Carbonic anhydrase (Nce103p): an essential biosynthetic enzyme for growth of *Saccharomyces cerevisiae* at atmospheric carbon dioxide pressure

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The *NCE103* gene of the yeast *Saccharomyces cerevisiae* encodes a CA (carbonic anhydrase) that catalyses the interconversion of CO_2 and bicarbonate. It has previously been reported that *nce103* null mutants require elevated CO_2 concentrations for growth in batch cultures. To discriminate between 'sparking' effects of CO_2 and a CO_2 requirement for steady-state fermentative growth, we switched glucose-limited anaerobic chemostat cultures of an *nce103* null mutant from sparging with pure CO_2 to sparging with nitrogen gas. This switch resulted in wash-out of the biomass, demonstrating that elevated CO_2 concentrations are required even under conditions where CO_2 is produced at high rates by fermentative sugar metabolism. Nutritional analysis of the *nce103* null mutant demonstrated that growth on glucose under a non-CO_2-enriched nitrogen atmosphere was possible when the culture medium was provided with L-aspartate, fatty acids, uracil and L-argininine. Thus the main physiological role of CA during growth of *S. cerevisiae* on glucose-ammonium salts media is the provision of inorganic carbon for the bicarbonate-dependent carboxylation reactions catalysed by pyruvate carboxylase, acetyl-CoA carboxylase and CPSase (carbamoyl-phosphate synthetase). To our knowledge, the present study represents the first full determination of the nutritional requirements of a CA-negative organism to date.

Key words: carbon dioxide, carbonic anhydrase, carboxylase, carboxylation, CPSase, *Saccharomyces cerevisiae*.

INTRODUCTION

Carbonic anhydrase (CA; EC 4.2.1.1) is a ubiquitous enzyme present in organisms belonging to all branches of the evolutionary tree, from archaea to mammals. CA catalyses the reversible hydration of CO_2 to $HCO_3^- + H^+$. This reaction can also occur spontaneously when the CO_2 concentration is sufficiently high. In nature, CO_2 concentrations are strongly influenced by factors such as pH, temperature and presence of other solutes [1]. Based on protein structure, CAs are divided into four classes: α, β, γ and δ [2]. The differences in tertiary and quaternary structures among the four classes of CAs constitute a spectacular case of convergent evolution, in which the same catalytic activity has evolved via several independent, evolutionarily very ancient ways [2]. This suggests that CA-catalysed interconversion of CO_2 and HCO_3^- is important for all forms of life.

Although universal, the α-class is best characterized in mammals, whereas isoenzymes of the β-class have been described in higher plants, algae, fungi, archaea and bacteria. Known members of the γ-class are only prokaryotes and the δ-class has only one member, the CA of the diatom *Thalassiosira weissflogii* [2,3]. Despite its widespread occurrence, CA has been deeply investigated in only a few organisms. Much research has been performed on mammalian cells because some CA isoenzymes are expressed at increased levels in tumours [4]. In mammals, CA is expressed in almost all tissues. Proposed functions include oxygen transport between lungs, red blood cells and tissues, pH regulation, ion exchange in the kidney and electrical activity in the retina and nervous system [5,6]. Another situation in which the role of CA has been extensively investigated is in autotrophic organisms, where CA is mostly related to the provision of bicarbonate for carbon fixation [7,8].

In contrast with the systems mentioned above, very little is known about the physiological role of CAs in heterotrophic microbes. Several studies on bacteria indicate that loss of CA activity impairs aerobic growth, unless the air is supplemented with extra CO_2 [9–12]. Apparently, at elevated CO_2 concentration, spontaneous hydration may obviate the need for CA-catalysed bicarbonate formation. This has led to the hypothesis that CA is involved in providing HCO_3^- for carboxylation reactions, such as the anaplerotic replenishment of C_4 intermediates, citrulline biosynthesis or fatty acid elongation. Similar biosynthetic functions have been proposed for mammalian CA [13–15] but, to our knowledge, the nutritional requirements of CA-deficient mutants have hitherto not been fully investigated for any heterotrophic organism.

In the budding yeast *Saccharomyces cerevisiae*, CA is encoded by *NCE103*, a gene that was formerly reported to be involved in non-classical protein secretion [16]. Aerobic growth was also impaired in an *S. cerevisiae nce103* null mutant, but could be restored by heterologous expression of CA-encoding genes from alfalfa (*Msca1*) or *Homo sapiens* (CAII) [17,18]. Furthermore, expression of the *S. cerevisiae NCE103* gene in *Escherichia coli* yielded CA activity [18].

It was initially proposed that the aerobic growth defect of an *S. cerevisiae nce103* null mutant reflected sensitivity to molecular oxygen [17]. However, a recent study [19] demonstrated that aerobic growth of an *nce103* null mutant was possible when the air was enriched with CO_2. We recently showed that *NCE103* is transcriptionally down-regulated at high CO_2 concentrations, irrespective of the oxygen concentration in the cultures [20]. This CO_2-dependent transcriptional down-regulation of *NCE103* and also of CA activity was recently confirmed [19]. High-throughput transcriptional studies revealed that *NCE103* is induced by a

Abbreviations used: AIR, 5′-phosphoribosyl-5-aminoimidazole; CA, carbonic anhydrase; CP, carbamoyl-phosphate; CPSase, CP synthetase; TCA, tricarboxylic acid.

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variety of natural stresses, including high pH [21,22], as well as in respiratory-deficient mutants [23]. However, an *nce103Δ* mutant did not show sensitivity to growth at pH 7, the maximum pH value that enabled growth for both mutant and wild-type strains [17].

Despite the recent studies on the regulation of *NCE103* and the phenotype of null mutants, the physiological role of CA in *S. cerevisiae* remains unclear. Therefore the aim of the present study was to determine the biochemical basis for the observed CO_2 requirement of CA-deficient yeast strains. Moreover, we investigated whether this CO_2 requirement merely occurs during the start-up phase of batch cultures, where metabolically generated CO_2 is not sufficient to compensate for the high CO_2 requirement of the mutant [9,24], or whether it also occurs during steady-state growth under conditions where fermentative sugar metabolism leads to sustained endogenous CO_2 production.

EXPERIMENTAL

Strains and maintenance

The *S. cerevisiae* strain CEN.PK113-7D (MATa *MAL2*-8^c *SUC2*) was kindly provided by Dr P. Kötter (der Johann Wolfgang Goethe-Universität Frankfurt, Frankfurt, Germany). It was maintained as 2 ml aliquots of frozen stock cultures in YPD medium (1 %, w/v, yeast extract, 2 %, w/v, peptone and 2 %, w/v, glucose) supplemented with 30 % (v/v) glycerol. One aliquot was used to inoculate precultures (100 ml) for chemostats and shake-flask cultivations.

Construction of an *nce103Δ* deletion strain

Construction of an *NCE103* disruptant in the auxotrophic strain CEN.PK113-7D was carried out by PCR-targeting with the *kanMX4* module, flanked by short homology regions of the gene [25]. The disruption cassette was obtained by amplifying the *KanMX4* module from the plasmid pUG6 [25] with the oligonucleotides NCE103.D-F (5′-ATCAACTACAGCTAAGACTACAAATTTCAATTATTACACATCAGCAGCGAAGCTTCGTACGC-3′) and NCE103.D-R (5′-TTCTATTTCAATGAATATTATATAAGTATATCGGTGAGGCTAAAAGCATAGGCCACTAGTGGATCTG-3′). PCR was performed with Expand High Fidelity PCR system (Roche, Indianapolis, IN, U.S.A.) following the programme: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 58 °C for 1 min and 72 °C for 3 min and a final step of 72 °C for 7 min. Amplicons were purified with the GenElute kit (Sigma). Exponentially growing YPD cultures of the strain CEN.PK113-7D were transformed with 5 μg of the purified DNA cassette [26]. The recovery incubation after the heat shock was performed in 25 ml screw-cap tubes flushed with CO_2 for 5 min (HoekLoos, Schiedam, The Netherlands), and the selection was on YPD plus G418 plates inside an airtight jar that contained an atmosphere with less than 0.1 % O_2 and 18 % CO_2 (Anaerocult A system; Merck, Darmstadt, Germany). Verification of correct *NCE103* replacement was carried out by diagnostic PCR. The oligonucleotides used were NCE103.TEST-F (5′-TTTCTAACCATCACCCCGC-3′) and KanA (5′-CGCACGTCAAGACTGTCAAG-3′), for testing the correct insertion of the *KanMX4* module in the 5′ flank of *NCE103*, and KanB (5′-TCGTATGTGAATGCTGGTCG-3′) and NCE103.TEST-R (5′-GGATCATGTGCCTTTGCC-3′), for the 3′ flank.

Batch and chemostat cultivation

Batch cultivations were performed in 250 ml shake flasks at 30 °C and 200 rev./min in orbital incubators. Cells were grown in a synthetic medium with $(NH_4)_2SO_4$ as nitrogen source [27], supplemented with 20 g · l^{-1} glucose. When L-aspartic acid was used as the nitrogen source, it was added at 30 mM (final concentration) and the sulphate concentration was adjusted by addition of K_2SO_4 (38 mM). All media were adjusted to pH 6.5 with 2 M KOH.

Chemostat cultivation was performed at 30 °C in laboratory fermenters (Applikon Biotechnology, Schiedam, The Netherlands) with a working volume of 1 litre as described previously [28]. Cultures were fed with the same media as described above, but the glucose concentration was lowered to 7.5 g · l^{-1} to ensure glucose-limited growth [29]. The dilution rate was set to 0.10 h^{-1}. The pH was measured on line and kept constant at 5.0 by the automatic addition of 2 M KOH with the use of an Applikon ADI 1030 biocontroller. Stirrer speed was 800 rev./min and the gas flow was 0.5 litre · min^{-1}. For anaerobic cultivation, ergosterol (10 mg · l^{-1}) and Tween 80 (420 mg · l^{-1}) were added, and the medium vessel was flushed with N_2. Cultures were sparged either with air, N_2, CO_2 or with a mixture containing 79 % N_2 and 21 % O_2 (HoekLoos, Schiedam, The Netherlands). Cultures were monitored as described previously [20]. In CO_2-enriched cultures, respiratory quotients could not be calculated due to the absence of nitrogen (used as reference gas). Therefore oxygen consumption was estimated by assuming a 100 % carbon balance.

Analysis of nutritional requirements on agar plates

For growth on plates, precultures grown at elevated CO_2 concentrations were washed with water and adjusted to $A_{600} = 0.2$. Then, 5 μl was spotted on to agar plates (2 %) of the indicated media and extended across a 9 cm^2 surface with an inoculation loop. Spotted dilution experiments [30] were not possible due to a hydrophobic layer formed on top of fatty-acid-supplemented plates. Plates were incubated at 30 °C for 3–5 days under a normal air atmosphere or, instead, in a jar with an atmosphere consisting of 5–7 % O_2 and 7–9 % CO_2 (Anaerocult C system; Merck). Where indicated, the synthetic medium was supplemented with the following nutritional complements: adenine (20 mg · l^{-1}), uracil (20 mg · l^{-1}), L-arginine (20 mg · l^{-1}) and a fatty acid mixture containing myristic, stearic and palmitic acids (30 mg · l^{-1} each) and Tween 80 (10 g · l^{-1}) as surfactant. Adenine, uracil and L-arginine were added from filter-sterilized concentrated stock solutions [31]. The fatty acid mixture was added from a 100-fold-concentrated stock solution prepared under sterile conditions.

RESULTS

Metabolic CO_2 cannot fulfil the inorganic carbon demand of an *nce103* null mutant

S. cerevisiae mutants lacking CA activity (encoded by *NCE103*) require a CO_2-enriched atmosphere for growth [17]. This CO_2 requirement is independent of the availability of oxygen [19]. Similar CO_2 requirements have been reported for several CA-deficient bacteria [10–12] and even for wild-type *E. coli* [24].

Attempts to start up chemostat cultures of the *nce103Δ* strain by sparging with air (aerobic cultures) or nitrogen gas (anaerobic cultures) were unsuccessful. However, glucose-limited chemostat cultures of *nce103Δ* grown in CO_2-enriched chemostat cultures (sparged either with a mixture containing 79 % CO_2 and 21 % O_2 or with pure CO_2) exhibited similar biomass yields as corresponding cultures of the parental strain CEN.PK113-7D (*NCE103*) (Table 1). Apparently, as previously demonstrated in shake-flask cultures [19], elevated CO_2 concentrations are sufficient to sustain glucose-limited growth of *nce103Δ S. cerevisiae* in glucose-limited chemostats.

Table 1 Biomass yields and biomass specific rates of alcoholic fermentation ($q_{ethanol}$) of the *S. cerevisiae* strain CEN.PK113-7D (wild-type) and the isogenic mutant *nce103Δ* in aerobic and anaerobic chemostat cultures sparged (0.5 litre · min^{-1}) with high concentrations of CO_2

Data represent the means ± S.D. for two independent chemostat cultures.

Genotype	Sparging gas	Biomass yield ($g_{biomass}/g_{glucose}$)	$q_{ethanol}$ (mmol · h^{-1} · g^{-1})
Wild-type	79 % CO_2 + 21 % O_2	0.38 ± 0.05	0.0 ± 0.0
nce103Δ	79 % CO_2 + 21 % O_2	0.33 ± 0.018	0.0 ± 0.0
Wild-type	CO_2	0.09 ± 0.004	10.4 ± 1.1
nce103Δ	CO_2	0.09 ± 0.00	10.0 ± 0.1

In some studies, the CO_2 requirement of CA-deficient microorganisms has been interpreted in terms of growth initiation: at low inoculum densities and in lag-phase cultures, endogenous CO_2 production may be insufficient to build up the critical level required for growth [32,33]. Consequently, in the absence of CA, the threshold HCO_3^- concentration required to 'spark' growth is not reached. This model is supported by the observation that high-density inocula relieved the CO_2 requirement of a CA-deficient mutant of *Ralstonia eutropha* [9] and shortened the lag phase of *E. coli* in minimal medium [32].

To investigate whether the phenotype of *S. cerevisiae nce103* null mutant is due to a 'sparking' effect of CO_2, chemostat cultures sparged with a high concentration of CO_2 were switched to nitrogen gas (anaerobic cultures) or air (aerobic cultures). In anaerobic as well as aerobic cultures, this switch resulted in termination of growth, as evident from the wash-out of the biomass and the increase in the residual glucose concentration (Figure 1). This indicates that, even in cultures that are vigorously respiring or fermenting, the inorganic carbon requirement of *nce103Δ S. cerevisiae* cannot be met by endogenous CO_2. We also observed a delay between the biomass decay and the theoretical wash-out curve (Figure 1), an effect that probably responded to the time needed to replace all the dissolved CO_2 in the culture broth by nitrogen or air.

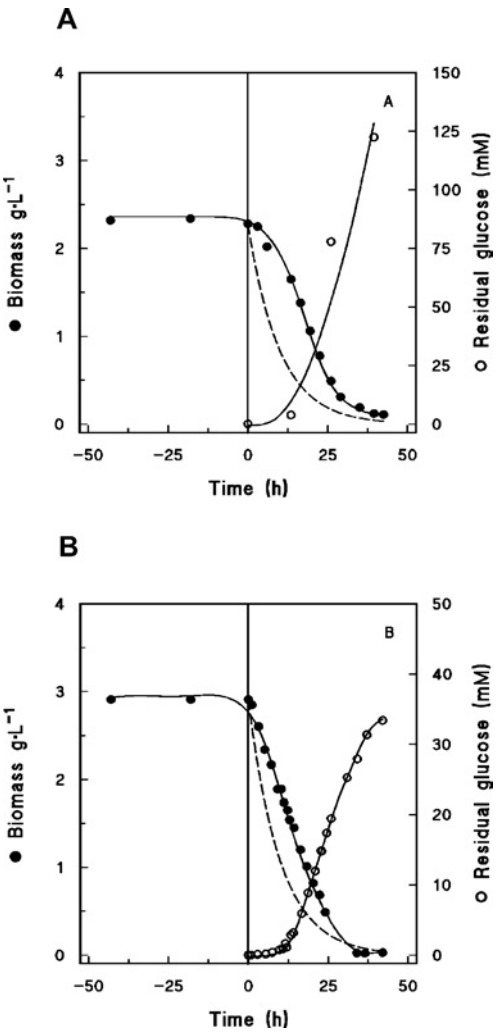


Figure 1 Anaerobic (100 % CO_2) (A) and aerobic (79 % CO_2/21 % O_2 mixture) (B) chemostat cultivations of *S. cerevisiae nce103Δ* null mutant

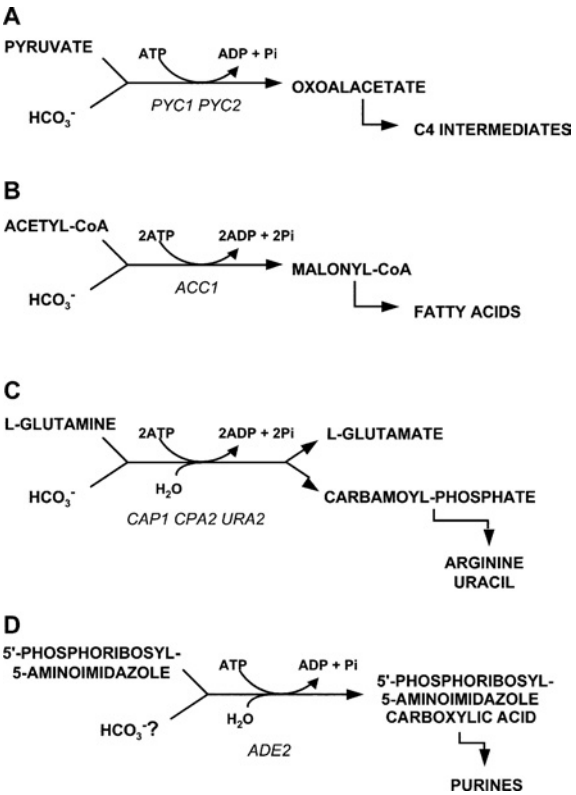
At zero time (vertical line), the gas supply was switched to 100 % N_2 (**A**) or air (**B**). ●, biomass (dry weight); ○, residual glucose concentration; - - - - -, theoretical wash-out curve. Independent replicate experiments gave identical results.

Nutritional complementation of *nce103* null mutants at atmospheric CO_2 pressures

The CO_2 requirement of CA-deficient microorganisms has led to the suggestion that the enzyme may be needed to provide bicarbonate for heterotrophic carboxylation reactions [10,18]. Indeed, non-identified CO_2-requiring mutants of several microbial species could be nutritionally complemented by addition of TCA (tricarboxylic acid) cycle intermediates, amino acids, purines and other metabolites whose biosynthesis require HCO_3^--dependent carboxylations [33]. However, addition of these compounds did not restore growth at atmospheric CO_2 partial pressures of several CA-deficient bacterial strains [9,12,24].

Before experimentally addressing the nutritional complementation of *S. cerevisiae nce103Δ* strains, we made an inventory of possible bicarbonate-dependent carboxylations during growth on glucose (Scheme 1). The two pyruvate carboxylases (Scheme 1A), encoded by the *PYC1* and *PYC2* genes, are exclusively responsible for replenishing the TCA cycle intermediates used for biosynthesis during growth of *S. cerevisiae* on sugars [34,35]. Addition of L-aspartate can nutritionally complement *pyc1 pyc2* null mutants by providing an alternative source of C_4-compounds [35]. Acetyl-CoA carboxylase (Scheme 1B), encoded by the *ACC1* gene [36], provides malonyl-CoA required for fatty acid biosynthesis. Acetyl-CoA carboxylase-deficient mutants can be nutritionally complemented by addition of long-chain (C_{14}–C_{16}) unsaturated fatty acids [36,37]. CPSase (carbamoyl-phosphate synthetase; Scheme 1C) catalyses the HCO_3^--dependent formation of CP from glutamine. CP is a precursor for L-citrulline, L-arginine and pyrimidines. Consequently, *S. cerevisiae* mutants that are unable to synthesize CP require either L-arginine or L-arginine and uracil for growth [38]. A final reaction that was considered is the carboxylation of AIR (5′-phosphoribosyl-5-aminoimidazole), a step in the pathway of purine biosynthesis (Scheme 1D). It is unclear if the substrate for AIR carboxylation is HCO_3^- or CO_2. However, in adenine-deficient mutants unable to catalyse this reaction, adenine can be substituted by gassing the cultures with 30 % CO_2 [39]. We therefore decided to also take into account a possible purine requirement of *nce103Δ S. cerevisiae*.

To investigate whether, at atmospheric CO_2 pressures, Nce103p is required to provide bicarbonate for any or all of the processes mentioned above, the prototrophic reference strain *S. cerevisiae* CEN.PK113-7D (*NCE103*) and the isogenic *nce103Δ* mutant were tested for aerobic growth on glucose synthetic medium plates supplemented with all possible combinations of the



Scheme 1 Metabolic reactions involving inorganic carboxylations in *S. cerevisiae* (see text)

(**A**) Pyruvate carboxylase (EC 6.4.1.1), (**B**) acetyl-CoA carboxylase (EC 6.3.4.14), (**C**) CPSase (EC 6.3.5.5) and (**D**) AIR carboxylase (EC 4.1.1.21). The genes encoding for the different enzymes are depicted in italics.

end-products corresponding to the pathways mentioned above: L-aspartic acid (used as nitrogen source), long-chain fatty acids, L-arginine, uracil and adenine (see the Experimental section). Growth of the reference strain was not affected by the addition of these additional nutrients. However, under atmospheric pressure, good growth of the *nce103Δ* mutant was observed only when L-aspartate, fatty acids, L-arginine and uracil were all present (Figure 2, panel 31). The additional supply of adenine did not have a clear influence (Figure 2, panel 32), indicating that purine biosynthesis is not affected during growth of the *nce103Δ* strain at atmospheric CO_2 pressure. Very slow residual growth was observed with L-aspartate, fatty acids and L-arginine (i.e. without inclusion of uracil; Figure 2, panel 15).

DISCUSSION

In previous studies, CO_2 dependence of *nce103Δ* mutants was shown in aerobic, but not in anaerobic cultures [17,19]. However, in those studies, anaerobic growth was investigated in closed systems, from which CO_2 produced during fermentation could not escape, thus leading to a CO_2-enriched environment [17]. Our experiments with anaerobic chemostat cultures demonstrate that CA is essential for growth in anaerobic cultures sparged with nitrogen gas. Apparently, even in these vigorously fermenting yeast cells (fermentation rates of 10 mmol $\cdot$ g^{-1} $\cdot$ h^{-1} [40]), loss of CO_2 from the cells via diffusion is too fast to allow for a sufficient rate of spontaneous intracellular formation of bicarbonate. These observations show that, in *S. cerevisiae*, absence of CA yeast does not merely result in a requirement for 'sparking' amounts of CO_2, as reported for many microorganisms [24,32,33,41], but instead causes a true CO_2 requirement for steady-state growth at atmospheric CO_2 pressures.

Although biosynthetic bicarbonate requirements have often been implicated in the phenotype of CA-deficient microorganisms [10,18,24], we are not aware of previous studies in which the biosynthetic requirements of a CA-negative microorganism have been fully determined. We have demonstrated that the requirement of CA-deficient *S. cerevisiae* for elevated CO_2 concentrations originates from three bicarbonate-dependent carboxylation reactions: pyruvate decarboxylase, acetyl-CoA carboxylase and CPSase (Scheme 1 and Figure 2). CA-deficient mutants could only be grown at atmospheric CO_2 partial pressures when products of the pathways in which these three enzymes participate were added to synthetic growth media (Figure 2). These observations demonstrate that, as long as cultures are not enriched with CO_2, yeast CA is a key biosynthetic enzyme during growth on glucose.

S. cerevisiae nce103Δ strains showed residual growth in the absence of uracil, but not in the absence of L-arginine (Figure 2). In *S. cerevisiae*, as well as in many other organisms, there are two enzymes for CP synthesis: CPSase A, which is primarily regulated based on L-citrulline and L-arginine status of the cells, and CPSase P, which is mainly regulated by pyrimidine requirement [38]. However, CP from the two enzymes can be used in both pathways [38]. Therefore the residual growth of the *nce103Δ* strain in the absence of uracil may reflect a low rate of CP biosynthesis driven by spontaneous bicarbonate formation, combined with a higher affinity of pyrimidine biosynthesis for CP [42]. CA-deficient mutants did not show an adenine auxotrophy for aerobic growth (Figure 2), suggesting that the carboxylation of AIR may depend on molecular CO_2 rather than on HCO_3^-.

Even after 'full' nutritional supplementation (i.e. addition of L-aspartic acid, fatty acids, uracil and L-arginine), growth of *nce103* null mutants was slower than that of the reference strain. We observed a similar reduction in specific growth rate when *acc1* mutants (lacking the main acetyl-CoA carboxylase in *S. cerevisiae* [43]) were supplemented with fatty acids (results not shown). This suggests that the reduced growth rate of nutritionally supplemented *nce103* mutants was due to limitations in fatty acid uptake or processing. However, these observations should be interpreted with some caution as a mutant lacking the whole *ACC1* gene could not be rescued by fatty acid supplementation, indicating that its role extends beyond fatty acid synthesis [44].

The contribution of CA to growth will depend on the growth substrates. For example, *nce103* mutants did not require L-aspartic acid for growth on ethanol (results not shown). This result was anticipated, since the glyoxylate cycle rather than pyruvate carboxylase is responsible for the synthesis of C_4-compounds during growth on ethanol and acetate. CA can be anticipated to play a key role when urea is the nitrogen source. Urea is metabolized to NH_3 and CO_2 in a carboxylation reaction catalysed by bicarbonate-dependent urea carboxylase [45]. Lack of *NCE103* might therefore lead to insufficient HCO_3^- supply for urea utilization. This possibility was not followed up in detail as we anticipated interference of the presence of the nitrogen sources L-aspartate and L-arginine in the growth media.

The metabolic role of yeast CA can probably be extended to other organisms. CO_2-requiring mutants (called 'CO_2 mutants') of *E. coli*, *Neurospora crassa* and other microorganisms have been known for a long time [35]. In some of these mutants, addition of TCA-cycle intermediates, amino acids or other compounds could replace CO_2 [46]. The yeast *Cyniclomyces guttulatus* (formerly *Saccharomycopsis guttulatus*), which occurs in the digestive tract of rabbit, mouse and other animals, also requires CO_2 for growth

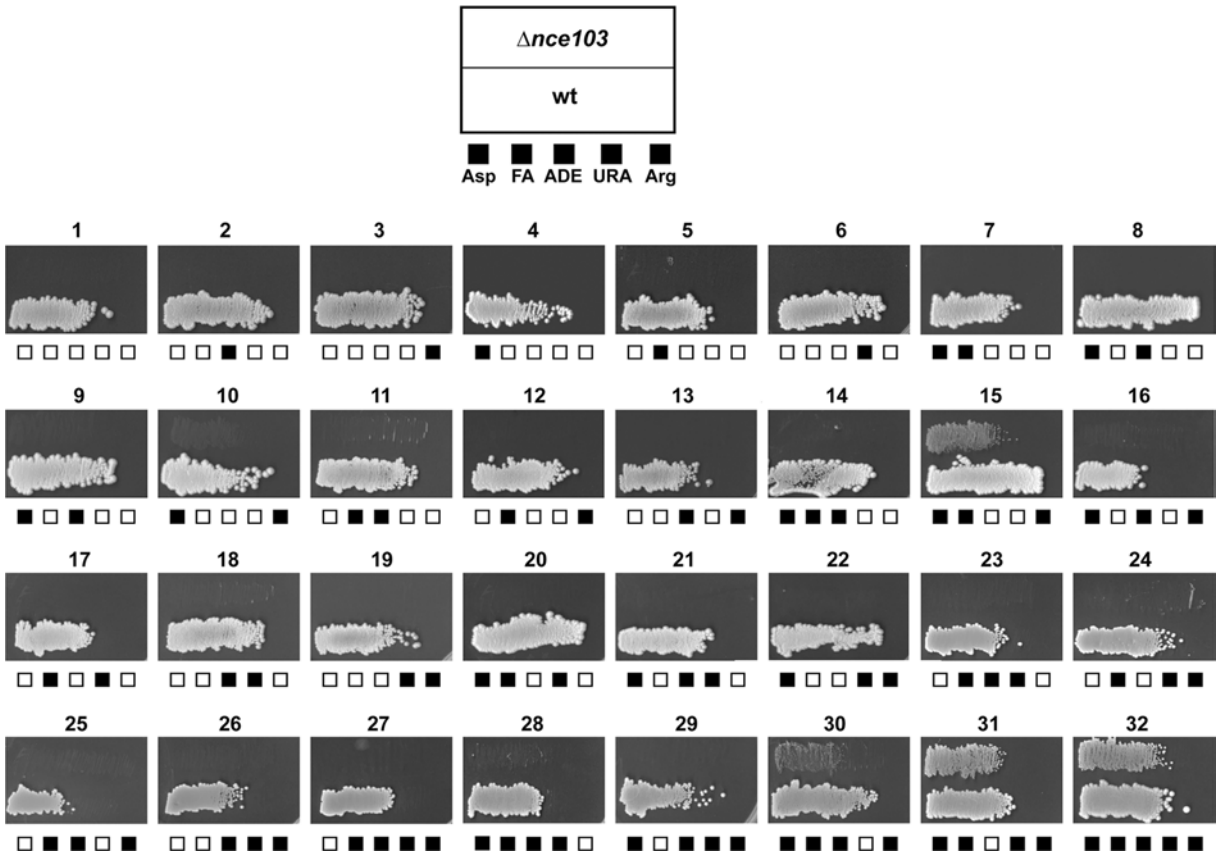


Figure 2 Aerobic growth of the prototrophic *S. cerevisiae* reference strain CEN.PK113-7D (wt; lower) and the isogenic mutant *nce103Δ* (upper) in MM-glucose plus different nutritional supplements

Stationary phase precultures were diluted to $A_{600} = 0.2$, plated and incubated at 30°C for 5 days. For each panel, the included components are indicated by closed squares. Asp, L-aspartic acid used as nitrogen source; FA, fatty acids; ADE, adenine; URA, uracil; Arg, L-arginine (see the Experimental section).

[47]. Remarkably, this requirement can be circumvented by the use of an enriched growth medium [48]. On the basis of our results, we speculate that evolution in CO_2-rich environments such as animal intestines may have yielded microorganisms that are naturally CA-deficient.

Studies with the green alga *Chlamydomonas reinhardtii* showed a correlation between CA gene expression and supply of NH_4^+, a nitrogen source that stimulates anaplerosis [49]. In addition, previous studies showed that exposure of mammalian cells to acetazolamide and other CA inhibitors led to a decrease in CP synthesis [13] and reduced carbon flux towards lipids and TCA cycle intermediates [14,15]. In addition to its proposed role in pH control, the biosynthetic role of CA may be relevant in cancer cells, in which CA is highly expressed [4]. In this respect, it is of interest to note that glucose dissimilation in many tumours occurs predominantly via homolactate fermentation [50] and thus does not lead to the production of large amounts of CO_2.

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