The *Saccharomyces cerevisiae RTG2* Gene Is a Regulator of Aconitase Expression Under Catabolite Repression Conditions

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ABSTRACT

The *ACO1* gene, encoding mitochondrial aconitase of *Saccharomyces cerevisiae,* is required both for oxidative metabolism and for glutamate prototrophy. This gene is subject to catabolite repression; the *ACO1* mRNA level is further reduced when glutamate is supplied with glucose. To further explore regulation of *ACO1* expression, we have screened for mutations that reduce expression of an *ACO1-lacZ* fusion borne on a multicopy vector. We identified a gene required for wild-type expression of *ACO1* only under catabolite repression conditions. Sequencing of the corresponding cloned gene revealed that it is identical to *RTG2* previously cloned as a pivotal gene in controlling interorganelle retrograde communication. Cells containing either the original *rtg2-2* mutation or a null *rtg2* allele are not petite but show a residual growth on minimum glucose medium with ammonium sulfate as the sole nitrogen source. This growth defect is partially restored by supplying aspartate or threonine, and fully with glutamate or proline supplement. Surprisingly, this phenotype is not observed on complete medium lacking either of these amino acids. In addition, a genetic analysis revealed an interaction between *RTG2* and *ASP5* (encoding aspartate amino transferase), thus supporting our hypothesis that *RTG2* may be involved in the control of several anaplerotic pathways.

ACONITASE (citrate [isocitrate] hydro-lyase; EC 4.2.1.3) is an enzyme located mainly in the mitochondrial matrix, where it is involved in the Krebs cycle (KREBS and LOWENSTEIN 1960), catalyzing the reversible isomerization of the tricarboxylic acids (TCA) citrate and isocitrate via *cis*-aconitate (ROSE and O'CONNELL 1967; VILLAFRANCA and MILDVAN 1971). In *Saccharomyces cerevisiae,* as in other eukaryotic cells, all the enzymes of the TCA cycle are encoded in the nucleus and are transported into the mitochondrial matrix. The mitochondrial aconitase of *S. cerevisiae* is encoded by the *ACO1* gene, previously cloned by GANGLOFF *et al.* (1990) by complementation of the *glu1-1* mutation (OGUR *et al.* 1964). In yeast cells, aconitase activity is also involved in the glyoxylate cycle, and the extramitochondrial aconitase activity probably fulfills this role (DUNTZE *et al.* 1969), although it does not seem to be located in peroxisomes where the glyoxylate cycle is thought to take place in yeasts (KAWAMOTO *et al.* 1977; WALES *et al.* 1980; MCCAMMON *et al.* 1990) as in plants and other fungi (ZIMMERMAN and NEUPERT 1980; TOLBERT 1981; WANNER and THEIMER 1982; TRELEASE 1984; DE BELLIS *et al.* 1990; REYNOLDS and SMITH 1995). Moreover, the genetic origin of this extramitochondrial aconitase is still not understood. A dual cell location has also been described for citrate synthase activity, for which three functional genes, *CIT1, CIT2,* and *CIT3,* have been characterized (KIM *et al.* 1986; ROSENKRANTZ *et al.* 1986; BÉCAM *et al.* 1995). However, the extramitochondrial citrate synthase activity (CS2, encoded by *CIT2*), which operates as part of the glyoxylate cycle, was clearly localized in peroxisomes (LEWIN *et al.* 1990). Interestingly, the effects of a *CIT1* or *ACO1* disruption on citrate synthase or aconitase activity, respectively, are very different. In the latter case, both mitochondrial and extramitochondrial aconitase activities are abolished (GANGLOFF 1990). On the contrary, in a *cit1* strain, it has been found that *CIT2* transcription and CS2 activity are increased (LIAO *et al.* 1991) and that this regulation (termed retrograde) depends on two genes, *RTG1* and *RTG2* (LIAO and BUTOW 1993).

The role of the TCA cycle is twofold. First, it is oxidative, generating NADH, that drives mitochondrial respiration and hence the synthesis of ATP. Second, the Krebs cycle provides the carbon skeletons used in many anabolic pathways, including α-ketoglutarate, the main precursor of glutamate. Thus, in *S. cerevisiae,* citrate synthase and aconitase are required both for glutamate prototrophy and for efficient utilization of nonfermentable carbon sources (OGUR *et al.* 1964; OGUR *et al.* 1965; KIM *et al.* 1986; GANGLOFF *et al.* 1990), implying that aconitase, like citrate synthase, will be highly regulated. It has been reported for the bacterium *Bacillus subtilis* that levels of citrate synthase and aconitase are

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downregulated by glucose alone (catabolite regulation) and synergistically by glucose and a glutamate source (HANSON and COX 1967). ROSENKRANTZ *et al.* (1985) have shown that, in the case of aconitase, this control is exercised at the transcriptional level. In *S. cerevisiae*, a similar control pathway operates for citrate synthase (KIM *et al.* 1986) and for aconitase (GANGLOFF *et al.* 1990). Moreover, GANGLOFF *et al.* (1990) found a sequence in the *ACO1* promoter corresponding to the inverted upstream activating sequence UAS2 UP1 (GUARENTE *et al.* 1984; FORSBURG and GUARENTE 1988), which has been shown to be the HAP2/3/4/5 protein-responsive site of the *CYC1* gene (OLESEN *et al.* 1987; FORSBURG and GUARENTE 1989; MCNABB *et al.* 1995). Accordingly, GANGLOFF (1990) showed that expression of the *ACO1* gene is under the control of both *HAP2* and *HAP3* genes (*HAP4* and *HAP5* were unknown at that time).

To study further the regulation of aconitase expression, we sought mutants affected in *ACO1* expression by using *lacZ* reporter constructs to mimic the regulation of *ACO1*. We have identified and cloned a nuclear gene required for a normal level of *ACO1* expression under catabolite repression conditions and found it to be identical to *RTG2* (LIAO and BUTOW 1993). Furthermore, we performed a genetic study on the relationships between genes *RTG2* and *ASP5* (encoding aspartate amino transferase), and found *rtg2* to partially suppress the *asp5* mutation.

MATERIALS AND METHODS

Strains and media: The *S. cerevisiae* strains used in this study are presented in Table 1. All of the strains listed are isogenic to the reference strain S288C, except STX32-10A and SFY/Z-50.

Standard rich (YPD), synthetic complete (SC), synthetic minimal (SD) presporulation (SP1) and minimal sporulation (AcK) media were described previously (SHERMAN *et al.* 1986). Other media were SD + X (like SD but including nutrient(s) X), SC −Y (like SC but lacking nutrient(s) Y) and X-Gal medium. X-Gal plates contained 70 mM potassium phosphate (pH 7.0), 0.17% yeast nitrogen base without amino acid and ammonium sulfate, 0.5% $(NH_4)_2SO_4$, 2% glucose, 40 μg/ml X-Gal, 20 μg/ml uracil, 60 μg/ml leucine and 20 μg/ml histidine.

Plasmids: The *ACO1-lacZ* fusion contains a 794-bp *Eco*RI-*Eco*RV fragment of the 5′ region of *ACO1* plus sequences encoding the first 49 amino acids fused in frame to the *lacZ* gene of *Escherichia coli*. The multicopy version was directly obtained by inserting the 794-bp *Eco*RI-*Eco*RV fragment from pSE-31 (GANGLOFF *et al.* 1990) between the *Eco*RI and *Sma*I sites of YEp358 (MYERS *et al.* 1986). The *ACO1-lacZ* fusion was also placed in an integrative vector bearing the *HIS3* marker and derived from pAT153 (TWIGG and SHERRAT 1980). This last plasmid was used to integrate the *ACO1-lacZ* fusion at the *ACO1* chromosomal locus. Southern analysis was performed to confirm that the integration event had occurred and that the *ACO1* chromosomal copy was intact. Such strains are designated "/Z" (see Table 1).

The yeast genomic library (CEN BANK, ATCC 37415) (ROSE *et al.* 1987) was constructed in the centromeric vector YCp50. YCp50-3A is an isolate from this library containing an 8-kbp fragment (3A). YCp50-3A1 and YCp50-3A2 were obtained by deletion of the 4.8-kbp *Sal*I fragment and the 4.8-kbp *Eco*RI fragment, respectively, from the 3A insert (see Figure 3A). The 1.7-kbp *Bgl*II-*Eco*RI, 3.4-kbp *Sal*I-*Kpn*I, 4.4-kbp *Bgl*II-*Kpn*I subclone fragments from 3A were inserted between the corresponding sites of the centromeric vector pRS316 (SIKORSKI and HIETER 1989) to generate pRS-3A3, pRS-3A4 and pRS-3A5, respectively (see Figure 3A). To construct pRS-Δrtg2 in which the *RTG2* gene is disrupted by the *TRP1* marker, the 750-bp *Eco*RI-*Sal*I fragment from the 3A5 subclone was replaced by the 800-bp *Eco*RI-*Sal*I *TRP1*-bearing fragment from YDp-W (BERBEN *et al.* 1991).

Genetic manipulations: Yeast transformations were carried out either by the spheroplast method described by BEGGS (1978) and modified by BURGERS and PERCIVAL (1987), or by the lithium method reported by ITO *et al.* (1983) and modified by GIETZ *et al.* (1992). Crosses, diploid selection, sporulation and tetrad analysis were carried out by standard procedures (SHERMAN *et al.* 1986). Colony hybridization was performed as previously described (HANAHAN and MESELSON 1980).

Southern analysis and DNA sequencing: *S. cerevisiae* DNA was prepared by the method of SHERMAN *et al.* (1986), digested with appropriate restriction enzymes, subjected to electrophoresis through 1% agarose gels and blotted onto nitrocellulose as described by SOUTHERN (1975). Blots were hybridized with purified random priming probes (FEINBERG and VOGELSTEIN 1984). Sequencing was carried out using dideoxy chain termination (SANGER *et al.* 1977). The *Sal*I-*Eco*RI fragment from 3A5 insert was sequenced directly on plasmids pRS-3A3 and pRS-3A4 (see Figure 3A) with the universal 20-mer reverse and SK primers.

Enzyme assays: Crude extracts were prepared from 100 ml cultures harvested in the early log phase. Cells were suspended in potassium phosphate buffer (20 mM, pH 7.4) supplemented with 1 mM phenylmethylsulfonyl fluoride, and broken with glass beads (0.5 mm diameter) by vortexing. Whole cell extracts were obtained following successive centrifugations of 5 min at 1200 × *g* and 20 min at 30,000 × *g*. Protein concentration was determined by the bicinchoninic acid-using method described by SMITH *et al.* (1985).

Aconitase activity was assayed by an adaptation of the method described by FANSLER and LOWENSTEIN (1969). Specific activities are given as nanomoles of *cis*-aconitate transformed per minute per milligram of protein. Assays were performed at 22° in duplicate or triplicate and results were averaged. This regimen produced activity measurements that were reproducible within 20%.

Quantitative β-galactosidase assays were carried out either on colonies grown on SC −Ura plates and suspended in 1 ml Z buffer so that OD_{600} was about 2 units, or on yeast strains grown overnight in the appropriate selective synthetic medium, diluted in fresh selective medium, and grown to log phase (OD_{600} = 1). After cell permeabilization as previously described (GUARENTE 1983), β-galactosidase assays with the colorimetric substrate o-nitrophenyl-β-D-galactopyranoside were performed as described by MILLER (1972). Activities are given as Miller units. Assays were carried out in duplicate or triplicate and averaged, and were reproducible within 20%.

Isolation of *ACO1* expression mutants: Strains FY1A and FY1B bearing pYACO1′Z were mutagenized with ethyl methane sulfonate (FINK 1970) to ~50% survival and plated on 0.45 μm nitrocellulose filters laid onto SC −Ura plates. After 48 hr of growth, filters were transferred to minimal glucose plates containing the β-galactosidase chromogenic substrate (X-Gal plates). About 20,000 colonies were screened (10,000 from each strain) and 227 pale blue or white colonies were identified. Of these 227 colonies, only eight strains consistently displayed reduced levels of β-galactosidase activity. Only

TABLE 1

Yeast strains used

Strain	Genotype	Source
FY1A	*MATa ura3-52 leu2Δ1*	Laboratory collection
FY1B	*MATα ura3-52 leu2Δ1 his3Δ200*	Laboratory collection
1B-1	*MATα ura3-52 leu2Δ1 his3Δ200 rtg2-2*	This study
FY1B/Z	*MATα ura3-52 leu2Δ1 his3Δ200 ACO1-lacZ-HIS3*	This study[a]
1B-1/Z	*MATα ura3-52 leu2Δ1 his3Δ200 rtg2-2 ACO1-lacZ-HIS3*	This study[a]
FY23	*MATa ura3-52 leu2Δ1 trp1Δ63*	B. DUJON (Institut Pasteur)
FY231	*MATa/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/HIS3 trp1Δ63/TRP1 rtg2-2/RTG2*	This study[b]
FY231-00	*MATa/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/HIS3 trp1Δ63/TRP1 rtg2-2/rtg2-2*	This study[c]
FY231-10	*MATa/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/HIS3 trp1Δ63/TRP1 rtg2-2/RTG2*	This study[d]
FY231-11	*MATa/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/HIS3 trp1Δ63/TRP1 RTG2/RTG2*	This study[e]
FYC5	*MATa ura3-52 trp1Δ63 his3Δ200*	Laboratory collection
FYC5/Z	*MATa ura3-52 trp1Δ63 his3Δ200 ACO1-lacZ-HIS3*	This study[a]
FYC5-V1	*MATa ura3-52 trp1Δ63 his3Δ200 rtg2::TRP1*	This study
FYC5/Z-V1	*MATa ura3-52 trp1Δ63 his3Δ200 ACO1-lacZ-HIS3 rtg2::TRP1*	This study[a]
STX32-10A	*MATα ura3 his3 arg8 asp5*	Berkeley collection
SFY/Z-50a	*MATa ura3-52 trp1Δ63 his3 asp5 ACO1-lacZ-HIS3*	This study[a,f]
SFY/Z-50α	*MATα ura3-52 trp1Δ63 his3 asp5 ACO1-lacZ-HIS3*	This study[a,f]

[a] Strains bearing the *ACO1-lacZ* fusion integrated at the *ACO1* chromosomal locus.
[b] Formed by mating 1B-1 and FY23.
[c] Formed by mating two *rtg2-2* segregants from FY231.
[d] Formed by mating a *rtg2-2* segregant and a wild segregant from FY231.
[e] Formed by mating two wild segregants from FY231.
[f] Segregants from STX32-10A × FYC5/Z.

five of these (all bearing the mating type α) appeared to carry chromosomal lesions; these were subjected to preliminary genetic and phenotypic analysis.

RESULTS

Isolation of a mutant with altered expression of *ACO1*: To further explore *ACO1* gene expression regulation, we screened for mutations that would affect *trans*-acting regulatory proteins and reduce expression of an *ACO1-lacZ* fusion borne on a 2μ *URA3* vector. Mutant clones giving rise to pale blue or white colonies on minimal glucose XGal plates were isolated (see MATERIALS AND METHODS). Eight mutants with reduced or abrogated β-galactosidase expression were further analyzed; three exhibited a plasmid-linked phenotype and were presumed to be *ACO1-lacZ* structural gene mutants. The remaining five mutants derived from FY1B (*i.e.*, bearing the mating type α) and were named 1B-1, 1B-3, 1B-24, 1B-35 and 1B-45. Preliminary genetic and phenotypic analysis revealed that 1B-3 and 1B-24 bore mutations not affecting *ACO1-lacZ* expression at all but probably the number of copies of the 2μm-based plasmid. Moreover, 1B-35 and 1B-45 proved to be *pet* mutants with complex phenotypes for which a strategy for gene cloning was difficult to set up. So we decided to further study the mutant 1B-1, the only one left with the expected phenotype.

Phenotypic characterization: The levels of expression of the *ACO1-lacZ* fusion were examined in wild-type and mutant strains bearing the integrated fusion; the results are presented in Table 2. To confirm that expression of *ACO1* was affected, aconitase activity was assayed in whole-cell extracts from FY1B and 1B-1 strains cured of any *ACO1-lacZ* fusion and grown in YPD medium. The results obtained corroborated those observed with the reporter gene, although the decrease in activity for aconitase was lower than for β-galactosi-

TABLE 2

***ACO1-lacZ* fusion and aconitase expression in wild FY1B and mutant 1B-1 strains**

Enzyme activity[a]	Strain			
	FY1B		1B-1	
	Glucose	Lactate	Glucose	Lactate
β-galactosidase[b]	42	153	3	177
Aconitase[c]	18 ± 4	110 ± 21	6 ± 3	135 ± 25

[a] Average values determined on three independent experiments.
[b] β-galactosidase activities are given as Miller units and were measured as described in MATERIALS AND METHODS after growth in SC −His medium containing either 2% glucose (catabolite repression conditions) or 2% lactate (derepression conditions).
[c] Aconitase activities are given as nmol of *cis*-aconitate transformed per min per mg of protein and were determined on crude extracts of the strains grown to early log phase either on YPD (glucose) or on YPL (lactate).

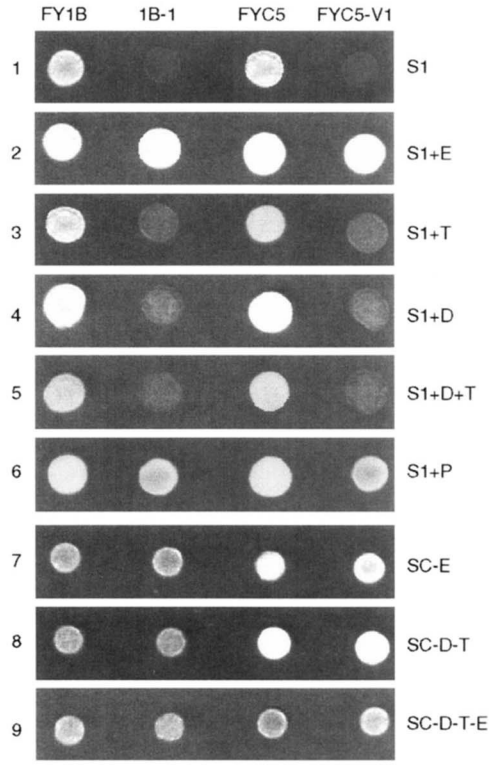

FIGURE 1.—Growth phenotypes of strains bearing either the original *rtg2-2* mutation (1B-1) or the *rtg2* null allele (FYC5-V1), and additional growth requirements. Wild-type and mutant strains were grown overnight in liquid YPD culture, washed twice in sterile water and resuspended to 2×10^6 cells/ml in water. For each strain, 5 μl drops (10^4 cells) were laid onto different synthetic minimal (SD) and complete (SC) glucose media. S1 medium is SD plus uracil, histidine, leucine and tryptophan (1); same as (1) plus glutamate (2); same as (1) plus threonine (3); same as (1) plus aspartate (4); same as (1) plus aspartate and threonine (5); same as (1) plus proline (6); SC medium lacking glutamate (7); SC medium lacking aspartate and threonine (8); SC medium lacking aspartate, threonine and glutamate (9).

dase (Table 2). The same experiments were carried out in the absence of catabolite repression by replacing the 2% glucose by 2% lactate, a nonfermentable carbon source. Results show that no repression phenotype is observed in these conditions (Table 2), *i.e.*, the 1B-1 strain does not exhibit any decrease in aconitase or β-galactosidase activities compared to the wild-type strain.

Since an aconitase-deficient strain is characterized by a petite phenotype, the mutant strain 1B-1 was plated onto various rich media supplemented with a nonfermentable carbon source, *i.e.*, either 2% lactate (YPL), 2% glycerol, 2% potassium acetate or 3% ethanol. Strain 1B-1 proved to be able to grow fully on these different media.

However, the 1B-1 mutant grew very poorly compared with wild-type strain FY1B on synthetic minimal glucose medium supplemented with uracil, leucine and histidine (SD + Ura + Leu + His). To understand the origin of this growth defect, we tested each of the other nutrients present in the SC medium (*i.e.*, other essential amino acids and adenine) as well as proline (for reasons explained in DISCUSSION) for the ability to restore normal growth on this minimal medium. The results showed that either aspartate or threonine could partially restore growth without being additive, and that either glutamate or proline could fully restore the growth of the 1B-1 mutant to wild-type levels (Figure 1). Surprisingly, no growth defect was observed on synthetic complete medium lacking either one or all of the three amino acids (aspartate, threonine and glutamate) (Figure 1). Since a strain harboring the *glu1-1* mutation or a null allele of *ACO1* is unable to grow on SC −Glu medium, these results suggest that there is a reduced but significant *ACO1* expression in the 1B-1 mutant cells that is reflected in the low aconitase activity detected in this mutant strain (Table 2).

TABLE 3

***ACO1* expression decrease phenotype segregates as a single nuclear locus**

	Aconitase activity[b]					
	Tetrad					
Segregant[a]	1	2	3	4	5	6
a	4	14	23	8	30	13
b	12	27	6	28	24	30
c	27	33	17	6	8	8
d	30	9	5	33	8	24

Values are nmol of *cis*-aconitate transformed/min per mg of protein.

[a] Segregants of the FY231 diploid strain resulting from a cross between 1B-1 and FY23 for which aconitase activities (in the same units) were respectively 6 and 28.

[b] Aconitase activities were assayed on crude extracts of the segregants grown on YPD to early log phase.

Genetic characterization: To determine whether these phenotypes were due to a single genetic lesion, 1B-1 was crossed with the wild-type strain FY23, generating the FY231 diploid strain. Segregants were analyzed after sporulation and tetrad dissection. Aconitase activities were determined on crude extracts of segregants (from six tetrads dissected) grown to early log phase on YPD culture medium, and the same segregants were also tested for their ability to grow normally on SD medium supplemented with uracil, histidine, leucine and tryptophan (SD + Ura + His + Leu + Trp). The results showed that both phenotypes segregated $2^+:2^-$ and cosegregated in all six tetrads (Table 3 and Figure 2A), suggesting that a single mutation caused these phenotypes. To confirm these results, the study was extended to tetrads resulting from the cross between 1B-1/Z and FYC5/Z (see Table 1), where each segregant bore the integrated *ACO1-lacZ* fusion and could be subjected directly to β-galactosidase assays. In each of the 18 tetrads analyzed, two segregants displayed low β-galactosidase activity and grew poorly on SD + Ura + His + Leu + Trp medium, thereby confirming that a

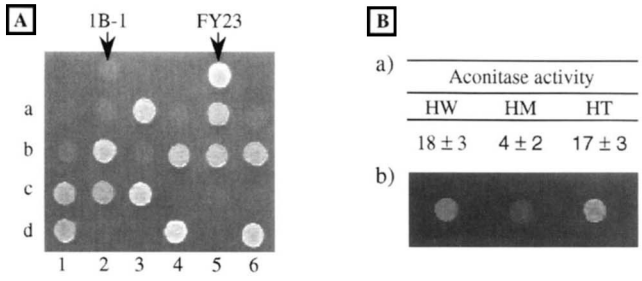

FIGURE 2.—Genetic characterization. (A) Poor growth phenotype on synthetic minimal glucose media segregates as a single nuclear locus. Segregants analyzed in Table 3 (letters from a to d refer to segregants and numbers from 1 to 6 refer to tetrads) were subjected to a growth test as described in the legend of Figure 1, on SD medium supplemented with uracil, histidine, leucine and tryptophan. Comparison between Table 3 and Figure 2A show that the two phenotypes cosegregate. (B) Dominance/recessiveness analysis. Homozygous wild *RTG2/RTG2* FY231-11 (HW), heterozygous *RTG2/rtg2-2* FY231–10 (HT) and homozygous mutant *rtg2-2/rtg2-2* FY231–00 (HM) diploids were formed by mating segregants from FY231. (a) *rtg2–2* mutation is recessive for the reduced aconitase expression phenotype. The values shown here represent nmol of *cis*-aconitate formed per min per mg of protein ± SE of three independent experiments. (b) *rtg2-2* mutation is recessive for residual growth phenotype. These diploids were subjected to a growth test as described in the legend of Figure 1 on SD medium supplemented with uracil and leucine.

single mutation was responsible for both phenotypes. For reasons that will become clear below, the mutation was termed *rtg2-2*.

To determine whether the *rtg2-2* mutation was recessive, homozygous mutant *rtg2-2/rtg2-2* (FY231-00), heterozygous *RTG2/rtg2-2* (FY231-10) and homozygous wild *RTG2/RTG2* (FY231-11) diploids were formed by mating segregants from FY231 (see Table 1). These diploids were tested for their ability to grow normally on SD + Ura + Leu medium, and aconitase activities were assayed on crude extracts of each of them grown to early log phase on YPD medium. Results (Figure 2B) indicated that the *rtg2-2* mutation was clearly recessive for both phenotypes.

Cloning *RTG2* and chromosomal location: Twelve Ura^+ transformants of the 1B-1 mutant able to grow normally on SD + His + Leu medium were selected after transformation with a YCp50-based library (ROSE *et al.* 1987). Complementation in all 12 transformants proved to be conferred by the same plasmid, YCp50-3A. YCp50-3A restored aconitase activity to wild-type levels (Figure 3A). After subcloning experiments (see MATERIALS AND METHODS), the minimal complementing region proved to be a 4.4-kbp *Bgl*II-*Kpn*I fragment (subclone 3A5, Figure 3A). This fragment maps to the left arm of chromosome *VII* (between the *HXK2* and *HAP2* loci) by hybridization to λ clones ATCC 70008 and 70713.

In addition, total DNA from wild-type strain FY1B was digested with eight different restriction enzymes, blotted onto nitrocellulose paper and probed with the 4.3-kbp *Eco*RV-*Eco*RV fragment (Figure 3B). The deduced genomic pattern was identical to that determined by restriction analysis of the cloned insert 3A, and indicated that no recombination event had occurred during the cloning steps. Moreover, the size of fragments generated by digestion with *Eco*RI and *Hin*dIII restriction enzymes corresponded to those expected from the *Eco*RI/*Hin*dIII physical map of the yeast genome (OLSON *et al.* 1986; RILES *et al.* 1993).

Disruption of the *RTG2* gene and characterization of the Δ*rtg2* strain: Subcloning experiments and complementation tests revealed that a 0.7-kbp *Eco*RI-*Sal*I fragment within the 3A5 subclone was probably essential for complementation of the 1B-1 mutant (Figure 3A). To perform the disruption of the corresponding gene, we replaced this fragment by the 0.8-kbp *Eco*RI-*Sal*I *TRP1* selectable marker to form pRS-Δ*rtg2* (see MATERIALS AND METHODS). After checking that this new plasmid was unable to complement the β-galactosidase activity decrease and residual growth phenotypes in mutant strains 1B-1/Z and 1B-1, respectively (Figure 3A), the newly generated 4.5-kbp *Kpn*I-*Spe*I *rtg2::TRP1*-bearing linear fragment from pRS-Δ*rtg2* was used to transform strains FYC5 and FYC5/Z to tryptophan prototrophy. We confirmed that the tryptophan-independent transformants carried the disrupted allele by performing Southern analysis of genomic DNA from both untransformed and transformed cells. The resulting *rtg2*-disrupted strains were termed FYC5-V1 and FYC5/Z-V1 (see Table 1).

To characterize these disrupted strains, we measured β-galactosidase activity in the yeast strains FYC5/Z and FYC5/Z-V1, as well as aconitase activity on crude extracts of FYC5 and FYC5-V1 grown to early log phase on glucose (YPD) and lactate (YPL) media (Table 4). As expected, both enzyme activities were decreased in the disrupted strains but this phenotype was observed only under catabolite repression conditions as seen for the strain bearing the original *rtg2-2* mutation. However, we noted that the aconitase activity decrease appeared to be more severe in the disrupted strain since no activity was detectable. Moreover, growth tests performed on different synthetic minimal or complete glucose media showed that the FYC5-V1 strain behaved like mutant 1B-1 (Figure 1). Finally, FYC5-V1, like the 1B-1strain, was able to grow fully on rich medium supplemented with various nonfermentable carbon sources (*i.e.,* lactate, glycerol, potassium acetate, or ethanol) (data not shown).

However, to exclude the possibility that subclone 3A5 bore an extragenic suppressor of the *rtg2-2* mutation, we crossed FYC5/Z-V1 with the *rtg2-2* mutant strain 1B-1/Z. The resulting diploid was sporulated and subjected to tetrad analysis. β-galactosidase activity was assayed in the segregants from 18 tetrads that were likewise tested for their ability to grow on SD + Ura + His + Leu + Trp medium. The segregation pattern was $0^+:4^-$ for

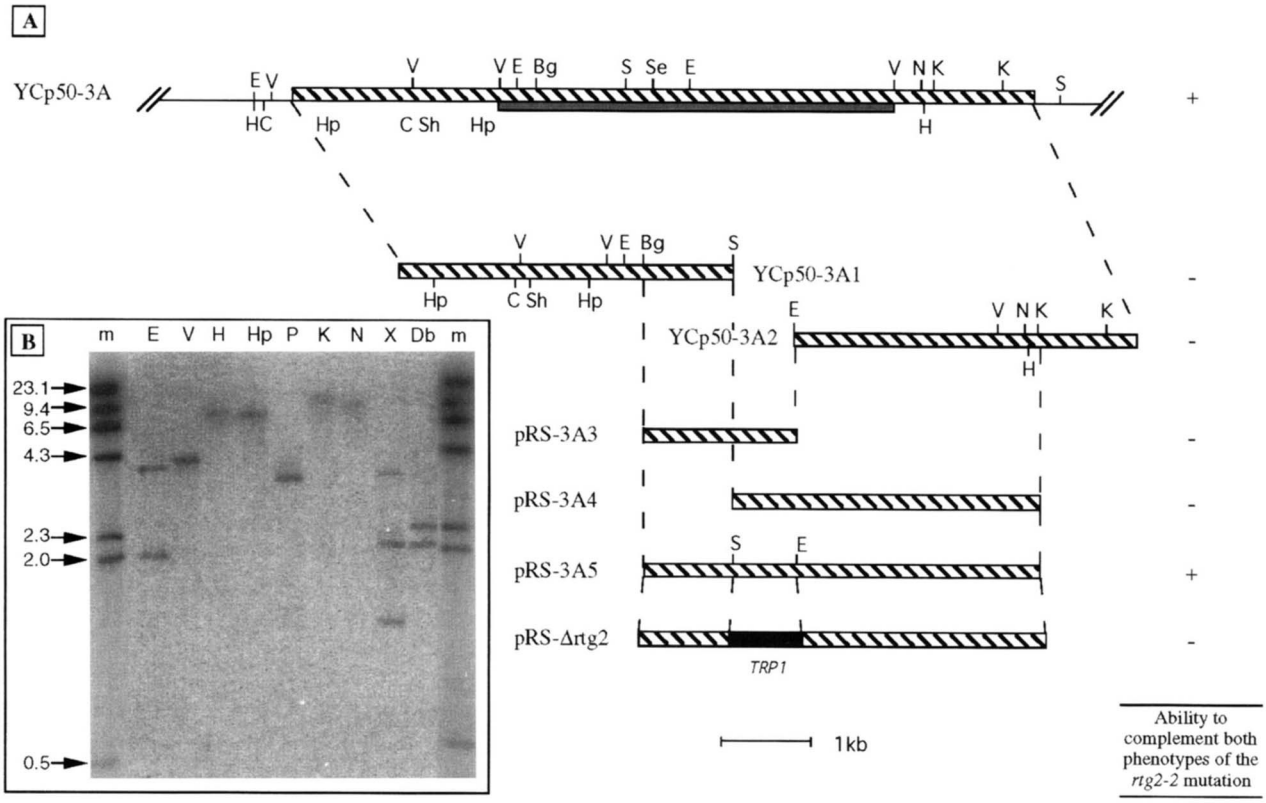

FIGURE 3.—Restriction map and subcloning analysis of the genomic area around the *RTG2* locus. (A) Restriction map of the genomic clone able to complement the *rtg2-2* mutation and subcloning experiments. Hatched boxes indicate DNA insert (3A) or subclones (3A1–3A5), and thin lines indicate vector DNA. Gray box below the map of insert 3A represents the DNA fragment used to probe the Southern blot. Plasmid YCp50-3A as well as plasmids bearing various DNA fragments from the insert were used to transform mutant strains (1B-1/Z and 1B-1) and tested for their ability to complement the β-galactosidase decrease phenotype (in 1B-1/Z strain) and poor growth phenotype (in 1B-1 strain). Each construction was either able (indicated as +) or unable (indicated as −) to complement both phenotypes. (B) Southern analysis of genomic DNA. Wild-type DNA (from strain FY1B) was cut with different restriction enzymes (Db means a double *Eco*RI + *Hin*dIII digestion), and the resulting DNA fragments were hybridized at high stringency to the ^{32}P-labeled *Eco*RV-*Eco*RV fragment on a Southern blot. Arrows on the left indicate the DNA size (in kbp) standards of lanes m (λ DNA cut with *Hin*dIII). Restriction site abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nco*I; P, *Pst*I; S, *Sal*I; Se, *Spe*I; Sh, *Sph*I; V, *Eco*RV; X, *Xba*I.

both phenotypes in all tetrads, thus confirming that we had not cloned an extragenic suppressor and consequently that FYC5/Z-V1 (and FYC5-V1 too) did bear a null allele (*rtg2::TRP1*) of the *RTG2* gene.

Sequence analysis of the 3A5 subclone DNA: We determined the sequence of this 3A5 region as described in MATERIALS AND METHODS. Approximately 500-base readings were obtained, and computer searches of available databases from EMBL+GenBank revealed that these sequences belonged to the *RTG2* gene, previously cloned by LIAO and BUTOW (1993). This gene is presumed to be a pivotal gene in controlling retrograde communication between mitochondria and the nucleus (*i.e.*, the fact that expression of some nuclear genes is sensitive to the functional state of mitochondria). *RTG2* encodes a protein of 394 amino acids of unknown function and was shown to affect the mRNA retrograde expression of the *CIT2* gene, which encodes the peroxisomal isoform of citrate synthase (CS2) (LIAO and BUTOW 1993).

Genetic relationships between *rtg2* and *asp5*: *ASP5* encodes aspartate aminotransferase, which catalyzes transamination from glutamate onto oxaloacetate to form α-ketoglutarate and aspartate. An *asp5* mutant is therefore auxotrophic for aspartate; it was observed that this auxotrophy is best scored on media lacking both aspartate and threonine (JONES and FINK 1982). Consequently, owing to the aspartate, threonine, and glutamate requirements of the *rtg2* mutants, we looked for interactions between the *rtg2* and *asp5* mutations. For this purpose, the 1B-1/Z strain was mated to strain SFY/Z-50a (see Table 1). After sporulation of the resulting diploid strain, the spores of 11 complete tetrads were characterized by determining their mating type and their ability to grow on SC media lacking either uracil (SC −U), histidine (SC −H), leucine (SC −L), tryptophan (SC −W), or both aspartate and threonine (SC −D −T). As expected, the segregation pattern, for each tetrad, was 2^a:2^α, 0^+:4^- on SC −U medium, 4^+:0^- on SC −H medium, and 2^+:2^- on SC −L, SC −W, and SC −D −T media. However, some spores grew slowly on SC −D −T, so that the segregation pattern became

TABLE 4

Δ*rtg2* strain is characterized by an *ACO1* transcription decrease under catabolite repression conditions

Strain	β-galactosidase activity[a] (SC-His[c])	Aconitase activity[b] YPD[c]	Aconitase activity[b] YPL[c]
FYC5/Z	44		
FYC5/Z-V1	8		
FYC5		28	328
FYC5-V1		0	371

[a] Average values given as Miller units and determined on three independent experiments.

[b] Values shown here represent nmol of *cis*-aconitate formed per min per mg of protein and were determined from two independent experiments under catabolite repression conditions (YPD) and from only one under derepression conditions (YPL).

[c] All cultures were performed to early log phase.

$3^+:1^-$ for eight of the 11 tetrads after several days at 28°. To test the hypothesis that *rtg2-2* was suppressing the *asp5* mutation and to pursue the study further, the genetic analysis was repeated but the original *rtg2-2* mutation was replaced by the *rtg2::TRP1* null mutation, so that *rtg2* segregants could be identified directly by tryptophan prototrophy. The diploid strain resulting from a cross between FYC5/Z-V1 and SFY/Z-50a (see Table 1) was sporulated and subjected to tetrad analysis. Growth tests (on the above-mentioned media) were carried out for segregants from 12 complete tetrads. Results showed that these tetrads were all tetratype, but that the $2^+:2^-$ segregation pattern on SC −D −T medium remained $2^+:2^-$ even after several days at 28°. These tetrads were kept on a YPD plate at 4°. After several weeks, we observed that for each tetrad, three spores were white and one was pink like the *asp5* parent (Figure 4). The pink/red coloration is well-known for *ade2* and *ade1* strains and is due to the accumulation in these mutants of a red pigment that apparently derives from phosphoribosylaminoimidazole (also termed AIR), a substrate of the enzyme encoded by *ADE2*. AIR is carboxylated by this enzyme to form phosphoribosylaminoimidazolecarboxylate (CAIR), which in turn is a substrate, with aspartate and ATP, of the enzyme encoded by *ADE1*, leading to phosphoribosylsuccinocarboxamide aminoimidazole (SAICAR) synthesis (JONES and FINK 1982). Thus, the pink coloration is not unexpected for cells deficient in aspartate, since this deficiency could result in reduced conversion of CAIR to SAICAR, thereby resulting in an *ade1* phenocopy (JONES and FINK 1982). The single pink spore clone of each tetrad shown in Figure 4 bears only the *asp5* mutation (indicated by *a*). Therefore, the segregant bearing both *rtg2::TRP1* and *asp5* mutations (indicated by *b*) is systematically white, just like the wild-type *RTG2 ASP5* spore (indicated by *c*), and that bearing the single *rtg2::TRP1* mutation (indicated by *d*). Thus, this result suggests that in the presence of the *rtg2* null allele, the *asp5* strain is able to synthesize enough aspartate to prevent the accumulation of AIR in the purine biosynthetic pathway, but not enough to support growth on SC media lacking aspartate. Consequently, this result confirms the partial suppression of *asp5* by the *rtg2-2* allele, initially observed on SC −D −T medium. Similar results for pigmentation were later seen in the cross involving the original *rtg2-2* and *asp5* mutations. To know if this suppressor effect was reciprocal, we measured the β-galactosidase activity in each segregant from six of the 12 tetrads shown in Figure 4. No evidence for other than $2^+:2^-$ segregation was seen for β-galactosidase expression (for all six tetrads), indicating that this partial metabolic suppression was probably "unidirectional" (data not shown).

DISCUSSION

In this paper, we present the isolation and identification in *S. cerevisiae* of a nuclear gene required to maintain, under catabolite repression conditions, a wild-type level of expression of the *ACO1* gene, encoding mitochondrial aconitase. A mutant was isolated, after EMS mutagenesis, displaying a second phenotype associated to the aconitase activity decrease: a partial growth defect on synthetic minimal glucose medium that was partially and fully overcome by aspartate and glutamate, respectively. Sequence analysis revealed that the gene we cloned by complementation of the growth defect was identical to *RTG2*, previously cloned, along with *RTG1*, by LIAO and BUTOW (1993) as genes required for *CIT2* retrograde regulation.

***RTG* genes and retrograde communication:** *RTG1* encodes a protein of 177 amino acids resembling members of the bHLH family of transcription factors (LIAO and BUTOW 1993). *RTG2* encodes a protein of 394 amino acids of unknown function and was recently shown to be related to bacterial phosphatases and to contain an ATPase domain of the Hsp70/actin/sugar kinase superfamily (KOONIN 1994). The term "retrograde communication" describes the fact that the mitochondrial state can influence expression of specific nuclear genes, like *CIT2*, which encodes peroxisomal citrate synthase (CS2) that operates as part of the glyoxylate cycle (ROSENKRANTZ *et al.* 1986; LEWIN *et al.* 1990). The *CIT2* transcription is elevated six- to 30-fold in a ρ° petite compared with isochromosomal respiratory competent (ρ^+) cells and this effect can be mimicked by the absence of a functional *CIT1* gene encoding mitochondrial citrate synthase (CS1) (LIAO *et al.* 1991). Recently, CHELSTOWSKA and BUTOW (1995) showed that *RTG* genes are also required for expression of genes encoding peroxisomal proteins.

***RTG2* regulates *ACO1* expression:** On the one hand, the finding that *RTG2* also affects *ACO1* expression sup-

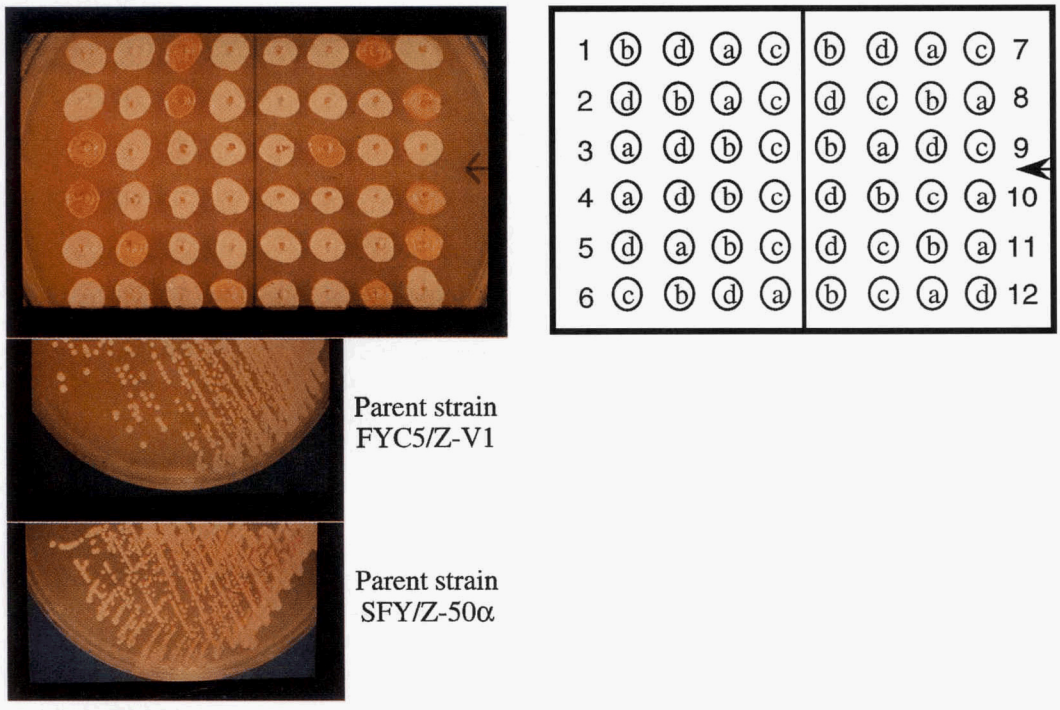

FIGURE 4.—Evidence of a partial metabolic suppression of *asp5* by the *rtg2* null allele. Strain FYC5/Z-V1 (*rtg2::TRP1*) was mated to strain SFY/Z-50α (*asp5*) and the resulting diploid was sporulated and subjected to tetrad analysis. Twelve tetrads were kept at 4° on YPD plate, and the pink coloration appeared progressively over several weeks. Numbers from 1 to 12 refer to tetrads, and letters from a to d refer to segregants as follows: single *asp5* mutant (a); double *rtg2::TRP1, asp5* mutant (b); wild-type *RTG2, ASP5* segregant (c); single *rtg2::TRP1* mutant (d).

ports the suggestion of LIAO and BUTOW (1993) that *RTG1* and *RTG2* act at a critical juncture between the glyoxylate and TCA cycles. On the other hand, this result indicates that *RTG2* might not necessarily be a specific actor of the retrograde regulation. Indeed, in contrast to the "*CIT1-CIT2* system" where the *CIT2* transcription and CS2 activity are stimulated when mitochondrial function is altered, an *aco1* strain does not display any aconitase activity (GANGLOFF 1990) although an extramitochondrial one has been found in wild-type cells (DUNTZE *et al.* 1969). Extramitochondrial aconitase, whose origin is still not understood, is supposed to be involved in the glyoxylate cycle. Moreover, we measured β-galactosidase activity in isogenic ρ^+ and ρ° strains transformed with a plasmid bearing the *ACO1-lacZ* fusion and no significant difference was observed (data not shown). The absence of aconitase activity in *aco1* cells indicates that not only the TCA but also the glyoxylate cycle is altered in the mutant as it is for cells bearing a double disruption of *CIT1* and *CIT2*. Consequently, it appears that *ACO1* is required for a functional glyoxylate cycle, either directly or indirectly. Thus, it seems that *RTG2* is related to genes required for expression of a fully functional glyoxylate cycle rather than to genes whose expression is elevated when mitochondrial function is impaired. However, it is likely that the role of *RTG2* is not restricted to the control of the glyoxylate cycle. Indeed, the fact that aspartate could partially restore the growth defect of an *rtg2* strain on synthetic minimal media, together with the recent finding that pyruvate carboxylase activity is reduced to 50% in Δ*rtg* mutants (SMALL *et al.* 1995), suggests that these mutants may be depleted for oxaloacetate. Thus it seems that *RTG2* may also affect expression of enzymes that catalyze reactions leading to oxaloacetate synthesis or oxaloacetate consumption, such as aspartate aminotransferase, encoded by *ASP5*. This hypothesis is supported by the partial suppression effect between *rtg2* and *asp5* mutations, which gives evidence of direct or indirect interactions between the corresponding genes.

Finally, it appears that *RTG2* acts at the juncture between the Krebs cycle and the anaplerotic pathways leading to TCA intermediate synthesis.

Auxotrophies and nutrient requirements of *rtg2* mutants: It is currently accepted that the glutamate requirement phenotype for aconitase-deficient cells is due to an α-ketoglutarate depletion (OGUR *et al.* 1964), but its understanding needs that of nitrogen metabolism. The nitrogen source used in synthetic minimal and complete media is usually ammonium sulfate, and utilization of ammonia occurs exclusively by its incorporation into glutamate and glutamine (MAGASANIK 1992), the main donors of cellular nitrogen (REITZER and MAGASANIK 1987). This incorporation requires a key metabolite that is α-ketoglutarate (MITCHELL and MAGASANIK 1983; MITCHELL 1985; WIAME *et al.* 1985; FOLCH *et al.* 1989; MILLER and MAGASANIK 1990; MAGA-

SANIK 1992). Therefore, it was theoretically possible to restore growth of the *rtg2* mutants on minimal media by supplying a nitrogen source able to give rise to glutamate without passing through α-ketoglutarate. Since proline is degraded directly to glutamate within mitochondria in a two steps (COOPER 1982), we expected that proline, when added to 0.1% w/v, would enable *rtg2* mutants to grow at wild-type rate on minimal media. This result was obtained and confirms that the pool of α-ketoglutarate is limited in *rtg2* mutants. Since proline is absent from the SC medium, the normal growth of the *rtg2* mutants observed on SC medium lacking glutamate is probably due to the cumulative effect of several nutrients, among which could be arginine, since it can be degraded into proline (MIDDELHOVEN 1964). However, we showed that arginine alone fails to restore the growth defect probably because its degradation pathway requires α-ketoglutarate as the amino group acceptor in the conversion of ornithine to glutamate-γ-semialdehyde catalyzed by ornithine transaminase (MIDDELHOVEN 1964).

Aspartate was able to partially restore the growth of *rtg2* mutants on minimal medium, while LIAO and BUTOW (1993) indicated that, like glutamate, it fully restores the growth of the null *rtg2* mutant. This difference was unexpected and could be due to the presence of different auxotrophic markers, *i.e.*, to a different composition of the minimal media.

In addition, we have shown that the partial restoration of the growth observed with aspartate is also obtained with threonine but without additive effect, indicating that the pathway by which the restoration occurs is the same for aspartate and threonine. This is not surprising because aspartate is the precursor of threonine (and methionine) via homoserine. Moreover, it has been observed that *asp5* mutants can be fed by a combination of threonine and methionine (JONES and FINK 1982) and that the aspartate auxotrophy of the *asp5* mutants is best scored on media lacking both aspartate and threonine. Aspartokinase, the first enzyme of the common pathway for synthesis of threonine and methionine from aspartate, is end-product inhibited by threonine (but not by methionine) and repressed fairly strongly (four- to sevenfold) by addition of threonine, but only weakly (30%) by addition of methionine (JONES and FINK 1982). Therefore, threonine could restore the growth of the *rtg2* mutants not by leading to aspartate synthesis but by preventing the consumption of the decreased pool of aspartate. Altogether, this could explain why methionine is not able to partially restore the growth of *rtg2* mutants on minimal media. In line with these results, succinate (10 mM) was also able to partially suppress the growth defect of *rtg2* mutants (data not shown).

Ability of the *rtg2* mutants to utilize nonfermentable carbon sources: We have shown that strains bearing either the original *rtg2-2* mutation or an *rtg2* null allele grow fully on rich media with glycerol, lactate, ethanol or acetate as carbon source, in contrast to strains bearing the *glu1-1* mutation or an *aco1* null allele, which do not grow at all on these rich media. However, LIAO and BUTOW (1993) showed that an *rtg2* null mutant does not grow on minimal acetate/ammonia medium even when supplemented with glutamate. We have confirmed this observation and noted that this carbon source utilization defect is specific for acetate and ethanol (not seen with lactate or glycerol) and observed only on synthetic minimal media (and not on SC media) (data not shown).

The inability of cells to grow on acetate is diagnostic of some defect in the TCA cycle (KIM *et al.* 1986; MCALISTER-HENN and THOMPSON 1987; KISPAL *et al.* 1989; CUPP and MCALISTER-HENN 1991). For *rtg2* mutants, this phenotype, like the growth defect phenotype, is observed only on minimal media. This means that, in complete media, several nutrients contribute to restoring a normal pool of some Krebs cycle intermediates (such as α-ketoglutarate and probably oxaloacetate) and thus to the recovery of a fully functional TCA cycle. When cells are shifted from a complete to a minimal medium, the absence of some metabolites necessarily requires some anabolic pathways, and the fact that *rtg2* mutants behave differently on these two types of media indicates that at least some of these pathways are controlled (directly or indirectly) by *RTG2*. This is consistent with the suggestion of SMALL *et al.* (1995) that phenotypes for the Δ*rtg* mutant cells could result from the cumulative effect of many small changes in the enzyme composition of the cell.

In any case, the elucidation of the specific role of the *RTG* genes requires the identification of new target genes as well as the physiological conditions under which the *RTG* genes operate for each one of these targets. Furthermore, it will be of great interest to determine whether *RTG2* is an actor of the synergistic regulation by glucose and glutamate of the *ACO1* gene.

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