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**Title:** *In vivo* emergence of high-level resistance during treatment reveals the first identified mechanism of amphotericin B resistance in *Candida auris*

**Keywords:** *Candida auris,* amphotericin B, resistance, *ERG6*, *in vivo* evolution

**Running Title:** Mutations in *Candida auris ERG6* confer amphotericin B resistance

**Authors:** Jeffrey M. Rybak[a, *], Katherine S. Barker[a], José F. Muñoz[b], Josie E. Parker[c], Suhail Ahmad[d], Eiman Mokaddas[d,e], Aneesa Abdullah[e], Rehab S. Elhagracy[f], Steve L. Kelly[c], Christina A. Cuomo[b], P. David Rogers[a, *]

[a] Department of Pharmacy and Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, Tennessee, USA
[b] Broad Institute of MIT and Harvard, Cambridge, MA, USA
[c] Centre for Cytochrome P450 Biodiversity, Institute of Life Science, Swansea University Medical School, Swansea, UK
[d] Department of Microbiology, Faculty of Medicine, Kuwait University, Jabriya, Kuwait
[e] Department of Microbiology, Ibn Sina Hospital, Shuwaikh, Kuwait
[f] Department of Hematology, Kuwait Cancer Control Center, Shuwaikh, Kuwait

*Corresponding Authors:
P. David Rogers, Pharm.D., Ph.D.
Member and Chair
Endowed Chair in Pharmaceutical Sciences
Department of Pharmacy and
Pharmaceutical Sciences
St. Jude Children's Research Hospital
262 Danny Thomas Place, Mail Stop 313
Memphis, TN 38105
dave.rogers@stjude.org
t. 901.595.7516
f. 901.595.3125

Jeffrey M. Rybak, Pharm.D., Ph.D.
Instructor
Department of Pharmacy
and Pharmaceutical Sciences
St. Jude Children's Research Hospital
262 Danny Thomas Place, Mail Stop 313
Memphis, TN 38105
jrybak@stjude.org
t. 901.595.2368
f. 901.595.3125

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1 **ABSTRACT**

2 *Candida auris* has emerged as a healthcare-associated and multidrug-resistant fungal
3 pathogen of great clinical concern. While as much as 50% of *C. auris* clinical isolates
4 are reported to be resistant to amphotericin B, to date, no mechanisms contributing to
5 this resistance have been identified. We report here mutations in the *C. auris* sterol-
6 methyltransferase gene, *ERG6,* as the first identified mechanism of amphotericin B
7 resistance in this emerging pathogen and describe the clinical case in which this high-
8 level amphotericin B resistance was acquired *in vivo* during therapy. Whole genome
9 sequencing revealed the four *C. auris* isolates obtained from this single patient case to
10 be genetically related and identified a mutation in *ERG6* as being associated with
11 amphotericin B resistance. Cas9-mediated genetic manipulations confirmed this
12 mutation alone to confer a >32-fold increase in amphotericin B resistance, and
13 comprehensive sterol profiling revealed an abrogation of ergosterol biosynthesis and a
14 corresponding accumulation of cholesta-type sterols in isolates and strains harboring
15 the clinically derived *ERG6* mutation. Together these findings represent the first
16 significant step forward in the understanding of clinical amphotericin B resistance in *C.*
17 *auris.*

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18 **INTRODUCTION**

19 In little over a decade, *Candida auris* has transformed from a newly identified
20 species of *Candida* originally associated with infections of the auditory canal, to being
21 recognized by the Centers for Disease Control and Prevention (CDC) as the first fungal
22 pathogen to represent an urgent level of threat to public health.[1-4] Recently found to
23 be the cause of outbreaks of invasive candidiasis on multiple continents and having
24 been isolated from patients in more than 45 countries, *C. auris* readily colonizes
25 patients and disseminates easily within healthcare facilities, changing the paradigm of
26 healthcare-associated fungal infections.[4, 5] Moreover, *C. auris* frequently exhibits
27 reduced susceptibility to multiple agents of the already limited antifungal
28 armamentarium.[1, 4, 6] While limited epidemiological and clinical outcomes data
29 currently preclude the establishment of true clinical breakpoints for the assessment of
30 *C. auris* antifungal susceptibility, the CDC has defined tentative antifungal breakpoints
31 using susceptibility data from hundreds of clinical *C. auris* isolates and available
32 pharmacokinetic-pharmacodynamic data. Applying these tentative breakpoints, more
33 than 90% of *C. auris* isolates are resistant to fluconazole (modal minimum inhibitory
34 concentration [MIC] ≥256 mg/L), 30 to 50% are resistant to amphotericin B, and
35 approximately 5% are resistant to echinocandins.[4, 7] Unfortunately, *C. auris* has also
36 demonstrated the capacity to rapidly acquire resistance to antifungals *in vivo*, leaving
37 clinicians with no reliable option for the treatment of infections caused by this emerging
38 public health threat.[8] While the fluconazole resistance frequently identified among
39 clinical isolates of *C. auris* has previously been associated with mutations in both
40 *ERG11* and *TAC1B,* and resistance to echinocandins has been associated with

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41 mutations in *FKS1*, to date, no mechanisms contributing to clinical amphotericin B
42 resistance have been identified in *C. auris*.[4, 6, 9-11]
43 We present here a case where a patient receiving treatment for a fluconazole-
44 resistant *C. auris* infection subsequently acquired amphotericin B-resistant, and later
45 echinocandin-resistant, disease following multiple courses of antifungal therapy.
46 Leveraging the clinical isolates from this single case, whole genome sequencing,
47 comprehensive sterol profiling, and Cas9-mediated genetic manipulations, we have
48 identified the first known mechanism of clinical amphotericin B resistance in *C. auris*
49 conferred by mutations in the sterol-methyltransferase gene, *ERG6.* Furthermore, we
50 show that the observed mutations in *ERG6* result in abolished biosynthesis of
51 ergosterol, the target of amphotericin B, and these mutations alone abrogate the activity
52 of this antifungal agent.
53
54 **MATERIALS AND METHODS**
55
56 **Isolate, strains, and growth media used in this study.** Isolation and species-specific
57 identification of the four clinical *C. auris* strains isolated from the patient cases included
58 in this study was performed as described elsewhere [12]. All laboratory-derived strains
59 and clinical isolates are listed in Supplementary Table 1 and were grown in YPD liquid
60 medium (1% yeast extract, 2% peptone, 2% dextrose) at 35$^{o}$C in a shaking incubator
61 unless otherwise indicated. Stocks of all strains and clinical isolates were prepared with
62 50% sterile glycerol and were maintained at -80$^{o}$C.
63

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64 **Whole genome sequencing and variant identification.** Clinical isolates were cultured
65 from glycerol stocks in YPD liquid media at 35 $^{o}$C, and genomic DNA was extracted as
66 previously described [13]. Genomic libraries were constructed and barcoded using the
67 NEBNext Ultra DNA Library Prep kit (New England Biolabs, Ipswich, MA, USA) per
68 manufacturer's instructions. Genomic libraries were sequenced using the Illumina HiSeq
69 2500 platform with the HiSeq Rapid SBS Kit v2 as previously described [14]. Read
70 quality and filtering was performed using FastQC v0.11.5 and PRINSEQ v0.20.3
71 (21278185) using "-trim_left 15 -trim_qual_left 20 -trim_qual_right 20 -min_len 100 -
72 min_qual_mean 25 -derep 14". Then, paired-end reads were aligned to the *C. auris*
73 assembly strain B8441 (GenBank accession PEKT00000000.2; (30559369)) using
74 BWA mem v0.7.12 (19451168) and variants were identified using GATK v3.7
75 (20644199) with the haploid mode and GATK tools (RealignerTargetCreator,
76 IndelRealigner, HaplotypeCaller for both SNPs and indels, CombineGVCFs,
77 GenotypeGVCFs, GatherVCFs, SelectVariants, and Variant Filtration). Sites were
78 filtered with Variant Filtration using "QD < 2.0 || FS > 60.0 || MQ < 40.0". Genotypes
79 were filtered if the minimum genotype quality < 50, percent alternate allele < 0.8, or
80 depth < 10 (https://github.com/broadinstitute/broad-
81 fungalgroup/blob/master/scripts/SNPs/filterGatkGenotypes.py). Genomic variants were
82 annotated, and the functional effect was predicted using SnpEff v4.3T (22728672).
83
84 **Plasmid construction and repair template preparation.** CRISPR-Cas9-mediated
85 gene editing was performed using the transient episomal plasmid-based system

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86 described previously and the plasmid pJMR17v3 [15, 16]. Guide DNAs used as inserts
87 in pJMR17v3 and DNA primers used in repair template generation are listed in Table 2.
88
89 **Strain construction.** Electrocompetent cells were prepared as previously described
90 [10] and were mixed in an electroporation cuvette with ~10 μg appropriate repair
91 template DNA (containing the desired/introduced *ERG6* sequence) and ~10 μg
92 appropriate plasmid (cloned with the guide DNA matching the *ERG6* sequence of the
93 isolate being electroporated) prior to electroporation with a BioRad GenePulser
94 (BioRad, Hercules, CA). One milliliter 1M sorbitol was used to transfer transformation
95 mixtures from the cuvette to a culture tube containing 1mL YPD, and transformants
96 were allowed to recover for 4-6h at 35$^{o}$C with shaking. Aliquots of the recovered
97 transformants were spread on YPD agar plates supplemented with 200 μg/ml
98 nourseothricin (Nou200) and incubated at 35$^{o}$C until colonies formed. Single colonies
99 were picked from transformation plates and patched sequentially on YPD agar. Once
100 the *ERG6* sequence was verified by Sanger sequencing, desired colonies were cultured
101 in 2mL YPD overnight at 35$^{o}$C and subsequently streaked for single colonies on YPD
102 agar. Colonies were then replica plated on both YPD agar and Nou200 to confirm
103 nourseothricin susceptibility due loss of the pJMR17v3 plasmid.
104
105 **Sanger sequencing.** Genomic DNA was isolated from colonies with the appropriate
106 growth phenotype and was used to amplify the *ERG6* ORF in PCR with primers
107 CAU0013J01m and CAU0014J01m (Table 2) and Phusion Green master mix per
108 manufacturer's instructions (Thermo Scientific, Waltham, MA). PCR amplicons were

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109 then used as templates in Sanger sequencing reactions primed with sequencing
110 primers (Supplementary Table 2) and run on an 3730xl DNA Analyzer (Applied
111 Biosystem, Foster City, CA) using standard DNA sequencing chemistries.
112
113 **Comprehensive sterol profiling.** Laboratory-derived strains and the parental clinical
114 isolates were grown to the exponential-growth phase at 35ºC in RPMI liquid medium.
115 Alcoholic KOH was used to extract nonsaponifiable lipids. A vacuum centrifuge (Heto)
116 was used to dry samples, which were then derivatized by adding 100 μl 90% N,O-
117 bis(trimethylsilyl)-trifluoroacetamide-10% tetramethylsilane (TMS) (Sigma, St. Louis,
118 MO) and 200 μl anhydrous pyridine (Sigma) while heating at 80ºC for 2h as previously
119 described [10, 17]. Gas chromatography-mass spectroscopy (GC-MS) (with a Thermo
120 1300 gas chromatography system coupled to a Thermo ISQ mass spectrometer
121 (Thermo Scientific) was used to analyze and identify TMS-derivatized sterols through
122 comparison of the retention times and fragmentation spectra for known standards.
123 Sterol profiles for each sample were determined by analyzing the integrated peak areas
124 from GC-MS data files using Xcalibur software (Thermo Scientific). All sterol analysis
125 was performed in biological triplicate. Error bars for each data point represent the
126 standard deviations of results from three independent measurements of technical
127 replicates.
128
129 **MICs by Elipsometer test (E-test).** E-tests were performed to determine amphotericin
130 B MICs as per manufacturer's instructions (Biomerieux USA, Chicago, IL) with

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131 modifications as recommended by the Clinical Laboratory Standards Institute. All
132 susceptibility testing was performed in biological duplicate.
133
134 **RESULTS**
135
136 ***Patient case and antifungal susceptibility testing of clinical C. auris isolates.***
137 A 33-year old female receiving treatment for Hodgkin's lymphoma and advanced stage
138 nodular sclerosis was admitted to the hospital after complaints of respiratory distress. A
139 high-resolution chest CT revealed bilateral diffuse alveolar disease, ground glass
140 opacities, and mild right-sided pneumothorax. The patient was empirically initiated on
141 broad spectrum antimicrobials including meropenem, linezolid, and liposomal
142 amphotericin B (dosed at 5mg/kg) (**Figure 1**). Both bronchoalveolar lavage fluid and
143 endotracheal tube cultures would subsequently grow *C. albicans*, and liposomal
144 amphotericin B was continued for a total of 3 weeks. The patient's condition improved,
145 and chemotherapy was resumed approximately one month after the completion of
146 amphotericin B therapy.
147 Subsequently, the patient developed dyspnea and alveolar hemorrhage and
148 treatment with meropenem and methylprednisolone was initiated. Cultures of
149 endotracheal tube (ETT) secretions grew yeast (Isolate 1) initially identified as *Candida*
150 *famata* by Vitek2, but later revealed to be *C. auris* by internal transcribed spacers (ITS)
151 sequencing. When applying the tentative CDC *C. auris* breakpoints, Isolate 1 was
152 resistant to fluconazole but susceptible to caspofungin and amphotericin B. A 2-week
153 course of caspofungin treatment was initiated, to be followed by a 2-week course of

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154 liposomal amphotericin B (5mg/kg/day) when ETT cultures remained positive. Following
155 this course of amphotericin B, all respiratory cultures remained negative for *C. auris*,
156 however the patient remained on broad spectrum antibiotics including piperacillin-
157 tazobactam, amikacin, and colistin for a complicated course of treatment for a
158 carbapenem-resistant *Pseudomonas aeruginosa* respiratory tract infection.

159 One month later, the patient developed a urinary tract infection and cultures grew
160 yeast initially identified as *Candida haemulonii*, but later confirmed as *C. auris* by ITS
161 sequencing (Isolate 2). Isolate 2 was highly resistant to both fluconazole and
162 amphotericin B but remained susceptible to caspofungin (**Table 1**), and a 2-week
163 course of caspofungin was initiated. After the completion of caspofungin treatment,
164 urine cultures again grew *C. auris* (Isolate 3), and a 3-week course of liposomal
165 amphotericin B (5 mg/kg/day) was initiated. Ten days into this course of amphotericin B,
166 urine cultures again grew *C. auris* (Isolate 4). Intriguingly, while Isolate 4 was resistant
167 to both fluconazole and caspofungin, it had regained susceptibility to amphotericin B
168 (**Table 1**). Shortly after the completion of amphotericin B therapy, the patient was
169 transferred to another medical facility for further treatment and was lost to follow-up.
170

171 ***Whole genome sequencing.***

172 Whole genome sequencing was performed as previously described on the four
173 isolates and revealed all isolates to belong to Clade I (subclade b), with four or fewer
174 single-nucleotide polymorphisms or insertions/deletions (indels) separating any two
175 isolates, consistent with all isolates being genetically related (**Table 2**).[11] All isolates
176 were found to have mutations previously associated with fluconazole resistance in the

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177 *ERG11* and *TAC1B* genes, encoding the Y132F and A583S amino acid substitutions,
178 respectively. The two isolates exhibiting high-level (MIC >32mg/L) amphotericin B
179 resistance, Isolates 2 and 3, were both found to have an indel mutation in the sterol-
180 methyltransferase gene, *ERG6*, resulting in an early stop codon and likely nonsense
181 transcript (YY98V*). Intriguingly, the terminal amphotericin B-susceptible isolate, Isolate
182 4, was found to retain this indel mutation in *ERG6,* but have also acquired a duplication
183 of two nucleotides, resulting in a full length *ERG6* transcript with 3 altered amino acid
184 residues (encoding RYY97LVS). Isolate 4 was also found to have a novel mutation
185 (encoding D642Y) in hot-spot 1 of the gene encoding β-D-glucan synthase, *FKS1*.
186
187 ***Impact of ERG6 mutations on Amphotericin B susceptibility.***
188 To test the influence of the identified mutations in *C. auris ERG6* on amphotericin B
189 susceptibility, a transient Cas9-mediated *C. auris* genetic manipulation system was
190 used to perform allelic exchange. Transformations were performed by electroporation,
191 two independent transformants were obtained for each allele exchange, and all
192 transformants were confirmed by Sanger sequencing as previously described [10, 11].
193 Introduction of the YY98V* encoding indel mutation into the *ERG6* gene of Isolates 1
194 and 4 resulted in a ≥32-fold increase in amphotericin B MIC in both backgrounds
195 (**Figure 2**) and no difference was observed between biological replicate transformants
196 (data not shown). Conversely, introduction of the wildtype (matching the antifungal
197 susceptible B8441 reference sequence) *ERG6* sequence into both Isolates 2 and 3
198 resulted in a complete restoration of amphotericin susceptibility (≥32-fold reduction in
199 MIC), confirming the role of this mutation in the high-level resistance observed.

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200

201 ***Comprehensive sterol profiling.***

202 As amphotericin B is known to exert an antifungal effect by directly binding to the
203 predominant sterol of most medically relevant fungi, ergosterol, and the YY98V*
204 encoding mutation in *ERG6* was hypothesized to result in an abrogation of sterol-
205 methyltransferase activity, we next performed comprehensive sterol profiling to
206 investigate changes in cellular sterol composition using methods as previously
207 described [11]. Consistent with previously reported *C. auris* cell sterol compositions, all
208 amphotericin B-susceptible isolates and strains were observed to have ergosterol as the
209 predominant sterol (>60%), with ergosta-type sterols (such as ergosta-5,7-dienol and
210 ergosta-5,8,22,24(28)-tetraenol), and early sterols (such as lanosterol and zymosterol)
211 comprising the majority of remaining sterols (**Figure 3**). Conversely, all isolates and
212 strains with the amphotericin B resistance-conferring YY98V* mutation in *ERG6* were
213 found to have sterol profiles completely devoid of ergosta-type sterols. Instead,
214 cholesta-5,7,24-trienol was found to be the most abundant sterol (>60%) in these
215 samples, with the remainder of sterols being comprised of other cholesta-type sterols
216 (such as cholesta-5,8,22,24-tetraenol and cholesta-7,24-dienol) and early sterols. Thus,
217 the observed resistance to amphotericin B correlates directly with the abrogation of the
218 production of ergosterol observed.

219

220 **DISCUSSION**

221 The patient case presented here exemplifies the challenges antifungal resistance
222 poses to clinicians treating patients with *C. auris* infections, and for the first time

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223 identifies mutations in the *C. auris* sterol-methyltransferase gene, *ERG6,* as a
224 mechanism conferring high-level amphotericin B resistance. These findings were
225 supported by allelic replacement studies. The *ERG6* mutations described here resulted
226 in a dramatic shift in *C. auris* cell sterol profiles, diverting sterol production toward
227 cholesta-type sterols more similar to those found in mammalian cell membranes.
228 Intriguingly, while we have previously reported the combination of mutations in *Candida*
229 *glabrata ERG2* and *ERG6* as being associated with both sterol profiles favoring
230 cholesta-type sterols and increased amphotericin B resistance, the level of amphotericin
231 B resistance observed in *C. glabrata* clinical isolates was significantly lower (MICs of 1
232 to 4 mg/L) than that which we observed in the *C. auris* clinical isolates in these studies
233 (MIC >32 mg/L)[18]. It remains to be seen whether this stark difference in the magnitude
234 of amphotericin B resistance is due to differences in other cell membrane lipids or
235 corresponds to how these distantly related species of *Candida* respond to the stress
236 induced by amphotericin B. Further research is needed to determine the prevalence of
237 *ERG6* mutations among amphotericin B-resistant clinical isolates of *C. auris.*
238
239 **NOTES**
240
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242 or the decision to submit the work for publication.
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249

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252 the editors consider relevant to the content of the manuscript have been disclosed.
253

254 ***Institutional review board statement*****:** The clinical samples were obtained after
255 obtaining verbal consent only as part of routine patient care and diagnostic work-up for
256 the isolation and susceptibility testing of bacterial and fungal pathogens. The study was
257 approved by the Health Sciences Center Ethical Committee, Kuwait University
258 (approval letter VDR/EC/3724).
259

260 ***Informed consent statement*****:** The need for informed consent was waived by the
261 Health Sciences Center Ethical Committee, Kuwait University as the results are
262 reported on deidentified samples without revealing patient identity.
263

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265 conception of experimental designs and methodological development. SA, EM, AA, and
266 RSE oversaw or contributed to the clinical and microbiological care of the patient
267 described in this work. JMR, KSB, JFM, JEP, SA, EM, AA, and CAC performed
268 experiments and analyzed data. JMR, SA, SLK, CAC, and PDR oversaw and the

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269 planning and execution of these studies. JMR, SLK, CAC, and PDR contributed to the
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271
272

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## FIGURES

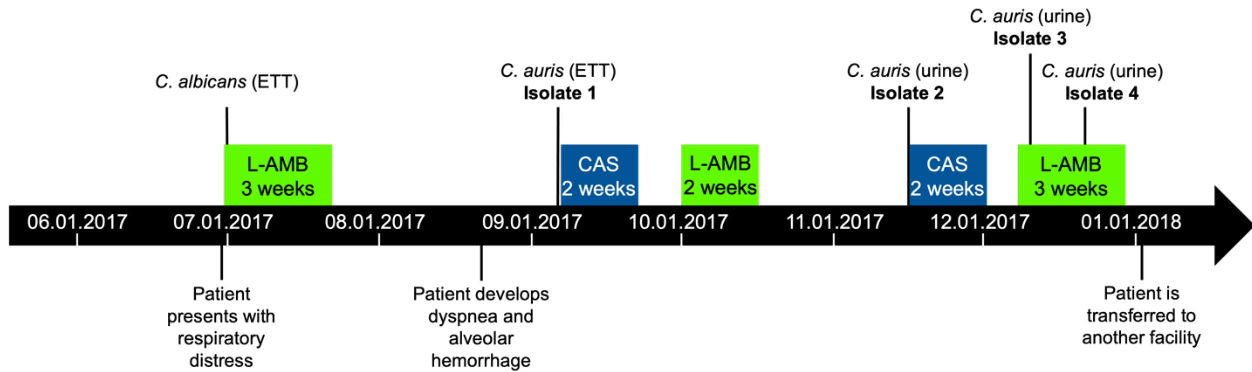


**Figure 1. High-level amphotericin B resistance develops during treatment of a *C. auris* invasive infection.** Timeline for the isolation of *Candida* clinical isolates and antifungal treatment administered to the patient. L-AMB: liposomal amphotericin B; CAS: caspofungin; ETT: endotracheal tube

| Gene ID | Gene Name | Mutation Type | Amino acid Substitution(s) | Gene Description | Isolate 1 | Isolate 2 | Isolate 3 | Isolate 4 |
|---|---|---|---|---|---|---|---|---|
| B9J08_005340 | *ERG6* | Indel | R97fs | Ortholog(s) have sterol 24-C-methyltransferase activity, role in ergosterol biosynthetic process and endoplasmic reticulum, lipid droplet localization | | ■ | ■ | ■ |
| B9J08_005340 | *ERG6* | Indel | Y98fs | Ortholog(s) have sterol 24-C-methyltransferase activity, role in ergosterol biosynthetic process and endoplasmic reticulum, lipid droplet localization | | | | ■ |
| B9J08_003401 | - | SNP | M1768K | Ortholog(s) have calcium channel activity, role in calcium ion import across plasma membrane, calcium ion transport, conidium formation, fungal-type cell wall organization, unidimensional cell growth and plasma membrane localization | | | ■ | |
| B9J08_000964 | *FKS1* | SNP | D642Y | Putative 1,3-beta-D-glucan synthase | | | | ■ |
| B9J08_001448 | *ERG11* | SNP | Y132F | Putative sterol 14-demethylase activity; mutations involved in fluconazole resistance | ■ | ■ | ■ | ■ |
| B9J08_004820 | *TAC1B* | SNP | A583S | Has domain(s) with predicted DNA binding, DNA-binding transcription factor activity, RNA polymerase II-specific, zinc ion binding activity and role in regulation of transcription, DNA-templated, transcription, DNA-templated | ■ | ■ | ■ | ■ |

**Figure 2. Whole genome sequencing reveals an association between mutations in *C. auris ERG6* and high-level amphotericin B resistance.** Non-synonymous mutations differing between *C. auris* clinical isolates in this study, and those previously associated with antifungal resistance are shown with gene identifier, gene name, mutation type, encoded amino acid substitution(s), and gene description (as listed on the Candida Genome Database). Boxes filled in blue indicate the presence of the listed mutation in the corresponding clinical isolate.

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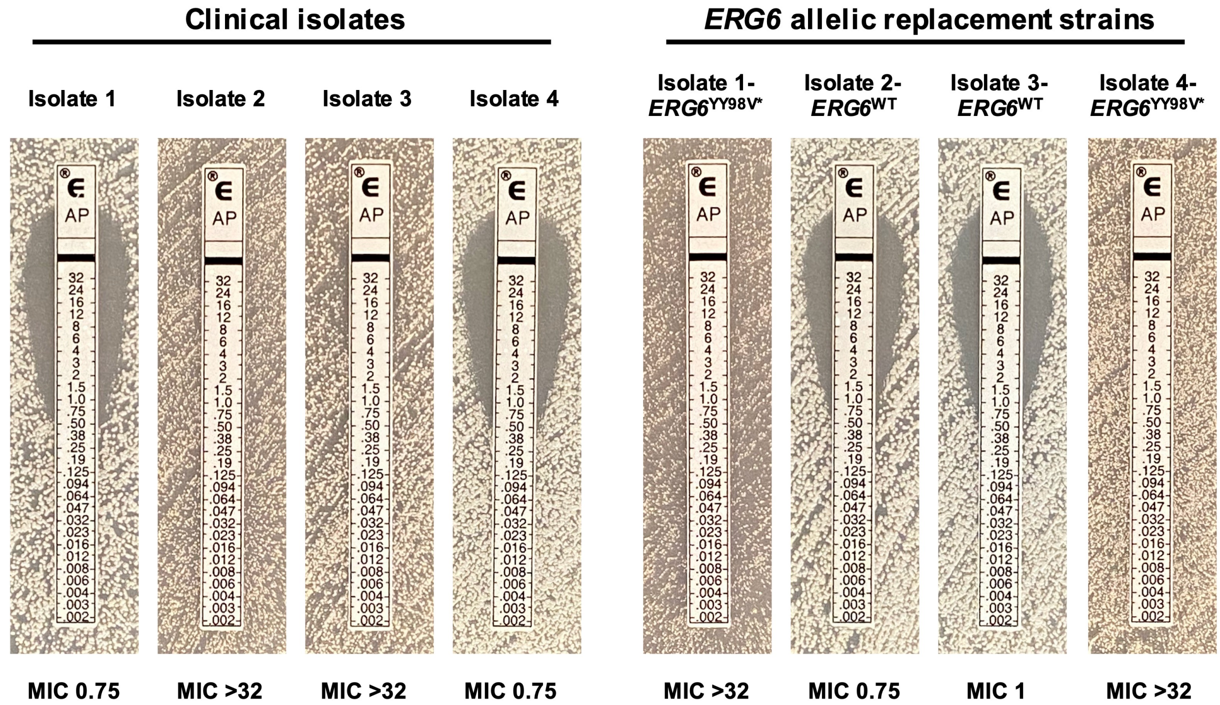


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379 **Figure 3. Mutations in *C. auris ERG6* confer high-level amphotericin B resistance.**
380 Amphotericin B MIC as determined by Etest for each of the clinical isolates and the
381 *ERG6* allelic replacement strains constructed in these studies are shown.
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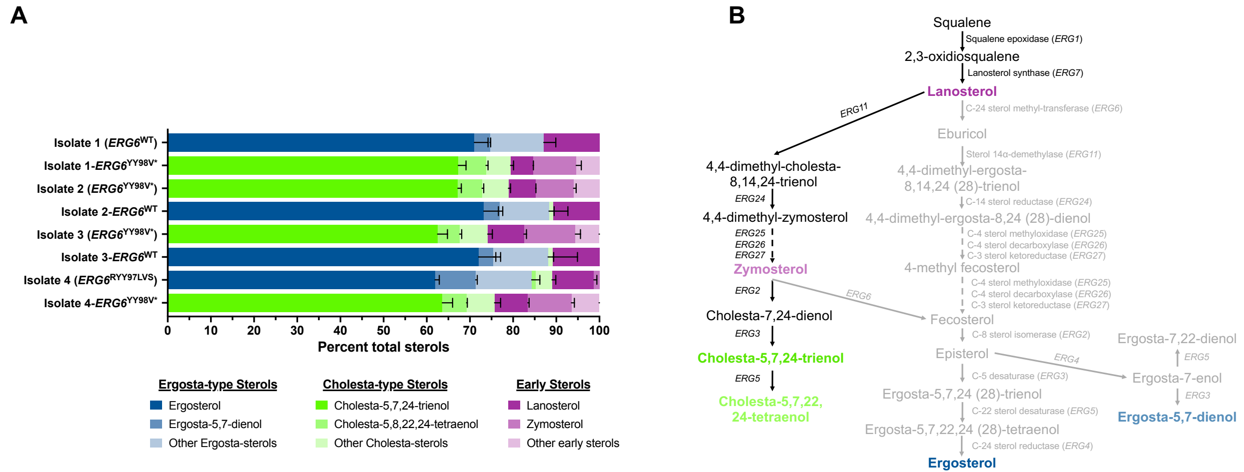


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397 **Figure 4. Mutations in *C. auris ERG6* result in an abrogation of ergosterol**
398 **biosynthesis and accumulation of cholesta-type sterols. A)** Comprehensive sterol
399 profiles of each of the clinical isolates and the *ERG6* allelic replacement strains
400 constructed in these studies. Sterol profiles are shown with each sterol represented as
401 the proportion of total cell sterols. Error bars represent the standard deviation from three
402 independent biological samples. **B)** Putative *C. auris* sterol biosynthesis pathway
403 demonstrating diversion of sterol production towards cholesta-type sterols upon loss of
404 Erg6 activity. Sterols shown in green, blue, and purple correspond to cholesta-type,
405 ergosta-type, and early sterols as shown in comprehensive sterol profiling.

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406 **TABLES**
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408
409 **Table 1. Clinical antifungal susceptibilities for *C. auris* isolates.**
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| | Clinical Antifungal MIC (mg/L) | | |
|---|---|---|---|
| Clinical Isolate | Amphotericin B | Fluconazole | Caspofungin |
| Isolate 1 | 0.75 | **256** | 0.75 |
| Isolate 2 | **>32** | **96** | 0.38 |
| Isolate 3 | **>32** | **96** | 0.38 |
| Isolate 4 | 0.75 | **256** | **4** |

MIC: minimum inhibitory concentration; MIC shown in **bold** exceed tentative CDC breakpoints

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