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Glucose Metabolism in the Yeast *Schwanniomyces castellii*: Role of Phosphorylation Site I and an Alternative Respiratory Pathway

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Glucose metabolism in a Crabtree-negative yeast, *Schwanniomyces castellii*, and a cytochrome *b*-deficient mutant of this strain was investigated in chemostat culture. The wild-type and mutant strains exhibited the same behavior. Oxidative metabolism was observed when the substrate uptake rate (qS) was low. Fermentative metabolites were excreted when the qS value was higher than 0.40 $g \cdot g^{-1} \cdot h^{-1}$, indicating the occurrence of a respirofermentative metabolism; however, the respiratory quotient (RQ) remained near 1. When fermentation occurred, the cytochrome pathway was repressed but not the salicylhydroxamic acid (SHAM)-sensitive pathway. The presence of an alternative SHAM-sensitive respiratory pathway and the presence of phosphorylation site I in all metabolic conditions explained the RQ value of 1 and accounted for high biomass yields in oxidative metabolism conditions (0.62 $g \cdot g^{-1}$ for the wild-type strain and 0.31 $g \cdot g^{-1}$ for the cytochrome *b*-deficient mutant strain).

An antimycin-insensitive, hydroxamate-sensitive alternative respiratory pathway has been described in *Schwanniomyces castellii* CBS 2863 yeast (15, 16, 23). This pathway branches off from the classical cytochrome pathway at the ubiquinone level and is not coupled to phosphorylation (31). On the inner mitochondrial membrane, two NADH dehydrogenase activities are present that differ from those of respiratory chain complex I (31); these activities are not coupled to phosphorylation but join the respiratory chain at the ubiquinone level. One of these dehydrogenase activities faces externally and the other internally.

In continuous cultures of *S. castellii* CBS 2863, glucose metabolism changes with D. For low D (up to 0.18 h^{-1}), metabolism is oxidative and $Y_{X/S}$ is maximal (0.62 $g \cdot g^{-1}$). For D higher than 0.18 h^{-1}, metabolism is respirofermentative and $Y_{X/S}$ remains high (0.50 $g \cdot g^{-1}$) (5, 6). This strain is considered Crabtree negative (12, 23). Several studies concerning the balance between oxidative and fermentative metabolisms have been performed in continuous culture on *Saccharomyces cerevisiae* (Crabtree positive) and *Candida utilis* (Crabtree negative). Continuous culture of *S. cerevisiae* showed that regulation of glucose metabolism is based on limitation of cell respiratory capacity (18, 22, 26).

In the present work we have studied the influence of the SHAM-sensitive pathway and phosphorylation site I on the physiological characteristics of *S. castellii*. Experiments were performed in continuous culture when metabolism was either oxidative or respirofermentative.

MATERIALS AND METHODS

Abbreviations. D is an abbreviation for dilution rates (h^{-1}) and TCA is an abbreviation for tricarboxylic acid cycle; qO_2 is the specific oxygen consumption rate (microliters of $O_2 \cdot hour^{-1} \cdot milligram\ [dry\ weight]^{-1}$), $Y_{X/S}$ is the biomass yield (grams [dry weight] · gram of $glucose^{-1}$), and qS is the specific substrate (glucose) uptake rate (grams of glucose · gram $[dry\ weight]^{-1} \cdot hour^{-1}$). X is biomass, SHAM is salicylhydroxamic acid, and OD is optical density.

Strains. The wild type of *S. castellii* Capriotti, included in the species *S. occidentalis* Klöcker according to Kreger van Rij's new classification (19), is kept under the name *S. castellii* CBS 2863 at the Centraalbureau voor Schimmelcultures in Delft, The Netherlands. The DR12 mutant, obtained and described by Poinsot et al. (24), is deficient in cytochrome *b*.

Growth conditions. The carbon source was glucose. The growth medium was the synthetic G medium containing (in grams per liter) KH_2PO_4 (1), $(NH_4)_2SO_4$ (2), $NH_4H_2PO_4$ (6), NaCl (0.1), and $MgSO_4 \cdot 7H_2O$ (0.5). The medium also contained (in micrograms per liter) $CaCl_2 \cdot 2H_2O$ (0.1), H_3BO_3 (500), $CuSO_4 \cdot 5H_2O$ (40), KI (100), $MnSO_4 \cdot H_2O$ (400), Na_2MoO_4 (200), $ZnSO_4$ (400), $FeCl_3 \cdot 6H_2O$ (200), calcium pantothenate (2,000), thiamine (2,000), inositol (2,000), pyridoxine (2,000), nicotinic acid (500), and biotin (20). Precultures were carried out in Erlenmeyer flasks filled to 1/10 of their capacity and under aerobic conditions on a shaker (80 oscillations per min; amplitude, 7 cm) at 28°C. The medium was buffered to pH 5.4 with a 0.1 M disodium phosphate-tartaric acid buffer.

Chemostat cultures were carried out in a Biostat E bioreactor (Braun, Melsungen, Germany). Exposed results were provided from two distinct experimentations on each strain. The working volume of the culture was 2.5 liters. The yeasts were grown at 28°C with an airflow of 2 vol/vol/min for the wild type and 1 vol/vol/min for the DR12 strain. Dissolved oxygen tension with a stirrer speed of 700 rpm was always higher than 80% of air saturation. pH was automatically controlled at 5.4 by the addition of 2 N NaOH. The glucose concentration in the reservoir was 10 $g \cdot liter^{-1}$. Previous research (5) has shown that cultures are glucose limited in these conditions. Oxygen and carbon dioxide were analyzed in the dried gas exhausted from the fermentor by using a paramagnetic oxygen analyzer (755A Beckman) and an infrared carbon dioxide analyzer (870 Beckman).

Cell population determination. Cell growth was monitored by measuring the OD at 420 nm with a Beckman DU7 spectrophotometer. At each D, when the culture was in a steady-state condition, the proportionality between dry weight and OD was established. The dry weight of parallel samples varied by less than 1% for the wild type and by 0.6% for the mutant strain. One OD unit represents $0.22 \pm 0.01\ g \cdot liter^{-1}$ between D = 0.10 h^{-1} and D = 0.36 h^{-1} for the wild strain and $0.20 \pm 0.01\ g \cdot liter^{-1}$ between D = 0.08 h^{-1} and D = 0.20 h^{-1} for the mutant strain.

Control of mutant stability. In the case of the mutant strain, insensitivity to KCN was controlled at each dilution rate.

Oxygen consumption measurements. Total qO_2 and maximal capacity of respiratory pathways were determined polarographically on the whole cells with a Clark-type oxygen electrode as described by Dubreucq et al. (16). KCN (0.25 mM) was used as a specific inhibitor of the cytochrome pathway, and SHAM (1.2 mM) was used as a specific inhibitor of the alternative pathway.

Protein determination. Protein contents in the cells were determined according to the method described by Stickland (29), with bovine serum albumin (Sigma) as the standard. Protein contents in the enzymatic fractions were deter-

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mined according to the method described by Bradford (7), with bovine serum albumin as the standard.

Analysis of glucose and metabolites. Analyses were performed on the supernatant after centrifugation (11,000 × *g*, 5 min) of a culture sample. Glucose concentration was determined by using a glucose enzymatic color kit (Biotrol, Paris, France). Acetate, ethanol, glycerol, and acetaldehyde were separated by an FAM-PAK high-performance liquid chromatography column (Waters model 600E) in ion exclusion mode. Before injection, samples were filtered by using a Millipore filter (pore size, 0.45 μm). The mobile phase was 3 mM orthophosphoric acid (Merck). Flow rate was 1 ml · min^{-1} at 40°C. Detection was performed by refractometry (Waters model 410). The lower detection level of the high-performance liquid chromatograph was 0.1 g · $liter^{-1}$. Pyruvate, citrate, oxaloacetate, 2-oxoglutarate, succinate, and L-malate concentrations were determined according to the method described by van Urk et al. (30).

Carbon recoveries. Carbon recoveries were calculated during steady-state experiments. The amount of carbon produced which formed part of each of the products (CO_2 [dry weight], acetaldehyde, acetate, and ethanol) was compared to the substrate carbon (glucose) and expressed in the same units, grams of carbon formed or consumed · gram [dry weight]$^{-1}$ · $hour^{-1}$, as follows: $C_{glucose} = 0.40$ qS (grams of glucose · grams [dry weight]$^{-1}$ · h^{-1}); $C_{CO_2} = 0.27\ q_{CO_2}$ (grams of CO_2 · grams [dry weight]$^{-1}$ · h^{-1}); $C_{ethanol} = 0.52\ q_{ethanol}$ (grams of ethanol · grams [dry weight]$^{-1}$ · h^{-1}); $C_{acetate} = 0.40\ q_{acetate}$ (grams of acetate · grams [dry weight]$^{-1}$ · h^{-1}); $C_{acetaldehyde} = 0.54\ q_{acetaldehyde}$ (grams of acetaldehyde · gram [dry weight]$^{-1}$ · h^{-1}); and $C_X = 0.49\ q_X$ grams of X · gram [dry weight]$^{-1}$ · h^{-1}). The theoretical equation for transformation of glucose into biomass (17) enables determination of the coefficient 0.49 (12/24.4): $1.8n\ CH_2O + 0.8n\ O_2 + 0.19n\ NH_4 \rightarrow n(CH_{1.7}O_{0.5}N_{0.19}) + 1.5n\ H_2O + 0.8n\ CO_2$.

Cell fractionation. Protoplasts were prepared by zymolyase treatment and disrupted by osmotic shock according to the method described by Bruinenberg et al. (8) and modified according to the method described by Zimmer (31). The resulting fraction, called the F_T fraction, contained mitochondria and cytoplasm but no cellular membrane.

Enzyme assays. Enzyme activities were assayed at 25°C on F_T fractions using a Beckman DU7 spectrophotometer. F_T fractions were conserved at −20°C after preparation in order to break organelles. Reaction rates were proportional to the amount of enzyme added. The assay mixtures for the individual enzymes are described below.

(i) Glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The assay mixture contained 50 mM Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 0.4 mM NADP, and 5 mM glucose-6-phosphate. The reaction was started by the addition of F_T fraction (8).

(ii) Phosphofructokinase (EC 2.7.1.11). The assay mixture contained 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.15 mM NADH, 2 mM fructose-6-phosphate, 0.2 mM dithiothreitol, 0.15 mM ATP, 0.9 U of aldolase, 10 U of triose phosphate isomerase, and 0.8 U of glycerol-3-phosphate dehydrogenase. The reaction was started by the addition of fructose-6-phosphate (9).

(iii) Pyruvate kinase (EC 2.7.1.40). The assay mixture contained 50 mM potassium phosphate buffer (pH 6.45), 8 mM $MgCl_2$, 0.3 mM NADH, 25 mM phosphoenolpyruvate, and 27.5 U of lactate dehydrogenase. The reaction was started by the addition of phosphoenolpyruvate (25).

(iv) Alcohol dehydrogenase (EC 4.1.1.1). The assay mixture contained 50 mM sodium pyrophosphate buffer (pH 8.7), 0.1 mM ethanol, and 0.6 mM NAD. The reaction was started by the addition of F_T fraction (4).

(v) Pyruvate dehydrogenase (EC 1.2.4.1). The assay mixture contained 50 mM Tris-HCl buffer (pH 7.8), 1 mM $MgCl_2$, 0.5 mM NAD, 1 mM cysteine, 0.2 mM thiamine pyrophosphate, and 0.1 mM coenzyme A. The reaction was started by the addition of F_T fraction (27).

(vi) Isocitrate dehydrogenase (EC 1.1.1.42). The assay mixture contained 50 mM Tris-HCl buffer (pH 7.5), 1 mM $MnSO_4$, 0.25 mM NADP, 0.3 mM dithiothreitol, and 1.6 mM isocitrate. The reaction was started by the addition of isocitrate (27).

(vii) 2-Oxoglutarate dehydrogenase (EC 1.2.4.2). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.8), 1 mM $MgCl_2$, 0.5 mM NAD, 1 mM cysteine, 0.2 mM thiamine pyrophosphate, 0.1 mM coenzyme A, and 0.2 mM 2-oxoglutarate. The reaction was started by the addition of F_T fraction (27).

(viii) Succinate dehydrogenase (succinate-phenazine methosulfate oxidoreductase [EC 1.3.99.1]). The assay mixture contained 50 mM Tris-HCl buffer (pH 7.2), 20 mM succinate, 0.6 mM phenazine methosulfate, 3 mM sodium azide and, 0.08 mM 2,6-dichlorophenol indophenol. The reaction was started by the addition of 2,6-dichlorophenol indophenol and phenazine methosulfate (2).

(ix) Malate dehydrogenase (EC 1.1.1.37). The assay mixture contained 50 mM potassium phosphate buffer (pH 6.5), 0.8 mM NADH, and 1 mM oxaloacetate. The reaction was started by the addition of F_T fraction (25).

(x) NADH dehydrogenase (EC 1.6.99.3). The assay mixture contained 50 mM potassium phosphate buffer (pH 6.0), 5 mM potassium cyanide, 1 mM potassium ferricyanide, and 0.15 mM NADH. The reaction was started by the addition of F_T fraction (8). This method allowed intramitochondrial and extramitochondrial NADH dehydrogenase activities to be assayed.

(xi) Cytochrome *c* oxidase (EC 1.9.3.1). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0) and 0.04 mM reduced cytochrome *c* (cytochrome *c* was stoichiometrically reduced by ascorbate). The reaction was started by the addition of F_T fraction (8).

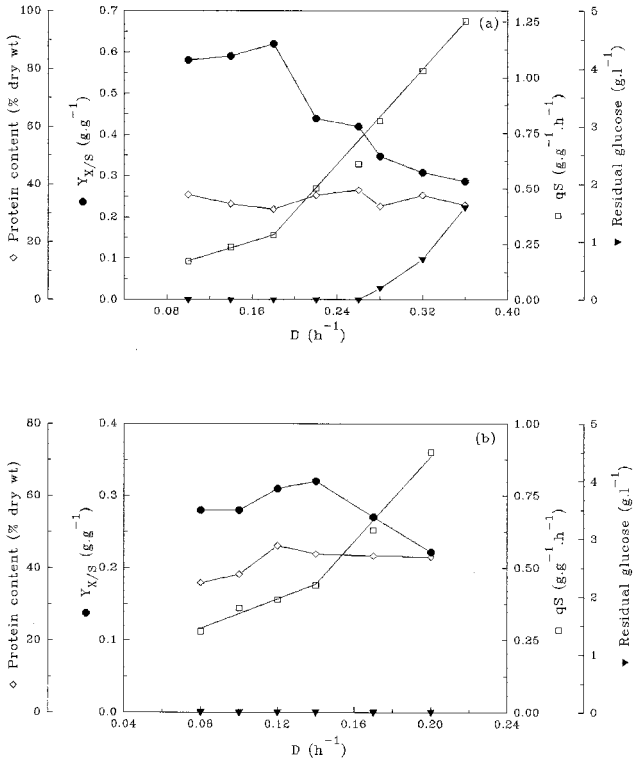

FIG. 1. $Y_{X/S}$, qS, residual glucose, and protein content as functions of D in glucose-limited cultures of *S. castellii* CBS 2863 (a) and DR12 (b).

(xii) NADH-cytochrome *c* reductase (EC 1.6.2.1). The assay mixture contained 50 mM Tris-HCl buffer (pH 7.2), 0.06 mM NADH, 3 mM sodium azide, and 0.06 mM ferricytochrome *c*. The reaction was started by the addition of F_T (10). This method theoretically allowed the activity of the respiratory chain from NADH dehydrogenases up to cytochrome *c* to be assayed.

(xiii) Succinate-cytochrome *c* reductase. The assay mixture contained 50 mM Tris-HCl buffer (pH 7.2), 3 mM succinate, 3 mM sodium azide, and 0.08 mM ferricytochrome *c*. The reaction was started by the addition of F_T (10). This method theoretically allowed the activities of complexes II and III of the respiratory chain to be assayed.

RESULTS

q(S) and $Y_{X/S}$ in steady-state cultures. In *S. castellii* CBS 2863, qS increased linearly as a function of D with a change in slope at D = 0.18 h^{-1} (Fig. 1a). Up to D = 0.18 h^{-1}, the slope was 1.47 ± 1.10 g of glucose · g (dry weight)$^{-1}$, $Y_{X/S}$ was maximal (0.62 g · g^{-1}) and metabolite excretion could not be detected: metabolism was fully oxidative. Above D = 0.18 h^{-1}, the slope of qS versus D was significantly different (5.58 ± 0.89 g of glucose · g [dry weight]$^{-1}$); $Y_{X/S}$ decreased to 0.35 g · g^{-1} at D = 0.28 h^{-1}; ethanol was excreted; metabolism was respirofermentative. The protein content did not change with D: the mean value was 33.8% ± 2.7% (dry weight).

Mutant strain DR12 exhibited the same behavior, except that the slope changed at D = 0.14 h^{-1} (Fig. 1b). Up to this value, the slope of qS as a function of D was 2.55 ± 0.28 g of glucose · g (dry weight)$^{-1}$, and $Y_{X/S}$ was 0.31 g · g^{-1}. Above D = 0.14 h^{-1}, the slope of qS versus D was significantly higher (7.68 ± 1.96) and $Y_{X/S}$ decreased to 0.22 g · g^{-1} at D = 0.20 h^{-1}. As in the wild-type strain, the protein content of DR12 did not change with D: the mean value was 41.6% ± 3.5% (dry weight).

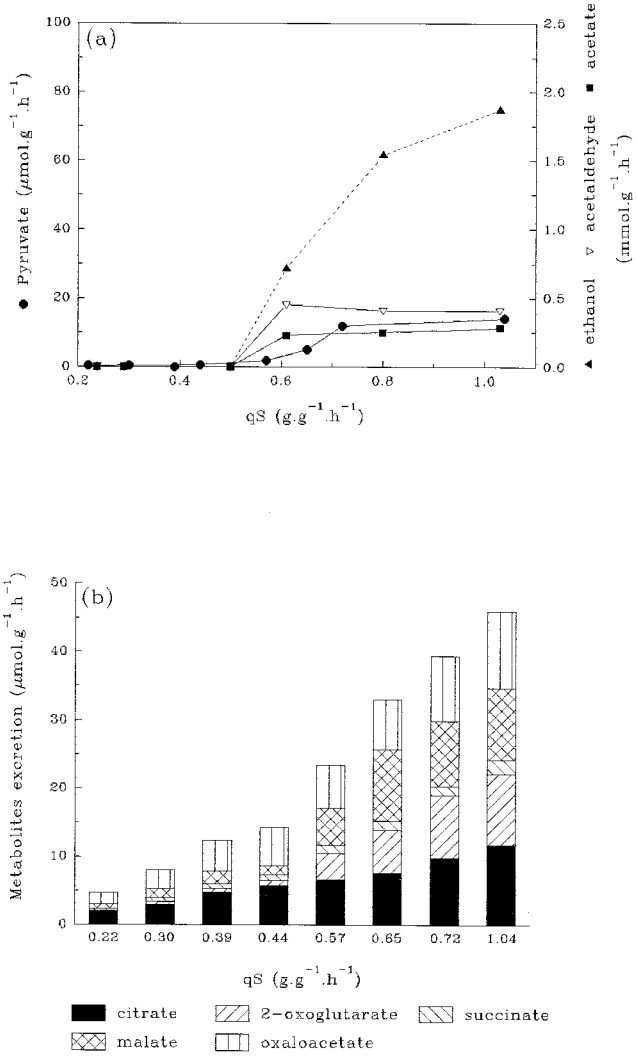

FIG. 2. Metabolite excretion as a function of qS in glucose-limited cultures of *S. castellii* CBS 2863. (a) Pyruvate, ethanol, acetaldehyde, and acetate excretion rates are shown. (b) Citrate, 2-oxoglutarate, malate, succinate, and oxaloacetate excretion rates are shown.

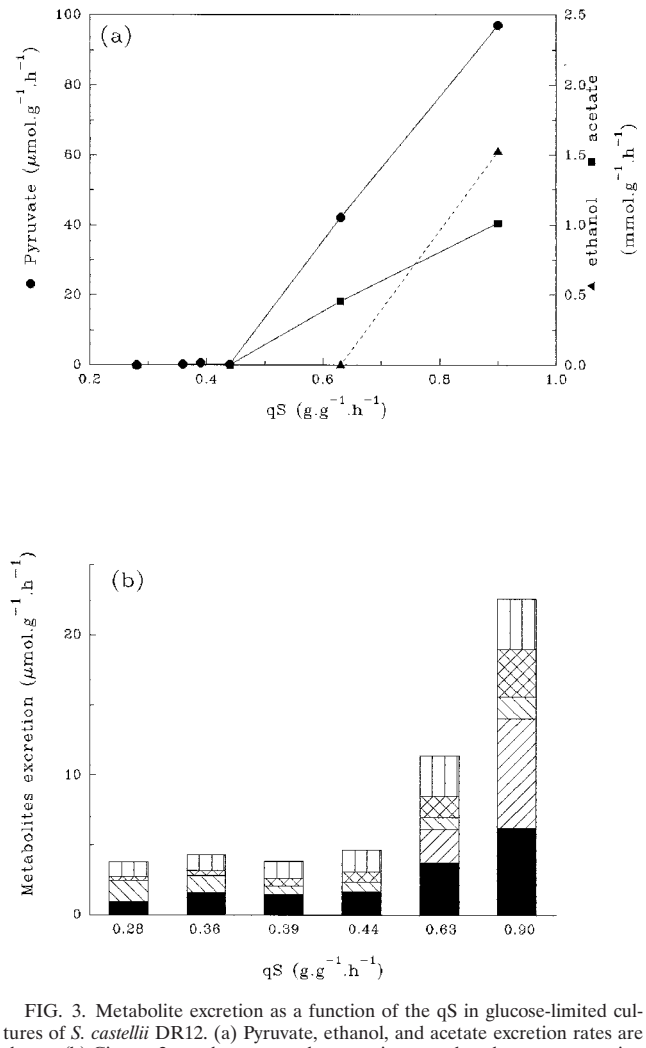

FIG. 3. Metabolite excretion as a function of the qS in glucose-limited cultures of *S. castellii* DR12. (a) Pyruvate, ethanol, and acetate excretion rates are shown. (b) Citrate, 2-oxoglutarate, malate, succinate, and oxaloacetate excretion rates are shown. Metabolites are as defined in Fig. 2b.

In both strains, the change in the slope of qS versus D was observed at the same value of qS: near 0.40 $g \cdot g^{-1} \cdot h^{-1}$.

Metabolite production in steady-state cultures. Analysis of the culture fluid revealed the presence of different metabolites. The data were expressed in micromoles or millimoles per gram (dry weight) as a function of qS in order to compare the biological activities of the two strains (Fig. 2 and 3). Up to qS = 0.40 $g \cdot g^{-1} \cdot h^{-1}$ pyruvate, the end product of glycolysis at the branching point of the oxidative and the fermentative pathways, was excreted at a low level in both strains (Fig. 2a and 3a). Above qS = 0.40 $g \cdot g^{-1} \cdot h^{-1}$, pyruvate excretion increased by a factor of 26 in the wild type and by a factor of 323 in the DR12 strain. Significant increases in ethanol and acetate excretion also occurred in both strains (Fig. 2a and 3a). Acetaldehyde was detected only for the wild-type strain. For qS values near 1 $g \cdot g^{-1} \cdot h^{-1}$, the DR12 strain excreted fourfold more acetate per gram (dry weight) than the wild type. Among TCA intermediate metabolites (Fig. 2b and 3b), excretion of malate, 2-oxoglutarate, and citrate increased with an increasing qS above 0.40 $g \cdot g^{-1} \cdot h^{-1}$ (Fig. 2b and 3b), showing a saturation of TCA. These three TCA metabolites require NAD directly or indirectly for their oxidation by TCA enzymes. Thus, the amount of NAD is likely insufficient to allow total oxidation of TCA metabolites.

These results confirm that for both strains, oxidative metabolism was observed when qS was below 0.40 $g \cdot g^{-1} \cdot h^{-1}$; above this value, a respirofermentative metabolism occurred.

Carbon recoveries. Studies conducted during steady-state conditions included the drawing up of the carbon balance (Fig. 4). The carbon content in yeast was estimated according to the theoretical equation given in Materials and Methods. The mean value was 49%. Glycerol, pyruvate, and TCA metabolites were not taken into account because they represented less than 10^{-3}% of the carbon content. In the case of the wild type, carbon recovery was 2% higher than the theoretical value (mean value of seven dilution rates), whereas the recovery was 17% lower for the mutant strain (mean value of six dilution rates). This gap could be explained by the choice of the same theoretical mean value of the carbon content in yeast. However, results showed that there is no accumulation of storage carbohydrates.

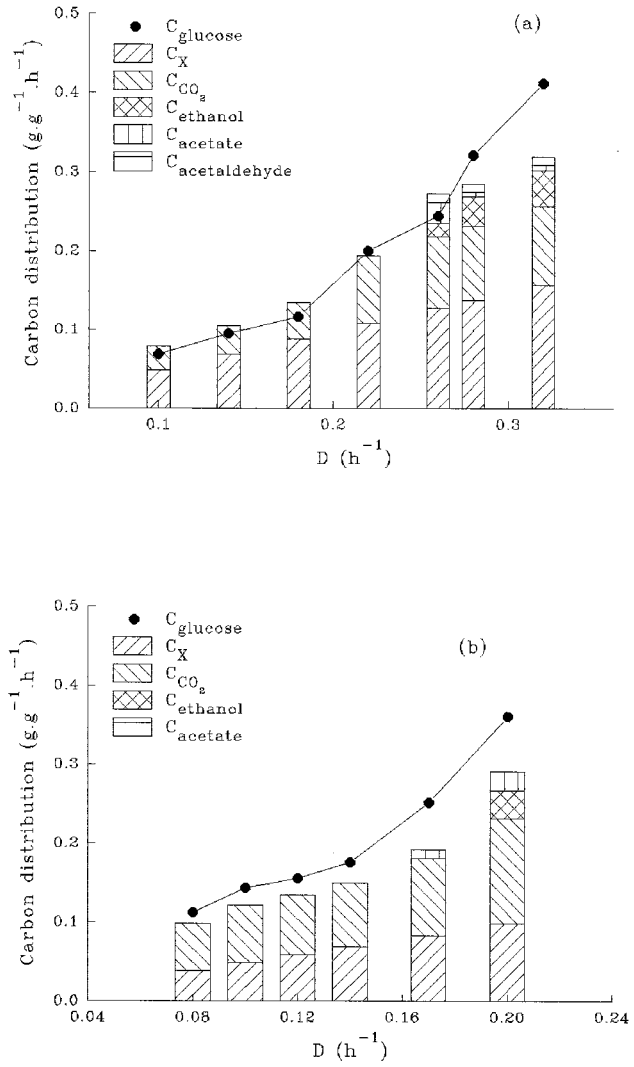

FIG. 4. Development of glucose consumption and product formation rates expressed in carbon element as a function of D in glucose-limited chemostat cultures of CBS 2863 (a) and DR12 (b). The estimated error for C_{CO_2} is ≤ 2% (mean of five measurements). The standard deviations for $C_{glucose}$, $C_{ethanol}$, $C_{acetate}$, $C_{acetaldehyde}$, and C_X are ≤5, ≤2, ≤3, ≤2, and ≤1%, respectively (means of five measurements).

To complete this study, we investigated the oxidative metabolism at D = 0.14 h^{-1} in both strains and the respirofermentative metabolism at D = 0.30 h^{-1} in the wild type and D = 0.20 h^{-1} in the mutant strain. The mutant strain washed out at D = 0.23 h^{-1}.

Respiratory pathways and enzyme levels in steady-state cultures. Either in oxidative or respirofermentative metabolic conditions, total qO_2 was higher for DR12 than for the wild type. In the wild type, total qO_2 decreased by 35% between D = 0.14 h^{-1} and D = 0.30 h^{-1} (Table 1). At D = 0.30 h^{-1}, cytochrome pathway capacity was negligible and the SHAM-sensitive pathway was the only active pathway. In opposition to our previous hypothesis (5), it appeared that the cytochrome pathway and not the SHAM-sensitive pathway was repressed in respirofermentative metabolic conditions. In the mutant strain (cytochrome *b* deficient), total qO_2 was similar at both dilution rates (Table 1), and only the alternative pathway was active. In both strains, total qO_2 was at least 130 μl of $O_2 \cdot h^{-1} \cdot mg$ (dry weight)$^{-1}$ in respirofermentative metabolism. These high qO_2 values were caused by the presence of the SHAM-sensitive pathway, which is not as sensitive to glucose repression as is the cytochrome pathway.

Cytoplasmic enzyme activities were higher in the DR12 strain than in the wild type (Table 2) except for pyruvate kinase activity. However, pyruvate kinase activity was always higher than phosphofructokinase activity and did not constitute the limiting steps of glycolysis. In both strains, pyruvate kinase activity was independent of the type of metabolism, whereas glucose-6-phosphate dehydrogenase and phosphofructokinase activities were slightly lower when the metabolism was respirofermentative. Only alcohol dehydrogenase activity increased significantly in both strains when the metabolism was respirofermentative, showing the triggering of fermentative pathways.

When the metabolism was fully oxidative, TCA activities were higher in the DR12 strain than in the wild type (Table 2). In DR12, all activities were more than twofold lower in respirofermentative metabolism. In the case of the wild type, only isocitrate dehydrogenase and malate dehydrogenase activities were significantly lower (Table 2).

Respiratory chain activities decreased in respirofermentative metabolism (Table 2). Decrease of NADH dehydrogenase activity confirmed the lower NADH reoxidation rate observed during the study of the excretion of TCA metabolites. Decrease of cytochrome *c* oxidase activity confirmed the repression of the oxidative activities in respirofermentative metabolism. In the DR12 strain, NADH-cytochrome *c* reductase activity was present when no succinate-cytochrome *c* reductase activity was detectable. This suggests the existence of a NADH-cytochrome *c* reductase activity which is different from that of the respiratory chain complex III. It could be a contaminating microsomal activity (13) or an activity located in the outer mitochondrial membrane (3, 11).

DISCUSSION

The present study indicated that the SHAM-sensitive respiratory pathway was always present in *S. castellii* whatever the growth conditions. This respiratory pathway allowed the activity of phosphorylation site I. The activity of phosphorylation site I (28) explained the high $Y_{X/S}$ of *S. castellii* CBS 2863 (0.62 $g \cdot g^{-1}$) and of the mutant strain (0.31 $g \cdot g^{-1}$) in oxidative conditions.

In respirofermentative metabolic conditions, i.e., when qS was higher than 0.40 $g \cdot g^{-1} \cdot h^{-1}$, the cytochrome pathway

TABLE 1. Maximum capacity of the different respiratory pathways at different D in glucose-limited steady-state cultures of *S. castellii* CBS 2863 and DR12[a]

Respiratory pathway	Respiratory capacity (μl of $O_2 \cdot h^{-1} \cdot mg$ [dry weight]$^{-1}$) in strain:			
	CBS 2863		DR12	
	0.14 h^{-1} D	0.30 h^{-1} D	0.14 h^{-1} D	0.20 h^{-1} D
Total qO_2	200	130	242	217
Cytochrome pathway	112	12	0	0
SHAM-sensitive pathway	74	85	288	290

[a] Measurements were done with glucose as substrate. Inhibitors used were potassium cyanide (0.25 mM), SHAM (1.25 mM), and sodium azide (0.16 mM). Respiration attributed to each pathway was calculated as follows: qO_2(cytochromic pathway) = qO_2(total) − qO_2(cyanide); and qO_2(SHAM-sensitive pathway) = qO_2(cyanide + azide) − qO_2(cyanide + SHAM + azide).

TABLE 2. Specific enzyme activities at different D in glucose-limited steady-state cultures of *S. castellii* CBS 2863 and DR12[a]

Enzyme	Enzyme activity (nmol · min^{-1} · mg of protein^{-1}) in strain:			
	CBS 2863		DR12	
	0.14 h^{-1} D	0.30 h^{-1} D	0.14 h^{-1} D	0.20 h^{-1} D
Cytoplasmic activities				
Glucose-6-phosphate dehydrogenase	768 ± 133	386 ± 76	963 ± 20	731 ± 29
Phosphofructokinase	107 ± 27	83 ± 9	314 ± 141	116 ± 10
Pyruvate kinase	1198 ± 174	1168 ± 156	518 ± 61	586 ± 57
Alcohol dehydrogenase	7 ± 1	40 ± 24	39 ± 12	109 ± 22
TCA activities				
Pyruvate dehydrogenase	7 ± 2	4 ± 2	32 ± 3	3 ± 1
Isocitrate dehydrogenase	80 ± 11	19 ± 8	262 ± 14	135 ± 17
2-Oxoglutarate dehydrogenase	8 ± 2	11 ± 1	25 ± 5	2 ± 1
Succinate dehydrogenase	12 ± 4	10 ± 3	51 ± 21	9 ± 1
Malate dehydrogenase	2573 ± 613	1378 ± 349	2550 ± 986	1263 ± 89
Respiratory chain activities				
NADH dehydrogenase	1414 ± 213	809 ± 146	1451 ± 85	788 ± 118
NADH-cytochrome *c* reductase	148 ± 49	114 ± 51	89 ± 12	43 ± 12
Succinate-cytochrome *c* reductase	43 ± 7	31 ± 8	nd[b]	nd[b]
Cytochrome *c* oxidase	59 ± 12	24 ± 7	157 ± 51	58 ± 7

[a] Each result is the means (± standard deviation) of four measurements (two measurements on two samples). Specific activities were measured on F_T fractions from cells of *S. castellii* CBS 2863 and DR12. Proportionality between specific activity and F_T volume has been verified for each enzyme.
[b] nd, not detectable.

was the only one repressed in *S. castellii* CBS 2863. The SHAM-sensitive pathway was insensitive to glucose repression in both the wild-type and mutant strains.

In oxidative conditions, the maximal capacity of the SHAM-sensitive pathway, expressed by the qO_2, was 3.5-fold higher for the DR12 strain (288 μl of $O_2 \cdot h^{-1} \cdot$ mg [dry weight]$^{-1}$) than for the wild strain (74 μl of $O_2 \cdot h^{-1} \cdot$ mg [dry weight]$^{-1}$), whereas total qO_2s were similar (240 and 200 μl of $O_2 \cdot h^{-1} \cdot$ mg [dry weight]$^{-1}$, respectively). At the same time, glycolytic flux (expressed by the qS value) was twofold higher in the DR12 strain than in the wild type for the same D (0.14 h^{-1}). One of the key enzymes of glycolysis regulation, phosphofructokinase, presented a threefold higher activity in the DR12 strain than in the wild type. The activities of the TCA enzymes were higher in the DR12 strain. All these results showed that, in continuous culture, the mutant strain metabolized glucose about twofold quicker than the wild type for the same D (0.14 h^{-1}). Most of the enzyme activity levels (e.g., cytoplasmic and TCA) were higher in the DR12 strain. This observation could partially explain the higher protein content in the mutant strain (41.6%) compared to the wild type (33.8%).

Unlike other strains (*S. cerevisiae* or *C. utilis*), phosphorylation site I was always present in *S. castellii*. In *S. cerevisiae*, phosphorylation site I was not present in the exponential growth phase and appeared only after a long stationary phase (21). In *C. utilis*, phosphorylation site I was present only for cells grown under suboptimal conditions in batch culture (20) or under various limitations in continuous culture (114, 30). The presence of phosphorylation site I and of the SHAM-sensitive pathway, which was insensitive to glucose repression, explained an RQ close to 1 even in respirofermentative conditions. Classification of *S. castellii* as a Crabtree-negative strain has to be discussed. This strain is situated at an intermediate level between Crabtree-negative and Crabtree-positive strains. Indeed, the enzymatic systems specifically involved in the cytochrome pathway are repressed by glucose as in Crabtree-positive strains, but those involved in both the cytochrome and SHAM-sensitive pathways are not. Furthermore the RQ value remains near 1 as in Crabtree-negative yeasts.

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