Cardiolipin Is Not Required to Maintain Mitochondrial DNA Stability or Cell Viability for *Saccharomyces cerevisiae* Grown at Elevated Temperatures* 

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In eukaryotic cells, the phospholipid cardiolipin (CL) is primarily found in the inner mitochondrial membrane. *Saccharomyces cerevisiae* mutants, unable to synthesize CL because of a null allele of the *CRD1* gene (encodes CL synthase), have been reported with different phenotypes. Some mutants, when grown on a nonfermentable carbon source at elevated temperatures, exhibit mitochondrial DNA instability, loss of viability, and significant defects in several functions that rely on the mitochondrial energy transducing system (ETS). These mutants also lack the immediate precursor to CL, phosphatidylglycerol (PG), when grown on glucose as a carbon source. Other mutants show reduced growth efficiency on a nonfermentable carbon source but much milder phenotypes associated with growth at elevated temperatures and increased levels of PG when grown on glucose. We present evidence that mitochondrial DNA instability, loss of viability, and defects in the ETS exhibited at elevated temperatures by some mutants are caused by the reduced expression of the *PET56* gene in the presence of the *his3Δ200* allele and not the lack of CL alone. We also found that PG is present and elevated in all *crd1Δ* strains when grown on glucose. A supermolecular complex between complex III and complex IV of the mitochondrial ETS detected in wild type cells was missing in all of the above *crd1Δ* cells. The level of components of the ETS was also reduced in *crd1Δ* cells grown at elevated temperatures because of reduced gene expression and not reduced stability. These results suggest that all phenotypes reported for cells carrying the *his3Δ200* allele and lacking CL should be re-evaluated.

Cardiolipin (CL) is a phospholipid uniquely found in membranes engaged in oxidative phosphorylation and photophosphorylation such as the cytoplasmic bacterial membrane and the inner membrane of chloroplasts and mitochondria (1, 2). Biochemical evidence indicates that it plays both a structural and functional role in many multimeric complexes of the energy transducing system (ETS) associated with inner mitochondrial membrane (3). When compared with a wild type *CRD1* (gene encoding CL synthase) strain, *Saccharomyces cerevisiae* mutants lacking CL synthase (*crd1Δ*, null in the expression of this enzyme) grow slower and to a lower cell density at the optimal temperature of 28–30 °C on a nonfermentable carbon source such as glycerol/ethanol (4–9). These mutant strains lacking CL have elevated levels of phosphatidylglycerol (PG), the precursor of CL, when grown on a nonfermentable carbon source. However, there has been disagreement with regard to several other phenotypes reported for *crd1Δ* strains when grown on glucose as a carbon source or at elevated temperatures on glucose or glycerol/ethanol. In some genetic backgrounds, *crd1Δ* strains contain little if any PG when grown on glucose at any temperature (5, 8). Because neither PG nor CL is required for growth of yeast on glucose, such conditions have been suggested as a model for studying yeast lacking these anionic lipids (8). When some *crd1Δ* strains were grown at elevated temperatures for extended periods of time, they displayed increased mitochondrial DNA (mtDNA) instability, reduced oxidative phosphorylation capacity, reduced function of the ADP/ATP translocator, loss of viability, reduced mitochondrial membrane potential, and increased mitochondrial membrane permeability (6, 8, 10). However, other *crd1Δ* strains were reported to contain high levels of PG under all growth conditions (4, 9) and were only mildly sensitive to growth at elevated temperatures (4).

A likely possibility for the phenotypic differences among *crd1Δ* strains is the genetic backgrounds in which this mutation was placed. We noted that *crd1Δ* strains also carrying the *his3Δ200* allele displayed significantly more severe phenotypes at elevated growth temperatures (6, 8, 10) than strains without the *HIS3* allele (4). The *his3Δ200* allele is a complete deletion of the gene that extends into the common promoter element for the *HIS3* gene and the divergently expressed *PET56* gene (11). This deletion results in an 80% reduction in expression of mRNA for the Pet56p. Pet56p is imported into the mitochondrial large ribosomal subunit (12). Null mutants in *PET56* are defective in assembly of the mitochondrial large ribosomal subunit and do not grow on nonfermentable carbon sources as well as other mutants lacking CL (4).
able carbon sources, but partial loss of PET56 expression results in slowed growth, mtDNA instability, and loss of cell viability at elevated temperatures (12, 13) similar to the properties of crd1Δ strains containing the his3Δ200 allele (8). We report here that many of the severe phenotypes ascribed to cells lacking CL, particularly when grown at 37°C, are because of a synergistic interaction of the crd1Δ mutation with the reduced expression of the PET56 gene in a his3Δ200 background. In addition, we report the presence of elevated PET56 expression in crd1Δ strains when grown on glucose. Finally, we show that a supermolecular complex between complex III (cytochrome bc1 complex) and complex IV (cytochrome c oxidase complex) of the inner mitochondrial membrane, originally detected in a crd1Δ his3Δ200 background (9), is because of lack of CL and not the genetic background.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Growth Conditions—**YPH500 (ade2–101, his3Δ200, leu2Δ1, lys2–801, trp1Δ63, ura3–52, MATα) and YZD2 (ade2–101, his3Δ200, leu2Δ1, lys2–801, trp1Δ63, ura3–52, crd1Δ::HIS3, MATα) with the entire CRD1 open reading frame disrupted as described previously (9) were used in this study. The complete CRD1 coding sequence was replaced in DIL (his3Δ–11, leu2–3,112, ura3–52, 251,328,372, MATα) to make the crd1Δ::HIS3 strain YZD5. YZD5 was constructed by homologous recombination with a polymerase chain reaction product of the HIS3 gene flanked by 5’ and 3’ regions that lie outside of the CRD1 gene coding sequence. Amplification was accomplished from HIS3-containing plasmid pRS903 (14) with primers 5’-ATGATGGAGGGCTGAGCCCATTGAC-3’ and 5’-GGTCAAAATAATCGAGGCAATGAGGCCGTTGAGCAGCTTTGCTCCTAATTACGTGATCCTCTCCTTCCTGAGGAATGCTGTTGATCTGACGTTGTTGATATAACCTACATAAAAACAAACATCTTTCTCAATTCGGTGCAGCAATTAAATGAAAAGTTGATCAAGGACCCCTTTCAAAAAAGATCGCAATATTGGGATGCAGGCTAG-3’ (regions flanking CRD1 homology are in capital letters, and HIS3 homologous regions are in lowercase letters). Yeast transformation was carried out using the Alkali-Cation™ yeast transformation kit (Bio 101, Inc.). A crd1Δ derivative was selected on histidine-drop-out minimal medium plates containing glucose. Polymerase chain reaction with three sets of primers confirmed the disruption. The crd1Δ derivative (HS2D of strain HMD22 (ade2–101, ura3–52, leu2Δ1, lys2, his3Δ3, HSD2) of strain HMD22 (his3Δ–11, leu2–3,112, ura3–52, MATα) was kindly provided by Dr. Thomas Fox, Cornell University) was constructed in a similar manner. In HMD22, the coding sequence of the mtDNA-encoded COX2 gene was replaced with the chromosomally encoded ARG8 gene that was recoded for translation in the mitochondrial matrix (15) thus generating PET56lacZ, URA3, 2μa, and COE1 origin was derived from pMA109 (16). A 1.5-kb DNA fragment, including the promoter and encoding the 5’-mRNA upstream untranslated leader (UTL) along with the initiation codon of COX4, was isolated by polymerase chain reaction and ligated to the open reading frame of lacZ thus generating PET56lacZ.

**Cell Viability and Retention of the Mitochondrial Function Assays—**Cells with different genotypes were grown at 37°C in rich media (1% yeast extract and 2% peptone) plus either a fermentable (YPD, 2% glucose) or nonfermentable (YEPG, 1% ethanol and 3% glycerol) carbon source for 100 h. An equal number of cells determined by microscopy was then plated at 30°C either on YPD to determine cell viability in the presence of mitochondrial function, or on YPEG to assess mitochondrial function. The number of colonies formed on YPD was taken as the number of viable cells in the culture. The ratio of the number of cells that formed colonies on YPEG versus the number on YPD plates was used as an index of retention of mitochondrial function and mtDNA stability.

**Phospholipid Analysis—**To prepare phospholipids for analysis by mass spectrometry, cells were grown at 30°C in YPD to late exponential phase and homogenized with a solution of CH2O·OH, HCI, 0.1 N HCl (10:5:4) in a Mini-Beadbeater™ (BioSpec Products) at maximal setting for 5 min. Mitochondria were also isolated from cells as described below, and the phospholipids were extracted using the above mentioned solvents. Extracted lipids were analyzed by mass spectrometry as described previously (17).

**In Vivo Labeling of Mitochondrial Translation Productions—**In vivo pulse-labeling of mitochondrial translation products with [35S]methionine was done essentially as described in Ref. 18. Briefly, cells were grown at 30°C in standard minimal medium containing 1% ethanol, 3% glycerol to mid-log phase, collected, resuspended in the same volume of fresh YPD, pre-warmed to 37°C, and incubated for an additional 2 h at 37°C. After cycloheximide treatment for 5 min, [35S]methionine was added. Labeling was stopped after 1 h by adding chase solution (1% casamino acids, 2 mg/ml Na2SO4). Cells were broken using a Mini-Beadbeater™, and crude mitochondria were collected by centrifugation at 20,000 × g for 10 min at 4°C. Pellets were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Radioactivity was quantified using a phosphorimaging device (Bio-Rad).

**Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)—**Sample preparation and BN-PAGE were carried out essentially as described previously (9). Briefly, mitochondria (75 μg of protein) were solubilized with 1% digitonin-containing buffer. After solubilization, samples were centrifuged at 125,000 × g for 30 min. Sample buffer (1.5 g of 5% (w/v) Serva Blue G in 500 mM aminocaproic acid) was added to the supernatant, and electrophoresis was carried out in a 4–8% polyacrylamide gradient mini-gel (8.7 × 13.3 cm). Proteins were transferred to nitrocellulose sheets for Western blot analysis and located using monoclonal antibodies or polyclonal antibody (from Dr. Gottfried Schatz, Biozentrum, Basel, Switzerland) specific for Coxp (cytochrome c oxidase subunit 3 of complex IV) or polyclonal antibody (from Dr. Gottfried Schatz, Biozentrum, Basel, Switzerland) specific for Cbp (cytochrome b subunit of complex III). Final detection was with secondary antibodies linked to horseradish peroxidase using chemiluminescence (Pierce) and a Bio-Rad FX. High molecular weight markers from Amersham Biosciences were used as standards.

**β-Galactosidase Assay—**β-Galactosidase activity was assayed as described previously (19). In brief, yeast cells were harvested by centrifugation, washed, and resuspended in 100 mM sodium phosphate, pH 7.0. Cells were disrupted in a Mini-Beadbeater™. β-Galactosidase activity was analyzed in cell lysates and calculated as Miller units.

**Other Methods—**The isolation of mitochondria was accomplished essentially by the method of Glick and Pon (20) from cells grown in YPD or YPEG. Protein samples were subjected to SDS-PAGE in 4–20% gradient Ready Gels and electrophoretically transferred to nitrocellulose sheets for Western blot analysis as described previously (21). Antibodies specific for Cox1–4p were purchased from Molecular Probes, and antibody against Arg6p was provided by Dr. Thomas Fox. After treatment with the designated antibodies, protein bands were visualized by chemiluminescence and detected with a Bio-Rad FX.

**RESULTS**

**Growth Rate and Final Cell Density of crd1Δ Strains—**Cells with different genotypes were grown at 30 or 37°C in either YPD or YPEG. Growth rates for crd1Δ strains carrying either the his3Δ-11,15 allele or the his3Δ200 allele were nearly the same as their respective isogenic CRD1 strains when grown on glucose as a carbon source at 30°C (Fig. 1A). However, strains carrying his3Δ-11,15 (DL1 and YZD5) grew much faster and reached higher final cell densities than strains carrying his3Δ200 (YPH500 and YZD2). CRD1 and crd1Δ strains carrying his3Δ-11,15 also showed no growth differences on glucose at 37°C (Fig. 1B). However, in the his3Δ200 background there was a significant reduction in growth rate and final cell density attained by crd1Δ cells when grown in YPD at 37°C compared with the parental CRD1 cells. In both his3 genetic backgrounds, CRD1 cells grew considerably faster and to a higher final cell density than crd1Δ cells when grown in YPEG at 30°C as has been observed previously (5, 7, 9), but strains carrying his3Δ-11,15 exhibited better growth than their corresponding CRD1 and crd1Δ cells with the his3Δ200 allele (Fig. 1C). When grown in YPEG at 37°C, crd1Δ cells were even more severely compromised in both genetic backgrounds (Fig. 1D).

**Cell Viability and Retention of the Mitochondrial Function—**Because strains carrying the his3Δ200 allele (and thus also the pet56 defective) grew much poorer than cells that were wild type for PET56 (his3Δ-11,15 allele), we investigated whether the
The difference was because of reduced cell viability and/or mtDNA instability for the his3Δ200-containing strains. The results in Fig. 2 are the average of three individual experiments showing the percentage of plated cells that formed colonies and were, therefore, viable after 100 h at 37 °C. CRD1 cell viability, when grown in YPD as measured by the number of colonies formed as a percentage of cells plated to YPD medium, ranged from 85 to 95% and was set at 100%. In some cases, cells grown in YPEG showed a higher plating efficiency resulting in an apparent viability over 100%.

There was little statistical difference in viability between crd1Δ and CRD1 cells with a high transcription level of PET56 (YZD5 and DL1, respectively) when grown in YPD and plated on YPD plates (Fig. 2, first bar in each strain set), whereas crd1Δ cells (YZD2) with a low transcription level of PET56 showed a 40% reduction in cell viability compared with its CRD1 parental strain (YPH500). When grown in YPEG at 37 °C and plated on YPD plates at 30 °C (Fig. 2, second bar in each strain set) to determine cell viability independent of loss of mitochondrial function, YZD5 maintained almost the same viability as the parental strain DL1. On the other hand, YZD2 showed 70% reduced viability compared with its wild type parental strain (YPH500), which maintained high viability. YZD2 (pet56) grown at 37 °C either in YPD (Fig. 2, third bar in each strain set) or YPEG (Fig. 2, fourth bar in each set) showed a dramatic loss of mitochondrial function as evidenced by low plating efficiency (<2%) at 30 °C on YPEG. Even YPH500 (pet56) maintained only 40% viability under these conditions as compared with DL1 and YZD5 (PET56), which showed little or no loss of mitochondrial function. When YPH500 and YZD2, carrying the his3Δ200 allele with its associated defective pet56, were supplied with a plasmid-borne copy of PET56, cell viability for both the CRD1 and crd1Δ strains was significantly increased particularly when they were plated on YPEG for which mitochondrial function is required.

The stability of mtDNA during growth in YPEG at 37 °C was determined by comparing the ratio of colonies formed at 30 °C on YPEG versus YPD plates. A lower ratio indicates decreased mtDNA stability. These ratios were high for his3–11,15 PET56 strains (DL1 background) but were significantly reduced for his3Δ200 pet56 strains (YPH500 background) with the latter crd1Δ strain even more compromised than its CRD1 parental strain (Fig. 3). When the latter strains were supplied with a plasmid-borne copy of PET56, the ratios were significantly increased indicating that mtDNA stability was increased. Analysis of cells for plasmid retention by plating to minimal media with or without leucine indicated that about 80% of the cells had retained the PET56-containing plasmid (data not shown). This is probably the reason that there was not complete complementation of YPH500 and YZD2 by the plasmid as shown in Figs. 3 and 4.

Reduced Levels of ETS Proteins in crd1Δ Cells—The steady state levels of the Cox1–4p subunits of cytochrome c oxidase

Fig. 1. Growth curves in YPD and YPEG. DL1 (CRD1, ●), YZD5 (crd1Δ in DL1, ○), YPH500 (CRD1, △), YZD2 (crd1Δ in YPH500, ▱) were pre-grown overnight in YPD or YPEG at 30 °C to A600 of 0.8 and diluted with pre-warmed YPD or YPEG, respectively, to an A600 of 0.04. Absorbance readings were taken at the indicated times. The experiment was performed in duplicate three times, and this is one of the typical results. Growth temperature and growth media are indicated at the top of each panel.

Fig. 2. Cell viability after prolonged growth. The indicated strains were grown at 37 °C in YPD or YPEG for 100 h. An equal number of cells grown in YPD was plated at 30 °C either on YPD agar plates (first bar in each set) to determine cell viability independent of loss of mitochondrial function or on YPEG agar plates (third bar in each set) to assess mitochondrial function. Similarly, cells grown in YPEG were plated for growth at 30 °C either on YPD agar plates (second bar in each set) to assess cell viability or on YPEG agar plates (fourth bar in each set) to assess mitochondrial function. The data are an average of three separate experiments with the error bars indicated.
and the Cobp subunit of cytochrome bc₁, were determined in the CRD1 cells relative to CRD1 cells grown at 37 °C in YPEG. 

CrdΔ cells (Fig. 4, lane 2) with the his3–11,15 background showed reduced levels of the mtDNA-encoded subunits Cox1–3p and Cobp as well as the nuclear-encoded Cox4p relative to the CRD1 parental strain (Fig. 4, lane 1). However, in crdΔ his3Δ200 cells there was almost no detectable Cox1–3p, Cobp, and Cox4p as shown by Western blotting (Fig. 4, lane 4 versus lane 3). Relative to the internal control (Porin), the absolute amount of ETS proteins was lower in YPH500 than in DL1.

A similar pattern was observed if the capacity of mitochondria to synthesize protein after growth at 37 °C was tested in whole cells with different genetic backgrounds. [35S]Methionine incorporation was followed in the presence of cycloheximide as described under “Experimental Procedures.” Crude mitochondria were isolated, and 30 μg of mitochondrial protein were subjected to SDSPAGE. Quantification of the radioactivity was performed by using a phosphorimaging device (Bio-Rad). The positions of various mitochondrial-encoded proteins (23, 24) are indicated on the left side. Lane 5 shows the results of exposing lane 4 five times longer.

**Reduced Level of Supermolecular Complex in crdΔ Cells**—Digitonin extracts of mitochondria isolated from CRD1 cells carrying the his3–11,15 allele (DL1) subjected to BN-PAGE showed 60–85% of supermolecular complex formation (∼1000 kDa) between complex IV (Fig. 7, lane 1) and complex III (lane 3) as detected by antibodies specific for Cox3p and Cobp, respectively. Another 10–40% of the signal showed a smaller complex that contained both components of complex III and IV. However, no homodimers of either complex III (660 kDa) or complex IV (440 kDa) were observed. On the other hand, extracts of mitochondria from crdΔ cells (YZD5) displayed around 50% of much smaller sized bands that reacted uniquely with antibody specific for a component of either complex IV (Fig. 7, lane 2) or complex III (lane 4). The mobility of these smaller species is consistent with those of a homodimer of complex IV or a homodimer of complex III. The remainder of the signal seen for extracts of mitochondria from crdΔ indicated a complex containing both Cox3p and Cobp but significantly smaller than the 1000-kDa supermolecular complex observed in CRD1 cells. The lack of supermolecular complex and, instead, the presence of individual homodimers of complex III and complex IV in crdΔ cells carrying the PET56 allele are similar to that observed in crdΔ cells carrying the his3Δ200 allele at 37 °C (Fig. 5). 

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**Fig. 3.** Assessment of mtDNA stability after growth in YPEG. The indicated strains carrying either the CRD1 or crdΔ allele were grown in YPEG at 37 °C for 100 h. An equal number of cells from each culture was then plated on YPD and YPEG agar and grown at 30 °C, and mtDNA retention was calculated as the number of colonies formed on YPEG plates divided by the number of colonies formed on YPD plates.

**Fig. 4.** Steady state levels of ETS protein subunits in mitochondria isolated from CRD1 and crdΔ strains. DL1 (lane 1), YZD5 (lane 2), YPH500 (lane 3), and YZD2 (lane 4) were grown in YPEG at 37 °C for 46 h. Mitochondria were isolated, and 20 μg of protein was subjected to SDS-PAGE followed by Western blot analysis using antibodies specific for the indicated proteins. The mitochondrial outer membrane protein porin was used to verify that the same amount of mitochondria (lanes 1 and 3 versus lanes 2 and 4, respectively) was loaded to each lane.

**Fig. 5.** Reduced level of mitochondrial protein synthesis in crdΔ cells. DL1 (lane 1), YZD5 (lane 2), YPH500 (lane 3), and YZD2 (lanes 4 and 5) were pre-grown at 30 °C in standard minimal medium with ethanol/glycerol and labeled at 37 °C with [35S]methionine in the presence of cycloheximide as described under “Experimental Procedures.” Crude mitochondria were isolated, and 30 μg of mitochondrial protein were subjected to 12% SDS-PAGE. Quantification of the radioactivity was performed by using a phosphorimaging device (Bio-Rad). The positions of various mitochondrial-encoded proteins (23, 24) are indicated on the left side. Lane 5 shows the results of exposing lane 4 five times longer.
which revealed the complete absence of the 1000-kDa species in crd1Δ cells.

Presence of PG in crd1Δ Strains Grown in YPD—Strains carrying the crd1Δ allele in a his3Δ200 background have been reported to lack detectable PG in addition to CL when grown in YPD (5, 8). Using [32P]Pi-labeled cells and thin layer chromatography, a radiolabeled phospholipid with the expected mobility of PG was readily detected in crd1Δ cells in either a his3Δ200 (9) or HIS3 background (4). To further verify the presence of PG in crd1Δ cells, total phospholipid was isolated from cells and mitochondria of crd1Δ and CRD1 strains grown in YPD at 30 °C and subjected to analysis by mass spectrometry. As expected, PG was barely detectable in the spectrum (Fig. 8A) of mitochondrial phospholipids extracted from YPH500 (CRD1). Prominent peaks were observed for phosphatidic acid. However, analysis of mitochondrial phospholipids extracted from YZD2 (crd1Δ) clearly showed prominent peaks (Fig. 8B) for PG consistent with the radiolabeling experiments (4, 9). The same results were seen for extracts of phospholipids from whole cells and mitochondria of DL1 and YZD5 (data not shown).

FIG. 6. Reduced expression of P_cox4-lacZ and P_cox2-ARG8m reporter genes in crd1Δ cells at 37 °C. A, DL1 and YZD5 cells were grown in uracil dropout media with glucose as a carbon source at either 30 or 37 °C for 48 h. Cells were broken, and β-galactosidase activity was assayed (see “Experimental Procedures”). B, HMD22 and HSD2 cells were grown in complete synthetic media containing glucose at 30 or 37 °C for 48 h. Mitochondria were isolated from cells, subjected to SDS-PAGE, and analyzed by Western blotting for the level of the mitochondria-encoded Arg8mp expressed from the COX2 promoter. Porin was included as a control for level of mitochondria loaded per lane.

FIG. 7. Lack of supermolecular complex formation in crd1Δ cells. DL1 (lanes 1 and 3) and YZD5 (lanes 2 and 4) were grown in YPEG at 30 °C and harvested in the exponential phase of growth. Mitochondria were isolated, solubilized by digitonin, and displayed (75 µg of protein/lane) by BN-PAGE as described under “Experimental Procedures.” The specificity of the antibodies used (lanes 1 and 2, Cox3p; lanes 3 and 4, Cobp) in each blot is indicated. The mobility of standard proteins of the indicated molecular mass is on the left.

Strains of S. cerevisiae unable to synthesize CL because of the crd1Δ allele were reported to have different phenotypes. The genetic backgrounds and the nature of the crd1Δ alleles differed among these strains. A strain with a complete deletion of the CRD1 gene displayed more severe phenotypes at elevated temperatures when grown on a nonfermentable carbon source and failed to accumulate detectable levels of PG when grown on a fermentable carbon source (6, 8, 10). A strain with an insertion in the CRD1 gene contained significant levels of PG when grown on all carbon sources and displayed much less severe phenotypes when grown at elevated temperatures on a nonfermentable carbon source (4). Therefore, the strain carrying a complete deletion is more compromised for mitochondrial function especially at elevated growth temperatures. Although the genetic backgrounds of the above strains are similar, a major difference is that the complete deletion strain carries the his3Δ200 allele (deletion of HIS3), whereas the insertionally inactivated strain carries the wild type HIS3. The major difference in these strains is at elevated growth temperatures particularly when a nonfermentable carbon source was employed. We noted that the commonly used his3Δ200 allele results in disruption of the promoter of the adjacent PET56 gene and also results in mitochondrial dysfunction particularly when grown on nonfermentable carbon sources at elevated temperatures (11, 13).

Because crd1Δ strains carrying the his3Δ200 allele displayed growth properties similar to strains with reduced Pet56p expression alone, we postulated that the different phenotypes reported for crd1Δ strains are due to the his3Δ200 and not the crd1Δ allele. To address this question, we constructed a complete deletion of the coding region of CRD1 in two different genetic backgrounds, one with the his3Δ200 allele (YPH500) and the other with the his3Δ11,15 (point mutations in HIS3) allele (DL1). We then compared the properties of these two mutants along with their respective parental strains to establish the molecular basis for the differences reported in phenotypes for crd1Δ strains. In some experiments we introduced a plasmid-borne copy of PET56.

The growth properties of CRD1 and crd1Δ in the DL1 background were essentially identical when grown in YPD at either 30 or 37 °C, although growth at 37 °C was slower, and the final cell density attained was lower. The YPH500 background strains grew less well than the DL1 background strains even in YPD at 30 °C, but only at 37 °C was there significantly poorer growth of the crd1Δ strain when compared with the CRD1 strain. Similar poorer growth in YPEG was observed for YPH500 background strains versus DL1 background strains at both temperatures, but the crd1Δ strains grew less well than the CRD1 strains in both genetic backgrounds at 30 °C, which was markedly accentuated at 37 °C. Although growth for both crd1Δ strains was poor at 37 °C in YPEG, we noted that the DL1 mutant formed colonies when seeded as single cells on agar plates under these conditions, whereas the YPH500 mu-
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Fig. 8. Analysis of phospholipid extracts by mass spectrometry. Spectra showing phosphatic acid (PA) and phosphatidylglycerol (PG) species contained in total lipid extracts of mitochondria isolated from YPH500 (A) and YZD2 (B). The notations in the parentheses indicate the fatty acids esterified to each phospholipid.

tant did not, as has been reported previously for hisΔ200 crd1Δ strains (6, 7). Further studies confirmed that DL1 strains maintained viability and mtDNA stability (as indicated by colony formation on YPEG plates) after growth for long periods in YDP or YPEG at both temperatures. The parental YPH500 strain maintained both viability and mtDNA stability when grown at 37 °C in YPD but lost mitochondrial function without losing cell viability when grown at 37 °C in YPEG. The crd1Δ derivative of YPH500 lost viability and mtDNA stability in YPD, which was very much accentuated in YPEG at 37 °C explaining the lack of colony-forming ability under the latter conditions. The poor viability properties of YPH500 and YZD2 were largely corrected by introducing a plasmid-borne copy of PET56 confirming that the synergistic effects of the crd1Δ and pet56 mutations were the primary reason for the poor growth properties rather than the lack of CL. Complete correction did not occur probably because of some loss of the plasmid during growth on rich medium. Therefore, lack of CL alone does not result in mtDNA instability or loss of viability at elevated growth temperatures as reported previously (6, 8).

The poor growth of crd1Δ strains in YPEG, particularly at 37 °C, has been attributed to a requirement for CL to support the components of the mitochondrial inner membrane ETS. CL is required for the stability of a supermolecular complex between complex III and IV of this system because lack of this complex was found independent of the his3 allele but dependent on the crd1Δ allele (reported here and in Ref. 9). However, previous results with respect to mitochondrial energy production efficiency (8, 10) could be partially or completely due to reduced levels of the components of the ETS. The latter appears to be a factor particularly for YPH500 mutant strains grown at 37 °C in YPEG. Under these conditions, the steady state levels of ETS components were barely detectable after 46 h of growth, and the ability to synthesize new components was lost within the first 2 h of growth. The DL1 mutant was also somewhat compromised but not to the extent of the YPH500 mutant. For the DL1 mutant, the basis for reduced levels of ETS components appears to be reduced synthesis rather than instability of the organized components. Fusion of the promotors and UTLs of the COX4 gene (nuclear-encoded and translated in the cytoplasm) or COX2 gene (mtDNA-encoded and translated in the mitochondria) to normally stable soluble proteins (β-galactosidase or Arg8p, respectively) also showed reductions of 60–70% in their steady state levels in a crd1Δ strain consistent with the reduced amount of the normal gene products. We have shown that the molecular basis for the lack of mitochondrial function in pgs1Δ strains (lacking PG-P synthase, PG and CL) is the drastically reduced translation of the mRNA of the COX1–4 and COB genes in a his3Δ200 (21) and his3–11,15 background. These two reporter constructs are poorly expressed in pgs1Δ strains, but Arg8p expressed from the nuclear gene is present in normal amounts in the mitochondria. Therefore, lack of CL may result in lower translation levels for ETS components, and any conclusions concerning the direct role of CL in supporting the ETS must consider effects on the expression of its components.

Strains carrying the crd1Δ and hisΔ200 alleles have been reported to lack both CL and its precursor PG when grown in YPD (5, 8). Such strains were suggested as a model to study cell processes in the absence of these two phospholipids (8). Paradoxically, these cells contain significant functional amounts of the ETS components and have functional mitochondria, whereas pgs1Δ strains, which cannot synthesize PG or CL, lack ETS components and functional mitochondria in both the his3–11,15 and his3Δ200 genetic backgrounds (21). However, in our hands the crd1Δ strains reported here as well as a crd1Δ derivative (4) of YPH98 (ura3–52, lys2–801, ade2–101, leu2Δ1, trp1Δ1) when grown in glucose have a level of PG comparable with the wild type level of PG plus CL. Because PG was previously identified based on radiolabeling followed by mobility

\[ M. \text{ Zhang and W. Dowhan, unpublished result.}\]

\[ X. \text{ Su and W. Dowhan, unpublished data.}\]
on thin layer chromatography (4, 9), we confirmed by mass spectrometry the presence of highly elevated levels of PG in all crd1Δ strains relative to the levels in the parental CRD1 strains. Possibly, detection by iodine staining followed by chemical phosphate determination was not sensitive enough to detect PG in other studies (5, 8).

In summary, we have shown that CL is required for optimum cell growth on a nonfermentable carbon source and that PG is present in all crd1Δ/H9004 strains independent of growth conditions where it partially substitutes for CL. These results further support the conclusion that lack of both PG and CL leads to totally dysfunctional mitochondria (21) and suggest that much of the reduced mitochondrial function in crd1Δ strains may be because of reduced expression of ETS components and/or their reduced ability to organize components of the ETS into larger supermolecular complexes. The instability of mtDNA previously attributed to lack of CL (6, 8) is because of the accentuated inherent instability of mtDNA in a pet56 background. Additional putative roles for CL in mitochondrial functions (8, 10), such as protein import, ADP/ATP translocator function, higher order association of the translocator, membrane potential levels, membrane permeability, respiratory rate, ATPase activity, and others, must now be re-investigated in a PET56 genetic background. These studies are currently under way.

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REFERENCES