

# Complementation of the yeast deletion mutant $\Delta NCE103$ by members of the $\beta$ class of carbonic anhydrases is dependent on carbonic anhydrase activity rather than on antioxidant activity

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In recent years, members of the  $\beta$  class of CAs (carbonic anhydrases) have been shown to complement  $\Delta NCE103$ , a yeast strain unable to grow under aerobic conditions. The activity required for complementation of  $\Delta NCE103$  by tobacco chloroplast CA was studied by site-directed mutagenesis. E196A (Glu<sup>196</sup> → Ala), a mutated tobacco CA with low levels of CA activity, complemented  $\Delta NCE103$ . To determine whether restoration of  $\Delta NCE103$  was due to residual levels of CA activity or whether it was related to previously proposed antioxidant activity of CAs [Götz, Gnann and Zimmermann (1999) *Yeast* **15**, 855–864], additional complementation analysis was performed using human

CAII, an  $\alpha$  CA structurally unrelated to the  $\beta$  class of CAs to which the tobacco protein belongs. Human CAII complemented  $\Delta NCE103$ , strongly arguing that CA activity is responsible for the complementation of  $\Delta NCE103$ . Consistent with this conclusion, recombinant NCE103 synthesized in *Escherichia coli* shows CA activity, and  $\Delta NCE103$  expressing the tobacco chloroplast CA exhibits the same sensitivity to H<sub>2</sub>O<sub>2</sub> as the wild-type strain.

**Key words:** aerobic growth, bicarbonate, carbonic anhydrase,  $\Delta NCE103$ , lipid biosynthesis, oxidative stress.

## INTRODUCTION

Carbonic anhydrase (CA, carbonate hydrolase; EC 4.2.1.1) is a zinc-containing enzyme, which catalyses the reversible hydration of CO<sub>2</sub> according to the following reaction: CO<sub>2</sub> + H<sub>2</sub>O ↔ HCO<sub>3</sub><sup>-</sup> + H<sup>+</sup>. This enzyme has been found in a variety of organisms, including animals, plants, bacteria and members of the Archaea domain. Four different classes of CAs ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) have been identified based on their origin and structural features.  $\alpha$  and  $\gamma$  CAs function solely as monomers or trimers respectively. In contrast, the basic unit of  $\beta$  CA is, generally, a dimer, and enzymes of this class are dimers, tetramers, hexamers or octamers [1]. The three-dimensional structure of the  $\alpha$ ,  $\beta$  and  $\gamma$  classes reveals that, in spite of their unrelated tertiary and quaternary structures, their active sites share several common features. Thus the basic catalytic mechanisms for these three classes are, probably, very similar [2]. CA has been shown to play multiple roles in animals, such as CO<sub>2</sub> exchange, pH regulation and provision of inorganic carbon substrate for carboxylation reactions in animals [3], as well as for C<sub>4</sub> plants and cyanobacteria [4,5].

In the budding yeast *Saccharomyces cerevisiae*, *NCE103*, a gene encoding a protein related to the  $\beta$  class of CAs, was first implicated in the non-classical export pathway [6]. Although the overall degree of identity with other  $\beta$  CAs is rather low, a multiple sequence alignment reveals that NCE103 contains all of the residues that form the active site of  $\beta$ -class CAs, with the sole exception of Tyr-204 (pea CA numbering), which is replaced by a phenylalanine residue [7]. However, no CA activity was detected in crude extracts from wt (wild-type) yeast using an electrometric assay [8]. Deletion of *NCE103* led to a growth-defect phenotype under aerobic conditions, but not under anaerobic conditions. The  $\Delta NCE103$  strain was also reported to exhibit enhanced sensitivity to H<sub>2</sub>O<sub>2</sub> when compared with the wt strain. Given the lack of

CA activity, it was concluded that loss of the putative antioxidant activity, and not CA activity, was most probably responsible for the  $\Delta NCE103$  phenotype [8]. Interestingly, expression of the  $\beta$  CAs from alfalfa, tobacco, or *Escherichia coli* restores normal growth under aerobic conditions [8–10]. These findings raised the possibility that these three  $\beta$  CAs possess an antioxidant activity similar to that proposed for NCE103. However, analysis of the primary structures of these three enzymes and NCE103 failed to reveal a known protein motif in addition to the CA motif. Moreover, attempts to detect catalase or peroxidase activity with the tobacco chloroplast CA were unsuccessful [9].

To investigate the proposed antioxidative role of  $\beta$  CAs, we performed an in-depth characterization of  $\Delta NCE103$  complementation by  $\beta$  CAs. In addition, the enzymic properties of NCE103 were more fully analysed. In the present study, we report that  $\Delta NCE103$  does not show enhanced sensitivity to H<sub>2</sub>O<sub>2</sub> or peroxynitrite. Moreover, expression of the tobacco chloroplast CA in an H<sub>2</sub>O<sub>2</sub>/peroxynitrite-sensitive yeast strain does not affect its sensitivity to these oxidants. Using a stopped-flow spectrophotometric method, we further demonstrate that NCE103 has significant CA activity. By introducing point mutations in the tobacco chloroplast CA, we attempted to show that complementation of the *NCE103* deletion in yeast is dependent on CA activity. Finally, this conclusion came from the demonstration that an active form of the structurally unrelated human  $\alpha$  CAII complemented  $\Delta NCE103$ .

## EXPERIMENTAL

### Strains and media

The *S. cerevisiae* strains CEN.PK2-1C (MATa; *ura3-52*; *trp1-289*; *leu2-3\_112*; *his3Δ1*; *MAL2-8<sup>c</sup>*; *SUC2*) and CEN.HE28-1A

Abbreviations used: CA, carbonic anhydrase; SD, synthetic dextrose; SG, synthetic galactose; SNP, sodium nitroprusside; wt, wild-type.

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(CEN.PK; Mat a; *ura3-52*; *his3Δ1*; *leu2-3-112*; *trp1-289*; *YNL036w::KAN*) were obtained from the European *S. cerevisiae* Archives for Functional Analysis. The gene deleted in CEN.HE28-1A, *YNL036w*, is synonymous with *NCE103*. In the present study, CEN.PK2-1C and CEN.HE28-1A are referred as wt and  $\Delta NCE103$  respectively. Both strains were grown in YPAD [2% (w/v) yeast extract, 1% peptone, 0.01% adenine and 2% (w/v) dextrose], YPAG [2% yeast extract, 1% peptone, 0.01% adenine and 2% (w/v) galactose], SD (synthetic dextrose) medium, SD-Ura (SD medium lacking uracil) or SG (synthetic galactose) medium lacking uracil (SG-Ura).  $\Delta NCE103$  was grown under anaerobic conditions in still liquid cultures, or on plates by using the Anaerocult A system (EM Science, Gibbston, NJ, U.S.A.). The strains BY4741 (MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and *tsa1Δtsa2Δ* (MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *tsa1::KAN*; *tsa2::LEU2*) were kindly provided by Dr D.-Y. Jin (University of Hong Kong, Hong Kong). Both strains were grown in YPD (2% yeast extract, 1% peptone and 2% dextrose) or YPG (2% yeast extract, 1% peptone and 2% galactose). Cell transformation was performed using the lithium acetate method (pYES2/NT C vector system; Invitrogen). Cultures were grown at 30 °C. Peroxynitrite was purchased from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals were from Sigma.

### Sensitivity to oxidants

For the spot assay, yeast was grown in YPAG or YPG until  $A_{600}$  reached 0.5; the cultures were then exposed to different concentrations of  $H_2O_2$  for 2 h. Serial dilutions were made and 5  $\mu$ l was spotted on YPAG or YPG plates to assay colony survival. Plates were incubated at 30 °C under aerobic or anaerobic conditions according to the experimental requirements. The drop-out assay was performed as described by Götz et al. [8]. Briefly, yeast was grown at 30 °C for 2 days in SD medium in still liquid cultures, and approx.  $10^6$  cells were plated on SD plates. Different concentrations of peroxynitrite were dropped on to small filter-paper discs placed on the surface of the agar. Plates were incubated at 30 °C under anaerobic conditions for 2 days. For the growth curve assay, yeast was grown overnight in YPG and diluted to an  $A_{600}$  of 0.2. Diluted cultures were treated with either  $H_2O$  or 2 mM SNP (sodium nitroprusside), and yeast growth in liquid medium was monitored at 30 °C under aerobic conditions.

### Gene cloning and site-directed mutagenesis

The *NCE103* gene was PCR-amplified from *S. cerevisiae* genomic DNA using the primer set ACTAGGATCCATGAGCGCTACCGAATCTTC (forward) and TAACTCGAGAAGTATATCGGTGAGGCTAAAAC (reverse). The forward primer introduced a *Bam*HI site (underlined) immediately upstream of Met-1, and the reverse primer introduced an *Xho*I site (underlined) downstream of the native stop codon. No introns were present in the *NCE103* gene. Human CAII cDNA and the V143Y CAII mutant cDNA were PCR-amplified from pJRC36 [11] and pDS14 [12] respectively. Both plasmids were gifts from Dr J. R. Casey (University of Alberta, Alberta, Canada). The primer set utilized to amplify both fragments was ACTAGAATTCATGTCTCATCTACTGGGGTACGGC (forward) and ACAC~~TC~~TAGATTATTTGAAGGAA-GCTTTGATTTGC (reverse). The forward primer introduced an *Eco*RI site immediately upstream of Met-1, and the reverse primer introduced an *Xba*I site downstream of the native stop codon. The tobacco CA cDNA (CA1) was PCR-amplified from a tobacco cDNA library (cv. Xanthi nc [NN]) as described by Slaymaker et al. [9] with the primer set CGTGGATCCATGGAATTGC-AATCATCA (forward) and CTCGGCGAATTCGCTTCATAC-

GGAAAGAGA (reverse). The forward primer removed the chloroplast transit peptide as predicted by Majeau and Coleman [13], introducing a methionine and in-frame *Bam*HI site upstream of Glu-101 in the full-length preprotein. The reverse primer introduced an *Eco*RI site downstream of the native stop codon. All PCR products were cloned directly into pGEM-T (Promega) according to the manufacturer's instructions, and their sequences were confirmed by automated sequencing. To introduce point mutations in the tobacco chloroplast cDNA, site-directed mutagenesis was performed using the Quik Change site-directed mutagenesis kit (Stratagene) and the appropriate primers from Sigma-GenoSys. Mutations were confirmed by automated sequencing.

### Expression and purification of recombinant proteins

cDNAs encoding the tobacco chloroplast CA, its mutated versions and NCE103 were subcloned into the *Bam*HI and *Eco*RI-*Not*I sites of the *E. coli* expression vector pET28a (Novagen), which introduces an N-terminal His<sub>6</sub> leader peptide to the coding region. The plasmids were transformed into *E. coli* strain BL21(DE3), and bacteria were grown at 27 °C for 4–6 h after induction with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside. The protein present in the soluble fraction was then affinity-purified on Ni<sup>2+</sup>-nitrilotriacetate agarose (Novagen) in the presence of 8 mM 2-mercaptoethanol according to the manufacturer's instructions.

### Yeast complementation

Complementation of the yeast strain  $\Delta NCE103$  was performed as described by Slaymaker et al. [9]. Briefly, cDNAs encoding the tobacco chloroplast CA and its mutated versions were subcloned into the *Bam*HI and *Eco*RI-*Not*I sites of the galactose-inducible yeast expression vector pYES/NT-C (Invitrogen) to generate pYES-CA (wt), pYES-DN [D154N (Asp<sup>154</sup> → Asn)], pYES-EA (E196A), pYES-YF (Y197F) and pYES-CS (C215S). cDNAs encoding the human CAII and the V143Y CAII mutant were subcloned into the same vector using the *Eco*RI and *Xba*I sites to generate pYES-hCAII and pYES-hVY respectively. After transforming  $\Delta NCE103$  with the appropriate plasmid, transformants were selected under anaerobic conditions at 30 °C on SD-Ura. Uracil-positive  $\Delta NCE103$  transformants were tested for aerobic growth on SG-Ura versus SD-Ura. In some cases, complementation was confirmed under aerobic conditions on YPAG.

### Enzymic activity and steady-state kinetics

Enzymic rates of CO<sub>2</sub> hydration catalysed by recombinant enzymes were measured with HiTech SF61DX2 spectrophotometer as described previously [14]. The buffer/pH indicator pairs and detection wavelengths utilized were 40 mM Bicine/60  $\mu$ M *m*-Cresol Purple/2.5 mM EDTA, pH 9.0 (578 nm) or 40 mM Mops/30  $\mu$ M *p*-nitrophenol/2.5 mM EDTA, pH 7.0 (400 nm). The catalytic constants  $k_{cat}$  and  $K_m$  were estimated by non-linear least-squares fits to  $v/[E]$  versus [CO<sub>2</sub>] data using Origin 6.0 (Microcal, Northampton, MA, U.S.A.). Protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories), with BSA as a standard. The concentrations utilized were 1  $\mu$ M (pH 9) and 2  $\mu$ M (pH 7) for D154N, C215S and NCE103; 500 nM (pH 9) and 1  $\mu$ M (pH 7) for Y197F; 500 nM (pH 9 and 7) for E196A; 200 nM (pH 9) and 375 nM (pH 7) for the wt enzyme.

### Western-blot analysis

$\Delta NCE103$  transformed with pYES-CA, pYES-DN, pYES-EA, pYES-YF or pYES-CS was grown in SG-Ura liquid medium for

2 days at 30 °C under anaerobic conditions. Protein extracts were prepared by disrupting yeast cells with glass beads, as described by Dunn and Wobbe [15]. Protein (30  $\mu\text{g}$ ) was boiled for 10 min in the presence of 2% (w/v) SDS and 0.1 M dithiothreitol, resolved by SDS/PAGE and transferred on to a nitrocellulose membrane for 2 h at 35 mA at room temperature (25 °C) in a buffer comprising 50 mM Tris base, 40 mM glycine, 0.04% SDS and 20% (v/v) methanol. The membrane was blocked overnight at 4 °C with blocking buffer [TTBS (100 mM Tris, pH 7.5/154 mM NaCl/0.1% Tween 20), 3% (w/v) BSA and 3% (w/v) non-fat milk], and then incubated with 10 ml of TTBS containing 5  $\mu\text{l}$  of a rabbit polyclonal antibody against the pea chloroplast CA [16] or 5  $\mu\text{l}$  of a rabbit polyclonal antibody against the human CAII (Novus Biologicals, Littleton, CO, U.S.A.), for 1 h at room temperature. After four washes with TTBS at room temperature, the blot was incubated in blocking buffer with 1:5000 anti-rabbit secondary antibody for 1 h at room temperature. The membrane was finally washed four times with TTBS and visualized using the Western Lightning™ Chemiluminescence reagent (PerkinElmer LifeSciences, Great Shelford, Cambridge, U.K.).

## RESULTS

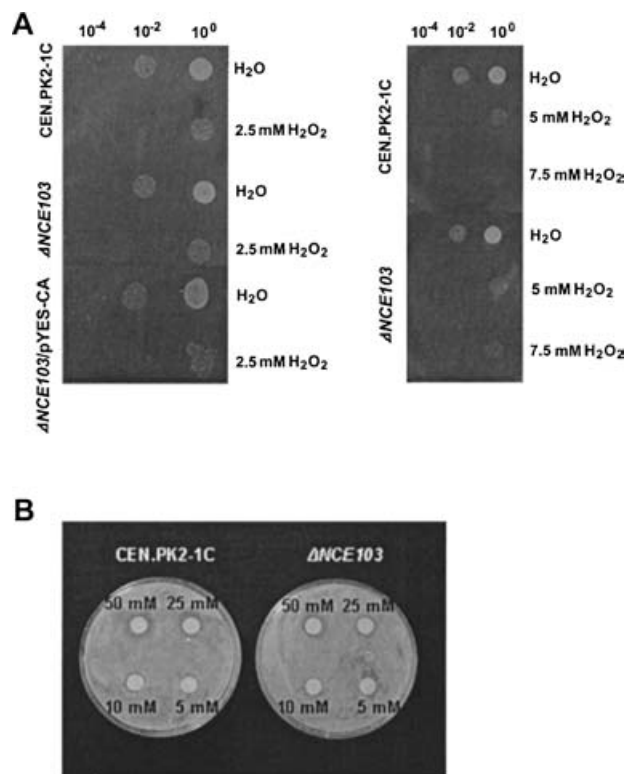
### Sensitivity of $\Delta NCE103$ to oxidants

$\Delta NCE103$  was previously reported to have enhanced sensitivity to  $\text{H}_2\text{O}_2$  [8]. To confirm this finding, wt and  $\Delta NCE103$  cells were exposed to 0, 2.5, 5 and 7.5 mM  $\text{H}_2\text{O}_2$  for 2 h, and their survival was assessed by serial dilutions on YPAG plates. No differences between wt and  $\Delta NCE103$  were observed (Figure 1A). Earlier studies have also suggested that the tobacco chloroplast CA has antioxidant activity, based on its ability to enable  $\Delta NCE103$  to grow under aerobic conditions [9]. However,  $\Delta NCE103$  transformed with the tobacco chloroplast CA (pYES-CA) showed the same sensitivity as wt and  $\Delta NCE103$  to 2.5 mM  $\text{H}_2\text{O}_2$  (Figure 1A); this suggests that expression of the tobacco chloroplast CA does not confer protection against oxidative stress. Similar results were obtained when  $\text{H}_2\text{O}_2$  sensitivity was tested using the drop-out assay (results not shown, [8]). Peroxynitrite, a product of superoxide and NO, was tested as an alternative oxidant in the drop-out assay. For  $\text{H}_2\text{O}_2$ , no differences between wt and  $\Delta NCE103$  were detected (Figure 1B).

To examine further whether expression of the tobacco chloroplast CA protects yeast against oxidative stress, the deletion strain *tsa1*  $\Delta$  *tsa2*  $\Delta$  was transformed with either pYES-CA or the empty vector pYES2/NT-C and then treated with  $\text{H}_2\text{O}_2$  or the NO donor SNP. *tsa1* and *tsa2* encode two members of the peroxiredoxin family in *S. cerevisiae*, and their deletion has been shown to confer enhanced sensitivity to oxidants [17]. As shown in Figure 2(A), treatment with 5 mM  $\text{H}_2\text{O}_2$  confirmed that *tsa1*  $\Delta$  *tsa2*  $\Delta$  is more susceptible to  $\text{H}_2\text{O}_2$  when compared with the parental wt strain BY4741 in the spot assay [17]. Transformation with pYES2/NT-C did not alter *tsa1*  $\Delta$  *tsa2*  $\Delta$  sensitivity. Indeed, transformation with pYES-CA not only failed to confer any protection, but also seemed to enhance *tsa1*  $\Delta$  *tsa2*  $\Delta$  sensitivity to  $\text{H}_2\text{O}_2$ . Similar results were obtained with SNP. As described previously, *tsa1*  $\Delta$  *tsa2*  $\Delta$  was more susceptible to 2 mM SNP when compared with BY4741 (results not shown, [17]), and this susceptibility was not affected by transformation with pYES-CA (Figure 2B). Taken together, these results suggest that neither NCE103 nor the tobacco chloroplast CA confers protection against oxidative stress.

### Complementation of $\Delta NCE103$ and determination of CA activity

Since the tobacco chloroplast CA does not appear to possess antioxidant activity, we investigated whether its ability to comple-

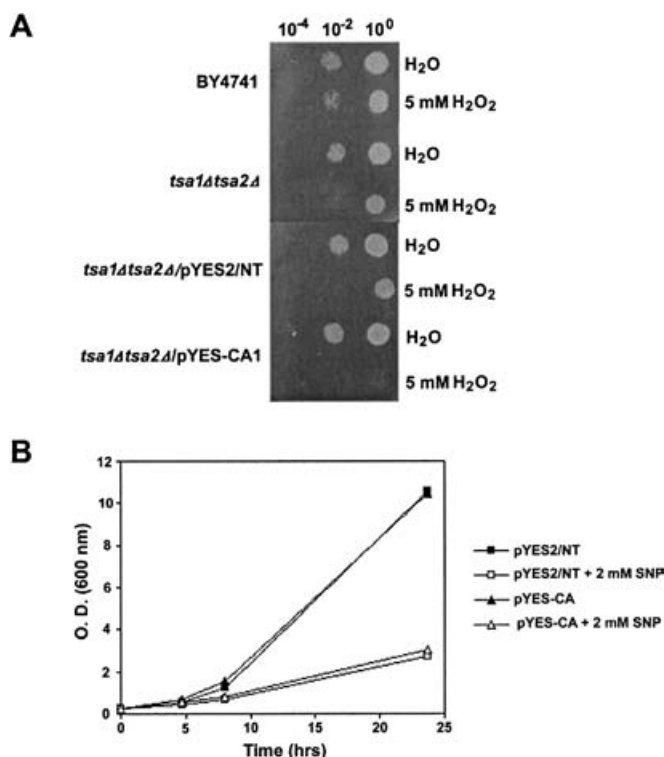


**Figure 1** NCE103 and the tobacco chloroplast CA do not show antioxidant activity in a  $\Delta NCE103$  background

(A) Sensitivity to  $\text{H}_2\text{O}_2$ . The indicated strains were grown anaerobically in YPAG liquid medium until  $A_{600}$  reached 0.5. Wt,  $\Delta NCE103$  and/or  $\Delta NCE103$ /pYES-CA cells were then treated with  $\text{H}_2\text{O}$  or 2.5, 5 or 7.5 mM  $\text{H}_2\text{O}_2$  for 2 h. Serial dilutions were spotted on YPAG plates to estimate the level of survival. Plates were photographed after incubation at 30 °C under anaerobic conditions for at least 4 days. (B) Sensitivity to peroxynitrite. wt and  $\Delta NCE103$  were grown anaerobically in SD liquid medium for 2 days at 30 °C, and then cells were plated on SD plates. Peroxynitrite (5, 10, 25 and 50 mM) was added in drops on to small filter-paper discs. Plates were photographed after incubation at 30 °C under anaerobic conditions for 2 days. The size of the halo surrounding the paper discs indicates the sensitivity of strains to the oxidant.

ment  $\Delta NCE103$  growth under aerobic conditions was due to its CA activity or some currently undefined activity. To distinguish these possibilities, site-directed mutagenesis was performed on four different amino acid residues in the mature chloroplast CA (Figure 3). The D154N and C215S mutations involve residues in the active site that were previously shown to reduce CA activity significantly [18,19]. The E196A mutation changes a residue that does not belong to the active site but also compromises CA activity, probably by altering the structure of the active site [20]. The Y197F mutation was made to investigate the only amino acid difference between the active site of NCE103 and those of other  $\beta$  CAs.

Complementation analysis revealed that E196A and Y197F restored  $\Delta NCE103$  growth under aerobic conditions, whereas D154N and C215S did not (Figure 4A). The level of expression for each construct was then assessed by Western-blot analysis. As shown in Figure 4(B), two major bands of 34 and 36 kDa were detected in yeast expressing the wt CA. The molecular masses of these bands are approx. 2 and 4 kDa greater than that of mature tobacco chloroplast CA (results not shown); this discrepancy may reflect differences in protein processing between yeast and plants. Since the expression construct did not encode a transit sequence, the unexpected appearance of a second protein band in the yeast extract could be due to post-translational modification and/or



**Figure 2** Tobacco chloroplast CA does not protect an oxidant-sensitive yeast strain against oxidative stress

(A) Sensitivity to  $H_2O_2$ . The indicated strains were grown aerobically in YPAG liquid medium. Cells ( $A_{600} = 0.5$ ) were treated with  $H_2O$  or 5 mM  $H_2O_2$  for 2 h, and serial dilutions were spotted on YPAG plates to estimate survival. Plates were photographed after incubation at 30 °C under aerobic conditions for 2 days. (B) Sensitivity to peroxyntirite. The strain  $tsa1\Delta tsa2\Delta$  transformed with the indicated plasmids was grown overnight in YPG liquid medium and then diluted to an  $A_{600}$  0.2. Cells were treated with  $H_2O$  or 2 mM peroxyntirite and cell growth (determined using  $A_{600}$ ) was monitored at 30 °C under aerobic conditions.

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10      20      30      40      50      60
MSTASINSLTI SPAQAS LKKPTRPVAFARLSNSSSSTSVPSLIRNEPVFAAPTPIINPI
70      80      90      100     110     120
LREEMAKESYEQAIAALEKLLSEKGLGPIAAARVDQITAEQLQSSDGSKPFDPVEHMKAG
130     140     150     ↓ 160     170     180
FIHFTEKYENKPNALYGLSKGQSPKFMVFACSDSRVCPSEHVLNFPGEAFVVRNIANMV
190     ↓↓200     210     N ↓ 220     230     240
PAYDKTRYSGVGAALIEYAVLHLKVENIVVIGHSAACGLKGLMSLPADGSESTAFIEDVWK
AF      S
250     260     270     280     290     300
IGLPKAKVQGEHVDKCFADQCTACEKEAVNVSLGNLLTYPFVREGLVKRTLALKGGHYD
310     320
FVNGGFELWGLEFLSPSLV

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**Figure 3** Tobacco chloroplast CA protein sequence

The complete sequence of the tobacco chloroplast CA is shown [13]. The sequence corresponding to the transit peptide is underlined, and the residues that form the active site are shown in bold. Arrows indicate the amino acids that were changed by site-directed mutagenesis, and their replacements are shown underneath.

partial degradation. Whereas E196A, Y197F and the wt CA were present in detectable levels, D154N and C215S were not detected in the soluble fraction. Thus, due to the presumed instability of D154N and C215S, we could not determine whether mutations in the CA active site affected  $\Delta NCE103$  complementation. How-

ever, the E196A mutation, which was previously shown to compromise CA activity, did allow complementation of  $\Delta NCE103$  growth.

To assess the CA activity of the various mutant proteins, recombinant His-tagged E196A, Y197F, D154N, C215S, NCE103 and wt CA fusion proteins were expressed in *E. coli*. After affinity purification, their kinetic properties were determined using a stopped-flow spectrophotometric assay. As described previously for other  $\beta$  CAs [10,14,20], the steady-state kinetic parameters of the wt enzyme, Y197F and NCE103 were pH-dependent. For all three enzymes, enzymic activity (based on  $k_{cat}$  and  $k_{cat}/K_m$  values) was greater at pH 9 when compared with pH 7 (Table 1). In addition, NCE103 exhibited detectable CA activity. Despite having a rather low  $k_{cat}$ , its  $k_{cat}/K_m$  ratio, which is an index of the enzyme activity under physiological conditions (i.e. substrate concentration substantially below the  $K_m$ ), was significant at pH 9 and 7. Y197F also displayed significant  $k_{cat}/K_m$  ratios at both pH values. Similar to a previously characterized *Arabidopsis*  $\beta$  CA carrying an equivalent Y  $\rightarrow$  F mutation [14], it also shows substrate inhibition at pH 9 (Figure 4C), but not at pH 7 (Figure 4D). In contrast, the kinetic parameters for E196A, D154N and C215S suggest that these enzymes have very little CA activity. Of the three, however, only E196A exhibited detectable levels of activity at pH 7. Probably, this very low-level activity was sufficient to complement the growth of  $\Delta NCE103$ .

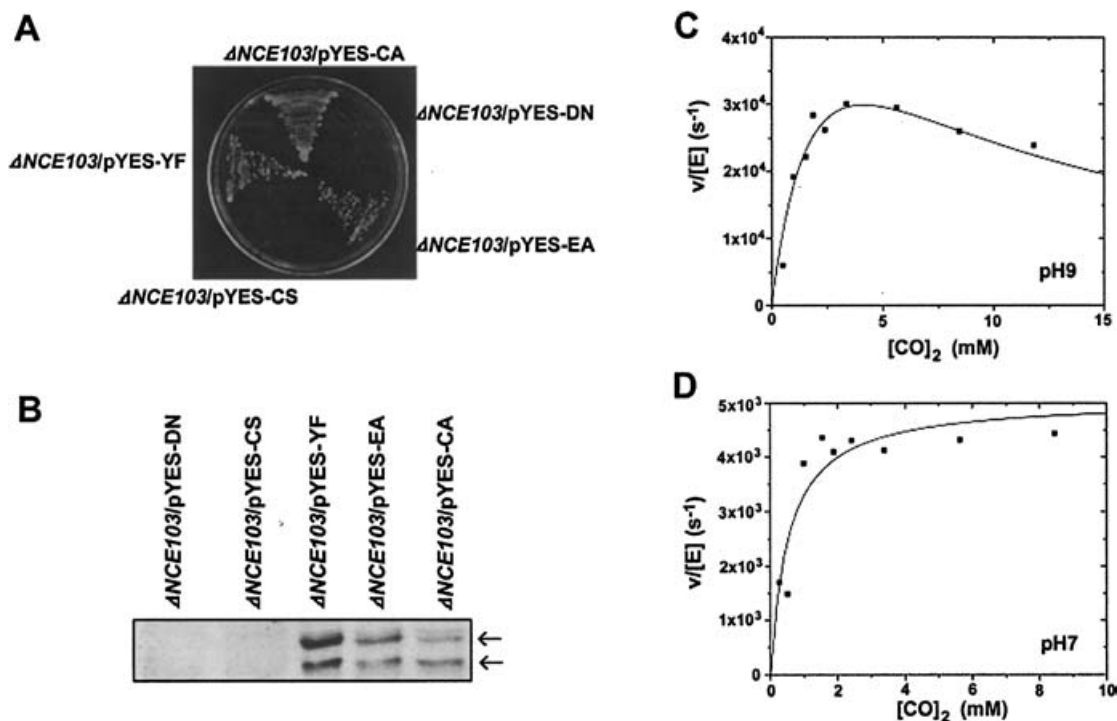
#### Complementation of $\Delta NCE103$ by the human CAII

To investigate further whether tobacco CA's ability to complement  $\Delta NCE103$  is due to its CA activity, we transformed  $\Delta NCE103$  with human CAII; this protein belongs to the  $\alpha$  class of CAs and is known to have high levels of CA activity [21]. Since the structure of  $\alpha$  class CAs is unrelated to that of the  $\beta$  class, human CAII would most probably lack any activity (besides CA activity) associated with  $\beta$  class CAs. As shown in Figure 5(A), transformation of  $\Delta NCE103$  with pYES-hCAII fully restored growth under aerobic conditions. V143Y, which is an enzymically inactive form of CAII [21], failed to complement  $\Delta NCE103$ . However, for D154N and C215S, expression of V143Y could not be detected by Western-blot analysis (Figure 5B).

#### DISCUSSION

In the present study, we investigated whether a previously proposed antioxidant activity of tobacco chloroplast CA was responsible for its ability to complement  $\Delta NCE103$  growth under aerobic conditions. Unlike a previous study [8], our efforts to detect a difference between the sensitivity of  $\Delta NCE103$  and wt yeast to oxidative stress were unsuccessful, implying that NCE103 does not have any antioxidant activity. Moreover, the tobacco chloroplast CA, which restores  $\Delta NCE103$  growth under aerobic conditions [9], did not increase the antioxidant capacity of  $\Delta NCE103$ , nor did it rescue  $tsa1\Delta tsa2\Delta$ , a yeast strain known for its enhanced susceptibility to  $H_2O_2$  and NO [17]. In addition to our findings, it was demonstrated recently that  $\Delta NCE103$  growth under aerobic conditions is restored by supplying the medium with increased concentrations of  $CO_2$  [22]. Thus it appears that growth of this mutant is not compromised by the presence of oxygen, but rather by the lack of sufficient  $CO_2$  in the medium.

Since these results argue that tobacco chloroplast CA's proposed antioxidant activity is not responsible for complementing  $\Delta NCE103$  growth under aerobic conditions, we tested whether its CA activity was required. Four different point mutations were introduced into the tobacco chloroplast CA by site-directed



**Figure 4** Characterization of wt tobacco chloroplast CA and the four site-directed mutants

(A) The wt tobacco chloroplast CA (pYES-CA) and mutant constructs E196A (pYES-EA) and Y197F (pYES-YF) complement  $\Delta NCE103$  growth.  $\Delta NCE103$  was transformed with the indicated plasmids, and transformants selected in SD-Ura medium under anaerobic conditions were replated on SG-Ura medium. The plate was incubated at 30 °C under aerobic conditions for 4 days. (B) Immunoblot analysis of wt tobacco chloroplast CA and the four mutants in yeast strain  $\Delta NCE103$ . The indicated strains were grown anaerobically in SG-Ura medium at 30 °C for 2 days. Total protein (30  $\mu$ g) extracted from each sample was resolved by SDS/PAGE (12% gel). After transfer to a nitrocellulose membrane, wt and mutant CAs were detected with an antibody raised against the pea chloroplast CA. Arrows indicate the position of the two major bands that were specifically recognized by the antibody. (C) Substrate inhibition of mutant Y197F. Kinetics of  $\text{CO}_2$  hydration catalysed by Y197F at pH 9. Reaction conditions were 25 °C, 40 mM Bicine/60  $\mu$ M *m*-Cresol Purple/2.5 mM EDTA (pH 9) and 500 nM enzyme. Data were fitted by least-squares regression to the equation  $\frac{v}{[E]} = \frac{k_{\text{cat}}[\text{CO}_2]}{K_m + [\text{CO}_2] + ([\text{CO}_2]^2/K_m)}$  [27]. Parameters for least-squares fit of these data are shown in Table 1. (D) Same as in (C) but performed at pH 7. Reaction conditions were 25 °C, 40 mM Mops/30  $\mu$ M *p*-nitrophenol/2.5 mM EDTA (pH 7) and 500 nM enzyme. Parameters for least-squares fit of these data are shown in Table 1.

**Table 1** Steady-state kinetic parameters of the tobacco chloroplast CA (wt), D154N, E196A, Y197F, C215S and yeast NCE103, determined by stopped-flow spectrophotometry at pH 9 and 7

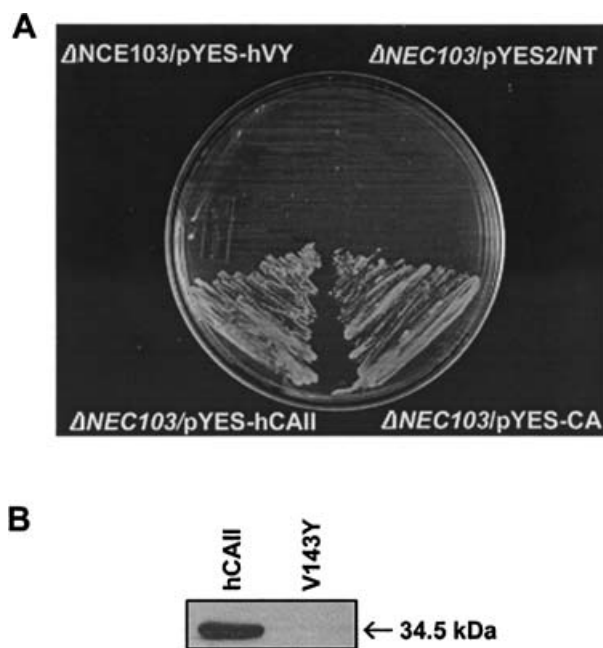
NCE103, the tobacco chloroplast CA, and its variants D154N, E196A, Y197F and C215S, were expressed in *E. coli* as His-tagged proteins. After affinity purification, their steady-state kinetic parameters  $k_{\text{cat}}$ ,  $K_m$  and  $k_{\text{cat}}/K_m$  were determined at pH 9 and 7 by stopped-flow spectrophotometry as described in the Experimental section. The protein concentrations were 1  $\mu$ M (pH 9) and 2  $\mu$ M (pH 7) for D154N, C215S and NCE103; 500 nM (pH 9) and 1  $\mu$ M (pH 7) for Y197F; 500 nM (pH 9 and 7) for E196A; 200 nM (pH 9) and 375 nM (pH 7) for the wt enzyme. ND, not detectable.

	pH 9			pH 7		
	$k_{\text{cat}}$ ( $\text{ms}^{-1}$ )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1} \cdot \text{s}^{-1}$ )	$k_{\text{cat}}$ ( $\text{ms}^{-1}$ )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1} \cdot \text{s}^{-1}$ )
wt	157.0 $\pm$ 4	3.4 $\pm$ 0.2	47.00 $\pm$ 2	52.00 $\pm$ 2	2.4 $\pm$ 2	22.00 $\pm$ 2
D154N	20.0 $\pm$ 20	23.0 $\pm$ 3	0.91 $\pm$ 0.04	ND	ND	ND
E196A	5.8 $\pm$ 0.4	19.0 $\pm$ 2	0.31 $\pm$ 0.1	0.18 $\pm$ 0.04	6.0 $\pm$ 3	0.03 $\pm$ 0.01
Y197F	67.0 $\pm$ 2	2.6 $\pm$ 0.1	26.00 $\pm$ 1	4.82 $\pm$ 0.03	0.6 $\pm$ 0.2	8.00 $\pm$ 2
C215S	5.1 $\pm$ 0.4	13.0 $\pm$ 2	0.40 $\pm$ 0.03	ND	ND	ND
NCE103	6.7 $\pm$ 0.04	0.1 $\pm$ 0.2	70.00 $\pm$ 10	1.09 $\pm$ 0.05	0.3 $\pm$ 0.1	0.40 $\pm$ 0.1

mutagenesis. D154N and C215S, which directly affect the CA active site, produced proteins with no CA activity at pH 7, as determined by stopped-flow spectrophotometry. These results are in good agreement with activities reported for equivalent mutations in spinach and pea chloroplast CAs [18,19]. E196A, which compromises CA activity without directly affecting the active site, generated a protein with low but detectable activity at this pH. Unfortunately, D154N and C215S did not accumulate to detectable levels in yeast; thus, their ability to complement

$\Delta NCE103$  could not be determined. In contrast, E196A was efficiently expressed in yeast, as was Y197F, which carries a mutation that reproduces the only amino acid difference between the active sites of NCE103 and other  $\beta$  class CAs. Both E196A and Y197F also restored  $\Delta NCE103$  growth under aerobic conditions.

Although the CA activity of E196A was substantially lower than that of Y197F, we suspected that it was sufficient to complement  $\Delta NCE103$ . Supporting this possibility was the discovery that NCE103 has CA activity, with a significant  $k_{\text{cat}}/K_m$  value at pH 9



**Figure 5** Human CA II, but not an inactive form, complements  $\Delta NCE103$

(A)  $\Delta NCE103$  was transformed with wt human CAII (pYES-hCAII), the inactive V143Y mutant (pYES-hVY), wt tobacco CA (pYES-CA) or empty vector (pYES2/NT). Transformants selected in SD-Ura medium under anaerobic conditions were re-plated on SG-Ura medium. The plate was photographed after incubation at 30 °C under aerobic conditions for 4 days. (B) Immunoblot analysis of hCAII and V143Y in yeast strain  $\Delta NCE103$ . The procedure was as indicated in Figure 4(B) but utilizing a polyclonal antibody raised against the human CAII (Novus Biologicals).

and 7. Thus NCE103 is probably a functional CA *in vivo*; this finding also indicates that results obtained with electrometrical CA assays should be interpreted cautiously. To demonstrate that CA activity is required for  $\Delta NCE103$  complementation, we tested whether growth of this mutant could be restored by expression of human CAII. Human CAII exhibits high levels of CA activity and is unlikely to possess any additional activity related to  $\beta$  CAs, since it belongs to the structurally unrelated  $\alpha$  CA class. Complementation of  $\Delta NCE103$  growth was observed in the presence of wt human CAII. Unfortunately, the inactive V143F form, which has essentially no CA activity [21] and was utilized as a negative control, was not expressed in yeast at detectable levels. In spite of the latter, these results support our hypothesis that CA activity is required for restoration of  $\Delta NCE103$  growth under aerobic conditions.

The mechanism by which CA activity allows yeast to grow under aerobic conditions is currently unknown. CA has been proposed to provide bicarbonate for various carboxylation reactions, such as those catalysed by pyruvate carboxylase in gluconeogenesis [3] or acetyl-CoA carboxylase in fatty acid synthesis [23,24]. In yeast mutants lacking CA activity, decreased production of bicarbonate might lead to a suppressed growth phenotype unless the CO<sub>2</sub> concentration in the medium becomes high enough to favour spontaneous bicarbonate production via the uncatalysed hydration reaction. Yeast grown in sugar-containing media utilize the glycolytic pathway, and thereby generate CO<sub>2</sub>, regardless of whether they are in aerobic or anaerobic conditions [25]. However, the CO<sub>2</sub> concentration in the medium will probably reach levels high enough to favour spontaneous bicarbonate production in a still liquid culture (anaerobic conditions) compared with the levels reached under continuous shaking (aerobic

conditions). Since  $\Delta NCE103$  grows anaerobically in still-liquid cultures or aerobically in liquid cultures with high concentrations of CO<sub>2</sub> [22], we propose that there is a minimum concentration of bicarbonate required for yeast growth. Aerobic conditions (shaking) would prevent  $\Delta NCE103$  growth because CO<sub>2</sub> would not accumulate in sufficient amounts to generate spontaneously the required concentration of bicarbonate. Under these conditions, the presence of a CA, such as NCE103 or a transgene, would be required to provide the necessary supply of bicarbonate. A similar situation was reported recently for mutant strain 25-1 of the facultative chemoautotroph *Ralstonia eutropha* H16. This strain is unable to grow at low CO<sub>2</sub> concentrations and bears a mutation in the *CAN* gene, which encodes a functional CA [22]. For NCE103, *CAN* exhibits a low but significant CA activity.

One possible mechanism through which reduced bicarbonate levels suppress growth of the  $\Delta NCE103$  mutant is by decreasing lipid synthesis. Tobacco plastidial CA was previously implicated in the incorporation of acetate into lipids [24]. Additionally, an *S. cerevisiae* strain carrying a temperature-sensitive mutation in the acetyl-CoA carboxylase gene, *acc1<sup>ts</sup>*, arrests at G<sub>2</sub>/M of the cell cycle when shifted to the restrictive temperature [26]. Acetyl-CoA carboxylase is a biotin-containing enzyme, which catalyses the carboxylation of acetyl-CoA to produce malonyl-CoA, a key intermediate in lipid biosynthesis. Thus the possibility that lipid biosynthesis is compromised in  $\Delta NCE103$  by an insufficient supply of bicarbonate under aerobic conditions, which would lead to growth arrest at G<sub>2</sub>/M, will be explored in future investigations.

In conclusion, by using different approaches we have generated evidence that strongly suggests that complementation of  $\Delta NCE103$  by  $\beta$  CAs depends on CA activity, rather than on a proposed antioxidative function. We further hypothesize that the requirement for CA activity is related to bicarbonate availability for biosynthetic reactions.

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