

Cyc2p is required for maintaining ionic stability and efficient cytochrome *c* import and mitochondrial function in *Saccharomyces cerevisiae*

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Abstract A previous study demonstrated that Cyc2p from *Saccharomyces cerevisiae* is a mitochondrial protein and that *cyc2* mutants contained only approximately 20% of the normal levels of cytochrome *c* due to a partial deficiency in mitochondrial import of apo-cytochrome *c*. We report herein that deletion of the entire gene results in defective mitochondrial function, as revealed by diminished growth on media containing non-fermentable carbon sources. This defect is exacerbated in hyper-ionic KCl media and at higher incubation temperatures, but is suppressed on media containing sorbitol, a non-ionic compound. We suggest that Cyc2p serves to maintain the osmotic stability of mitochondria, and its defect is exacerbated by KCl.

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Key words: *CYC2*; YOR037w; Yeast; Mitochondrion; Cytochrome *c*

1. Introduction

Eukaryotic cytochrome *c* is initially synthesized in the cytosol as apo-cytochrome *c*, lacking heme. After or during import into mitochondria, heme is covalently attached by thioether linkages to two cysteine residues in the apo-cytochrome *c*. This covalent attachment is catalyzed by the enzyme cytochrome *c* heme lyase (also denoted holo-cytochrome *c* synthase, EC 4.4.1.17), which is located in the mitochondrial intermembrane space, and which is encoded by the gene *CYC3* in the yeast *Saccharomyces cerevisiae* (for reviews, see [1,2]).

S. cerevisiae contains two forms of cytochrome *c*, iso-1-cytochrome *c* and iso-2-cytochrome *c*, which are encoded by the *CYC1* and *CYC7* genes, respectively, and which constitute 95% and 5%, respectively, of the total complement of cytochrome *c* in aerobically grown, derepressed cells [3–5]. The pathway for mitochondrial import and the requirements for the complete biosynthesis of cytochrome *c* have been systematically investigated by isolating and characterizing mutations that cause specific deficiencies. The genetic analysis of over 100 cytochrome *c* deficient mutants revealed that mutation of only three genes, *CYC1*, *CYC2*, and *CYC3*, resulted in greater than 75% deficiencies in the total amount of cytochrome *c*, without affecting the other cytochromes [6–9]. Certain *cyc1* mutants completely lacked iso-1-cytochrome *c*, but maintained the normal 5% level of iso-2-cytochrome *c*. Cer-

tain *cyc3* mutants completely lacked both iso-1-cytochrome *c* and iso-2-cytochrome *c*, a result that is expected in light of the fact that *CYC3* encodes cytochrome *c* heme lyase [10,11]. The *cyc2* mutants had substantial deficiencies of cytochrome *c*, but none of them completely lacked cytochrome *c* and all contained at least 10% of the total normal amount. The considerable and specific deficiencies of cytochrome *c* in *cyc2* mutants obviously suggested that *CYC2* encoded a protein important for the synthesis or regulation of cytochrome *c*.

Thus, the *CYC1*, *CYC7* and *CYC3* genes, whose functions are known, and the *CYC2* gene, which is the subject of this paper, are the only known genes that specifically and substantially affect cytochrome *c*. (Although these genes are considered to be specific, certain *cyc3* mutants, and double *cyc1 cyc7* mutants also lacked cytochrome *aa₃*, due to the secondary effect of cytochrome *c* deficiency; subunits I, II, and III of cytochrome *aa₃* are rapidly degraded in mutants completely lacking cytochrome *c* [12].) It is therefore important to determine the function of the *CYC2* gene, and to determine the mechanism by which it specifically controls only cytochrome *c*.

The cloning and characterization of *CYC2* was previously reported by Dumont et al. [13], who demonstrated by immunoblotting of subcellular fractions that Cyc2p is a mitochondrial protein, and that *cyc2* mutations leads to the accumulation of apo-cytochrome *c*. It was suggested that Cyc2p is required for the complete normal level of mitochondrial import of cytochrome *c*. Analysis of *CYC1⁺ cyc7-Δ* and *cyc1-Δ CYC7-H3* strains, which contain solely iso-1-cytochrome *c* and iso-2-cytochrome *c*, respectively, revealed that *cyc2* mutations diminished the level of holo-iso-1-cytochrome *c* more than the level of holo-iso-2-cytochrome *c* [13]. This difference is due to the finding that apo-iso-2-cytochrome *c* has a much longer half-life than apo-iso-1-cytochrome *c* [14,15].

Further studies revealed that Cyc2p was located in the inner mitochondrial membrane (M.E. Dumont, personal communication). Subsequently, the DNA sequence of *CYC2* (open reading frame YOR037w) was reported as part of the effort for sequencing chromosome XV [16]. The results of the ORF indicated that Cyc2p was 404 amino acids long, and not 168 amino acids as first suggested by Dumont et al. [13]. In addition, Cyc2p is 19% identical and 38% similar to EnvZp of *Escherichia coli* [17], although it is doubtful that these two proteins are physiologically related.

Our new results reported herein demonstrate that strains with a complete deletion, *cyc2-Δ2*, have a partial defect in mitochondrial function when grown at 37°C, in addition to a partial defect in mitochondrial import of cytochrome *c*. Furthermore, *cyc2-Δ2* strains grow poorly on hyper-ionic KCl media, suggesting that Cyc2p may be involved in maintaining mitochondrial integrity.

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2. Materials and methods

2.1. Genetic nomenclature

The symbol *CYC* denotes genes that significantly and presumably specifically affect cytochrome *c* in *S. cerevisiae*. The symbols *CYC1*⁺, *CYC7*⁺ and *CYC3*⁺ denote, respectively, the wild type alleles encoding iso-1-cytochrome *c* [4], iso-2-cytochrome *c* [5] and cytochrome *c* heme lyase [10]. The symbols *cyc1*-Δ, *cyc7*-Δ, etc., denote partial or complete deletions.

2.2. Construction of *cyc2*-Δ1 and *cyc2*-Δ2 strains

The *CYC2*⁺ normal strain B-7553 (*MATa CYC1*⁺ *cyc7*-738::*CYH2 ura3-52 his3*-Δ1 *leu2*-3,112 *trp1*-289 *cyh2*), described by Dumont et al. [13], served as the parental strain for generating the *cyc2*-Δ1 (B-10101 *MATa CYC1*⁺ *cyc7*-738::*CYH2 ura3-52 his3*-Δ1 *leu2*-3,112 *trp1*-289 *cyh2 cyc2*-Δ1::*LEU2*) and *cyc2*-Δ2 (B-10100 *MATa CYC1*⁺ *cyc7*-738::*CYH2 ura3-52 his3*-Δ1 *leu2*-3,112 *trp1*-289 *cyh2 cyc2*-Δ2::*URA3*) deletions by the one step gene replacement procedure. The *cyc2*-Δ1 deletion was made by disruption with a *LEU2* fragment exactly as the deletion previously denoted *cyc2*-Δ or *cyc2*::*LEU2* in the strain B-8132 [13]. (Thus, the independently constructed strains B-8132 and B-10101 should be identical.) The *cyc2*-Δ2 deletion was generated by replacing the 1543 bp *Bam*HI-*Kpn*I of *CYC2* fragment with a 1.1 kb *URA3* fragment obtained from the plasmid pAB1714. Yeast strains bearing the deletions were recovered by homologous recombination of linear fragments from the above constructs using standard yeast transformation procedures. The disruptions were confirmed by PCR analysis.

For the sake of brevity, the isogenic series of strains are abbreviated in this paper as follows: B-7553, *CYC2*⁺; B-10101, *cyc2*-Δ1; and B-10100, *cyc2*-Δ2.

2.3. Growth on media

The relative growth of the strains was estimated by inoculating dilute suspension of cell on the surface of YPD (1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose) and YPG (1% Bacto-yeast extract, 2% Bacto-peptone and 2% [v/v] glycerol) plates, and incubating the plates at 23°C, 30°C and 37°C, for 2–5 days. The concentrations of the various salts used were 1.2 M or 1.5 M KCl, 1 M NaCl, 0.36 M LiCl, and 1.5 M CaCl₂, where indicated. Sorbitol was also added to 1.0 M where indicated.

2.4. Low temperature spectroscopic and spectrophotometric analysis of intact cells

The yeast strains were grown on the surface of YP1%S (1% Bacto-yeast extract, 2% Bacto-peptone and 1% sucrose) plates at 23°C for 4 days, 30°C for 3 days, or 37°C for 2 days, which are slightly modified conditions of our standard procedure [8]. Total amounts of cytochrome *c* were determined by spectroscopic visual examination of intact cells at −196°C [18] and by comparing the intensities of the *c*_α bands at 547 nm to the *c*_α bands of strains having known amounts of cytochrome *c*. The levels of cytochromes *a*₃, *b*, *c* and *c*₁ were quantitatively estimated by absorbance recordings of intact cells at −196°C, using an Aviv model 14 spectrophotometer as described by Hickey et al. [19]. The relative amounts of cytochrome *c* were estimated from the heights of the *c*_α band after baseline corrections.

2.5. Determination of ATPase and NADH-cytochrome *c* reductase

The yeast strains were grown in liquid YPD at 23°C, 30°C and 37°C to early stationary phase. Mitochondria were prepared by the method of Faye et al. [20], except that zymolyase 20000 instead of glucylase was used for the preparation of spheroplasts. ATPase activity was assayed at 37°C by measuring inorganic phosphate released from ATP [21] in the presence or absence of 10 μg/ml oligomycin. NADH-cytochrome *c* reductase was assayed at 30°C by following the rate of reduction of ferricytochrome *c* at 550 nm. The reaction mixture contained 20 mM potassium phosphate buffer, pH 7.5, 0.08% ferricytochrome *c*, 5 × 10^{−4} M potassium cyanide and 50 μg of mitochondrial protein. The reaction was started by the addition of 0.5 μmol NADH. The increase in absorbance was measured against a reagent blank containing all of the components except mitochondria. The specific activity was calculated from the initial linear rate using a mM extinction coefficient of 18.5 for the difference in absorbance of oxidized and reduced cytochrome *c* at 550 nm. Protein concentrations were determined by the method of Lowry et al. [22].

3. Results

3.1. Sequence of *CYC2*

The normal sequence of Cyc2p, deduced from the YOR037w, is schematically presented in Fig. 1, along with the previously constructed truncated Cyc2-Δ1p. The *CYC2* gene encodes a protein of 404 amino acids, with a predicted molecular weight of 47 kDa, and with two potential transmembrane domains from amino acids 67–86, and 260–278. Sequence comparisons by Bestfit analysis (UWGC) revealed that Cyc2p is 19% identical and 38% similar to EnvZp, which is a regulator of porins in *Escherichia coli*. This sequence similarity between Cyc2p and EnvZp does not appear to be physiologically significant and there is no evidence that Cyc2p and EnvZp are functionally related.

3.2. The *cyc2*-Δ1 and *cyc2*-Δ2 mutant strains

The isogenic series of yeast strains contain the normal *CYC1* gene encoding iso-1-cytochrome *c*, but lacks iso-2-cytochrome *c* because of the *cyc7*-Δ deletion. The *cyc2*-Δ1 deletion, previously denoted *cyc2*-Δ or *cyc2*::*LEU2* [13], is inferred to encode a protein which contains the normal residues at amino acid positions 1–169, and abnormal residues at positions 170–173, but which lacks residues at positions 174–404 (Fig. 1). Thus, Cyc2-Δ1p contains only 43% of the amino-terminal portion of the normal Cyc2p protein. On the other hand, the *cyc2*-Δ2 deletion spans nucleotide positions −318 to 1225, including the entire translated region, resulting in the complete absence of the entire Cyc2p.

The overall function of the mitochondrial system was evaluated from the degree of growth on media containing non-fermentable substrates as the sole carbon and energy source. The growth of the strains at 23°C, 30°C and 37°C on YPD (glucose) and YPG (glycerol) media is shown in Fig. 2. The two mutants, *cyc2*-Δ1 and *cyc2*-Δ2, grew approximately the same as the normal *CYC2*⁺ strain on YPD medium at all three temperatures. In contrast, the *cyc2*-Δ2 mutant grew considerably less on YPG medium than either of the *CYC2*⁺ and *cyc2*-Δ1 strains, whose growth was approximately equivalent. In this respect, the *cyc2* point mutants resembles the *CYC2*⁺ and *cyc2*-Δ1 strains. The *cyc2*-Δ2 mutant grew less on YPG medium at all three incubation temperatures, but especially at 37°C. Thus, the *cyc2*-Δ2 mutation causes a substantial diminution of mitochondrial function. Clearly, the reduced *cyc2*-Δ2 function is not caused by the reduced amount of cytochrome

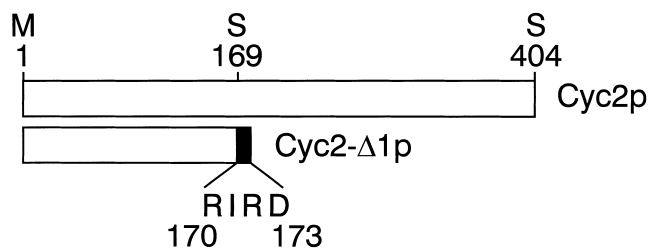


Fig. 1. Schematic representation of the normal Cyc2p and the truncated Cyc2-Δp proteins encoded by *CYC2*⁺ and *cyc2*-Δ1, respectively. The numbers denote amino acid positions. The *cyc2*-Δ1 mutation was formed by deleting the *Bam*HI fragment corresponding to positions 509–1225 as described by Dumont et al. [13], with A of the ATG initiator codon assigned as nucleotide position 1. The deduced carboxyl end of truncated Cyc2-Δ1p is shown for the amino residues at positions 170–173.

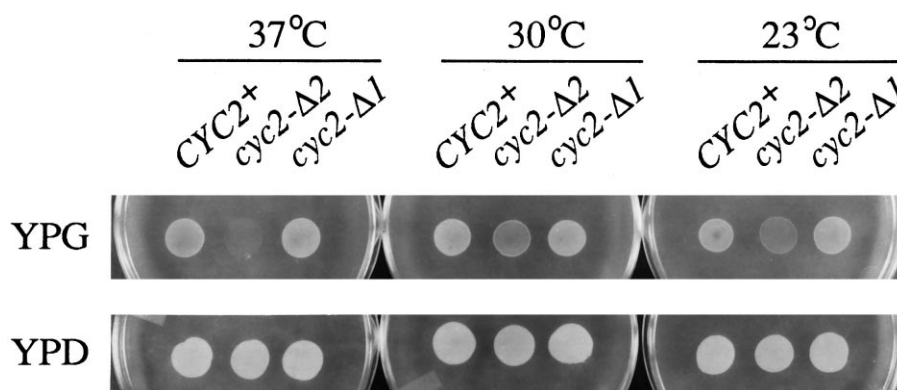


Fig. 2. Complete deletion of *CYC2* causes temperature sensitivity on glycerol media. Growth of the *CYC2*⁺, *cyc2-Δ1* and *cyc2-Δ2* strains (Table 1) on YPG (glycerol) and YPD (glucose) media at 37°C for 2 days, 30°C for 3 days, or 23°C for 4 days.

c, because the cytochrome *c* levels are the same in both of the *cyc2-Δ1* and *cyc2-Δ2* strains.

Also, it should be emphasized that growth on YPG medium is not appreciably diminished until the level of cytochrome *c* is below 1% of the normal level. For example, only slightly reduced growth on YPG medium is observed with *cyc1-Δ* *CYC7*⁺ strains that lack iso-1-cytochrome *c* but contain the approximately 5% normal level of iso-2-cytochrome *c* [3]. Also only slightly reduced growth on YPG medium is observed with *cyc1-948 cyc7-Δ* strains, which contain only approximately 2% of the normal level of *CYC1* mRNA and 5% of the normal amount iso-1-cytochrome *c* due to lack of TATA elements [23]. However, as expected, double *cyc1-Δ cyc7-Δ* mutants completely lacking cytochrome *c* do not grow on YPG medium [9].

3.3. Comparative analysis of the *CYC2*⁺, *cyc2-Δ1*, *cyc2-Δ2*, and *cyc2* point mutants

The levels of iso-1-cytochrome *c*, as well as the other cytochromes, were quantitatively estimated by low temperature (−196°C) spectrophotometric recordings of intact cells grown at 23°C, 30°C and 37°C. The results, summarised in Table 1, revealed that the two *cyc2-Δ1* and *cyc2-Δ2* strains both contained approximately 20% of the normal level, similar to *cyc2* point mutants previously examined [6–9]. Furthermore, this level of deficiency was observed for the strains grown at

30°C and 37°C. Thus, a low amount of cytochrome *c* is still produced in the strain containing a complete deletion of *CYC2*. The other cytochromes *a_{a3}*, *b*, and *c₁*, were approximately normal, also consistent with previous studies of *cyc2* point mutants.

The defect caused by the *cyc2-Δ2* deletion was further investigated by determining the rates of respiration, and activities of mitochondrial ATPase and NADH-cytochrome *c* reductase. The results show that respiration rates correlate with the growth on glycerol medium; the rate of respiration of *cyc2-Δ* grown at 37°C was decreased 31% of the value of the similarly grown *CYC2*⁺ and *cyc2-Δ2* strains. However, this diminished respiration is not sufficient to account for the diminished growth on glycerol medium, indicating a still unidentified temperature-sensitive block in electron transfer. Also, it should be noted that the rate of respiration is not strongly influenced by the level of cytochrome *c*; *cyc1-Δ* *CYC7*⁺ strains containing only approximately 5% of normal amount of total cytochrome *c* have approximately one-half of the normal level of respiration [18]. Mitochondrial ATPase activities for *CYC2*⁺, *cyc2-Δ1* and *cyc2-Δ2* strains were essentially the same and results indicated that the ATPase activities of the three strains grown at 30°C and 37°C temperatures were approximately the same, with and without added oligomycin (not shown). However, the NADH-cytochrome *c* reductase activities of both the *cyc2-Δ1* and *cyc2-Δ2* mutants

Table 1
Growth of *CYC2*⁺ and *cyc2-Δ2* strains on YPD and YPG media containing 1.5 M KCl, 1 M NaCl, or 1 M sorbitol

	30°C			37°C		
	<i>CYC2</i> ⁺	<i>cyc2-Δ1</i>	<i>cyc2-Δ2</i>	<i>CYC2</i> ⁺	<i>cyc2-Δ1</i>	<i>cyc2-Δ2</i>
YPD	4	4	4	4	4	4
YPD+KCl	3	3	<u>1</u>	1	1	<u>0</u>
YPD+NaCl	3	3	<u>1</u>	3	3	<u>1</u>
YPD+sorbitol	3	3	<u>3</u>	3	3	<u>3</u>
YPG	4	4	3	3	3	0
YPG+KCl	2	2	<u>0</u>	0	0	<u>0</u>
YPG+NaCl	1	1	<u>1</u>	0	0	0
YPG+sorbitol	3	3	3	2	2	<u>2</u>
Cytochrome <i>c</i> , YPD	100	20	20	100	—	20
Cytochrome <i>c</i> , YPD+sorbitol	100	—	20	—	—	—
Cytochrome <i>c</i> , YPD+KCl	100	—	< 5	—	—	—

The relative levels of iso-1-cytochrome *c* after growth on various media are presented at the bottom of the table.

Relative growth is indicated by 4 (normal) to 0 (no growth). Values that differ from the normal are indicated in boldface. The preferential inhibition by KCl or NaCl and suppression by sorbitol are highlighted with underlined and bold underlined numbers, respectively.



Fig. 3. Growth of the various strains under various conditions, demonstrating that the *cyc2-Δ2* mutant is sensitive to elevated growth temperature and the presence of KCl, and that the temperature sensitivity of *cyc2-Δ2* can be suppressed by sorbitol. Growth of *CYC2*⁺ and *cyc2-Δ2* strains is shown as follows: A, YPD, 30°C, 2 days; B, YPD, 37°C, 2 days; C, YPD, 38°C, 2 days; D, YPD+1.5 M KCl, 30°C, 6 days; E, YPD+1.5 M KCl, 37°C, 6 days; F, YPG, 30°C, 2 days; G, YPG, 37°C, 2 days; H, YPG, 38°C, 2 days; I, YPG+1.2 M KCl, 6 days, 30°C; J, YPG+1.0 M sorbitol, 30°C, 4 days; K, YPG+1.0 M sorbitol, 37°C, 4 days; and L, YPG+1.0 M sorbitol, 38°C, 4 days.

were approximately twice the normal level. The higher levels of NADH-cytochrome *c* reductase were observed in the *cyc2-Δ1* and *cyc2-Δ2* mutants grown at 30°C and 37°C, as well as at 23°C, establishing that the difference is reproducible.

3.4. Deletion of *CYC2* causes ionic sensitivity

Cyc2p and EnvZp do not appear to be functionally related, although they share 19% identity, and 38% similarity. Nevertheless, because EnvZp regulates porin in *E. coli*, we investigated the osmosensitivity of *cyc2-Δ* strains by examining growth on media containing several different salts at various concentrations. Growth characteristics of these strains, presented in Table 1, revealed that *cyc2-Δ2* strains are abnormally sensitive to K⁺ and Na⁺ salts. In addition, *cyc2-Δ2* strains are sensitive to Cu²⁺ salts, but not Ca²⁺ and Li⁺ salts (results not presented). The sensitivity of *cyc2-Δ2* to these salts were manifested on YPD medium, and was exacerbated at 37°C, and still further on YPG medium. The growth of the *cyc2-Δ1* strain was also sensitive to KCl, but to a lesser degree (not shown). Measurement of the cytochrome *c* content showed that a decrease in cytochrome *c* to 20% is evident in *cyc2-Δ2* strains. Cytochrome *c* levels decreased further from 20% to less than 5% in the presence of KCl. The rate of respiration in the presence of KCl was also considerably decreased at 37°C (not shown).

Further examination of *CYC2*⁺ and *cyc2-Δ2* strains on YPD and YPG containing KCl and sorbitol are presented in Fig. 3. The growth of *cyc2-Δ2* strains, as previously described, is diminished on YPG at 37°C (Fig. 3G), and increasing the growth temperature to 38°C exacerbated the temperature sensitivity of *cyc2-Δ2* (Fig. 3H). *Cyc2-Δ2* strains were not sensitive to hyperosmotic sorbitol media, and, importantly, 1.0 M sorbitol suppressed the diminished growth on YPG at 37°C, and partially at 38°C of *cyc2-Δ2* strains (Fig. 3K,L, respectively).

4. Discussion

We have shown that *cyc2* mutations are pleiotropic and that *CYC2* does not solely affect cytochrome *c*. *Cyc2* mutants contain low amounts of cytochrome *c*, which is at approximately 20% of the normal level in *CYC1*⁺ *cyc7-Δ* strains having either the *cyc2-Δ1* partial deletion or the *cyc2-Δ2* complete deletion; or in *CYC1*⁺ *CYC7*⁺ strains having certain *cyc2* point mutations. Dumont et al. [13] previously demonstrated that the diminished level of cytochrome *c* in the *cyc2-Δ1* mutant was due to a reduced rate of import of apo-cytochrome *c*. Most importantly, the *cyc2-Δ2* mutant, but not the *cyc2-Δ1* mutant or *cyc2* point mutants, was defective in mitochondrial

function, as indicated by diminished growth on media with non-fermentable carbon sources, such as YPG, especially at the higher incubation temperature of 37°C. The reduced growth on YPG cannot be explained by deficiencies of cytochromes or respiration. The growth of the *cyc2-Δ2* mutant was further diminished on YPG+KCl and YPD+KCl media. On the other hand, the diminished growth on YPG was reversed by sorbitol. Thus, the *cyc2-Δ2* mutant contains a mitochondrial defect that is enhanced by K⁺ and suppressed by sorbitol. In addition, both of the *cyc2-Δ1* and *cyc2-Δ2* mutants contained twice the activity of NADH-cytochrome *c* reductase.

The presence of a mitochondrial defect in the *cyc2-Δ2* mutant is unexpected since over one-half of the carboxy-terminal portion of the protein is absent in *Cyc2-Δ1p*. One could suggest that *Cyc2p* contains two or more domains with separate functions, and that the amino-terminal domain is necessary for an unknown process required for efficient mitochondrial function, whereas the carboxy-terminal domain is required for efficient import of cytochrome *c*. However, we favor the view that *Cyc2p* is a membrane component required for normal mitochondrial integrity, and its loss results in partial defects and stimulation of other mitochondrial processes. The phenotypic suppression of impaired growth by sorbitol suggests that the mitochondrial membrane is impaired in *cyc2-Δ2* mutants, resulting in the increased sensitivity to ionic conditions that causes reduced utilization of non-fermentable carbon sources. The amino-terminal portion of the protein in *cyc2-Δ2* mutants may be sufficient to prevent this osmotic sensitivity, but not sufficient for establishing the normal rate of apo-cytochrome *c* import.

It is interesting that *Cyc2p* has weak sequence homology to EnvZp, which is an outer membrane protein that regulates porins of *E. coli* in response to changes in osmolarity. EnvZp modulates the degree of phosphorylation of OmpRp in response to changes in the osmolarity of the growth environment. Phosphorylated OmpRp, in turn directly regulates the transcription of the porin genes *ompF* and *ompC*. Under conditions of low osmolarity *ompF* is activated, while under conditions of high osmolarity *ompC* is activated and *ompF* repressed [24–26]. Furthermore, EnvZp is a histidine kinase, and is phosphorylated at His²⁴³ [27], a residue that is not conserved in *Cyc2p*. Also, EnvZp, like *Cyc2p*, has two transmembrane domains. EnvZp probably functions as a dimer containing a leucine zipper [28], which involves leucine residues that are not conserved in *Cyc2p*. Furthermore we have demonstrated by Western blot analysis that deletion of *CYC2* did not affect the level of Por1p (data not shown). Therefore, if *Cyc2p* has a role in regulating Por1p, it is not at the tran-

scriptional level. While it is clear that Cyc2p and EnvZp are ancestrally related, and both are membrane proteins, we believe that they have diversified to the point where they do not carry out the same or similar functions.

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