000. All spectra reported in this work were acquired using a 45° carbon pulse and a 6-s delay between pulses to ensure nonsaturating conditions. All samples were maintained at 25 °C during data acquisition. The ^{13}C resonances were quantitated using NMR 286 software (Softpulse). All noise levels were normalized, and peak heights were used to determine the fractional enrichments of the carbon pools.

Overexpression of CS1 in Δrtg1 and Δrtg2 Cells. To recover uracil auxotrophs, the yeast strains COP161-1D (*Mat a, ade, lys, ura3 rtg2::URA3*) and COP161-2D (*Mat a, ade, lys, ura3 rtg2::URA3*) were treated with 5-fluoroorotic acid on YP (1% yeast extract, 2% bactopeptone) supplemented with 2% glucose. The uracil auxotrophic (COP161-1D/U and COP161-2D/U) colonies were used for further study.

The plasmid YEp352/CS1 is a 2μ shuttle vector containing the parental *CIT1* gene and the URA3 gene. This plasmid was transformed into COP161-1D/U and COP161-2D/U by using the LiCl procedure described by Ito *et al.* (1983) and selecting for URA$^+$.

Cells were grown to stationary phase and harvested by centrifugation at 4 °C. Cells were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 1 mM benzamidine and were disrupted with glass beads. The suspension was centrifuged in an Eppendorf centrifuge at maximum speed for 15 min at 4 °C. Citrate synthase assays of the supernatant were conducted by the spectrophotometric method of Srere (1969).

RESULTS

The metabolic pathways necessary for acetate utilization in yeast have been well established and are shown schematically in Figure 1. For growth on acetate a number of metabolic steps must occur: (1) acetate must be converted to acetyl-CoA; (2) the acetyl-CoA must be transported into mitochondria; (3) energy must be supplied via acetyl-CoA oxidation by the TCA cycle; and (4) C2 units must be assimilated by way of the glyoxylate/gluconeogenesis pathway. Carbon fixation for biosynthesis must occur by way of the glyoxylate cycle, in which two acetyl-CoAs are converted to succinate and NADH, another potential source of energy. All enzymes involved in these processes were

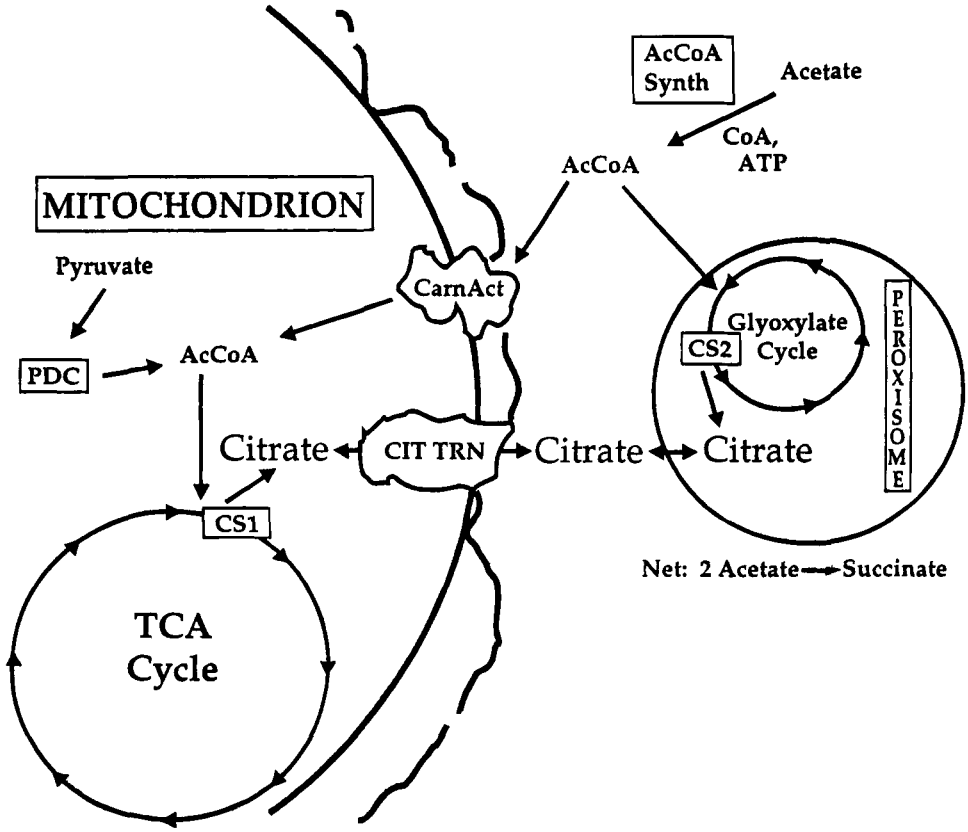

FIGURE 1: Schematic drawing of the pathway involved in acetate utilization in yeast.

Table 1: Selected Enzymatic Activities in *S. cerevesiae* COP161 Parental and Δ*rtg* Mutants[a]

enzyme	enzyme activity [nmol (mg of protein)$^{-1}$ min^{-1}] for strain			
	parental	Δ*rtg1*	Δ*rtg2*	Δ*rtg1*Δ*rtg2*
mitochondrial				
citrate synthase	265(8)	91(8)	188(8)	74(8)
aconitase	357(7)	316(7)	210(7)	287(7)
isocitrate dehydrogenase (NAD)	218(7)	223(7)	314(7)	207(7)
αKGDC	3(4)	3(4)	4(4)	2(4)
succinate thiokinase	7(1)	6(1)	5(1)	8(1)
succinate dehydrogenase	75(5)	73(5)	65(5)	35(5)
fumarase	386(6)	363(6)	339(6)	370(6)
malate dehydrogenase	1662(7)	1616(7)	1220(7)	1805(7)
pyruvate dehydrogenase complex	73(6)	54(6)	88(6)	61(7)
carnitine acetyltransferase	260(2)	259(2)	322(2)	260(2)
nonmitochondrial				
citrate synthase	15(4)	BDL(4)[b]	BDL(4)	BDL(4)
aconitase	9(4)	10(4)	14(4)	15(4)
isocitrate dehydrogenase (NAD)	6(7)	3(7)	3(4)	4(4)
fumarase	34(4)	37(4)	34(4)	37(4)
acetyl-CoA synthetase	163(2)	67(2)	54(2)	74(2)
isocitrate lyase	2(2)	2(2)	2(2)	2(2)
malate synthase	4(1)	5(1)	4(1)	3(1)
pyruvate carboxylase	8(2)	3(2)	4(2)	4(2)

[a] The numbers in parentheses indicate the number of sets of preparations on which each assay was performed. Assays were performed in triplicate in each case as described in Materials and Methods. Nonmitochondrial enzyme activities were measured in a postmitochondrial supernatant fraction. It can be seen that the number of sets (parental, Δ*rtg1*, Δ*rtg2*, Δ*rtg1*Δ*rtg2*) varied for the different enzymes as a greater number of enzymes were assayed. The relative values of enzyme activities in each set showed the same relationships as indicated by the averages of the values given here, even though the absolute values of enzyme activities between sets may vary as much as 30%. [b] BDL, below detection limit. The detection limit for CS activity is ~0.02 nmol min^{-1} (mg of protein)$^{-1}$.

measured in the Δ*rtg1*, Δ*rtg2*, and Δ*rtg1*Δ*rtg2* mutant strains (Table 1). When compared to levels in the parental strain, the only changes seen, aside from the absence of CS2, were a reduction in CS1 activity (~30−50%), a reduction in acetyl-CoA synthetase activity (~50%), a reduction in cytosolic (NAD)isocitrate dehydrogenase activity (~50%), and a reduction in pyruvate carboxylase activity (~50%). Succinate dehydrogenase was reduced in the double mutants only.

The ability of mitochondria to carry out oxidative phosphorylation was examined using a number of different Krebs TCA cycle substrates. Mitochondria from mutant cells did not show any significant deviation from normal values in the respiratory control ratio (RCR); the QO_2 values for

Table 2: Respiration of Isolated *S. cerevesiae* COP161 Parental and Δ*rtg* Mutants[a]

substrate	strain			
	parental COP161	Δ*rtg1*	Δ*rtg2*	Δ*rtg1*Δ*rtg2*
NADH				
RCR	2.86 ± 0.9	2.54 ± 0.4	2.85 ± 0.01	2.55 ± 0.17
QO_2	220 ± 12	175 ± 21	213 ± 5	227 ± 16
P/O	1.30 ± 0.13	1.68 ± 0.25	1.33 ± 0.31	1.57 ± 0.21
citrate				
RCR	1.86 ± 0.23	2.23 ± 0.5	2.04 ± 0.49	1.35 ± 0.06
QO_2	56.5 ± 18	57 ± 5	60 ± 14	41 ± 7
P/O	0.79 ± 0.05	0.84 ± 0.02	0.83 ± 0.02	0.85 ± 0.08
isocitrate				
RCR	1.78	1.78	1.70	1.50
QO_2	41	46	45	36
P/O	0.82	0.85	0.91	0.82
α-ketoglutarate				
RCR	4.64 ± 0.83	5.19 ± 0.52	4.99 ± 0.89	4.07 ± 0.55
QO_2	55 ± 5	79.5 ± 11	62 ± 2.5	77 ± 15
P/O	0.86 ± 0.16	0.86 ± 0.12	0.79 ± 0.07	0.83 ± 0.08
succinate				
RCR	1.52 ± 0.04	1.42 ± 0.01	1.51 ± 0.01	1.51 ± 0.03
QO_2	68 ± 13	63 ± 5	60 ± 5.5	65 ± 4.7
P/O	1.04 ± 0.32	1.03 ± 0.01	0.99 ± 0.1	1.00 ± 0.15
pyruvate/malate				
P/O	2.11 ± 0.13	1.79 ± 0.24	2.26 ± 0.07	1.6 ± 0.03
QO_2	82.5 ± 17	62 ± 10	93.5 ± 10	62 ± 6.51
P/O	0.97 ± 0.15	1.05 ± 0.27	1.04 ± 0.15	1.00 ± 0.15

[a] Assays were performed as described in Materials and Methods. Assays were performed in triplicate and are reported ±SE.

Table 3: Uptake of [^{14}C]Citrate by Isolated Mitochondria of COP161 Parental and Δ*rtg* Mutants[a]

cell line	citrate uptake [nmol (mg of protein)$^{-1}$ min^{-1}]
parental	13.2 ± 1.0
Δ*rtg1*	7.2 ± 2.0
Δ*rtg2*	9.6 ± 1.0
Δ*rtg1*Δ*rtg2*	6.0 ± 0.8

[a] Values shown were obtained in 30-s assays for three different preparations in triplicate as described in Materials and Methods.

Table 4: Oxidation of [2-^{14}C]Acetate by COP161 and Δ*rtg* Mutants[a]

strain	$^{14}CO_2$ production [dpm (g of wet cells)$^{-1}$]	%
parental	15 750	100
Δ*rtg1* mutant	11 710	74.4
Δ*rtg2* mutant	12 990	82.5
Δ*rtg1*Δ*rtg2*	9 010	57.2

[a] This procedure is described in Materials and Methods.

Table 5: Growth of COP161 Parental and Δ*rtg* Mutant Cells[a]

C source	strain	doubling time (h)	ΔG_{max} (Klett units)	
YP + raffinose	parental	2.75	420	
	Δ*rtg1*	2.95	412	(27 h)
	Δ*rtg2*	3.2	375	
	Δ*rtg1*Δ*rtg2*	3.5	393	
YP + EtOH:glycerol	parental	12	340	
	Δ*rtg1*	14	342	(54 h)
	Δ*rtg2*	15	230	
	Δ*rtg1*Δ*rtg2*	28	175	
YP + 1% Na acetate	parental	9.5	380	
	Δ*rtg1*	10.2	375	(50 h)
	Δ*rtg2*	13	330	
	Δ*rtg1*Δ*rtg2*	18	230	
YNB raffinose	parental	4.2	280	
	Δ*rtg1*	3.8	295	(24 h)
	Δ*rtg2*	11.4	200	
	Δ*rtg1*Δ*rtg2*	7.4	210	
YNB EtOH−glycerol	parental	12.3	255	
	Δ*rtg1*	14	250	(30 h)
	Δ*rtg2*		75	
	Δ*rtg1*Δ*rtg2*		85	

[a] Conditions for growth are described in the text. Klett units refer to the optical density (540 nm) at the stationary phase, and G_{max} is the optical density at the time indicated in parentheses for each carbon source.

NADH, isocitrate, citrate, α-ketoglutarate, succinate, and pyruvate/malate (Table 2); or the P/O values. These results indicated normally respiring mitochondria.

The data in Table 3 compare the uptake of citrate by the parental and mutant strains and show an approximate 50% decrease in mitochondrial citrate transport by the mutant strains. This observation is in agreement with our previous observations that CS1$^-$ yeast cells lack the ability to transport citrate (Grigorenko *et al.*, 1990; Sandor *et al.*, 1995). When stationary-phase cells were incubated with [2-^{14}C]acetate and malate, the mutant cells oxidized acetate to CO_2 at 60−70% the rate of the parental strain (Table 4).

Growth of the mutant strains on several carbon sources was followed on both rich (YP) and minimal (YNB) media (Table 5). All strains grew on rich media containing raffinose, ethanol−glycerol, or acetate as carbon sources. On minimal medium, however, slow growth of the mutant strains occurred as reported earlier (Liao & Butow, 1993). No growth of Δ*rtg2* or the double mutant took place on glycerol−ethanol in minimal media.

The overall metabolic patterns of these mutants was examined using [2-^{13}C]acetate and [2-^{13}C]propionate. NMR analyses of the ^{13}C metabolites formed from [2-^{13}C]acetate showed the same pattern of labeling (i.e., into sugars and TCA cycle intermediates) in both parental and mutant strains except for an apparent reduction in the amount of the precursors incorporated into intermediates in the mutant strains. Figure 2 shows a comparison of the parental strain and Δ*rtg1*.

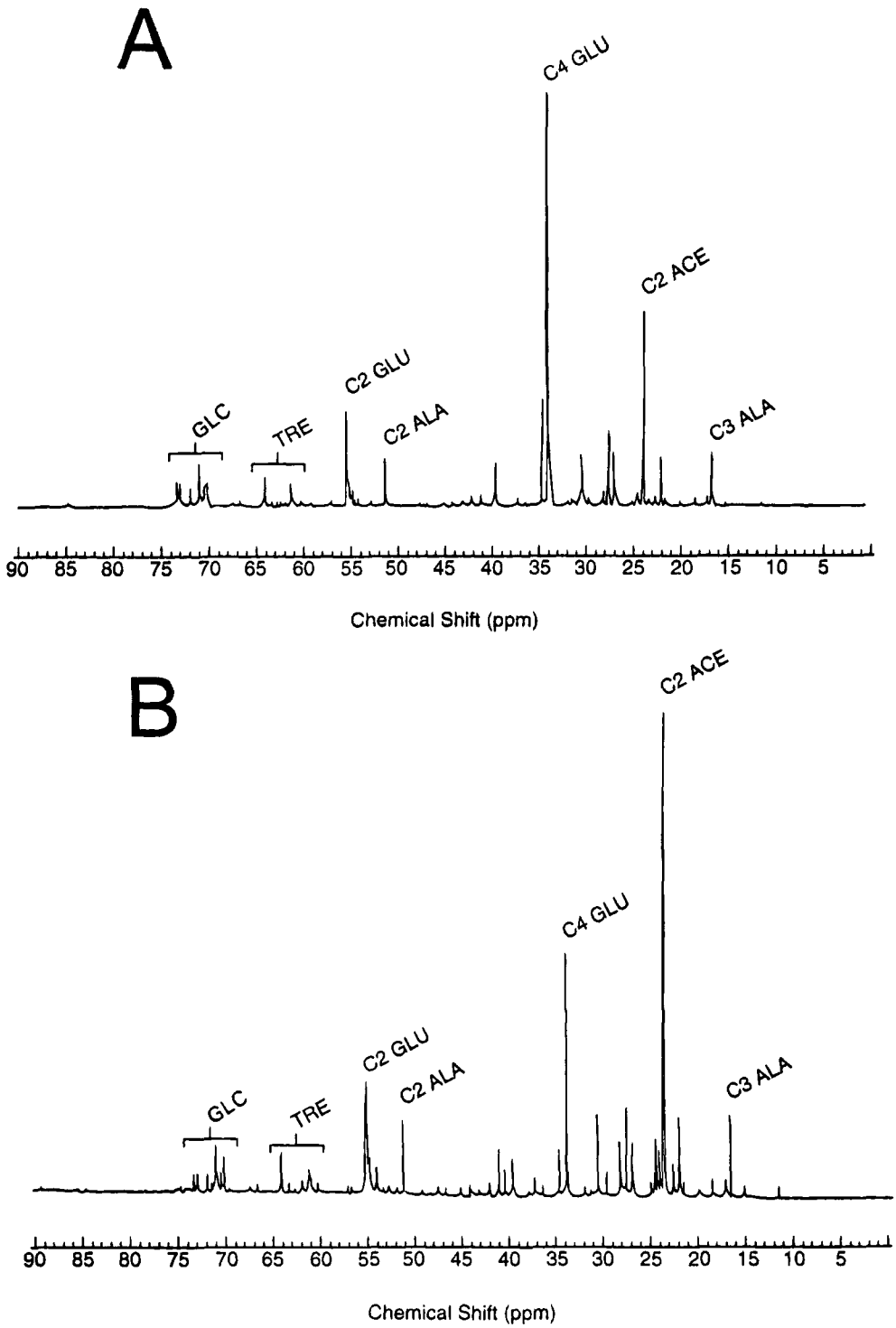

FIGURE 2: ^{13}C NMR spectra of parental and ΔRTG1 cells grown in [2-^{13}C]acetate. (A) Parental cells; (B) Δ*rtg1* cells. ALA, alanine; ACE, acetate; C4 GLU, C4 of glutamate; C2 GLU, C2 of glutamate; TRE, trehalose; GLC, glucose (chemical shift, 70−75 ppm).

When [2-^{13}C]propionate was used as a substrate, a shift in the labeling pattern for Δ*rtg2* and the double mutant was observed (Table 6). Both Δ*rtg1* and Δ*rtg2* showed glutamate/aspartate auxotrophy, and only Δ*rtg2* (and Δ*rtg1*Δ*rtg2*) showed a relative decrease in glutamate flux from propionate. It is difficult to ascribe the glutamate/aspartate auxotrophy phenotype to this difference in metabolism. When [3-^{13}C]-pyruvate was used as substrate, reduced incorporation into several metabolites was observed in the double mutant and in Δ*rtg2* (Table 7).

When CS1 was introduced into the Δ*rtg1* and Δ*rtg2* strains with a 2μ yeast−*Escherichia coli* shuttle vector, the transformants grew slowly on acetate (Figure 3). Growth on YP supplemented with acetate was better than on minimal medium supplemented with acetate. Furthermore, the transformant Δ*rtg2*/CS1 grew faster than Δ*rtg1*/CS1 on both acetate plates. The transformants retained their requirement for glutamic acid or aspartic acid.

CS1 activity in the parental and transformed strains is shown in Table 8. The CS1 activity in Δ*rtg1* is 140-fold greater than in either the untransformed Δ*rtg1* strain or the Δ*rtg1* strain transformed with the vector alone. Likewise,

Table 6: Glutamate Labeling Relative to C3 Alanine Labeling in COP161 and Δ*rtg* Mutants[a]

	C3 glutamate / C3 alanine	C4 glutamate / C3 alanine	C2 glutamate / C3 alanine
COP161	0.57	0.79	0.57
Δ*rtg1*	0.43	0.52	0.61
Δ*rtg2*	0.14	0.14	0.19
Δ*rtg1*Δ*rtg2*	0.08	0.13	0.13

[a] Cells were labeled with [2-^{13}C]propionate for 30 min as described by Sumegi *et al.* (1992).

Table 7: NMR Metabolite Peak Intensities of Parental and Δ*rtg* Mutants[a]

ppm	metabolite[b]	parental	Δ*rtg1*	Δ*rtg2*	Δ*rtg1*Δ*rtg2*
17.3	Ala 3	74.0	57.0	57.0	48.5
51.9	Ala 2	15.5	10.0	12.0	3.0
55.2	Glut 2	41.0	37.0	13	7.0
27.6	Glut 3	45.0	26.5	8	5.0
34.0	Glut 4	56.0	31.0	9	7.0
72.5	Gluc 2	37.5	15.0	7	3.0
21.0	Lac 3	9.0	6.0	5.5	2.0
24.2	Acet 2	7.0	5.5	5.5	4.0
27.3	Pyr 3	9.5	7.5	9.0	2.5

[a] Parental cells and the deletion mutants Δ*rtg1*, Δ*rtg2*, and Δ*rtg1*Δ*rtg2* were labeled with [3-^{13}C]pyruvate as described in Materials and Methods. Metabolic intermediates were quantified by a peak-picking routine using NMR 286 software. Peak intensity is expressed as arbitrary units from spectra which were identically scaled. [b] Ala, alanine; glut, glutamate; gluc, glucose; lac, lactate; acet, acetate; pyr, pyruvate. The number refers to the position of the labeled carbon atom.

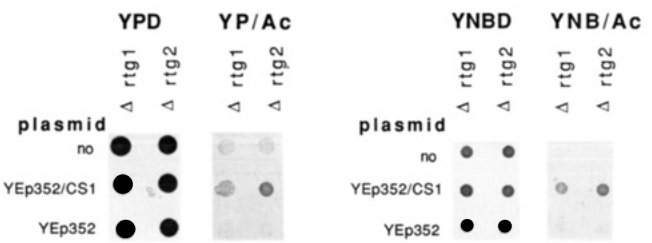

FIGURE 3: Growth of transformants and activity of CS1. (A) Strains COP161-1D/U (Δ*rtg1*) and COP161-2D/U (Δ*rtg2*) were each transformed with the plasmid YEp352/CS1 and with the vector YEp352. Aliquots of the transformation mixtures were dropped onto selective plates and incubated for 3 days on YPD and YP/acetate plates and 9 days on minimum plates (YNB) supplemented with acetate.

the CS1 activity in Δ*rtg2* is 290-fold greater than in either control strain.

DISCUSSION

As previously reported (Liao *et al.*, 1991), strains of *S. cerevesiae* which contain disruptions of the genes encoding the RTG1 and RTG2 proteins show greatly reduced expression of *CIT2* mRNA. We have confirmed that finding by demonstrating that such strains, when grown on raffinose, a carbon source which, unlike glucose, does not repress *CIT2* expression, have no measurable peroxisomal CS activity (CS2). These strains also exhibit the phenotypic characteristics of glutamate and aspartate auxotrophy as well as the inability to grow on acetate as a carbon source in minimal media. With these multiple phenotypic effects, it seemed possible that the missing RTG proteins might affect expression of other genes. This seemed especially true for *rtg1*, whose DNA sequence indicates a protein with close resemblance to the helix−loop−helix family of DNA binding proteins such as MyoD and Myc which have been shown to bind specifically to a common DNA sequence (Liao & Butow, 1993).

Table 8: Activity of Citrate Synthase [units min^{-1} (mg of protein)$^{-1}$ [a]

transformed plasmid	strain Δ*rtg1*	strain Δ*rtg2*
none	0.22 ± 0.001	0.013
YEp352	0.0245 ± 0.003	0.014 ± 0.015
YEp352/CS1	3.09 ± 0.3	3.8 ± 0.002

[a] Each value is the average of three assays ± SEM.

A possible explanation for these secondary phenotypic changes in RTG mutant strains might be inefficient functioning of the TCA cycle. Therefore, this study measured each of the eight enzymatic activities involved in the TCA cycle. Only CS1 was reduced ~50% of these enzymatic activities. The activities of these TCA cycle enzymes in the Δ*rtg* mutant strains, however, would be more than sufficient to support growth in otherwise wild-type strains since they are normally present at levels of activity at least an order of magnitude higher than required for oxygen consumption as measured in isolated mitochondria (Grigorenko et al., 1990).

Since no other significant problems in TCA cycle function were detected, alternative causes for the observed phenotypes were explored. As shown previously, reduction of CS1 levels to less than 50% activity had no effect on acetate growth (Grigorenko *et al.*, 1990). No difference was seen among the strains in the levels of glyoxylate cycle activity (other than CS2), the pyruvate dehydrogenase complex, or nonmitochondrial aconitase or fumarase. In addition, carnitine acetyltransferase, the enzyme responsible for making exogenously derived acetyl-CoA available to the TCA cycle, was equivalent in all four strains tested. Acetyl-CoA synthetase was reduced by 2−3-fold in the Δ*rtg* mutant cells, but as in the case of the reduction of CS1, this decrease is insufficient to explain the considerable differences in observed phenotype. Decreases in cytosolic activities of NAD isocitrate dehydrogenase (~50%) and cytosolic pyruvate carboxylase (~50%) were noted. A modest decrease in mitochondrial citrate transport would appear to be insufficient by itself to account for the observed phenotype change. The decrease in citrate transport supported previous work which shows the loss of mitochondrial citrate transport in CS1$^-$ strains (Grigorenko *et al.*, 1990).

Another possibility explored was that of a blockade in the electron-transport network or in the transfer of reducing equivalents generated by the turning of the TCA cycle to the process of oxidative phosphorylation. No significant differences were detectable in the rate of oxygen consumption, the respiratory control ratio, or the P/O ratio for any of the strains studied. Additionally, all strains oxidized various intermediates of the TCA cycle at the same relative rates. Although the enzymes unique to gluconeogenesis were not assayed, inspection of the NMR spectra obtained from extracts of Δ*rtg* strains which had been incubated with [2-^{13}C]acetate (Figure 2) indicated that *de novo* synthesis of sugars proceeded normally in these strains. Thus, in summary, no blockade in energy production, transport, or anabolism was detected in these strains.

The yeast cell is capable of the metabolic events involved in the catabolism of carbon compounds for the production

of energy as well as those events responsible for the fixing of carbon into precursors of anabolic reactions by many different routes. This observation is indicative of the considerable redundancy of metabolic pathways within the cell. Thus, the organism possesses the necessary flexibility to meet the challenges presented by its environmental milieu as the amounts of oxygen and nutrients vary.

The interaction between various metabolic pathways in yeast appears to have a 2-fold purpose. In addition to the obvious function of coordinating the many different routes through which metabolites flow in the course of cellular activity, yeast cells are capable of overcoming blockades which in less flexible systems would prevent an effective response to such challenges as the unavailability of easily utilizable carbon sources. It is evident that the full complexity of such interactions is not well understood, nor is it surprising that a genotypic change causing one observable mutation can effect other unpredicted phenotypic differences within the cell. Additionally, genotypic changes of two or more kinds which have the same primary phenotypic effect upon the organism can cause secondary metabolic changes which may be considerably different from each other. The strains examined in this study present such a case, where the disruption of genes controlling *CIT2* expression results in a profound phenotype change that is not observed when CS2 is absent due to disruption of the gene encoding it.

The "normal" growth conditions for yeast (i.e., not in the laboratory) may occur in the following manner: (1) fermentation of glucose, (2) utilization of fermentation products such as alcohol and glycerol and (3) utilization of acetate. In all these stages it is probable that adequate N sources are available. The gross metabolic changes include (1) repression of respiratory activity, (2) derepression of TCA cycle enzymes, and (3) induction of C2 fixation (glyoxylate cycle). Of all its available carbon sources during this process, the least "nutritious" is acetate, especially in the absence of other nutrient carbon sources. Thus, even alcohol, another two-carbon source, supplies more energy than acetate since its conversion to acetate entails the production of two NADHs. The glyoxylate cycle, even in its most induced state, does not supply adequate carbon to support any more than a much reduced growth potential. Thus, acetate growth on minimal media tests the vigor of all the metabolic systems of a yeast cell.

McCammon (personal communication) recently isolated a series of acetate nonutilizing (ACN) yeast mutants. McCammon's analysis of 104 ACN mutants revealed as many as 43 complementation groups. Of these, only 15 have been identified as either TCA cycle mutants, glyoxylate cycle mutants, or a group that involved novel regulatory proteins of a number of metabolic pathways. Several mutants that are not members of the ACN collection are also acetate$^-$ (McCammon, personal communication). The other 28 complementation groups may represent a minimal estimate of this class of mutants. It is apparent, therefore, that acetate utilization is a complex and tightly regulated process. It is also apparent that acetate metabolism is a "last ditch" effort at yeast cell survival and that any slight change in its ability to supply energy or carbon sources results in an inability of the cell to grow.

Thus, in the case of the $\Delta rtg1$ and $\Delta rtg2$ mutants of yeast, no one specific enzymic defect (other than an otherwise nonharmful absence of CS2 activity) seems to be responsible for the acetate$^-$ phenotype. Rather, the accumulation of a number of pleiotropic changes, such as decreases in the activities of CS1, citrate transport, and acetate activation cumulatively may have decreased the energetic and/or carbon-supplying ability below a threshold level sufficient for the cell to utilize acetate as a sole carbon source. Some support for these ideas comes from the surprising result that overexpression of CS1 in the RTG disruption strains was able to restore to some extent their ability to grow on acetate without affecting the glutamate/aspartate requirement. Because of the apparent pleiotropic effects of the *rtg* mutants, it is possible that the results seen here are due to changes of some unidentified factors. However, it should be emphasized that the inability of these cells to grow on acetate, as well as their auxotrophy for glutamate/aspartate, cannot be easily explained by the data in this study. Since there is no single enzyme involved in acetate metabolism, which is missing and could account for the acetate$^-$ phenotype, these results differ from those previously reported on that phenotype.

Several current metabolic principles, however, could be cited that may explain these results. First, it is clear that control of a metabolic pathway is distributed throughout the sequence of reactions of that pathway, and the degree of control varies between the steps, depending upon the conditions for growth or the response to stress (Srere, 1993, 1994). Second, it has been shown that many metabolic systems (like the TCA cycle) exist *in situ* as a complex of enzymes (a metabolon), and structural disruption of a metabolon with a change of enzyme activities can cause a large diminution of the flux of that pathway (Srere, 1993, 1994). Whether or not these explanations are valid for these mutants must await different metabolic approaches.

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