

# Combining Inhibitor Resistance-conferring Mutations in Cytochrome *b* Creates Conditional Synthetic Lethality in *Saccharomyces cerevisiae*\*<sup>§</sup>

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The mitochondrial cytochrome *bc*<sub>1</sub> complex is an essential respiratory enzyme in oxygen-utilizing eukaryotic cells. Its core subunit, cytochrome *b*, contains two sites, center P and center N, that participate in the electron transfer activity of the *bc*<sub>1</sub> complex and that can be blocked by specific inhibitors. In yeast, there are various point mutations that confer inhibitor resistance at center P or center N. However, there are no yeast strains in which the *bc*<sub>1</sub> complex is resistant to both center P and center N inhibitors. We attempted to create such strains by crossing yeast strains with inhibitor-resistant mutations at center P with yeast strains with inhibitor-resistant mutations at center N. Characterization of yeast colonies emerging from the cross revealed that there were multiple colonies resistant against either inhibitor alone but that the mutational changes were ineffective when combined and when the yeast were grown in the presence of both inhibitors. Inhibitor titrations of *bc*<sub>1</sub> complex activities in mitochondrial membranes from the various yeast mutants showed that a mutation that confers resistance to an inhibitor at center P, when combined with a mutation that confers resistance to an inhibitor at center N, eliminates or markedly decreases the resistance conferred by the center N mutation. These results indicate that there is a pathway for structural communication between the two active sites of cytochrome *b* and open new possibilities for the utilization of center N as a potential drug target.

The mitochondrial cytochrome *bc*<sub>1</sub> complex is an essential respiratory enzyme in oxygen-utilizing eukaryotic cells. The *bc*<sub>1</sub> complex oxidizes ubiquinol and reduces cytochrome *c* and couples this electron transfer reaction to proton translocation across the inner mitochondrial membrane, thereby forming the electrochemical gradient needed for the production of ATP (1).

In yeast, the *bc*<sub>1</sub> complex consists of 10 subunits, three of which carry redox prosthetic groups. Cytochrome *b* contains two b-type hemes, cytochrome *c*<sub>1</sub> contains a c-type heme, and

the Rieske protein contains an iron-sulfur cluster. The remaining seven proteins are supernumerary subunits that are not essential for the function of the enzyme because they are absent in the *bc*<sub>1</sub> complexes of bacteria (2, 3).

The core subunit of the *bc*<sub>1</sub> complex is cytochrome *b*, which is encoded on the mitochondrial genome. Cytochrome *b* forms the two reaction centers, center P and center N, which participate in the electron transfer activity of the *bc*<sub>1</sub> complex (1). Ubiquinol is oxidized at center P, the reaction center that is closer to the intermembrane space and thus to the positive side of the membrane. Ubiquinone is reduced at center N, the reaction center that is closer to the electronegative side of the membrane toward the mitochondrial matrix.

Both reaction centers are inhibited by specific inhibitors that are thought to mimic the natural substrates (4). Center P has medical relevance because it is the target of the antifungal and antimalarial drug atovaquone, a hydroxynaphthoquinone (5, 6). There are several mutations described in cytochrome *b* that render the pathogenic organisms resistant to atovaquone (7), and thus it would be desirable to develop drugs targeted to the *bc*<sub>1</sub> complex that are effective in atovaquone-resistant organisms or that are not likely to become ineffective due to acquired resistance. So far, center N has not been exploited as a drug target, although there are several naturally occurring inhibitors that block this site (8, 9).

Natural products that inhibit essential enzymes in parasites and fungi are of particular interest because the pharmacophores of these compounds may provide the starting point for the synthesis of drugs targeted to such pathogens, provided that structures can be designed with the appropriate discrimination between pathogen and host targets. We recently selected for *Saccharomyces cerevisiae* mutants with resistance to ilicicolin H, a new center N inhibitor isolated from the imperfect fungus *Cylindrocladium ilicicola* (10). One of the ilicicolin-resistant mutants, bearing an L198F cytochrome *b* mutation, was also resistant to two other potent center N inhibitors: funiculosin, an antibiotic produced by *Penicillium funiculosum* Thom (11), and antimycin, which is produced by various species of *Streptomyces* (12). Leu-198 is close to His-197, which is a ligand to the b<sub>H</sub> heme and close to the quinone ring of ubiquinone bound at center N (see below).

*S. cerevisiae* mutants resistant to center P inhibitors had been obtained in a screen performed decades ago (13–15), including yeast with F129L and L275F cytochrome *b* mutations conferring resistance to the center P inhibitor myxothiazol, an anti-

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fungal antibiotic produced by *Myxococcus fulvus*, a myxobacterium (15–17). The myxothiazol molecule contains two thiazole rings, and the part that structurally resembles ubiquinone and therefore is important for binding to the ubiquinol oxidation pocket at center P is the amide of the *E*- $\beta$ -methoxyacrylate pharmacophore (4).

Although there are numerous yeast strains with mutations that confer resistance to inhibitors acting at either center N or center P, currently there are no yeast strains where the *bc*<sub>1</sub> complex is resistant to both center N and center P inhibitors. To create strains containing resistance-conferring point mutations in both center N and center P, we crossed the haploid *S. cerevisiae* strain containing the ilicicolin resistance-conferring cytochrome *b* mutation L198F (located in exon 4) with strains containing myxothiazol resistance-conferring cytochrome *b* mutations, F129L (located in exon 1) and L275F (located in exon 6) (14). These three mutations were chosen for the crossings because they did not seem to have detrimental effects on respiration (10, 14).

The frequency at which “double resistant” colonies arise from such a cross will depend on the genomic distance between the resistance-conferring mutations, with the frequency increasing as the distance increases. As expected, the outcome of the crossing included diploid strains carrying no mutation in cytochrome *b* (i.e. the wild-type sequence was restored) or both mutations due to homologous crossing over, as well as either one of the parental mutations. When the phenotypes of the emergent strains were examined, we found that mutations that conferred resistance at either center N or center P when present as a single mutation in cytochrome *b* had antagonistic effects when present in combination such that resistance was eliminated or markedly decreased. This indicates that there is a structural communication between center P and center N and suggests that combinations of drugs targeted to center P and center N might be especially effective at preventing drug-resistant pathogens.

## MATERIALS AND METHODS

**Yeast Strains, Media, and Genetic Techniques**—The center N mutant strain used for crossing is W303-1B,  $\alpha$ , *ade2-1*, *his 3-11*, *his 3-15*, *trp1-1*, *leu 2-3*, *leu 2-112*, *ura 3-52*, *can 1-100* and contains the mutation L198F in cytochrome *b* (10). The two center P mutant strains used for crossing have the GM50-3C background (14, 15), *a*, *his1*, *trp2*, and contain either the mutation F129L or the mutation L275F. Media used for the growth of *S. cerevisiae* were YPD<sup>2</sup> and 2% glucose (Fisher Scientific); 1% yeast extract (United States Biological); 1% bacto-peptone (BD Biosciences); YPGA (YPD supplemented with 40 mg/liter adenine) (Sigma); YPGal (YPD supplemented with 2% galactose (Acros Organics) instead of glucose); N3 medium (non-fermentable carbon source) and 2% glycerol (LabChem Inc.); or 1% yeast extract, 1% bacto-peptone, 40 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 or base) prepared

according to the manufacturer's instructions (Bio 101, Inc.); and W0, 2% glucose, 0.67% yeast-nitrogen base without amino acids. For plates, 2% agar (Difco) was added.

Illicicolin H was obtained from the Merck sample repository, and myxothiazol was purchased from Sigma. The inhibitors were added as ethanolic solutions to agar-containing media at ~50 °C to obtain final concentrations of 5  $\mu$ M illicicolin H (10) and 4  $\mu$ M myxothiazol (14).

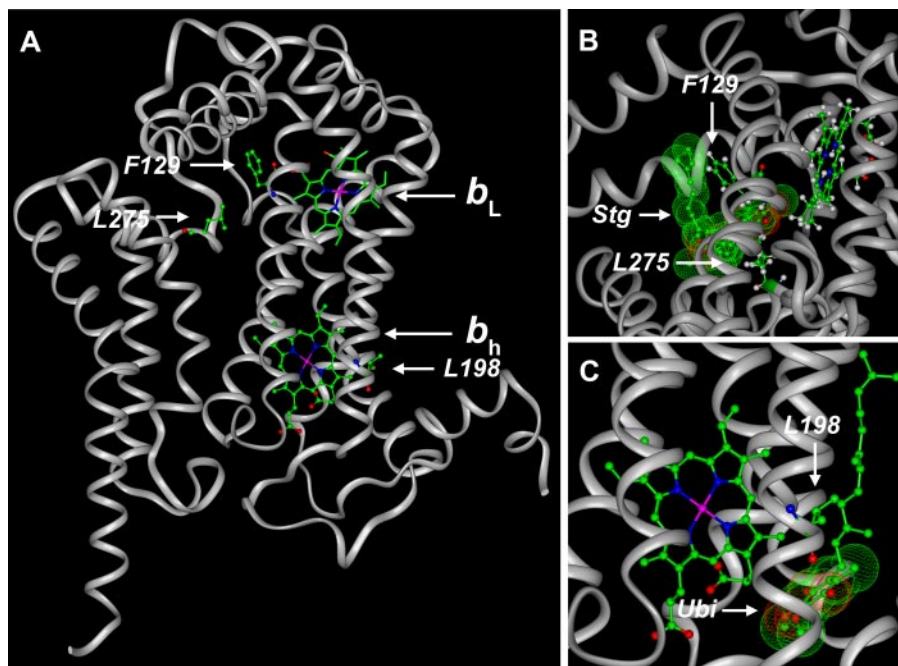
Strain L198F was crossed with strains F129L and L275F. To this end, 5-ml YPGA precultures of each strain were inoculated and incubated at 30 °C for 2 days. Approximately 100  $\mu$ l of each strain were added together in 5 ml of YPGA and incubated at 30 °C for several hours. Cells were recovered by brief centrifugation and left at 30 °C without shaking over night. The diploid strains were grown for at least 15 generations in W10 medium to obtain homoplasmic cells and then spread for single colonies on W0 medium. The emerging diploid colonies were then replica-plated on N3 medium, N3 medium supplemented either with 5  $\mu$ M illicicolin H or with 4  $\mu$ M myxothiazol, and N3 medium supplemented with both inhibitors in the above concentrations. Individual colonies of each type, i.e. sensitive, resistant to one inhibitor, and resistant to both inhibitors, were picked and used for further analysis.

**Sequencing**—The primers used for amplifying and sequencing exon 1 of the cytochrome *b* gene were: pMD26 (sense primer, upstream of ATG), 5'-TTT ATA TAT TTT TTA TTA ATT AAT ATA TAT AAA ATA TTA G-3'; pMD16 (antisense primer, the 3'-end covers the last two bases of exon 1), 5'-ATA ATA TAC TTA TAC TTG TCT CAC TC-3'. Additional sequencing primers are: pMD10 (sense primer), 5'-GAT ATT TAC ATG CAA ATG GTG C-3', and 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'. The primers for amplifying and sequencing exon 6 were: pMD15 (sense primer, in intron 5, the last 5 bases covering exon 6), 5'-GTT AAC ATA TAT AAA TTG TGT ACC-3', and pMD12 (antisense primer, 3'-end close to the stop codon), f5'-GAA TAA AAC ATT TTC AAT AGT AGA GAT AAC AGG-3'.

**Growth in Non-fermentable Medium**—The strains were grown to stationary phase in YPGA medium and washed, and the cultures were then used for the inoculation of non-fermentable medium (N3) to a starting  $A_{600}$  of 0.3. 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. Each yeast strain was inoculated and incubated in 500 ml of 2% YPGA, harvested in exponential phase, and used for mitochondrial membrane purification. Before breaking the cells, 1 mM diisopropylfluorophosphate was added to the buffer. The total buffer volume was twice the wet weight of the cell pellet. An equal volume of glass beads (Sigma, 425–600  $\mu$ 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.

<sup>2</sup> The abbreviations used are: YPD, yeast peptone dextrose; Ili, illicicolin; Myx, myxothiazol.



**FIGURE 1. Structure of cytochrome *b* showing center N and center P and the locations of the mutations that confer resistance to ilicicolin or myxothiazol.** Shown in *A* is the *S. cerevisiae* cytochrome *b* structure showing the location of the resistance mutations at center N and center P and the  $b_H$  and  $b_L$  hemes. Shown in *B* is center P with stigmatellin (*Stg*) bound and the Phe-129 and Leu-275 residues shown as ball and stick structures. The van der Waals radii of the stigmatellin atoms are also shown. Shown in *C* is the structure of center N with ubiquinone (*Ubi*) bound. The structure includes a portion of cytochrome *b* (gray) with Leu-198 shown as a ball and stick structure and the  $b_H$  heme and van der Waals radii of the quinone ring indicated. Carbon atoms are green, oxygen atoms are red, nitrogen atoms are blue, and the heme iron is pink. The figures were constructed from the coordinates of the yeast cytochrome  $bc_1$  complex crystal structure, Protein Data Bank code 1EZV (21).

**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  $25 \text{ mM}^{-1} \text{ cm}^{-1}$  at 563–578 nm (19).

**Measurement of Ubiquinol-Cytochrome *c* Reductase Activities and Inhibitor Titrations of Activities**—The assay buffer contained 50 mM potassium phosphate, pH 7.0, 250 mM sucrose, 2 mM EDTA, 1 mM  $\text{NaN}_3$ , and 0.01% dodecyl-maltopyranoside. Membranes were diluted to obtain a concentration of 5 nM cytochrome  $bc_1$  complex in assay buffer supplemented with 1 mM potassium cyanide and 30  $\mu\text{M}$  horse heart cytochrome *c*. The reaction was started by adding decyl-ubiquinol to a final concentration of 50  $\mu\text{M}$  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 DW2A<sup>TM</sup> spectrophotometer in the dual wavelength mode. An extinction coefficient of  $21.5 \text{ mM}^{-1}$  was used to calculate cytochrome *c* reduction at 550–539 nm (20). The activity of the  $bc_1$  complex after stirring 90 s without inhibitor was determined prior to each inhibitor titration. For each inhibitor concentration, measurements were taken in triplicate, and the average value was calculated. Turnover numbers were calculated on the basis of cytochrome *b* content. The non-inhibited enzymatic activity was set at 100%.

**Molecular Modeling**—The views of the cytochrome *b* subunit of the  $bc_1$  complex and of center P and center N of cytochrome *b* were constructed from the coordinates of the yeast

cytochrome  $bc_1$  complex (Protein Data Bank code 1EZV) with stigmatellin bound at center P (21) using the Discover 3<sup>®</sup> module within the Insight II<sup>®</sup> software package (Accelrys Inc., San Diego, CA).

## RESULTS

**Crossing Illicicolin *H*-resistant Yeast Strain L198F with the Myxothiazol-resistant Strains L129F and L275F**—We chose strain L198F as a representative for a center N mutant because the cytochrome *b* mutation it contains, leucine 198 to phenylalanine, is so far the only mutation known that confers resistance to three different center N inhibitors (10), which might be due to the fact that it is close to the ubiquinone binding pocket at center N (Fig. 1, *A* and *C*). Position 198 localizes to exon 4 of cytochrome *b* and is obviously important for the function of center N. Replacing leucine with phenylalanine did not impair respiration.

The center P mutants we picked for crossing carried the mutation

F129L or L275F in cytochrome *b* (Fig. 1, *A* and *B*). They were described in a previous study to confer resistance to the center P inhibitor myxothiazol (14). The mutation L275F has been widely described, and most respiratory organisms, except yeast, *Aspergillus* and *Neurospora crassa*, naturally carry a phenylalanine at this position (14). The Leu-275 amino acid position immediately follows the very conserved sequence motif known as the “PEWY loop” in center P that is crucial for ubiquinol oxidation as a ligand and proton acceptor (22). The mutation L275F is also known to confer resistance to atovaquone in the fungus *Pneumocystis jirovecii* (5, 23). Changing *S. cerevisiae* leucine 275 to phenylalanine rendered the protein highly resistant toward atovaquone (6), probably by steric interference stemming from the increased van der Waals radius of the aromatic ring.

The phenylalanine at position 129 is highly conserved among species. Mutating Phe-129 to arginine or lysine leads to impaired respiration (25), which is thought to be due to its postulated role in docking ubiquinol at center P (Fig. 1*B*). Its mutation to resistance-conferring amino acids, like leucine, mainly plays a role in plant pathogens (26).

To obtain strains that carry the L198F mutation in center N and a mutation in center P, the haploid strain L198F<sup>3</sup> was crossed with either haploid strain F129L or haploid strain L275F. The cells were grown for ~15 generations in minimal

<sup>3</sup> We have named the strains according to the cytochrome *b* mutation that they carry.

medium to obtain clones with homoplasmic mitochondrial DNA after potential homologous recombination events. This homologous recombination in the cytochrome *b* gene is illustrated in Fig. 2A, which shows the cytochrome *b* genes of the ilicicolin-resistant strain L198F and the myxothiazol-resistant strain F129L, whereas B shows the same scenario for strains L198F and L275F.

After crossing, the cells were spread on YPDA plates and replica-plated onto glycerol plates, glycerol plates containing ilicicolin H, glycerol plates containing myxothiazol, and gly-

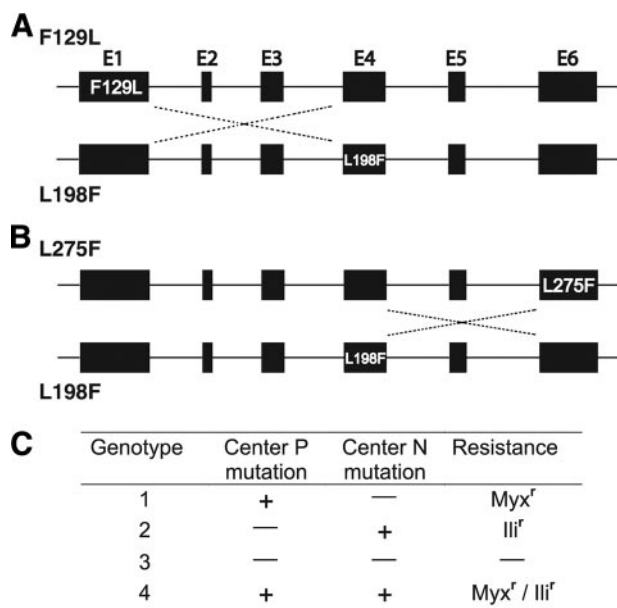
cerol plates with both drugs. The various theoretical cytochrome *b* genotypes and resistance phenotypes after crossing over are shown in Fig. 2C. One expected result is a diploid strain carrying the center P mutation only, resembling one parental strain. Another possibility is a diploid strain carrying the center N mutation, resembling the other parental strain. If there is only one crossing over event at the locus of one of the mutations, followed by homologous recombination, the expected outcome is a cytochrome *b* gene without any mutation, *i.e.* the wild-type sequence is restored, and a cytochrome *b* gene carrying both the L198F center N mutation and the center P mutation (either F129L or L275F, “double mutant”).

Obviously, double mutant strains can express resistance to both center P and center N inhibitors only if the combination of both mutations does not have a detrimental effect on respiration. In a previous study, it was speculated that the failure to obtain such double mutants using the strain L198F for crosses with different center P mutants conferring mucidin resistance might be due to respiratory deficiency (13). Therefore, in the current study, prior to testing their respiratory capacity and inhibitor resistance, the double mutant strains were first isolated in 10% glucose (*i.e.* pure fermenting conditions) and in the absence of drugs. In this way, and after complete mitotic segregation of the cell progenies, we obtained all strains with the different single or double mutated or wild-type cytochrome *b* genotypes depicted in Fig. 2C, both for the cross of L198F with F129L and for the cross of L198F with L275F. The presence of the mutations was verified by sequencing the cytochrome *b* genes. Importantly, for each cross, all the resulting diploid strains retained for phenotypic and biochemical analysis were isonuclear and differed only by the cytochrome *b* mutations.

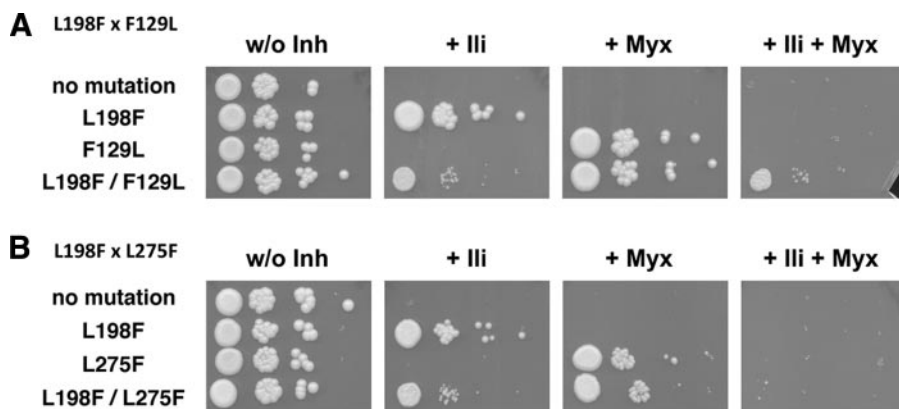
*Resistance Phenotypes of the Mutant Strains*—Serial dilutions of the diploid mutant strains resulting from the crosses were grown on plates containing glycerol and glycerol including either 5  $\mu\text{M}$  ilicicolin H or 4  $\mu\text{M}$  myxothiazol or both (*i.e.* 5  $\mu\text{M}$  ilicicolin H and 4  $\mu\text{M}$  myxothiazol). Each strain was applied on the plate in four different dilutions and tested for growth at 30 °C. The comparison with strains carrying single resistance-

conferring mutations and no mutations was necessary to evaluate the contribution of single mutations to the growth behavior of the strains carrying double mutations.

The results are depicted in Fig. 3. A shows the strains resulting from the cross of L198F x F129L, and B shows the strains resulting from the cross of L198F x L275F. All of the emergent strains grew equally well on glycerol alone, indicating no severe respiratory impairment, even for the double mutants. The ilicicolin plates revealed that the emergent strain with the L198F mutation showed the expected resistance to the drug, as seen by the same growth with 5  $\mu\text{M}$  ilicicolin in the plate (+*Ili*) as on glycerol alone (*w/o Inh*).



**FIGURE 2. Schematic representation of the yeast cytochrome *b* genes of the center P and center N mutant strains used in the crossing process, indicating the positions of the mutations and the possible outcomes regarding cytochrome *b*.** A and B depict the structures of the cytochrome *b* genes of the mutant strains that were crossed. The six exons of cytochrome *b* are labeled E1–E6, and the point mutations of the parent strains bearing a center N mutation, in both cases L198F, or a center P mutation, F129L (A) and L275F (B), respectively, are highlighted, and the possible crossing over events are indicated. C shows the corresponding outcome of the crossings regarding cytochrome *b*.



**FIGURE 3. Effect of the cytochrome *b* mutations on respiratory growth and resistance to ilicicolin H and myxothiazol.** The diploid strains resulting from the crossings of L198F x F129L (A) and L198F x L275F (B) were tested by serial dilutions on agar plates containing non-fermentable medium (glycerol) without inhibitor (*w/o Inh*) and with either ilicicolin H (+*Ili*) or myxothiazol (+*Myx*) or both inhibitors together (+*Ili* + *Myx*) added. Each strain was applied on the plate in four different dilutions, decreasing in cell density from left to right. The parent strains used for crossing are depicted on the upper left of each panel. The resulting cytochrome *b* mutant strains with their identifying mutations are listed to the left of the plates. Identical results were obtained in three repetitions.

## Conditional Synthetic Lethality in Cytochrome *b*

The two emergent strains with the myxothiazol resistance-conferring mutations, F129L and L275F, did not promote ilicicolin resistance, and like the strain with no mutation, failed to grow on the plate containing ilicicolin (Fig. 3, +*Ili*). Interestingly, after crossing the L198F strain with the F129L strain or with the L275F strain, the two double mutant strains (L198F/F129L and L198F/L275F) showed some resistance when grown on ilicicolin H plates, but the serial dilutions indicated that the double mutations were much less effective at conferring ilicicolin resistance when compared with the strain with the L198F mutation alone. This was true of the strains with double mutations emerging from either cross (Fig. 3, *A* and *B*). In other words, the presence of either the F129L or the L275F center P mutation compromised the efficacy of the L198F center N mutation in conferring ilicicolin resistance.

In contrast, the presence of the L198F center N mutation had no effect on the efficacy of the myxothiazol resistance-conferring F129L mutation in the strains emerging from the L198F x F129L cross. The yeast with the combination of L198F/F129L mutations was as resistant to myxothiazol as the yeast with the F129L mutation alone (Fig. 3*A*).

There was, however, a slight but consistent decrease in efficacy of myxothiazol resistance in the yeast with the L198F/L275F double mutations emerging from the L198F x L275F cross. This can be seen by comparing the growth on myxothiazol plates of the L198F/L275F double mutant strain with the L275F single mutant strain (Fig. 3*B*). It should be noted that although the growth differences seen here are subtle and represent only a single series of serial dilutions, they were consistently reproducible with multiple repetitions.

The most striking effect of combining the center N and center P mutations was revealed on the plates containing ilicicolin and myxothiazol. As expected, the wild-type yeast and single mutation bearing mutants could not grow on medium containing a combination of the two inhibitors (Fig. 3, *A* and *B*, +*Ili* + *Myx*). The yeast emerging from the cross of the L198F x F129L mutants and carrying the L198F/F129L double mutation grew slightly weaker in the presence of the two inhibitors than when ilicicolin was applied alone (Fig. 3*A*). This mimics the compromising effect of the F129L mutation on the resistance-conferring properties of the L198F mutation that was seen when this double mutant was grown in the presence of ilicicolin and is expected with the burden of two inhibitors applied at once.

The most dramatic effect of combining the center N and center P mutations was observed with the strain carrying the L198F/L275F double mutations, which could not grow at all when both drugs were applied (Fig. 3*B*). In this case, the combined compromising effect of the two mutations leads to a synthetic lethality on non-fermentable carbon sources containing ilicicolin + myxothiazol.

The same phenomenon occurred when we crossed the ilicicolin resistance-conferring center N mutation S20T with the F129L and L275F center P mutations. The presence of the F129L center P mutation in combination with the ilicicolin resistance-conferring S20T mutation significantly decreased the efficacy of ilicicolin resistance conferred by the latter mutation. This can be seen by comparing the serial dilutions of the

**TABLE 1**  
**Ubiquinol-cytochrome *c* reductase activities of mitochondrial membranes**

Shown are the ubiquinol-cytochrome *c* reductase activities of mitochondrial membranes prepared from the diploid strains resulting from the cross of L198F with F129L and L198F with L275F, respectively. WT, wild type. *cyt b*, cytochrome *b*.

| Resulting diploid strains ( <i>cyt b</i> mutation) | Activity |
|--|----------|
|  | $s^{-1}$ |
| <b>L198F x F129L</b>                               |          |
| WT (no mutation)                                   | 217 ± 5  |
| Illicolin <sup>r</sup> (L198F)                     | 205 ± 10 |
| Myxothiazol <sup>r</sup> (F129L)                   | 194 ± 8  |
| Ili <sup>r</sup> /Myx <sup>r</sup> (L1 98F/F129L)  | 145 ± 8  |
| <b>L198F x L275F</b>                               |          |
| WT (no mutation)                                   | 210 ± 11 |
| Illicolin <sup>r</sup> (L198F)                     | 207 ± 31 |
| Myxothiazol <sup>r</sup> (L275F)                   | 408 ± 10 |
| Ili <sup>r</sup> /Myx <sup>r</sup> (L1 98F/L275F)  | 319 ± 23 |

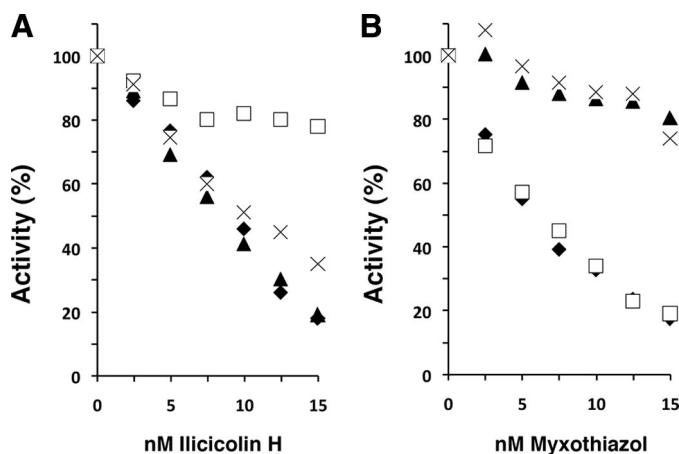
S20T/F129L mutant with the S20T mutant on plates containing ilicicolin (see supplemental Fig. 1*A*).

When the S20T mutation was crossed with the L275F mutation, the presence of both mutations resulted in less effective resistance against either ilicicolin or myxothiazol when compared with the resistance of either single resistance-conferring mutation. In addition, the strain containing the combined S20T/L275F mutations was not able to grow when both inhibitors were applied but could grow when either drug was added to the medium alone (supplemental Fig. 1*B*).

**Respiratory Competence of the Mutant Strains in Non-fermentable Medium**—When the strains emerging from the cross of the L198F x F129L and L198F x L275F mutant strains were grown in non-fermentable medium, the doubling times during exponential growth of all of the mutants were similar to those of the strains with no mutation (supplemental Fig. 2, *A* and *B*). The cell density as the cultures approached stationary phase was also similar for most of the mutants and the wild-type strains. The exception to this was the double mutant strain L198F/L275F, which reached stationary phase at a lower final cell density (supplemental Fig. 2*B*). This suggests that the combination of these two mutations might adversely affect the efficacy of energy transduction by the *bc*<sub>1</sub> complex in this mutant.

**Activities of the *bc*<sub>1</sub> Complexes in Mitochondrial Membranes of the Mutant Strains**—Ubiquinol-cytochrome *c* reductase activities were measured in mitochondrial membranes isolated from the various strains resulting from the crosses of the L198F x F129L and L198F x L275F mutant strains and are tabulated in Table 1. The activities of the *bc*<sub>1</sub> complexes in the emerging diploid strains that carried no mutation, whose genotypes are identical, were also identical, 217 when compared with 210  $s^{-1}$ . The ilicicolin H-resistant L198F strains emerging from the two crosses also displayed essentially identical activities of 205 and 207  $s^{-1}$ , which were not significantly different from the activities of the strains carrying no mutations. In contrast to the similar activities of the two ilicicolin H-resistant diploids, the diploid strain containing the L275F myxothiazol-resistant mutation had an activity of 408  $s^{-1}$ , which is almost twice as much as the activity of the diploid with no mutation emerging from the cross of L198F x L275F.

Interestingly, the combination of the L198F center N mutation and center P mutations emerging from both crosses

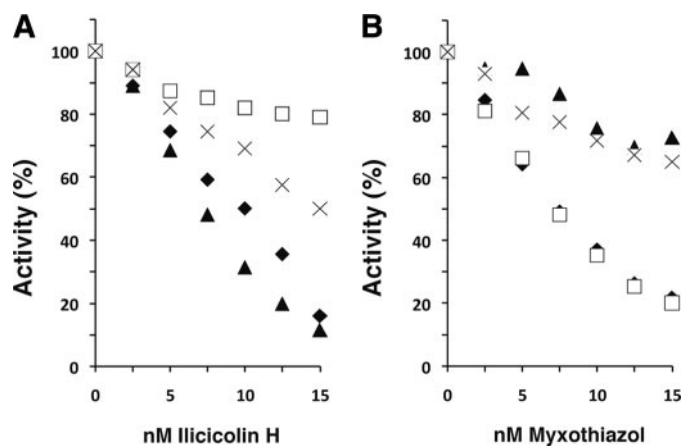


**FIGURE 4. Efficacy of inhibition by ilicicolin H and myxothiazol tested on mitochondrial membranes from the strains resulting from the crossing of L198F x F129L.** The ubiquinol-cytochrome *c* reductase activities of mitochondrial membranes of the strains resulting from the crossing of L198F x F129L mutants were measured in the presence of increasing concentrations of ilicicolin H (A) and myxothiazol (B). Activities are expressed as the percentage of the activity of the corresponding non-inhibited mitochondrial membranes, which are listed in Table 1. A shows the titration with ilicicolin H, in increments of 2.5 nM, up to 15 nM. B shows the myxothiazol titration of the same set of membranes. Data from the titration of membranes with no cytochrome *b* mutation are shown with *solid diamonds*, along with membranes with the L198F mutation (*open squares*), the F129L mutation (*solid triangles*), and the double mutation L198F/F129L (*Xs*).

adversely affected the activity of the *bc*<sub>1</sub> complex. The activity of the enzyme with the L198F/F129L double mutation was only about 67% of the activity of the enzyme with either of these single mutations. In addition, the combination of L198F/L275F mutations caused the high turnover of the enzyme with the L275F mutation to decline from 408 to 319 s<sup>-1</sup> (Table 1). This suggests that the L198F center N mutation exerts a long range effect on the rate of ubiquinol oxidation at center P. This might also account for the lower growth yield attributed to a decline in energy transduction efficacy in the *bc*<sub>1</sub> complex with the L198F/L275F mutation (supplemental Fig. 2A).

**Illicicolin H and Myxothiazol Resistance of the *bc*<sub>1</sub> Complexes in Membranes of the Mutant Strains**—To investigate the effects of mutations in one center of cytochrome *b* on the other center, the *bc*<sub>1</sub> complex activities of mitochondrial membranes of the mutants were titrated with each inhibitor and compared with the activities of membranes of the diploids that had no mutations. The data for the strains resulting from the cross of L198F x F129L are shown in Fig. 4. It can be seen in Fig. 4A that although the diploid with the myxothiazol resistance-conferring F129L mutation is just as sensitive to ilicicolin as the strain with no mutation, the strain with the L198F mutation is highly resistant toward ilicicolin H. Significantly, the diploid with the combination of L198F and F129L mutations is almost as sensitive to ilicicolin as the diploid strain with no mutations. This indicates a long distance impact of the F129L center P mutation on center N.

The myxothiazol titrations in Fig. 4B show that the F129L mutation confers myxothiazol resistance, and the L198F center N mutation does not affect the resistance conferred by the center P inhibitor. The diploid strain with the L198F/F129L mutations is just as resistant to myxothiazol as the strain with the F129L mutation alone. This result agrees with the growth of the



**FIGURE 5. Efficacy of inhibition by ilicicolin H and myxothiazol tested on mitochondrial membranes from the strains resulting from the crossing of L198F x L275F.** The ubiquinol-cytochrome *c* reductase activities of mitochondrial membranes of the strains resulting from the crossing of L198F x L275F mutants were measured in the presence of increasing concentrations of ilicicolin H (A) and myxothiazol (B). Activities are expressed as the percentage of the activity of the corresponding non-inhibited mitochondrial membranes, which are listed in Table 1. A shows the titration with ilicicolin H, in increments of 2.5 nM, up to 15 nM. B shows the myxothiazol titration of the same set of membranes. Data from the titration of membranes with no cytochrome *b* mutation are shown with *solid diamonds*, along with membranes with the L198F mutation (*open squares*), the L275F mutation (*solid triangles*), and the double mutation L198F/L275F (*Xs*).

resistant strains emerging from this cross on the plates containing the inhibitors (Fig. 3A).

The inhibitor titrations of *bc*<sub>1</sub> activities in mitochondrial membranes of the diploid strains resulting from the cross of L198F with L275F are shown in Fig. 5. As expected, the L198F center N mutation conferred significant resistance to ilicicolin (Fig. 5A). Strikingly, the L275F center P mutation seems to render the *bc*<sub>1</sub> complex hypersensitive toward ilicicolin H. In addition, the combination of the L275F center P mutation with the ilicicolin resistance-conferring L198F mutation partially eliminated ilicicolin resistance conferred by the latter mutation. These effects of the L275F mutation may account for the inability of the L198F/L275F double mutant to grow on plates when both drugs are applied together (Fig. 3B).

The titrations with myxothiazol of the strains emerging from the cross of the L198F x L275F mutants are shown in Fig. 5B. The L275F strain is resistant to myxothiazol, as expected, and the myxothiazol resistance is slightly compromised in the L198/L275F double mutant strain. In contrast to the increased sensitivity to ilicicolin conferred by the L275F mutation (Fig. 5A), the L198F strain is not hypersensitive toward myxothiazol, as indicated by identical titration curves for the diploid with the L198F mutation and the diploid with no mutation (Fig. 5B).

## DISCUSSION

Cytochrome *b* of the *bc*<sub>1</sub> complex is encoded on the mitochondrial genome and contains two active sites, center P where ubiquinol is oxidized and center N where ubiquinone is reduced (1). Both sites are essential for the activity of the enzyme, and there are numerous inhibitors that act at one of the sites and mutations that confer resistance to these inhibitors (4–6). To create yeast strains with mutations conferring resistance to inhibitors at both reactive sites, we created double

## Conditional Synthetic Lethality in Cytochrome *b*

mutant strains by crossing a strain carrying center N mutation L198F with the center P mutation strains F129L and L275F, respectively. We identified the diploid strains with double mutations by testing for resistance on inhibitor-containing glycerol plates. We then compared the inhibitor resistance, growth characteristics, and  $bc_1$  complex activities of the diploid strains containing double mutations with those of the diploid strains containing a single resistance-conferring mutation and those containing no mutations.

The strains containing the L198F/F129L and L198F/L275F double mutations did not impair respiratory-dependent growth on plates containing non-fermentable medium, although in previous studies, certain other mutations had detrimental effects on respiration (25). A lowered activity is not unusual for a cytochrome *b* mutant strain (9, 10, 25, 28) and might be seen as a trade-off between resistance and fitness, especially when the mutation is in center P, where the rate-limiting step occurs (29).

In the current study, the most notable change in activity was the L275F center P mutation, which increased the activity of the  $bc_1$  complex to twice that of the enzyme with no mutations. This effect was still present, albeit quenched, in the enzyme with a L198F/L275F double mutation. This came somewhat as a surprise because the mutations described so far in or near the PEWY loop motif, like mutation Y279C (25, 28, 30), usually show reduced activity. The PEWY loop, comprised of amino acids 271–274 of cytochrome *b*, is a conserved stretch of amino acids that is involved in ubiquinol oxidation and proton acceptance and thus pivotal for the function of center P (22).

Most organisms naturally carry a phenylalanine at position 275 (14). It is tempting to speculate that the increased activity that results from replacing leucine in the yeast cytochrome *b* background might be due to participation of the aromatic ring of the phenylalanine in docking ubiquinol for catalysis at center P. Whatever the basis for the enhancing effect of the L275F mutation on activity of the  $bc_1$  complex, it seems to be quite sensitive to the environment of the enzyme. Although the current activities were measured in mitochondrial membranes, in a previous study in which the enzyme with this mutation was purified from the membranes, the activity of the enzyme from the L275F mutant was identical to the activity of the enzyme from wild-type yeast (31).

The most striking finding from this study is that single mutations that confer resistance to inhibitors that act at center N or center P have a compromising effect on inhibitor resistance of diploid yeast containing combinations of mutations at the two reaction centers. Thus the F129L mutation at center P that confers myxothiazol resistance markedly decreased the ilicicolin resistance-conferring properties of the L198F mutation at center N. This was seen both in the growth characteristics on plates containing the inhibitors and in the inhibitor titrations of  $bc_1$  complex activities in mitochondrial membranes isolated from the L198F/F129L diploid strain.

A similar effect on inhibitor resistance was seen when the L198F mutation at center N was combined with the L275F mutation at center P. In this instance, the compromising effect was reciprocal in that the L275F mutation markedly decreased the ilicicolin resistance conferred by the L198F mutation, and

the L198F mutation slightly decreased the myxothiazol resistance conferred by the L275F mutation. Furthermore, the L275F mutation alone, which is located at center P, made the yeast hypersensitive to ilicicolin, which inhibits at center N. Consequently, the yeast strain with the L198F/L275F double mutation was unable to grow on plates containing ilicicolin + myxothiazol.

In the case of the center P and center N mutations characterized in this study, the interaction between the two centers appears to be asymmetric. For example, the F129L center P mutation has a much more pronounced effect on the ilicicolin resistance of the L198F mutation than does the L198F mutation on the myxothiazol resistance of the F129L mutation. This is seen in both the plate assays (Fig. 3) and the inhibitor titrations (Fig. 4). A similar asymmetry is seen in the interaction between the L275F center P mutation and the L198F center N mutation. In this case also, the center P mutation more strongly alters the resistance of the center N mutation than vice versa. Although it would not be warranted to extrapolate the results from this small number of mutations to all possible combinations of amino acid changes at the two centers, it is conceivable that the “structural plasticity” in center N that was noted in a previous study of inhibitor resistance (32) may allow for amino acid substitutions at this site without significant structural changes and hence without long range effects on center P.

Because the inhibitors share binding sites with the natural substrates, *i.e.* mimic these ligands (4, 6, 33), the enzyme from strains with double mutations provide a powerful tool to investigate how ligand binding and catalysis in one center have an effect on these processes in the other center. In this regard, we note that the effects of amino acid changes at one reaction center on binding of ligand at the other reaction center are reminiscent of the previously documented regulatory interactions between center N and center P (34, 35). In those studies, it was shown that binding of inhibitory ligands that mimic substrate at one center regulates half-of-the-sites reactivity at the other reaction center.

These interactions between the two reactions centers are quite striking because they span a considerable distance across the inner mitochondrial membrane. The distance between the backbone carbons of amino acids Leu-198 and Leu-275 is 26.3 Å, and the distance between those of Leu-198 and Phe-129 is 25.3 Å. A challenge for future experimentation will be to determine the amino acids that mediate this crucial communication over such a long distance. One possible approach to identify amino acids in the communication pathway would be to obtain revertants of the inhibitor sensitive double mutants that restore growth when both drugs are present.

The conditional synthetic lethality that we have described here also has profound implications for the design of drugs for treatment of pathogens such as *Plasmodium*, *Pneumocystis*, and *Toxoplasma gondii* infections because the  $bc_1$  complex is essential for viability in these pathogens. In the instance of *Plasmodium falciparum*, the parasite that causes malaria, it has been shown that the function of the  $bc_1$  complex is to ensure that ubiquinone remains oxidized to serve as an electron acceptor for dihydroorotate dehydrogenase (36).

Although there are currently drugs that target the ubiquinol oxidation center P in these organisms, there are currently no drugs directed to center N. It was shown in a previous study that species differences in sensitivity to natural product inhibitors acting at center N are sufficiently great to allow for successful drug design targeted to this center (32). If it is possible to design combinations of drugs targeted to center P and center N in these pathogens, it would be virtually impossible for the organism to develop resistance at the level of the  $bc_1$  complex to such drug combinations because a resistance-conferring mutation at one center would eliminate or significantly decrease the resistance conferred by a mutation at the other center.

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