

Investigating the Q_n Site of the Cytochrome bc₁ Complex in *Saccharomyces cerevisiae* with Mutants Resistant to Ilicicolin H, a Novel Q_n Site Inhibitor*

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The cytochrome bc₁ complex resides in the inner membrane of mitochondria and transfers electrons from ubiquinol to cytochrome c. This electron transfer is coupled to the translocation of protons across the membrane by the protonmotive Q cycle mechanism. This mechanism topographically separates reduction of quinone and reoxidation of quinol at sites on opposite sides of the membrane, referred to as center N (Q_n site) and center P (Q_p site), respectively. Both are located on cytochrome b, a transmembrane protein of the bc₁ complex that is encoded on the mitochondrial genome. To better understand the parameters that affect ligand binding at the Q_n site, we applied the Q_n site inhibitor ilicicolin H to select for mutations conferring resistance in *Saccharomyces cerevisiae*. The screen resulted in seven different single amino acid substitutions in cytochrome b rendering the yeast resistant to the inhibitor. Six of the seven mutations have not been previously linked to inhibitor resistance. Ubiquinol-cytochrome c reductase activities of mitochondrial membranes isolated from the mutants confirmed that the differences in sensitivity toward ilicicolin H originated in the cytochrome bc₁ complex. Comparative *in vivo* studies using the known Q_n site inhibitors antimycin and funiculosin showed little cross-resistance, indicating different modes of binding of these inhibitors at center N of the bc₁ complex.

The cytochrome bc₁ complex of *Saccharomyces cerevisiae* is a homodimeric protein embedded in the inner membrane of mitochondria (1). Each monomer consists of three catalytic subunits carrying prosthetic groups, which are cytochrome b with two b-type hemes, cytochrome c₁ and the Rieske iron-sulfur protein with its [2Fe-2S] cluster (2). Additionally, there are 7 supernumerary subunits lacking any cofactors (3). Cytochrome b is the only protein of the complex that is encoded by the organelle DNA; all the other subunits are encoded by the nucleus (4).

The bc₁ complex transfers two electrons from ubiquinol to two molecules of cytochrome c, while translocating four protons from the negative to the positive side of the membrane

via the protonmotive Q cycle mechanism (5). There are two catalytic sites involved in the Q cycle mechanism. The site where ubiquinol is oxidized, center P (Q_p site), and the site where quinone is reduced, center N (Q_n site), are located near the electropositive and electronegative sides of the membrane, respectively. Both sites are located on cytochrome b and have different heme prosthetic groups, heme b_L at center P and heme b_H at center N.

There are several compounds that inhibit specifically the Q_p or the Q_n site (6). One of the Q_p site inhibitors, atovaquone, is of pharmaceutical relevance because of its anti-protozoal and anti-fungal properties (7). The Q_p site has been characterized by applying Q_p site inhibitors, which show more structural homology to the Q_p site substrate ubiquinol than the few Q_n site inhibitors known to date.

It was shown recently that ilicicolin H, a new inhibitor isolated from the imperfect fungus *Cylindrocladium ilicicola*, acts at the Q_n site of the *S. cerevisiae* bc₁ complex (8). Previously, two other inhibitors produced by microorganisms have been known that act at the Q_n site; antimycin, which is a natural product of various species of *Streptomyces* (8–10), and funiculosin, an antibiotic produced by *Penicillium funiculosum* Thom (8, 11–14).

Ilicicolin H is a 5-(4-hydroxyphenyl)- α -pyridone with a decalin ring system. The 5-(4-hydroxyphenyl)-pyridone is the chromophore of tenellin, produced by the insect-pathogenic fungus *Beumeria bassiana* (8). Even though the structures of the three Q_n site inhibitors are clearly different, there are some similarities (see Fig. 1 in Ref. 8 for a comparison of the structures). Ilicicolin H possesses a phenol ring, a feature that is also present in antimycin. There is also a resemblance with funiculosin, which shares the pyridone ring system with ilicicolin H. Similar effects of ilicicolin H and funiculosin on the cytochrome b optical spectrum have been observed. It was therefore concluded that the pyridone ring system plays an important role for ilicicolin H and funiculosin binding (8).

In this study we wanted to shed some light on the Q_n site by applying the novel center N inhibitor ilicicolin H to isolate cytochrome b mutants in *S. cerevisiae*. From previous work, we know that antimycin displaces semiquinone from center N in the bovine bc₁ complex (15), and more recently we have found that all three of the center N inhibitors eliminate the EPR detectable signal attributed to the stable semiquinone at center N in the yeast enzyme. We thus anticipate that the location of

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resistance conferring mutations might identify amino acids that contribute to semiquinone stability at center N.

Seven cytochrome *b* mutations, differing from the wild-type protein by only one amino acid exchange, were obtained. The sequenced mutations were confirmed and assigned to the cytochrome *b* gene using genetic complementation techniques. Six of the seven mutations obtained in this study have not been described before. The effects of these mutations on the activity of the enzyme, as well as the sensitivity of the altered cytochrome *b* proteins toward ilicicolin H, were determined enzymatically. We also checked the mutant yeast strains for cross-resistance using antimycin and funiculosin.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Genetic Techniques—The wild-type strain used for the mutant selection was W303-1B, α , *ade2-1*, *his 3-11*, *his 3-15*, *trp1-1*, *leu 2-3*, *leu 2-112*, *ura 3-52*, *can 1-100*. The strains used for genetic mapping were KL14-4A/60, *a*, *his1*, *trp2*, *rho*⁰; RE11, *a*, *his1*, *trp2*, *rho*⁻; RD1, *a*, *his1*, *trp2*, *rho*⁻; PK1, *a*, *his1*, *trp2*, *rho*⁻. Media used for the growth of *S. cerevisiae* were YPD, 2% glucose (Fisher Scientific), 1% yeast extract (US Biological), 1% bactopectone (BD); YPDA, YPD supplemented with 20 mg/liter adenine (Sigma); YP10, 10% glucose, 1% yeast extract, 1% bactopectone, supplemented with adenine where needed; N3 medium (non-fermentable carbon source), 2% glycerol (LabChem Inc.), 1% yeast extract, 1% bactopectone, 20 mg/ml adenine, 50 mM phosphate buffer, pH 6.2; W10, 10% glucose, 0.67% yeast-nitrogen base without amino acids; CSM-media (complete supplement mixture without a certain amino acid) were prepared according to the manufacturer's instructions (Bio 101, Inc.); W0, 2% glucose, 0.67% yeast-nitrogen base without amino acids. For plates 2% agar (Difco) was added. Sporulation medium was 1% potassium acetate, 0.25% yeast extract and 0.1% glucose.

Antimycin was purchased from Sigma. Funiculosin was a gift of Novartis, Basel, Switzerland. Ilicicolin H was obtained from the Merck sample repository. The inhibitors were added as ethanolic solutions to agar-containing medium at ~50 °C to obtain final concentrations of 5 and 10 μ M ilicicolin H, 5 μ M funiculosin, and 0.1 μ M antimycin.

Isolation of Resistant Mutants—A YPD culture of the wild-type strain W303-1B was plated for single colonies on YPDA. Ten individual subclones were randomly picked and each subclone was inoculated into YP10 medium supplemented with adenine and incubated for 2 days at 30 °C. After washing, $\sim 5 \times 10^7$ cells from each subclone were spread on N3 plates containing 5 and 10 μ M ilicicolin H. Each subclone produced a different number of resistant colonies after 5–10 days incubation at 30 °C. Randomly picked colonies were subcloned on YPDA and then checked for ilicicolin H resistance and resistance to cycloheximide. This allowed separation of mutations affecting cell permeability.

The ilicicolin H resistant strains were crossed with the *rho*⁰ strain KL14-4A/60 to establish that the mutation was mitochondrial. The resulting diploid clones were sporulated and the dissected asci spores were tested for their ability to grow on N3 medium supplemented with ilicicolin H. The mutant strains were then mapped by crossing them with the *rho*⁻ tester strains

RE11, RD1, and PK1, which have retained different segments of the cytochrome *b* gene. The diploid strains were grown for at least 15 generations in W10, to get homoplasmic cells, and then spread for single colonies on W0. The amount of cells spread was calculated such that the cells were countable but yet statistically conclusive. Several plates were evaluated per clone. The resulting colonies were replica plated on N3 and N3 supplemented with ilicicolin H to determine the frequency of respiring clones rendered sensitive toward the drug by double homologous recombination (16, 17). All further experiments were carried out with the diploid strains resulting from the crossing of the resistant strains with KL14-4A/60, because the diploid strains then contained the mitochondrial genome of the resistant strains.

Sequencing—The primers used for amplifying and sequencing exon 1 of the cytochrome *b* gene were: pMD1 (sense primer): 5'-ATG GCA TTT AGA AAA TCA AAT GTG TAT TTA AG-3'; pMD2 (antisense primer): 5'-CCA TAA TAT AAA CCT TTA GCC ATA TGC-3'. The primers for amplifying and sequencing exon 4 were: pMD3 (sense primer): 5'-CTC AGT ATC TAA CCC TCT AAT CCA GAG ATT C-3'; pMD4 (antisense primer): 5'-ACC TAA AGT ATT AGG TGA ATA GAA TAC-3'.

Growth in Non-fermentable Medium—Diploid mutants were grown to log phase in YPD medium, washed, and the cells were then used for the inoculation of non-fermentable medium (N3) to a starting A_{600} of 0.15. Measurements in log phase were taken approximately every 90 min.

Isolation of Mitochondrial Membranes—Mitochondrial membranes were prepared as described elsewhere (18) with the following modifications. One liter of 2% YPD was inoculated with the corresponding strain and used for membrane purification. Before breaking the cells, 0.2% bovine serum albumin and 1 mM diisopropylfluorophosphate were added to the buffer. The total buffer volume was two times the wet weight of the cell pellet. An equal volume of glass beads (Sigma, 425–600 μ m) was then added. The last washing step was with 50 mM Tris, pH 8.0. Membranes were stored at -20 °C in 50 mM Tris, pH 8.0, containing 50% glycerol.

Determination of *bc*₁ Complex Concentrations—Cytochrome *b* concentration was determined from the difference spectrum of the sodium dithionite-reduced minus ascorbate-reduced enzyme using an extinction coefficient of 25 mM⁻¹ cm⁻¹ at 563–578 nm.

Ubiquinol-Cytochrome *c* Reductase Activity and Inhibitor Titrations—The assay buffer contained 50 mM potassium phosphate, pH 7.0, 250 mM sucrose, 0.2 mM EDTA, 1 mM NaN₃, and 0.01% Tween-20. Measurements were performed with membranes diluted to obtain a concentration of 10 nM cytochrome *bc*₁ complex in assay buffer supplemented with 1 mM potassium cyanide and 30 μ M cytochrome *c*. The reaction was started by adding decyl-ubiquinol (DBH₂) to a final concentration of 50 μ M after stirring the enzyme 90 s in the absence or presence of inhibitor. Reduction of cytochrome *c* was monitored at 550–539 nm with the Aminco DW2aTM spectrophotometer in the dual wavelength mode. An extinction coefficient of 21.5 mM⁻¹ was used to calculate cytochrome *c* reduction at 550–539 nm (19). The activity of the *bc*₁ complex after stirring 90 s without

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inhibitor was determined prior to each inhibitor titration. The activity of the membranes from each mutant was determined simultaneously with that of wild-type membranes. For each inhibitor concentration, measurements were taken in duplicate and the average value was calculated. Turnover numbers were calculated on the basis of cytochrome *b* content. The non-inhibited enzymatic activity was $65 + 6 \text{ s}^{-1}$, which was set at 100%.

Molecular Modeling—Molecular modeling was performed with a Silicon Graphics Octane2 workstation using the Discover 3[®] module within the Insight II[®] software package (Accelrys Inc., San Diego, CA). The view of the structure of center N was constructed from the coordinates of the yeast cytochrome *bc*₁ complex (Protein Data Bank code 1EZV) with stigmatellin bound at center P (20).

RESULTS

Selecting for *S. cerevisiae* Cytochrome *b* Mutations Conferring Resistance to the Novel *Q_n* Site Inhibitor Ilicicolin H—The novel inhibitor ilicicolin H was used to select for spontaneous mutants exhibiting resistance in *S. cerevisiae*. As a first step, an inhibitor concentration for plates had to be determined that would suppress the growth of wild-type cells, but would still allow mutants to survive. That threshold was between 5 and 10 μM ilicicolin H.

As described above, ten haploid W303-1B colonies were randomly picked and streaked on N3 plates containing 5 and 10 μM ilicicolin H to screen for resistant mutants. To differentiate mutations that modified cell permeability from the mutations in the cytochrome *b* gene, the mutants were tested for their resistance toward cycloheximide (21). Among the 52 randomly picked colonies from the ilicicolin H plates, 40 were resistant to cycloheximide concentrations up to 1 $\mu\text{g}/\text{ml}$ (data not shown). These were discarded.

Determining if the Mutation Is of Mitochondrial Origin—The cytochrome *bc*₁ complex in *S. cerevisiae* is composed of ten subunits, with cytochrome *b* being the only protein encoded by the mitochondrial (mt)² DNA. To rule out that the mutation was in one of the other subunits and hence of nuclear origin, the ilicicolin H-resistant, cycloheximide-sensitive clones were crossed with the *rho*^o strain KL14-4A/60. The resulting diploid progeny had the mtDNA of the corresponding resistant clone. These were then sporulated, and the spores of the dissected asci were tested for growth on drug plates. All twelve cycloheximide-sensitive strains showed a mitochondrial DNA segregation pattern of the ilicicolin H resistance (data not shown). The diploid-resistant clones resulting from these crosses were then used for most of the experiments unless otherwise specified.

Sequencing Exon 1 and Exon 4 of the Cytochrome *b* Gene from the Haploid-resistant Strains—It was known that previously identified mutations conferring resistance to *Q_n* site inhibitors are located in exon 1 or exon 4 of the cytochrome *b* gene. Both of these exons were thus sequenced from two directions in the ilicicolin H-resistant, cycloheximide-sensitive strains. Mutations were found in either exon 1 or exon 4. Table 1 shows the

TABLE 1

Cytochrome *b* mutations conferring ilicicolin H resistance in *S. cerevisiae*

Shown are the different mutations obtained after selection for ilicicolin H resistance on plates, the exon location and frequency of the mutation, and the growth characteristics of the yeast strains on a non-fermentable medium.

Mutation	Codon change	Exon location	Frequency	Growth
S20T	TCA ACA	1	1	Normal
S20L	TCA TTA	1	1	Impaired
Q22E	CAA GAA	1	3	Normal
Q22T	CAA CTA	1	2	Normal
G37D	GGT GAT	1	2	Impaired
G37S	GGT AGT	1	2	Normal
L198F	TTA TTT	4	1	Normal

different amino acid mutations, their position in the protein, followed by the codon that was changed. The selection yielded seven different mutations; serine at position 20 to threonine (S20T) or leucine (S20L), glutamine at position 22 to glutamate (Q22E) or threonine (Q22T), glycine 37 to serine (G37S) or aspartate (G37D), and leucine 198 to phenylalanine (L198F).

The most frequently occurring mutation was Q22E, which was obtained three times independently from different W303-1B subclones (Table 1). Q22T was obtained two times from independent subclones, while G37S and G37D were both obtained two times, each from the same original subclone. Further characterization of the mutant strains was performed with one representative for each mutation found.

The glycine at position 37 of the yeast cytochrome *b* seems to be a favorable location for mutations, since mutations at this position conferring resistance to antimycin and funiculosin have been described previously (22, 23). The mutation described previously was glycine to valine, which had been obtained in eight independent isolated mutants. It was concluded that at glycine 37 the mutated codon could give rise to at least seven different amino acids as a result of a single base change, but that some might not be found because they would confer a respiratory deficiency (22). One of the two glycine 37 mutations selected in this study, G37D, shows an impaired growth phenotype on plates containing a non-fermentable carbon source (Table 1).

Another strain, carrying the S20L mutation, also shows an impaired growth phenotype on non-fermentable medium. Interestingly, there was one additional mutation emerging at both sites, G37S and S20T, for which growth on glycerol plates is not hampered. In the case of G37D, the small, non-polar glycine is exchanged with aspartate, containing a charged polar side chain. That exchange obviously has a more detrimental effect than replacing the glycine with serine, which has an uncharged polar side chain. In the case of serine 20, it is a more subtle change to replace an amino acid with an uncharged polar side chain with an amino acid similar in size and characteristics, threonine, than with an amino acid with a non-polar side chain, leucine.

The remaining five resistant mutant strains show growth similar to that of the wild-type strain on non-fermentable medium (Table 1). The L198F mutation has been described before as a funiculosin resistance-conferring mutation (23). All of the other mutations are described here for the first time. Further characterization of the mutant strains was performed with one representative for each mutation found.

² The abbreviations used are: mt, mitochondrial; EPR, electroparamagnetic resonance.

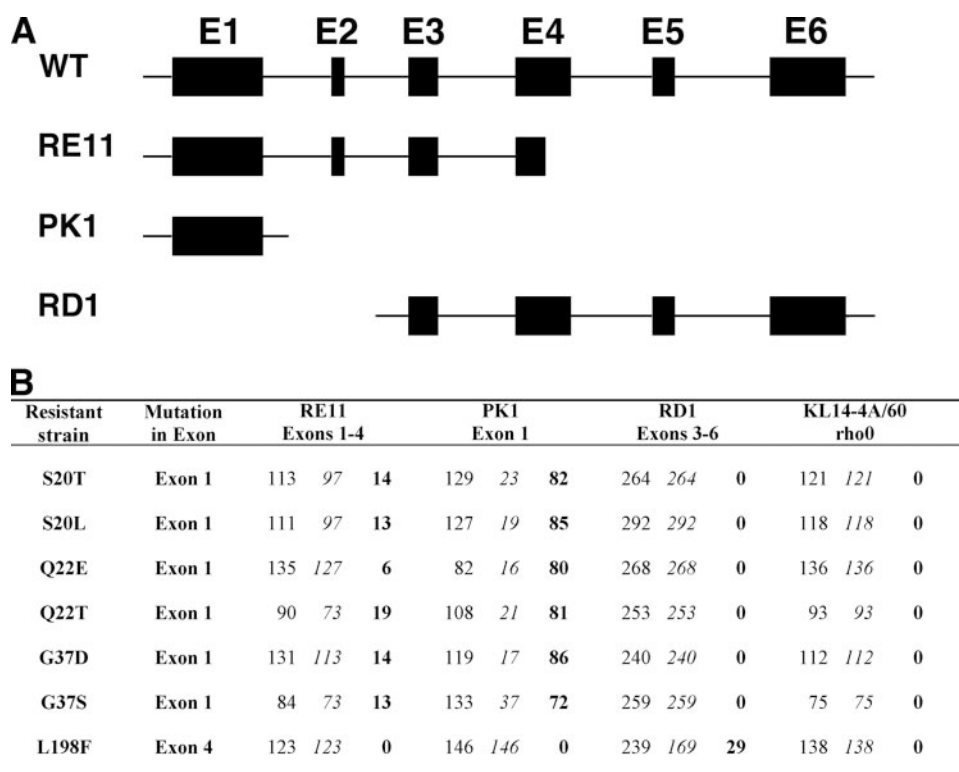


FIGURE 1. Schematic representation of the exon/intron structure of the cytochrome *b* gene of wild-type yeast and the ρ^- strains used for genetic mapping and the results of crossing the resistant strains with the ρ^- strains. Panel A depicts the structures of the cytochrome *b* genes of wild-type yeast and of the three different ρ^- strains, which contain defined limited regions of the cytochrome *b* gene and were used to localize the mutations conferring ilicicolin H resistance by crossing with the resistant strains. The six exons of the wild-type cytochrome *b* gene are labeled E1–E6 for comparison to the exons in the three ρ^- strains underneath. Panel B shows the results of crossing the haploid ilicicolin H-resistant strains to the ρ^- strains and a ρ^0 strain, KL14-4A/60, which is completely devoid of mitochondrial DNA, each cross being represented by three columns of numbers. For each cross, a countable amount of cells was spread on W0 plates, which have dextrose as a carbon source, and then replica-plated on glycerol-containing and drug-containing plates. The first number in each column is the number of respiratory-competent colonies growing on glycerol after replica plating from the W0 plates, where all of the colonies grew equally well. The number in *italics* indicates the number of colonies capable of growing on plates containing ilicicolin H. The **bold numbers** indicate the percentage of colonies rendered sensitive to ilicicolin H after the recombination event, proving the mutation is present in the corresponding exon.

Genetic Mapping of the Resistant Clones—To confirm that the mutations identified by sequencing are responsible for the resistance, and to exclude the possibility of additional mutations elsewhere in the cytochrome *b* gene, crosses between the ilicicolin H-resistant/cycloheximide-sensitive strains and relevant ρ^- tester strains were performed as described previously (16). By using ρ^- mutant strains containing only sections of the cytochrome *b* gene, mutations can be mapped to defined areas of the gene. These strains are deprived of large parts of mtDNA, and the remaining wild-type sequence is amplified until the mtDNA reaches its original size (17). This accounts for the different recombination frequencies observed for distinct ρ^- strains; the smaller the remaining, amplified fragment, the higher the frequency. Panel A in Fig. 1 shows schematically the exons (E1–E6) separated by introns of the wild-type cytochrome *b* gene, and underneath the regions that are covered by the ρ^- strains used in this study. Strain RE11 covers the first three exons and parts of exon 4. Strain PK1 covers exon 1, and RD1 consists of the last four exons.

The results of the crosses are shown in panel B in Fig. 1. The four columns represent the crossing of the three ρ^- strains

and one ρ^0 strain with the resistant strains. After ~15 generations in W10 medium to obtain progeny containing only homoplasmic cells, a countable, comparable amount of cells was spread on W0 plates and replica plated on non-fermentable glycerol medium, and on glycerol plates supplemented with 5 μ M ilicicolin H. In crossings with RE11, only the resistant strains carrying the mutation in exon 1 of the cytochrome *b* gene showed recombination, ranging from 6 to 19%. RE 11 covers parts of exon 4, but is lacking the area where mutation Leu-198 is localized, no crossing over was possible. In crossings with PK1, which only consists of exon 1 of the cytochrome *b* gene, the recombination efficiency ranges from 72 to 86% for the strains carrying the mutation in that part of the gene, 0% for L198F.

As mentioned above, the frequency of recombination changes significantly, depending on the size of the fragment. There was no homologous recombination when the resistant clones were crossed with RD1, except for the strain carrying the L198F mutation, an area that is covered by this ρ^- strain. Also shown are the results of the control crossings with the ρ^0 strain KL14-4A/60. The results of the crossings proved that the mutations by sequencing the cyto-

chrome *b* genes of the resistant strains were responsible for the resistance toward ilicicolin H.

Localization of the Amino Acids Altered in the Ilicicolin H-resistant Strains—The location of the amino acids that are mutated in the resistant strains at the Q_n site provides some insight into the basis for the inhibitor resistance. The secondary structure of the yeast cytochrome *b* is shown in Fig. 2A. The protein spans the inner mitochondrial membrane eight times with both N and C termini in the mitochondrial matrix. Most of the mutations are near the N terminus, except the mutation at position Leu-198, which is located in helix D.

The crystal structure of cytochrome *b* in Fig. 2B shows the mutational sites serine 20, glutamine 22, glycine 37, and leucine 198, in addition to ubiquinone and the b_H heme at center N. This view of the crystal structure shows that leucine 198, serine 20, and glutamine 22 are close (<5 Å) to the ubiquinone ring in the center N pocket. In contrast, glycine 37, which also confers resistance to several other center N inhibitors (22), is rather distant from the quinone ring (9.2 Å).

Growth on Plates Containing Inhibitor—After confirming the mutations by genetic crossing, the mutant strains were

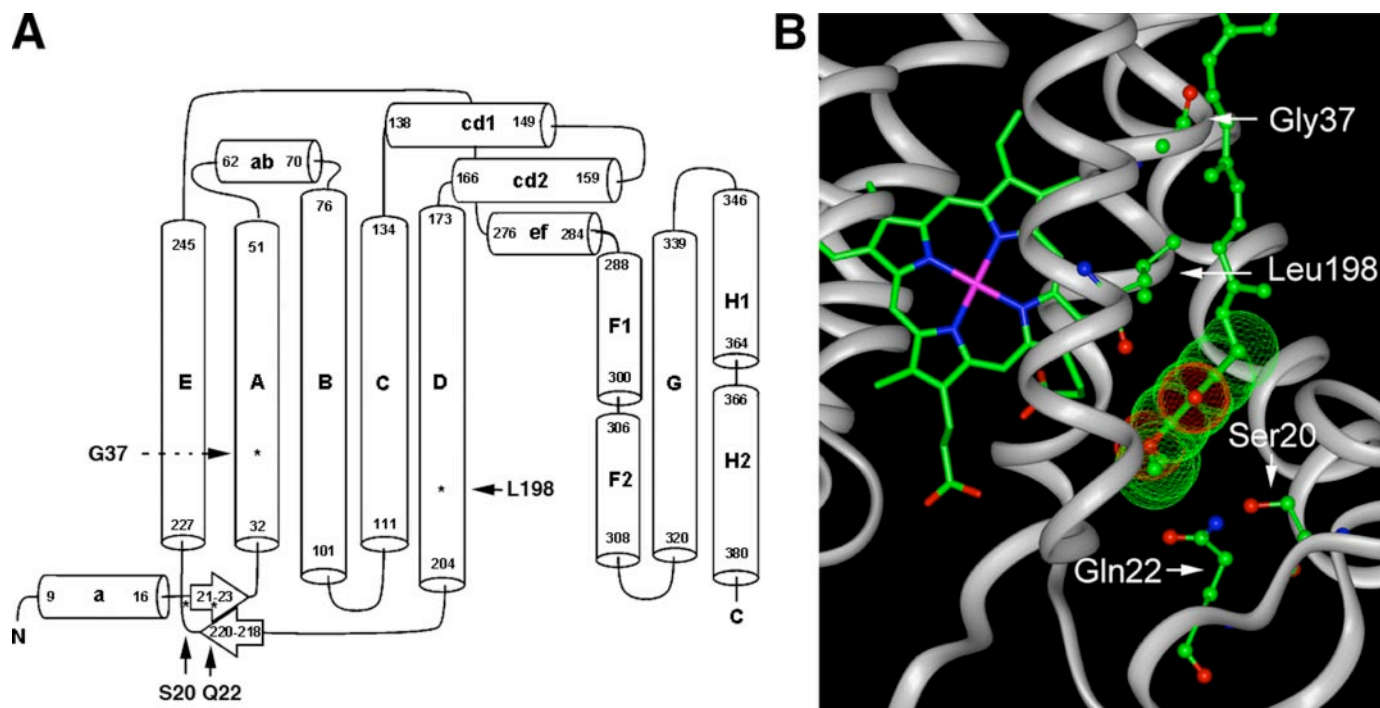


FIGURE 2. Secondary structure of yeast cytochrome *b* and a view of center N, highlighting the positions where mutations conferring resistance to ilicicolin H occurred. Shown in panel A is the *S. cerevisiae* cytochrome *b* secondary structure, modified from the chicken structure (2) to agree with numbering of the yeast protein. Asterisks indicate the amino acid positions in cytochrome *b* where mutations conferring resistance to ilicicolin H occurred. Shown in panel B is the structure of center N excerpted from the crystal structure of the yeast cytochrome *bc*₁ complex, 1EZV (20). The structure includes a portion of cytochrome *b* (gray) and the *b*_{L1} heme with ubiquinone bound and van der Waals radii of the quinone ring indicated. The four amino acids where mutational changes confer resistance to ilicicolin H are shown as ball and stick structures in cytochrome *b*. Carbon atoms are green, oxygen atoms are red, nitrogen atoms are blue, and the heme iron is pink.

tested for their growth characteristics. All of the strains grew normally on glucose. Their growth on plates containing non-fermentable medium is shown in Fig. 3. For this experiment, the resistant diploid strains resulting from the crosses with KL14-4A/60 were used. For comparison, strains were grown on plates supplemented with either 5 μ M ilicicolin H, 5 μ M funiculosin, or 0.1 μ M antimycin. For the latter two inhibitors, the appropriate concentration for plates was determined as described for ilicicolin H above.

The upper part of the panel in Fig. 3 shows the results of the incubation of the resistant clones at the normal 30 °C growth temperature, while the lower panels show the same subset of strains grown at 34 °C to check if the mutations rendered the *bc*₁ complex temperature-sensitive. The panels on the left show the growth of the various strains on the non-fermentable carbon source in the absence of any inhibitor. The figure shows the plates after 6 days of incubation, except for the antimycin plates, which are shown after fourteen days, because it took longer for the resistant cells to emerge with antimycin present.

For most of the strains there was not much difference if they were grown at 30 or 34 °C. The L198F-resistant strain did not grow on antimycin plates at 34 °C, but did grow at 30 °C. The S20L resistant strain, one of the slow-growers, which had problems growing at 30 °C on all plates, did not grow at all at 34 °C.

At 30 °C, the S20T, Q22E, Q22T, G37S, and L198F resistant strains grew at a rate similar to the wild-type strain when grown on glycerol alone. Their growth was also not impaired when grown on plates containing 5 μ M ilicicolin H, irrespective of whether the clones were grown at 30 or 34 °C. Except for the

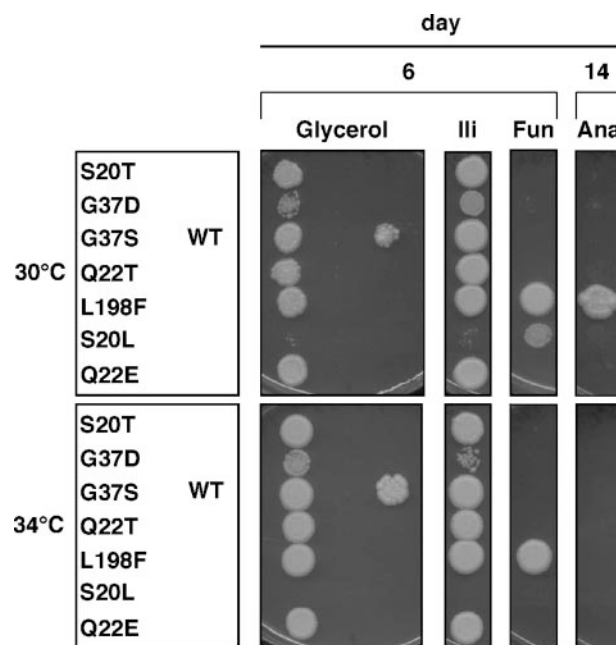


FIGURE 3. Effect of the cytochrome *b* mutations on respiratory growth and cross-resistance to other *Q*_n site inhibitors. The ilicicolin H-resistant diploid strains resulting from the crosses with KL14-4A/60 were tested on non-fermentable medium, glycerol, with or without ilicicolin H (Ili), funiculosin (Fun), or antimycin (Ana) added. The yeast strains with their identifying mutations are listed to the left of the plates. The upper panel shows growth at 30 °C; the lower panel shows the same strains grown at 34 °C. The wild-type strain (WT) did not grow on plates containing inhibitor (not shown). The plates were evaluated after 6 days, except for the antimycin plates, which were evaluated after 14 days because of weaker growth.

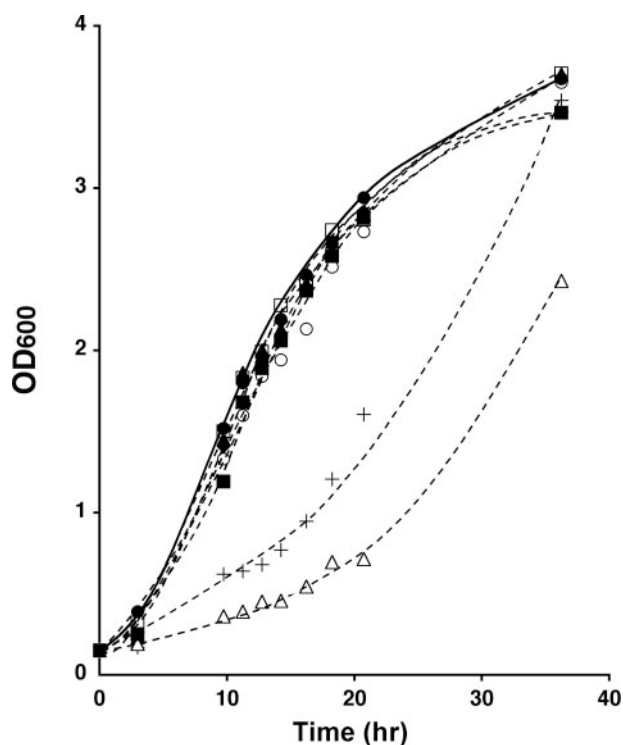


FIGURE 4. Growth of the resistant strains in non-fermentable liquid medium. The growth of the resistant strains at 30 °C was compared with that of the wild-type strain in N3 medium, which contains glycerol as a carbon source. The density of the cell suspensions was monitored at 600 nm (OD_{600}). The strains are WT (solid line, solid circles), S20T (solid diamonds), S20L (open triangles), Q22E (solid squares), Q22T (open squares), G37D (crosses), G37S (open circles), and L198F (solid triangles).

L198F mutation, which conferred ilicicolin H and funiculosin resistance at both 30 and 34 °C, there was no other mutation that conferred strong resistance to either funiculosin or antimycin.

The two strains with S20L and G37D cytochrome *b* mutations grew poorly on non-fermentable medium at 30 °C, the former being more impaired than the latter. The S20L strain showed weak ilicicolin H resistance and cross-resistance to funiculosin. Notably, the S20L strain grew better at 30 °C when funiculosin was present, but did not grow at 34 °C, regardless if inhibitors were added or not. The wild-type strain grew only on the glycerol plate when no inhibitors were present (results not shown).

Respiratory Competence in Liquid Medium—To compare the growth characteristics of the resistant clones, they were grown in the non-fermentable liquid N3 medium at 30 °C. The growth in liquid medium (Fig. 4) confirmed the results obtained with plates (Fig. 3). Two strains, S20L and G37D, showed impaired growth in the non-fermentable medium and, like on the plates, growth of the S20L strain was more impaired than G37D. The other mutant strains grew at rates similar to the wild-type strain.

Effect of the Cytochrome *b* Mutations on the Activity of the bc_1 Complex—To assess the impact of the mutations on the activity of the bc_1 complex, ubiquinol-cytochrome *c* reductase activity was measured in membranes isolated from the various strains. The activity of the bc_1 complex from the wild-type strain in the absence of inhibitor was 65 s^{-1} . The activities for the bc_1 com-

TABLE 2

Ubiquinol-cytochrome *c* reductase activities of mitochondrial membranes and sensitivity to ilicicolin H

Shown are the ubiquinol-cytochrome *c* reductase activities of mitochondrial membranes prepared from the resistant strains and the wild-type strain expressed as a percent of wild-type activity, which was $65 \pm 6 \text{ sec}^{-1}$. Also shown are the corresponding IC_{50} values for inhibition of the activities by ilicicolin H.

Strain	Activity	Ilicicolin IC_{50}
	%	nM
Wild type	100	12
S20T	95	>50
S20L	56	>50
Q22E	75	>50
Q22T	77	>50
G37D	52	34
G37S	80	26
L198F	110	20

plexes from the resistant strains in the absence of inhibitors are shown in Table 2. The activities of the bc_1 complexes in membranes from the S20T and L198F mutants seemed unaffected by the mutations. Membranes prepared from the Q22E, Q22T, and G37S mutants showed a loss of activity of 20–25%. The two strains that exhibited impaired growth in non-fermentable medium, G37D and S20L, had diminished activities, equivalent to ~50% of that of the wild-type strain.

Impact of the Cytochrome *b* Mutations on Sensitivity toward Ilicicolin H—To confirm that the resistance conferring mutations altered the efficacy of ilicicolin inhibition of bc_1 activity and to quantify the magnitude of any such changes, ubiquinol-cytochrome *c* reductase activities of mitochondrial membranes from the mutants were measured in the presence of increasing concentrations of ilicicolin H. The resulting inhibitor titration curves are shown in Fig. 5 and the IC_{50} values are compiled in Table 2. Whereas the ilicicolin H concentration required to inhibit 50% of the activity (IC_{50}) of the enzyme in membranes from the wild-type strain was 12 nM (Table 2), the IC_{50} values for the strains carrying the L198F, G37S, and G37D mutations increased to 20, 26, and 34 nM, respectively. The remaining four strains, carrying either the S20T, S20L, Q22E, or Q22T mutations exhibited IC_{50} values higher than 50 nM ilicicolin H. The titrations of the cytochrome *c* reductase activities with the inhibitor thus confirm *in vitro* the altered inhibitor sensitivity of these strains seen *in vivo*.

DISCUSSION

It was shown previously that ilicicolin H is a potent inhibitor of the Q_n site of the cytochrome bc_1 complex in yeast (8). Several cytochrome *b* mutations conferring resistance to Q_n site inhibitors, antimycin (22, 24, 25), funiculosin (13, 23, 26, 27), and diuron (22, 28) had been described, but many of the strains carrying these mutations are no longer available. We decided to use ilicicolin H for a mutant screen in *S. cerevisiae* to identify determinants of ligand binding, and to gain further insights into structure/function relationships at center N. Amino acids identified by mutational changes affecting ilicicolin H binding may also overlap the semiquinone binding site, since this inhibitor, like antimycin (15), apparently displaces the bound semiquinone.³

³ K. Zwicker, R. Covian, and B. L. Trumppower, unpublished results.

Ilicicolin H Resistance in Yeast

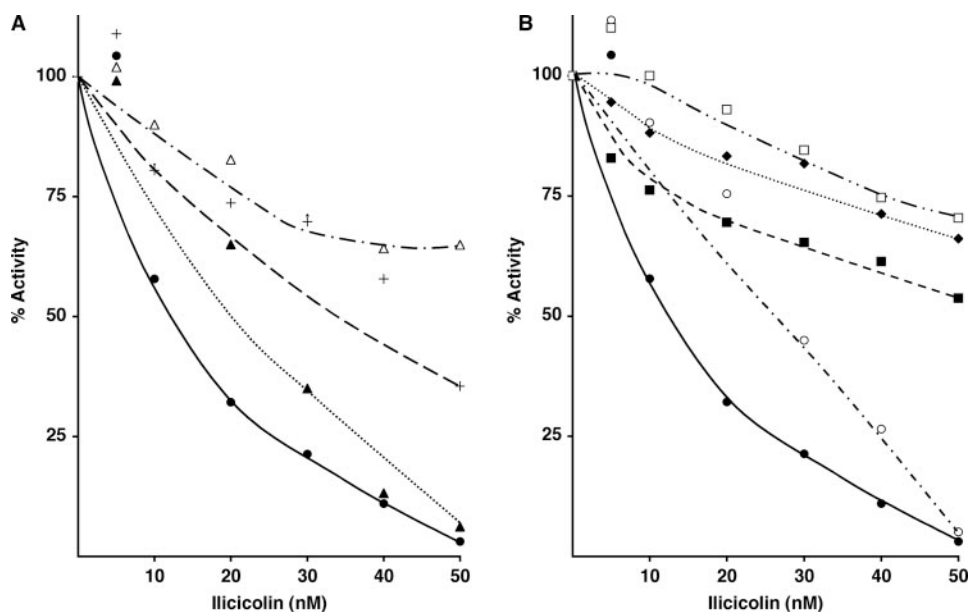


FIGURE 5. Ilicicolin H inhibition efficacy tested on mitochondrial membranes from wild-type and ilicicolin H-resistant strains. Ubiquinol-cytochrome *c* reductase activities of mitochondrial membranes of wild-type and mutant strains were measured in the presence of increasing concentrations of ilicicolin H. Activities are expressed as percentage of the activity of the corresponding non-inhibited mitochondrial membranes, which are listed in Table 2. For clarity, the results are presented in two graphs, *A* and *B*, with the data for the wild-type strain included in both for comparison. The data in *A* are for membranes from the wild-type strain (solid circles), and the S20L (open triangles), G37D (crosses), and L198F (solid triangles) mutant strains. The data in *B* are for the wild-type strain (solid circles), and the Q22T (open squares), S20T (solid diamonds), Q22E (solid squares), and G37S (open circles) mutant strains.

After an exhaustive mutant screen, the selection yielded seven different mutations. All of the mutations obtained are novel, except for L198F, which had been previously characterized as conferring funiculosin resistance (23). It appears that there are several hotspots within the cytochrome *b* gene where mutations occurred. These include serine at position 20, glutamine at position 22, and glycine at position 37. Two different mutational changes were found at each of these locations. The codons for these three amino acid positions are located in exon 1 of the cytochrome *b* gene, whereas the codon for leucine 198 is in exon 4. Genetic complementation tests were performed to exclude the possibility that there were additional mutations contributing to the ilicicolin H resistance.

The titration experiments with ilicicolin H confirmed that the cytochrome *bc*₁ complexes of the mutated strains were resistant to the inhibitor. The IC₅₀ values for inhibition of cytochrome *c* reductase activity increased from 12 nM in mitochondrial membranes from the wild-type strain to higher than 50 nM with membranes from the strains with mutations to serine 20 or glutamine 22.

Examination of the crystal structure of the yeast *bc*₁ complex confirmed that positions 20, 22, and 198 are in close proximity to the ubiquinone bound at the Q_n site, while glycine 37 is more distant from the Q_n site than the other three mutational locations. Substitution of this glycine may result in transmission of a conformational change through helix A into the center N pocket.

The strains with the S20L and G37D mutations grew poorly on a non-fermentable carbon source. In contrast, the S20T, Q22E, Q22T, G37S, and L198F mutant strains grew normally. In addition, the *bc*₁ activities in the membranes from the S20L and G37D mutants were decreased to approximately half of that of the wild-type membranes. The effect of the S20L muta-

tion on catalytic activity is not surprising, since the hydroxyl group of the serine side chain extends to within 5 Å of the ubiquinone ring, and its replacement with the more bulky leucine side chain might be expected to hinder ubiquinone access to the Q_n site by steric interference. It is interesting to note that the bovine *bc*₁ complex has leucine at the equivalent position and is highly resistant to ilicicolin H (8), but the activity of the bovine enzyme is not compromised. This difference may reflect differences in how ubiquinone is bound at center N in the yeast and bovine enzymes. In the yeast enzyme there is a water-mediated hydrogen bond between the quinone ring and histidine 202 (20), and it is conceivable that replacement of serine 20 with leucine could displace this water and thus interfere with ubiquinone reduction. In the bovine enzyme the equivalent histidine forms a hydrogen bond directly to the quinone ring, and the leucine does not interfere with this hydrogen bond (29). However, since ubiquinone reduction at center N is considerably faster than ubiquinol oxidation at center P (2), we cannot exclude the possibility that the S20L mutation has long-range structural effects on center P in the yeast enzyme.

It is also interesting that the Q22E substitution seems to be well tolerated, since the oxygen atom of the glutamine side chain is within 3.5 Å of the quinone ring (see Fig. 2) and one would expect that inserting a negatively charged carboxyl group in an equivalent position would destabilize semiquinone anion at the Q_n site. In future experiments, we plan to examine the stability of the EPR detectable semiquinone and the kinetics of cytochrome *b* reduction by ubiquinol at center N when center P is blocked in the *bc*₁ complex isolated from this mutant.

The *bc*₁ activities for the two mutations that impair growth on the non-fermentable medium are still unexpectedly high, since a residual activity of the *bc*₁ complex of around 30% should be sufficient to allow normal growth in non-fermentable medium. An explanation could be that these mutations either lead to an assembly defect or render the *bc*₁ complex unstable.

The mutant strains were tested for temperature sensitive growth by incubation at 34 °C. The S20L mutation prevented growth at 34 °C, consistent with an assembly or stability defect in this strain. Surprisingly, the growth of this mutant at 30 °C was notably better when funiculosin was present. This result, which was quite reproducible, suggests that this mutation allows this inhibitor to bind to the *bc*₁ complex in a manner that stabilizes the enzyme but does not inhibit its activity.

Although the G37D mutation did not appear to be temperature sensitive in the absence of inhibitor, it did grow more slowly at 34 °C when ilicicolin H was present, suggesting that

this mutation may also compromise bc_1 stability. The growth of this mutant at 30 °C was also somewhat better when ilicicolin H was present, suggesting a similar stabilizing and non-inhibitory binding resulting from this mutation.

Glycine 37 appears to be a "hotspot" for resistance-conferring mutations, which has been recognized as such for some time (22) and must exert its effects by transmitting a structural change over a considerable distance to modify the Q_n site. Theoretically, at least seven different amino acids are possible at this position by single nucleotide changes (22). Mutational changes to this glycine known so far are the alteration to valine (22), cysteine⁴ (resistant toward ilicicolin H and antimycin), and, from this study, serine and aspartate. It is striking that one amino acid exchange at this position, to serine, does not change the bc_1 activity significantly, whereas the other exchange, to aspartate, has pronounced effects on both activity and growth of the strain on non-fermentable carbon source. The same is true for position 20. Replacing serine 20, an amino acid with an uncharged polar side chain, with threonine, also carrying a hydroxyl group, is a more subtle change than substituting it with leucine, an amino acid with a non-polar side chain. By introducing other mutations at these two positions, we should be able to gain additional insights into the functionality of center N.

Information of the type gleaned in this study may also be useful for exploiting the Q_n site as a target for drugs against human and plant pathogens. To date, only the Q_p site has been successfully utilized in this regard (7, 30). Finding differences in the amino acids that are crucial for the function of center N between a pathogen and its host is an essential first step in the development of potential drugs, and this is facilitated by the availability of the cytochrome *b* sequences of a large number of species, including several pathogens (31). There are already some striking examples of species-specific Q_n site inhibitor effects. For example, the yeast cytochrome bc_1 complex is much more susceptible to inhibition by ilicicolin H than its bovine counterpart (8). It is also known that some fish mitochondria are highly resistant to funiculosin (13), while this Q_n site inhibitor is moderately effective against the yeast enzyme.⁴

Testing cross-resistance of Q_n site mutations with several Q_n site inhibitors offers the opportunity to identify similarities and differences in binding determinants. This is also useful information for the design of drugs specifically targeting the Q_n site. Ilicicolin H and funiculosin have the same effect on the heme b_H absorption spectrum, in which the α band undergoes a blue shift of ~1 nm (8). Thus it was concluded that the pyridone ring, a structural feature that ilicicolin H and funiculosin share (8), plays a crucial role in binding to the Q_n site, and cross-resistances between these two inhibitors were expected.

Several Q_n site cross-resistances have been previously described. For example the mutation glycine 37 to valine confers weak resistance to antimycin and funiculosin in mouse cytochrome *b* (32), and asparagine 31 to lysine in yeast cytochrome *b* is resistant to antimycin (33) and diuron (34). Also, all diuron resistant yeast mutants are cross-resistant to HQNO, a

quinone analog (22, 34, 35). We thus expected that the mutations found in this study would confer cross-resistance to the other Q_n site inhibitors.

The S20L mutation conferred resistance to funiculosin and the L198F mutation conferred resistance to all three inhibitors; ilicicolin H, funiculosin, as already described (23), and antimycin, a resistance that had not been connected to this mutation before. Otherwise there was a surprising lack of cross-resistance for the other mutations identified here. This suggests that there must be significant differences in the binding determinants for the Q_n site inhibitors.

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⁴ M. Ding and B. L. Trumpower, unpublished results.