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Role of Arg-166 in Yeast Cytochrome c_1*

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A systematic screen for dominant-negative mutations of the *CYT1* gene, which encodes cytochrome c_1, revealed seven mutants after testing ~10^4 *Saccharomyces cerevisiae* strains transformed with a library of mutagenized multicopy plasmids. DNA sequence analysis revealed multiple nucleotide substitutions with six of the seven altered Cyt1p having a common R166G replacement, either by itself or accompanied with other amino acid replacements. A single R166G replacement produced by site-directed mutagenesis demonstrated that this change produced a nearly nonfunctional cytochrome c_1, with diminished growth on glycerol medium and diminished respiration but with the normal or near normal level of cytochrome c_1 having an attached heme group. In contrast, R166K, R166M, or R166L replacements resulted in normal or near normal function. Arg-166 is conserved in all cytochromes c_1 and lies on the surface of Cyt1p in close proximity to the heme group but does not seem to interact directly with any of the physiological partners of the cytochrome bc_1 complex. Thus, the large size of the side chain at position 166 is critical for the function of cytochrome c_1 but not for its assembly in the cytochrome bc_1 complex.

The cytochrome bc_1 complex, also known as complex III of the respiratory chain or ubiquinol:cytochrome *c* oxidoreductase, is an oligomeric complex found in the inner mitochondrial membrane of eukaryotes and in the plasma membrane of bacteria (1–4). This complex transfers electrons from ubiquinol to cytochrome *c* and couples this transfer to a proton gradient across the inner mitochondrial or bacterial plasma membrane by a mechanism known as the proton motive Q cycle (2, 5–7). The prokaryotic and eukaryotic cytochrome bc_1 complexes contain three essential catalytic subunits having the following characteristic prosthetic groups: cytochrome *b* with two *b*-type hemes; cytochrome c_1 with a *c*-type heme; and the so-called Rieske protein that contains a high potential [2Fe-2S] cluster.

In addition to the three catalytic subunits, mitochondrial cytochrome bc_1 complexes from vertebrates (8) and the yeast *Saccharomyces cerevisiae* (9, 10) contain eight and seven additional subunits, respectively. X-ray crystallographic atomic structures have been determined for the soluble fragment of the Rieske protein at 1.5-Å resolution (11) and for entire mitochondrial cytochrome bc_1 complexes from bovine, chicken (12–14), and *S. cerevisiae* (15) at 2–3-Å resolution. The cytochrome bc_1 complexes are dimers with each monomer of the yeast complex consisting of the following 10 protein subunits (with the orthologous bovine subunits or synonyms shown in parentheses): Cyt1p (cytochrome c_1); COB (cytochrome *b*); Rip1p (ISP, Rieske protein); Cor1p (Core 1, SU1); Qcr2p (Core 2, SU2); Qcr6p (SU8); Qcr7p (SU6); Qcr8p (SU7); Qcr9p (SU10); and Qcr10p (SU11). In addition, the subunit SU9 is present in the vertebrate but not in the yeast cytochrome bc_1 complex. Furthermore, the protein prepared for determining the atomic structure of the yeast complex lacked Qcr10p (15). (The overall structure and components of the bovine cytochrome bc_1 complex are presented in Fig. 1.) SU11 of the bovine complex, orthologous to Qcr10p, forms a transmembrane helix that is bound on the outside of the complex to the helices of the Rieske protein and SU10 (Qcr9p) (14). Removal of SU10 did not affect enzymatic activity (8), but it may be important for the correct assembly of the complex (10). The similar overall structure of the yeast compared with the vertebrate complexes suggests that Qcr9p is associated with the complex in the same way (15). Furthermore, the relative positions and orientations of heme groups and the distances between the iron positions indicate that the yeast and vertebrate complexes are essentially the same, although the exact position, length, and conformation of connecting loops varied (15).

Cytochrome c_1 is responsible directly for the electron transfer reaction with cytochrome *c* by catalyzing the oxidation of ubiquinol and reduction of cytochrome *c* (16, 17). Like all other physiological partners, cytochromes *c* and c_1 interact with each other through electrostatic forces (18).

Although cytochrome c_1 is a mitochondrial protein, it is encoded by a nuclear gene, *CYT1*, translated in the cytosol, and subsequently imported in mitochondria, a process involving cleavage of a leader sequence (19, 20). In yeast, the cleavage of a 61-amino acid amino-terminal region from the 309-amino acid-long precursor results in a 248-amino acid-long mature form.

We have undertaken a genetic investigation of the functional requirements of amino acid residues of yeast cytochrome c_1 and of possible critical interactions between cytochromes c_1 and *c*. Although mutational analysis of cytochromes c_1 from yeast (17, 21–23)[1] and *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* (7, 24–27) has been used in several studies, we have elected to isolate and characterize dominant-negative mutants. The characterization of altered cytochromes c_1 generated by "random" mutagenesis may reveal functional requirements that are difficult to predict even with a detailed knowledge of the structure of the protein. However, the vast majority of nonfunctional proteins generated by random mutagenesis are

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[1] S. L. Hatch, D. A. Pearce, F. Sherman, and G. McLendon, unpublished result.

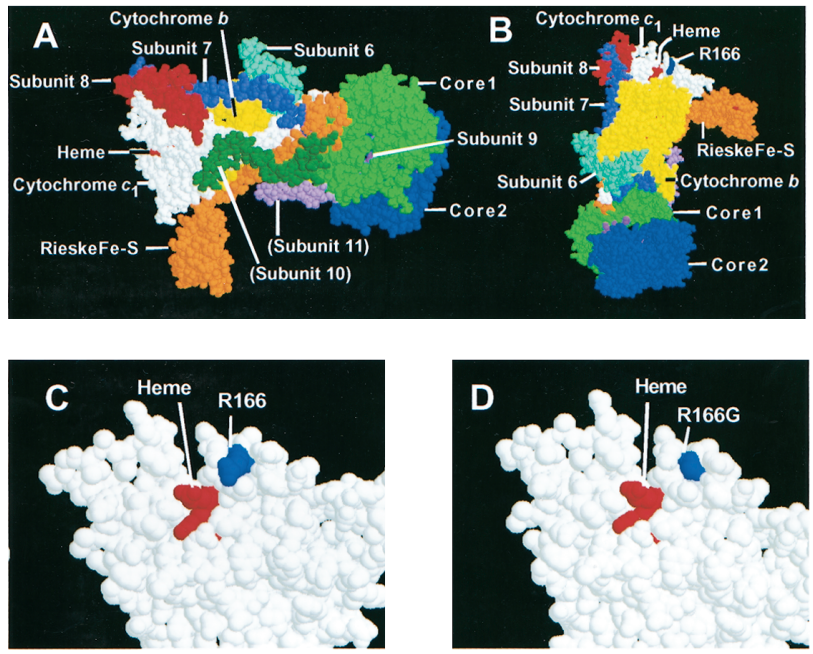

FIG. 1. **The bovine cytochrome bc_1 complex (14) shown in two orientations (*A* and *B*) and the position of Arg-166 (R166) (*C*) and R166G (*D*) in cytochrome c_1.** The three-dimensional structure of cytochrome bc_1 complex from *S. cerevisiae* has been published (15), but the coordinates will not be deposited until May 12, 2001 (1EZV, Protein Data Bank). We therefore have used the related bovine complex to depict the position of Arg-166 relative to the heme group and other components of the complex. Subunit 11 is not found in the yeast cytochrome bc_1 complex, and subunit 10 was not in the preparation that was used to determine the atomic structure of the yeast complex (15). The figure was generated with the molecular graphics program RasMol using the bovine cytochrome bc_1 coordinates (14).

defective for trivial reasons such as missense mutations that affect folding or assembly and nonsense mutations that produce truncated proteins. On the other hand, interesting nonfunctional but stable proteins can be detected by the dominant-negative genetic test (28). For example, if the overexpression of a *cyt1*-x mutation inhibits the function of the normal *CYT1*$^+$ chromosomal gene, then the *cyt1*-x allele most likely encodes a nonfunctional cytochrome c_1, which is competing with the normal form.

The systematic screen for dominant-negative mutations of the *CYT1* gene carried out in this study revealed that the R166G replacement caused a nearly nonfunctional cytochrome c_1, with diminished growth on glycerol medium and diminished respiration but with the normal or near normal level of cytochrome c_1 having an attached heme group. Furthermore, the small size of the side chain was responsible for the defect because R166K, R166M, or R166L replacements resulted in normal or near normal function of cytochromes c_1.

EXPERIMENTAL PROCEDURES

Numbering of Amino Acid Positions of Cytochrome c_1—The amino acid positions of yeast cytochrome c_1 are assigned in this paper according to the full-length precursor having 309 amino acid residues. For example, Arg-166 of yeast cytochrome c_1 corresponds to Arg-102 of bovine cytochrome c_1.

Media and General Methods—Standard YPD[2] (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose) and YPG (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% (v/v) glycerol) and synthetic media for the growth of yeast and the normal cultivation and manipulation of yeast strains have been described by Sherman (29). SG − uracil and SD − uracil designate synthetic media containing 3% glycerol and 2% glucose, respectively, and 12 other supplements (29) without uracil.

The relative growth of the strains was estimated by inoculating a dilute suspension of cells on the surface of YPD and YPG plates, incubating the plates at 23, 30, and 37 °C, and examining the plates daily for up to 5 days.

Yeast cells were transformed by the lithium-acetate method (30).

Methods used in the construction of plasmids including restriction enzyme digests, separation of plasmid DNA and restriction fragments on agarose gels, ligation of DNA fragments, and the isolation of plasmid DNA are described by Maniatis *et al.* (31). *Escherichia coli* transformations were performed with the $CaCl_2$ method (32). The polymerase chain reaction (PCR) was carried out as described by Saiki *et al.* (33).

TABLE I
List of oligonucleotides

Oligonucleotide number	Sequence
OL.ZA01	GGGGGCTCGGGATCCATAGACTATCTAAGC
OL.ZA02	GGGGGCTCGGTCGACTTGGGCAACATTTTG
OL.ZA03	GGGGGCTCGGTCGACAAAAAGAAACGAAAC
OL.ZA04	GGTATCACCGCATCGACT
OL.ZA05	GGTATCACCGCATTGACTTTACTCTAT
OL.ZA06	GCCGAAGCTATGGCCGCAGCTGAACAC
OL.ZA07	GAACAGGCTGCAGGAGCTGCCAATCAA
OL.ZA08	AACGAACAGGCTGCATTGGCTGCCAATCAAGGT
OL.ZA09	AACGAACAGGCTGCAATGGCTGCCAATCAAGGT
OL.ZA10	AACGAACAGGCTGCAAAAGCTGCCAATCAAGGT
OL.ZA14	TCCATTGCAATGGCATAGGTCTTGTTTGATGAC

The enzymes used in this study, *Bam*HI, *Sal*I, DNA polymerases, polynucleotide kinase, etc., were purchased from either New England Biolabs, Amersham Pharmacia Biotech, or U. S. Biochemical Corp. The media constituents were obtained from Difco or Roche Molecular Biochemicals. All other chemicals used were from Sigma. Agarose was obtained from Roche Molecular Biochemicals.

DNA sequencing of segments containing *CYT1* was carried out with the oligonucleotides OL.ZA01–OL.ZA04 (Table I) using the ABI PRISM dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase (Big Dye).

Yeast Strains and Plasmids—The yeast strains used in this study along with their complete and partial genotypes are presented in Table II. The normal *CYT1* strain B-7553 described by Dumont *et al.* (34) served as the parental strain for generating the *cyt1*-Δ::*TRP1* mutant B-9737 by the one-step gene replacement procedure. A 1.1-kilobase *Kpn*I-*Spe*I segment containing the *CYT1* gene in the plasmid pAB1192 was replaced with an 829-base pair *Kpn*I-*Spe*I segment containing the *TRP1* gene, resulting in a plasmid denoted pAB1193. B-7553 was transformed with a fragment from pAB1193 encompassing *cyt1*-Δ::*TRP1*, and the desired disruptant was confirmed by PCR analysis.[1]

The plasmids used in this study are listed in Table III, and some are described below.

Dominant-negative Mutants—A library of 1.5-kilobase *CYT1* segments containing *Bam*HI and *Sal*I sites and random alterations was generated from pAB1097 by error-prone PCR with oligonucleotides OL.ZA01 and OL.ZA03. PCRs were carried out separately with 0.05, 0.10, 0.15, and 0.20 mM $MnCl_2$. All reactions contained the appropriate amounts of DNA (200–600 ng), $MgCl_2$ (1.5–6.0 mM), oligos (4.0–6.0 pmol), dNTPs, buffer, and AmpliTaq DNA polymerase. The libraries of PCR segments were inserted in pAB1198, and the resulting plasmids were amplified in XL1-Blue. The plasmid libraries, obtained by differ-

[2] The abbreviations used are: YPD, yeast extract peptone dextrose medium; YPG, yeast extract peptone glycerol medium; PCR, polymerase chain reaction.

TABLE II
Yeast strains

Strain number	Complete genotype
B-7553	*MAT***a** *CYC1 cyc7-Δ::CYH2 cyh2 leu2–3,112 ura3–52 his3-Δ trp1–289 can1–100 CYT1*
B-9737	*MAT***a** *CYC1 cyc7-Δ::CYH2 cyh2 leu2–3,112 ura3–52 his3-Δ trp1–289 can1–100 cyt1-Δ::TRP1*

Strain number	Parental strain	Plasmid number	Abbreviated genotype
B-7553			*CYT1 ura3*
B-12705	B-7553	pAB1198	*CYT1 ura3* p[2 μ *URA3*]
B-12707	B-7553	pAB2580	*CYT1 ura3* p[2 μ *URA3 CYT1*]
B-9737	B-7553		*cyt1-Δ ura3*
B-13435	B-9737	pAB625	*cyt1-Δ ura3* p[*CEN6 URA3*]
B-13436	B-9737	pAB2306	*cyt1-Δ ura3* p[*CEN6 URA3 CYT1*]

TABLE III
Description of plasmids

Plasmid number	Description
pAA625	Also designated pRS316; a 4.9-kb[a] *CEN6 URA3* plasmid having *Bam*HI and *Sal*I cloning sites (35)
pAB1097	Also designated pMH101; A 5.9-kb plasmid derived from pUC13 having a 3.2-kb *Bam*HI-*Sal*I segment with *CYT1* (21)
pAB1198	Also designated YEp436; a 13.3-kb 2 μ plasmid having *LEU2-d* and *URA3* markers (36)
pAB2306	pAA625 (*CEN6 URA3*) with a 3.2-kb segment having *CYT1* inserted at *Bam*HI and *Sal*I cloning sites; used for generating site-directed mutants
pAB2580	pAB1198 (2 μ *URA3*) having a PCR-generated 1.5-kb segment with *CYT1* inserted at *Bam*HI and *Sal*I cloning sites; used for generating dominant-negative mutants
pAB2657–pAB2663,	Dominant-negative mutants (Table V)
pAB2665–pAB2672,	Site-directed mutants (Table VI)

[a] kb, kilobase.

TABLE IV
Growth of dominant-negative (B-13412–B-13418) and site-directed (B-13445–B-13452) mutants

Strain number	YPD			YPG			SD − uracil			SG − uracil		
	22 °C	30 °C	37 °C	22 °C	30 °C	37 °C	22 °C	30 °C	37 °C	22 °C	30 °C	37 °C
B-7553	++++	++++	++++	+++	+++	+++	0	0	0	0	0	0
B-12705	++++	++++	++++	+++	+++	+++	++++	++++	++++	++++	++++	++++
B-12707	++++	++++	++++	+++	+++	+++	++++	++++	++++	++++	++++	++++
B-13412	++++	++++	++++	+	++	++	++++	++++	++++	0	0	0
B-13413	++++	++++	++++	+	++	++	++++	++++	++++	±	±	±
B-13414	++++	++++	++++	+	++	++	++++	++++	++++	±	±	±
B-13415	++++	++++	++++	+	+	++	++++	++++	++++	±	±	0
B-13416	++++	++++	++++	++	++	++	++++	++++	++++	±	±	0
B-13417	++++	++++	++++	+	+	++	++++	++++	++++	±	+	±
B-13418	++++	++++	++++	+	+	+	++++	++++	++++	0	0	0
B-9737	++++	++++	++++	0	0	0	0	0	0	0	0	0
B-13435	++++	++++	++++	0	0	0	++++	++++	++++	0	0	0
B-13436	++++	++++	++++	++	++	++	++++	++++	++++	++++	++++	++++
B-13445	++++	++++	++++	±	±	±	++++	++++	++++	0	0	0
B-13446	++++	++++	++++	++	++	++	++++	++++	++++	+++	++++	++++
B-13447	++++	++++	++++	++	++	++	++++	++++	++++	++++	++++	++++
B-13448	++++	++++	++++	++	++	++	++++	++++	++++	+++	+++	+++
B-13449	++++	++++	++++	++	++	++	++++	++++	++++	+++	+++	++++
B-13450	++++	++++	++++	+++	++	++	++++	++++	++++	++++	++++	++++
B-13451	++++	++++	++++	++	++	++	++++	++++	++++	+++	+++	+++
B-13452	++++	++++	++++	+	+	+	++++	++++	++++	±	±	±

ent error-prone PCR conditions, were pooled and used to transform the B-7553 strain; the resulting transformants were plated on SD − uracil plates. Approximately 104 colonies from the SD − uracil plates were replica-plated on SG − uracil, SD − uracil, YPG, and YPD plates for the detection of dominant-negative mutants. A transformant was considered to be a dominant-negative mutant if it exhibited diminished growth on SG − uracil, if the corresponding Ura^- strain lacking the plasmid had normal growth on YPG, and if reintroduction of the plasmid in pAB1198 again resulted in a transformant with diminished growth on SG − uracil medium.

Oligonucleotide-directed Mutagenesis—Oligonucleotide-directed mutagenesis was carried out by the procedure described by Kunkel *et al*. (37) using the plasmid pAB2306 (Table III), *E. coli* strain CJ236 (*dut1 ung1 thi1 relA1*/pCJ105[Cm^R]) (38), and the oligonucleotides OL.Z05–OL.Z14 (Table I). The *E. coli* strain XL1-Blue (*supE44 hsd17 recA1 endA1 gyrA46 thi1 relA1 lac*−) was used for the amplification and storage of plasmids. The site-directed change was confirmed by DNA sequencing of the *CYT1* region.

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 (39). The levels of cytochromes aa_3, *b*, *c*, and c_1 were estimated in intact cells at −196 °C by spectroscopic visual examination (40) and by absorbance recordings using an Aviv model 14 spectrophotometer as described by Hickey *et al*. (41).

Rates of Respiration—Oxygen uptake was measured polarographically with a commercially available Teflon-covered Clark electrode, the Yellow Stone Instruments oxygen monitor (Yellow Springs, OH), as described previously (42), using 3-ml solutions of 44 mM KH_2PO_4, 1 mM glucose, and various amounts of washed yeast cells obtained from cultures grown to late stationary phase in YPD medium. [Q_{O_2} is expressed as microliters of oxygen consumed per hour per milligram of yeast, dry weight.

TABLE V
Amino acid replacements and codon changes in the dominant-negative mutations of CYT1

Strain and plasmid numbers						
B-13412 pAB2657 *cyt1–101*	B-13413 pAB2658 *cyt1–102*	B-13414 pAB2659 *cyt1–103*	B-13415 pAB2660 *cyt1–104*	B-13416 pAB2661 *cyt1–105*	B-13417 pAB2662 *cyt1–106*	B-13418 pAB2663 *cty1–107*
L36L CTC→CTA	A22A GCA→GCT	A22A GCA→GCT	G45G GGT→GGA	L36L CTC→CTA	L36L CTC→CTA	T33T ACT→ACC
G45G GGT→GGA	L36L CTC→CTA	L36L CTC→CTA	R166G AGA→GGA	R166G AGA→GGA	S56S TCC→TCA	L36L CTC→CTA
S49L TCG→TTG	S56S TCC→TCA	S56S TCC→TCA	A168A GCC→GCT	A168A GCC→GCT	R166G AGA→GGA	V41V GTT→GTC
S56S TCC→TCA	T63A ACC→GCC	T63A ACC→GCC			A168A GCC→GCT	N79D AAT→GAT
T63A ACC→GCC	R166G AGA→GGA	R166G AGA→GGA			G221G GGT→GGC	A103S GCC→TCC
R166G AGA→GGA	A168A GCC→GCT	A168A GCC→GCT				S106T TCT→ACT
A168A GCC→GCT						G157S GGC→AGC
						A167A GCT→GCC
						A168A GCC→GCT
						L195M TTG→ATG
						M225K ATG→AAG
						R227End AAG→TAG
						E237E GAA→GAG
						T240S ACC→TCC

RESULTS AND DISCUSSION

Dominant-negative Mutants—We undertook an extensive screen for dominant-negative mutations of the *CYT1* gene that encodes cytochrome c_1 with the aim of identifying amino acid residues that are critical for function but not for stability or incorporation into the cytochrome bc_1 complex. For example, residues that are on the surface of the complex and are required for interaction with their physiological partners would be expected to be revealed with dominant-negative mutations. Because such altered cytochromes c_1 are expected to be stable, they should be particularly amenable to biochemical studies.

In this study, we have used a library of multicopy plasmids containing a *CYT1* segment that was mutated randomly by error-prone PCR. The plasmids, which are derivatives of pAB1198 (Table III), are maintained at a high copy number in the strain B-7553 (Table II) because of the *URA3* marker and the 2 μ origin of replication. In addition, the *LEU2-d* markers can be used to produce an even higher copy number on medium lacking leucine.

The screen is based on the lack of utilizing a nonfermentable carbon source, glycerol, because of competition of an altered nonfunctional form for the wild-type cytochrome c_1. Because manifestation of dominant-negative mutations depends on the presence of the plasmid, the desired mutants can be differentiated conveniently from other glycerol-negative mutants such as commonly occurring ρ^- mutations.

Approximately 10^4 transformants containing the library of mutagenized plasmids were screened for diminished growth on synthetic medium lacking uracil in a plasmid-dependent manner. A total of 181 colonies with some degree of diminished growth was uncovered, but only 12 were almost completely negative. The *CYT1* region of seven of these was subjected to DNA sequencing. The seven strains were designated B-13412–B-13418; the corresponding plasmids were designated pAB2657–pAB2663; and the corresponding altered alleles were designated *cyt1-101–cyt1-107* (Tables IV and V). The growth of the strains under various conditions is presented in Table IV, and as an example, the growth of B-13415 on SG − uracil medium is presented in Fig. 2.

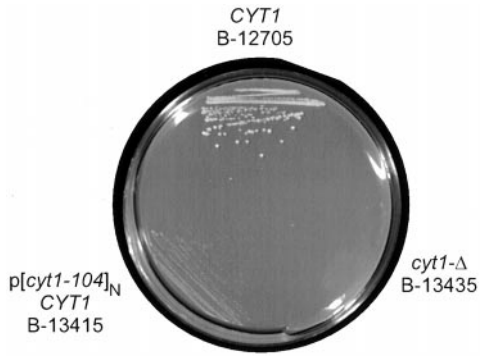

FIG. 2. Growth of the following strains on SG medium lacking uracil, demonstrating the dominant-negative effect of the R166G replacement: B-12705 (*CYT1 ura3* p[2 μ *URA3*]), the *CYT1* normal strain; B-13415 (*CYT1 ura3* p[2 μ *URA3 cyt1-104*]), the R166G dominant-negative mutant; and B-13435 (*cyt1*-Δ *ura3* p[*CEN6 URA3*]), a *cyt1*-Δ-deficient mutant.

The sequences of the *CYT1* region of the pAB2657–pAB2663 plasmids, presented in Table V, revealed multiple base pair substitutions with many of the changes common to more than one plasmid. The two plasmids pAB2658 and pAB2659 were identical, and all seven plasmids contained the neutral change A168A (GCC→GCT). Two different sets (six each) of the seven plasmids contained the neutral change L36L (CTC→CTA) and the radical change R166G (AGA→GGA), respectively. The plasmid pAB2663 contained 14 base pair substitutions including the formation of a UAG nonsense codon at amino acid position 227. The multiple and common base pair substitutions suggest that the altered PCR products may be related clonally, a result that would be expected if the DNA fragments were derived from common molecules because of low amounts of starting material. Thus, it is unclear which if any of the multiple directed changes occurred independently.

TABLE VI
Properties of mutants constructed by site-directed mutagenesis

Strain number	Plasmid	Allele	Oligonucleotide	Codon change	Amino acid changes	Cytochrome c_1	Growth on glycerol medium	Q_{O_2}
								μl/mg
B-9737						0	0	
B-13435	pAB625	No insert				0	0	9.5
B-13436	pAB2306	*CYT1*		None	None	+	+	50.4
B-13445	pAB2665	*cyt1–201*	OL.Z07	AGA → GGA	R166G	+	0	8.9
B-13446	pAB2666	*cyt1–202*	OL.Z08	AGA → TTG	R166L	+	+	
B-13447	pAB2667	*cyt1–203*	OL.Z09	AGA → ATG	R166M	+	+	
B-13448	pAB2668	*cyt1–204*	OL.Z10	AGA → AAA	R166K	+	+	
B-13449	pAB2669	*cyt1–205*	OL.Z06	ACC → GCC	T63A	+	+	
B-13450	pAB2670	*cyt1–206*	OL.Z05	TCG → TTG	S49L	+	+	
B-13451	pAB2671	*cyt1–207*	OL.Z06, OL.Z05	TCG → TTG, ACC → GCC	S49L, T63A	+	+	
B-13452	pAB2672	*cyt1–208*	OL.Z14	AGA → TAG	R227End	0	±	

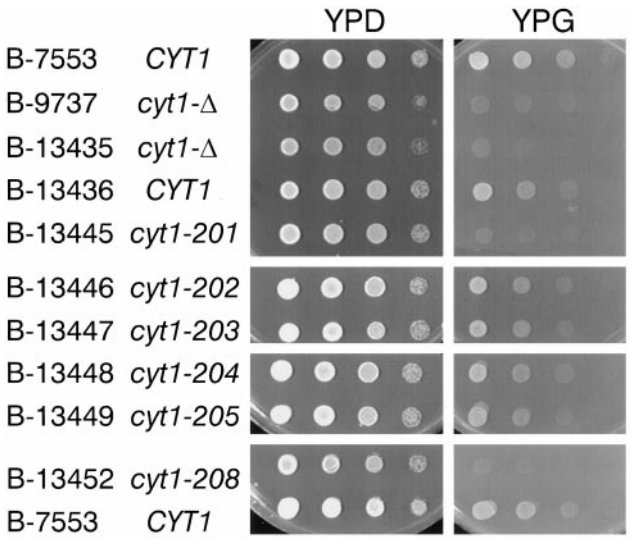

FIG. 3. Growth of 1:10 serial dilution of the following strains on YPD and YPG: B-7553 (*CYT1 ura3*); B-9737 (*cyt1*-Δ *ura3*); B-13435 (*cyt1*-Δ *ura3* p[*CEN6 URA3*]); B-13436 (*cyt1*-Δ *ura3* p[*CEN6 URA3 CYT1*]); B-13445 (*cyt1*-Δ *ura3* p[*CEN6 URA3 cyt1-201*] (R166G)); B-13446 (*cyt1*-Δ *ura3* p[*CEN6 URA3 cyt1-202*] (R166L)); B-13447 (*cyt1*-Δ *ura3* p[*CEN6 URA3 cyt1-203*] (R166M)); B-13448 (*cyt1*-Δ *ura3* p[*CEN6 URA3 cyt1-204*] (R166K)); B-13449 (*cyt1*-Δ *ura3* p[*CEN6 URA3 cyt1-205*] (T63A)); B-13452 (*cyt1*-Δ *ura3* p[*CEN6 URA3 cyt1-208*] (R227End)); and B-7553 (*CYT1 ura3*).

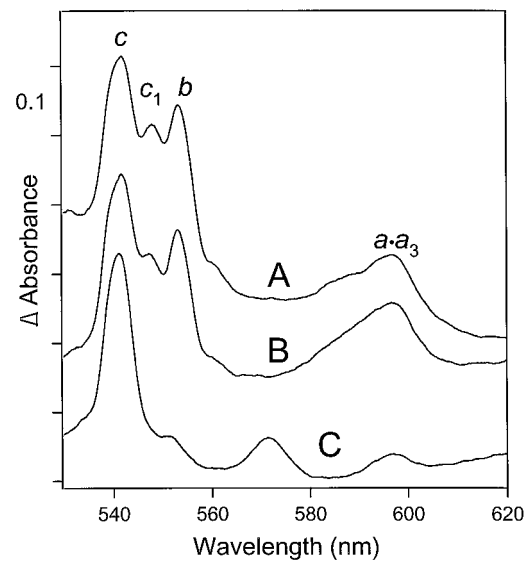

FIG. 4. Low temperature (−196 °C) spectrophotometric recordings of intact cells of the following isogenic strains: *A*, B-7553, *CYT1* (normal); *B*, B-13445, *cyt1-201* (R166G); and *C*, B-9737, *cyt1*-Δ (deficient mutant). The α-peaks of cytochromes $a \cdot a_3$, *b*, c_1, and *c* are located at 602.5, 558.5, 553.3, and 547.3 nm, respectively. The peak at ~575 nm in B-9737 (*C*) is caused by zinc protoporphyrin.

The results clearly established that at least the R166G replacement was responsible for the dominant-negative phenotype. This amino acid replacement occurred in six of the seven sequenced plasmids, pAB2657–pAB2662, and was the only amino acid change in the two plasmids pAB2660 and pAB2661. Furthermore, the only plasmid lacking the R166G replacement, pAB2663, contained the UAG nonsense mutation at amino acid position 227. The drastic nature of premature chain termination suggests that the R227End change in pAB2663 is responsible for the dominant-negative effect.

Site-directed Mutants—To confirm and extend these findings and to determine whether other replacements may confer a dominant-negative phenotype, the following changes were introduced in the single-copy *CEN6* plasmid by oligonucleotide-directed mutagenesis (Table VI): R166G; R166M; R166L; R166K; S49L; T63A; S49L and T63A; and R227End. Strain B-9737 (*cyt1*-Δ *ura3*) was transformed with each of the plasmids, and the transformants, B-13445–B-13452, were examined for growth on a variety of media at various temperatures and for the levels of the cytochromes aa_3, *b*, c_1, and *c*. The growth of B-13445 (R166G) and B-13452 (R227End) was diminished greatly on YPG medium, and the growth of B-13446 (R166L), B-13448 (R166K), B-13449 (T63A), and B-13451 (S49L, T63A) was diminished only slightly on SG − uracil medium (Tables IV and VI, Fig. 3). These results confirm that the R166G and R227End changes are responsible for the cytochrome c_1 defects and presumably for the dominant-negative phenotypes. Furthermore, the T63A replacement in pAB2657, pAB2658, and pAB2659 and the S49L replacement in pAB2657 are apparently innocuous. The detrimental effect of the R166G replacement was substantiated further from the diminished respiratory rate of strain B-13445, which was equivalent to the strain B-13435 lacking cytochrome c_1 (Table VI). Nevertheless, B-13445 (R166G) contained the nearly normal level of cytochrome c_1 as indicated by the α-peak in the low temperature (−196 °C) spectrophotometric recording (Fig. 4).

In contrast to the R166G replacement, the R166M, R166L, and R166K replacements at most only caused minor diminution of function as indicated by the normal or nearly normal level of growth on YPG medium (Table IV). This finding suggests that the large size but not the charge of the Arg-166 side chain is critical for maintaining the normal function of cytochrome c_1.

Yeast Arg-166 (Vertebrate Arg-102)—The importance of the yeast Arg-166 (or vertebrate Arg-102) along with the adjacent

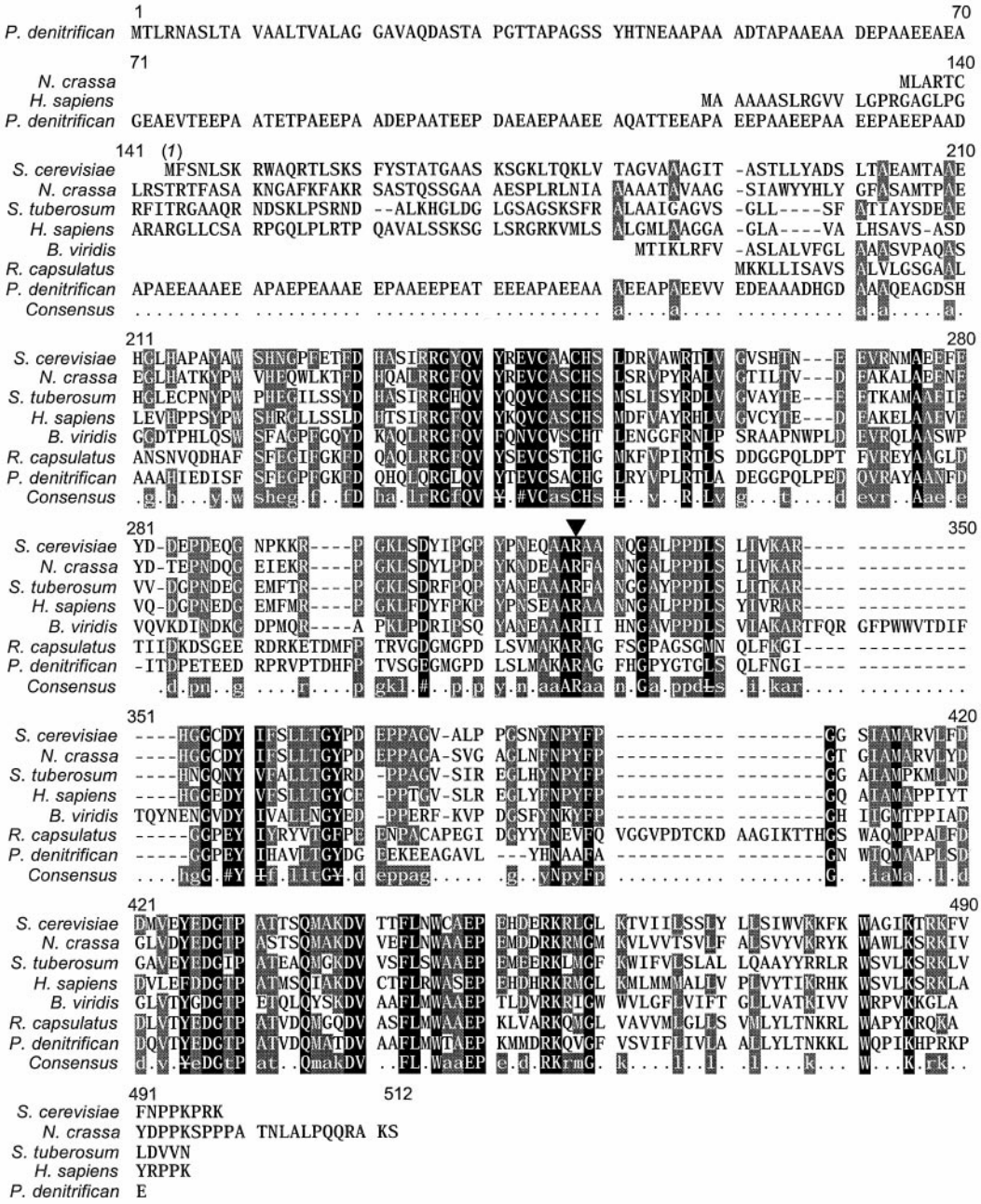

FIG. 5. **Amino acid sequence alignment of cytochromes c_1 from *S. cerevisiae*, *Neurospora crassa*, *Solanium tuberosum* (potato), *Homo sapiens*, *Blastochloris viridis*, *Rhodobacter capsulatus*, and *Paracoccus denitrificans*.** Highly conserved residues are *highlighted* in *black*, where *I* designates I or V; *L* designates L or M; *Y* designates Y or F; and # designates N, D, Q, E, B, or Z. Moderately conserved residues are *highlighted* in *gray*. The residue positions are *numbered* starting with the *P. denitrificans* sequence. Thus, residue position 1 of *S. cerevisiae* corresponds to residue position 144 of *P. denitrificans*. The conserved Arg-166 of *S. cerevisiae* is denoted at position 308 (▼).

Ala-165 residue is reflected by the phylogenetic conservation of these residues in cytochrome c_1 from all species including higher and lower eukaryotes and prokaryotes. As shown in Fig. 5, of the almost 250 residues, Ala-165 and Arg-166 represent two of the 32 absolutely conserved residues in all cytochromes c_1.

Insight into the function of Arg-166 is provided by considering its position in the cytochrome bc_1 complex and the proposed models of electron transfer. As shown in Fig. 1, Arg-166 is located on the surface of cytochrome c_1 in close proximity to the exposed pyrrole C corner of the heme group but not adjacent to any of the other components of the complex. The surface location of Arg-166 is consistent with proper assembly and stability of the altered R166G cytochrome c_1. Zhang *et al*. (13) suggested that electron transfer into cytochrome c_1 occurs through the D propionate and out of cytochrome c_1 through the C corner of the heme to cytochrome *c*. On the other hand, *in vitro* protection and cross-linking experiments suggested that at least two different regions of cytochrome c_1, encompassed by vertebrate positions 63–81 and 167–174, are folded together to form the cytochrome *c* binding site (43, 44). It remains to be seen if the R166G cytochrome c_1 still binds cytochrome *c* but is unable to transfer electrons. In this regard, an R166G replacement is not expected to disrupt the α-helical structure in this region, and it is unclear how R166G but not R166K, R166M, or R166L replacements could effect binding to cytochrome *c*.

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