## Title

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FUNGAL AND PARASITIC INFECTIONS

# *Candida albicans* Cannot Acquire Sufficient Ethanolamine from the Host To Support Virulence in the Absence of *De Novo* Phosphatidylethanolamine Synthesis

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**ABSTRACT** *Candida albicans* mutants for phosphatidylserine (PS) synthase (*cho1*ΔΔ) and PS decarboxylase (*psd1*ΔΔ *psd2*ΔΔ) are compromised for virulence in mouse models of systemic infection and oropharyngeal candidiasis (OPC). Both of these enzymes are necessary to synthesize phosphatidylethanolamine (PE) by the *de novo* pathway, but these mutants are still capable of growth in culture media, as they can import ethanolamine from media to synthesize PE through the Kennedy pathway. Given that the host has ethanolamine in its serum, the exact mechanism by which virulence is lost in these mutants is not clear. There are two competing hypotheses to explain their loss of virulence. (i) PE from the Kennedy pathway cannot substitute for *de novo*-synthesized PE. (ii) The mutants cannot acquire sufficient ethanolamine from the host to support adequate PE synthesis. These hypotheses can be simultaneously tested if ethanolamine availability is increased for *Candida* while it is inside the host. We accomplish this by transcomplementation of *C. albicans* with the *Arabidopsis thaliana* serine decarboxylase gene (*AtSDC*), which converts cytoplasmic serine to ethanolamine. Expression of *AtSDC* in either mutant restores PE synthesis, even in the absence of exogenous ethanolamine. *AtSDC* also restores virulence to *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ strains in systemic and OPC infections. Thus, in the absence of *de novo* PE synthesis, *C. albicans* cannot acquire sufficient ethanolamine from the host to support virulence. In addition, expression of *AtSDC* restores PS synthesis in the *cho1*ΔΔ mutant, which may be due to causing PS decarboxylase to run backwards and convert PE to PS.

**KEYWORDS** *Candida albicans*, mice, oropharyngeal, phosphatidylethanolamine, phosphatidylserine, serine decarboxylase, virulence

*Candida* spp. are human commensal fungi that colonize the gastrointestinal tract as well as vaginal and oral mucosa. They reside as oral flora in approximately 30 to 60% of healthy individuals and usually do not cause disease (1). However, in immunocompromised patients, *Candida* spp. are able to cause a common oral infection known as oropharyngeal candidiasis (OPC), or thrush (1, 2). OPC is one of the first indicators of suppression of adaptive immunity and is seen in up to 95% of AIDS patients at some time during their infection (3). Other risk factors for OPC include use of steroid inhalers or dentures, and it is becoming a common infection in recipients of solid-organ transplants (4–7). These infections are detrimental to the comfort of patients, causing difficulty in chewing and swallowing, and in severe cases will result in reduced food intake and weight loss (1). It is important to note that these oral infections can be refractory to medications and exhibit drug resistance, making treatment challenging (2,

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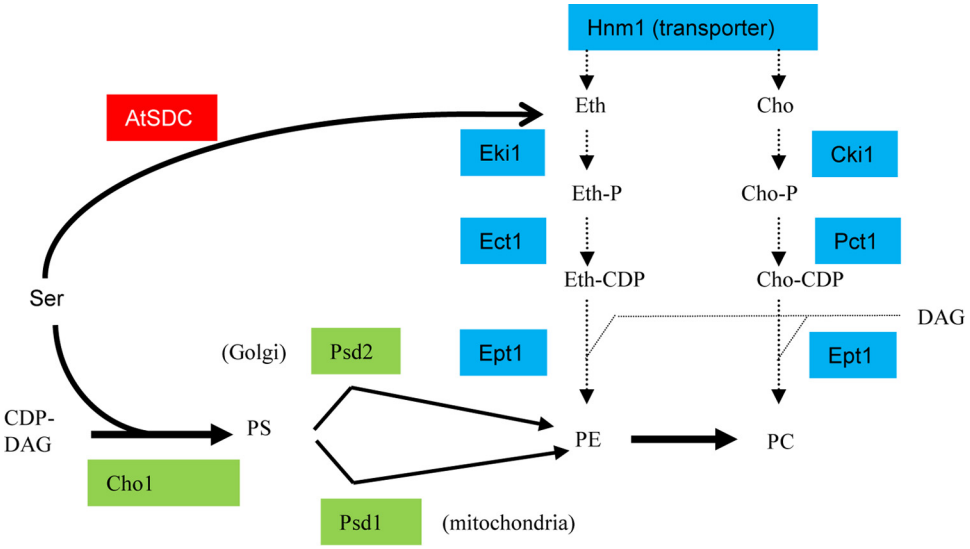


**FIG 1** Pathways for synthesizing aminophospholipids in *C. albicans*. Enzymes are named after their confirmed or predicted homologs in *S. cerevisiae*. The Kennedy pathway enzymes are shown in blue, and the *de novo* pathway proteins are shown in green. *Arabidopsis thaliana* serine decarboxylase (*AtSDC*) is shown in red. Ser, serine; Eth, ethanolamine; Cho, choline; Eth-P, phosphoethanolamine; Cho-P, phosphocholine; Etn-CDP, cytidyldiphosphate-ethanolamine; Cho-CDP, cytidyldiphosphate-choline; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CDP-DAG, cytidyldiphosphate-diacylglycerol; DAG, diacylglycerol.

8). Out of all *Candida* species, *C. albicans* is the species most commonly isolated from oral lesions (9).

*C. albicans* and related species are also capable of causing more serious systemic infections when they enter the bloodstream and deep organs, and these infections have a mortality rate of ~30% (10–12). Systemic infections tend to occur in neutropenic patients and are associated with intravascular catheters.

Previously, we discovered that mutations that block the *de novo* pathway for synthesizing the phospholipids phosphatidylserine (PS) and/or phosphatidylethanolamine (PE) compromise the virulence of *C. albicans* in a mouse model of systemic candidiasis and could serve as good drug targets (13). The *cho1*ΔΔ mutation disrupts PS synthase, the first step of the *de novo* pathway (Fig. 1, enzymes shown in green), which generates PS from cytidyldiphosphate-diacylglycerol (CDP-DAG) and serine (14, 15). A second pair of mutations (*psd1*ΔΔ *psd2*ΔΔ) blocks the second step of the pathway, whereby a pool of PS is decarboxylated to PE by Psd1p or Psd2p (16–18). The *cho1*ΔΔ mutant has a complete loss of PS and cannot make PE by this pathway, whereas the *psd1*ΔΔ *psd2*ΔΔ mutant cannot synthesize PE from PS and therefore accumulates PS (13, 19). In both mutants, PE can be synthesized via an alternative pathway called the Kennedy pathway (Fig. 1, enzymes shown in blue), where diacylglycerol (DAG) and ethanolamine are used to make PE (20) (Fig. 1). Thus, the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants are ethanolamine auxotrophs (13).

The *cho1*ΔΔ mutant is avirulent and the *psd1*ΔΔ *psd2*ΔΔ mutant is highly attenuated in a mouse model for systemic candidiasis (13). The *cho1*ΔΔ mutant exhibits more severe cell wall defects than the *psd1*ΔΔ *psd2*ΔΔ mutant, and this is manifested in increased exposure of cell wall β(1,3)-glucan (unmasking) to the immune system, as well as an altered cell wall structure, as seen by transmission electron microscopy (TEM) and increased chitin levels (13, 21, 22).

The avirulence of the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants is somewhat surprising, given that both mutants can grow in the presence of exogenous ethanolamine and host serum contains ~30 μM ethanolamine (23). Therefore, in this communication we specifically address the molecular mechanism by which these mutants are compromised for virulence *in vivo* using both systemic and OPC infection models.

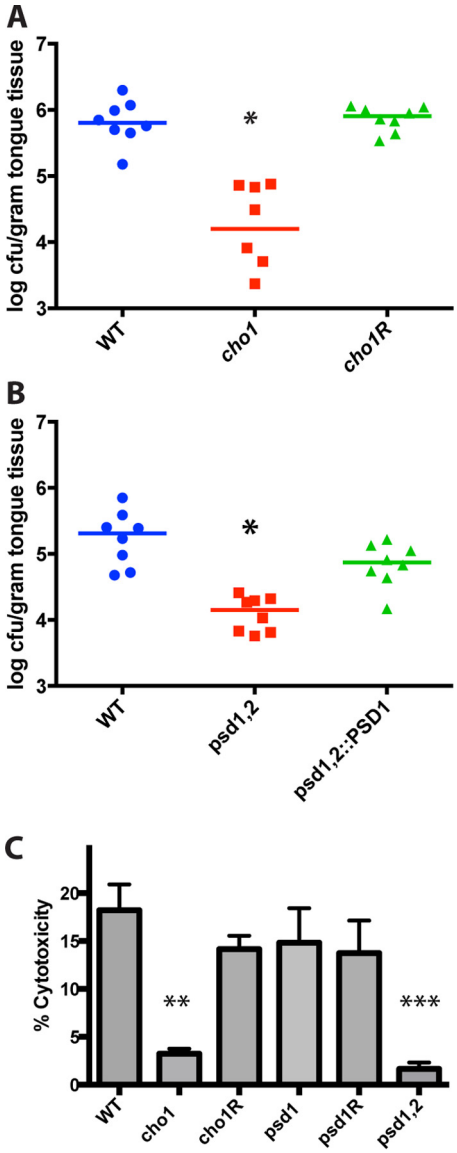


**FIG 2** *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants are attenuated for virulence during *in vivo* and *in vitro* models of OPC. Mice were inoculated orally with the wild type (WT) or *cho1*ΔΔ (cho1) or *cho1*ΔΔ::*CHO1* (cho1R) reintegrant strains (A) or the WT or *psd1*ΔΔ *psd2*ΔΔ (psd1,2) or *psd1*ΔΔ *psd2*ΔΔ::*PSD1* (psd1,2R) reintegrant strains (B). After 5 days their tongues were harvested, and the CFU/gram of tissue were measured. *, $P < 0.0002$ compared to the WT. (C) The strains were coincubated with FaDu cells, and the level of lactase dehydrogenase (LDH) released into the medium was measured as a proxy for FaDu cell damage. $P = 0.0007$ (**) and $P = 0.0005$ (***), both compared to the wild type.

## RESULTS

To determine if *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ strains have defects in oral infections by *C. albicans*, we compared these mutants to wild-type and reintegrant strains in the mouse OPC model. It was discovered that there was a decrease in pathogenicity for both *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ strains in OPC infections (Fig. 2A and B). The *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants both showed decreased colonization of the tongues of mice based on recovered CFU.

In addition to the mouse OPC model, a tissue culture model has been used to study the damage of oral epithelial cells (FaDu) by *C. albicans*. This can be measured based on the release of lactate dehydrogenase (LDH), a cytoplasmic enzyme, as a proxy for FaDu cell damage (24). When *C. albicans* strains are coincubated with FaDu cells, the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants show reduced damage of the epithelial cells (Fig. 2C).

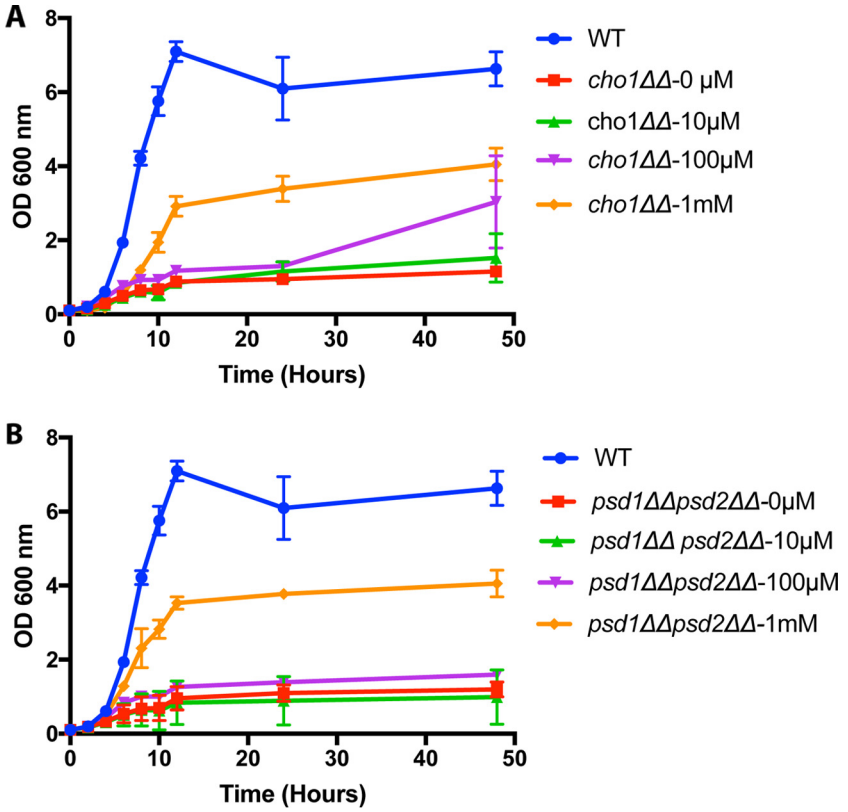


**FIG 3** Ethanolamine (1 mM) is required to support growth of the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants in minimal medium. The *cho1*ΔΔ (A) and *psd1*ΔΔ *psd2*ΔΔ (B) strains were grown overnight in YPD, diluted to an $OD_{600}$ of 0.1, and grown in minimal medium plus various levels of ethanolamine (Eth) at 37°C with shaking, and their growth was measured for up to 48 h. Growth is compared to that of the wild type (WT) with 0 $\mu$M ethanolamine.

**Loss of *de novo* PE synthesis compromises virulence because mutants cannot acquire sufficient ethanolamine in the host.** The *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ phospholipid biosynthesis mutants show decreased virulence in three models of virulence: murine systemic infection (13), murine OPC (Fig. 2A and B), and FaDu epithelial damage (Fig. 2C). The main feature shared by both mutants is the inability to synthesize PE from PS. However, it is not clear why loss of PE synthesis through the *de novo* pathway compromises virulence given the existence of a functional Kennedy pathway. One hypothesis is that PE synthesized by Psd1 or Psd2 is fundamentally different in composition or localization than PE synthesized through the Kennedy pathway, and therefore the Kennedy pathway PE cannot compensate for *de novo* pathway PE. A competing hypothesis is that PE from either pathway is sufficient, but *C. albicans* cannot acquire enough ethanolamine from the host to satisfy its PE requirement via the Kennedy pathway.

The way to test these competing hypotheses is to increase PE synthesis through the Kennedy pathway while *C. albicans* is in the host during an infection. This requires that we provide sufficient ethanolamine intracellularly to *C. albicans*, as it is possible that the amount of ethanolamine in the host is not sufficient to support growth or virulence of these mutants. The level of ethanolamine in mouse serum is estimated to be ~30 $\mu$M (23). However, we found that the level of ethanolamine required to support growth of these *Candida* mutants in culture media at 37°C is greater than 100 $\mu$M (Fig. 3). Therefore, the mutants may not be able to import sufficient ethanolamine from the host to support virulence.

In order to provide ethanolamine intracellularly, a codon-optimized serine decarboxylase enzyme derived from *Arabidopsis thaliana* (AtSDC) was expressed in our

*C. albicans* strains (25). Serine carboxylase activity is found in plants but not in mammals or fungi. AtSDC decarboxylates serine to ethanolamine within the cytoplasm, and the ethanolamine can be used by the Kennedy pathway to synthesize PE (Fig. 1). If the loss of virulence in both mutants is due to an inability to acquire sufficient ethanolamine from the host, then addition of *AtSDC* should restore ethanolamine prototrophy and virulence in mouse models of candidiasis. If, alternatively, PE must be made by the *de novo* pathway to support virulence because the Kennedy pathway-derived PE is insufficient, then *AtSDC* will not rescue the virulence defect for either mutant.

The ability of *AtSDC* to support PE synthesis by the Kennedy pathway was tested in two ways. First, it was found to restore ethanolamine prototrophy in the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ strains by supporting growth in minimal medium lacking ethanolamine (Fig. 4A to C). In fact, when grown in medium lacking ethanolamine, mutants expressing *AtSDC* grew at rates similar to that of the wild type and exhibited better growth than the parental mutant strain supplemented with ethanolamine (Fig. 4A to C). Second, thin-layer chromatography (TLC) confirmed that PE levels were restored in the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants growing in the absence of ethanolamine when *AtSDC* is expressed (Fig. 4D).

We then examined if addition of *AtSDC* would restore virulence for the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants in a mouse model of systemic infection (Fig. 5A and B). Indeed, the addition of this enzyme fully restored virulence to both the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants in the systemic infection model. The differences seen in the survival curves were reflected in fungal burden measurements (Fig. 5C). It is interesting that although both the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants had much lower fungal burden than the wild type, the *cho1*ΔΔ mutant exhibited a significantly lower fungal burden than the *psd1*ΔΔ *psd2*ΔΔ mutant (Fig. 5C).

We further determined if the *AtSDC*-expressing mutants would be restored for virulence in the OPC model. Indeed, when these strains were tested in this model, they revealed that the mutants expressing *AtSDC* had virulence levels similar to that of the wild type (Fig. 6A). In the previous experiments shown in Fig. 2, *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants were tested separately and with outbred (ICR) and inbred (Balb-c) mice, respectively; however, when compared in outbred mice (CD-1) in the same experiment, the *psd1*ΔΔ *psd2*ΔΔ mutant appears to be more virulent than the *cho1*ΔΔ mutant. Finally, we determined if the addition of *AtSDC* would restore the ability of the mutants to damage epithelial cells. This was found to be the case for both mutants (Fig. 6B). In both cases, the mutants with *AtSDC* caused as much damage as the wild type.

**Expression of AtSDC restores β(1,3)-glucan unmasking in the *cho1*ΔΔ mutant by restoring PS synthesis.** Previous work from Davis et al. (21) revealed that the *cho1*ΔΔ, but not *psd1*ΔΔ *psd2*ΔΔ, mutant exhibits increased exposure of β(1,3)-glucan (unmasking). These data showed that PS, but not PE, regulates unmasking. Therefore, complete restoration of virulence in the *cho1*ΔΔ mutant by *AtSDC* was unexpected, as AtSDC synthesizes ethanolamine specifically. Therefore, we measured whether β(1,3)-glucan unmasking in the *cho1*ΔΔ mutant would be affected by PE synthesis through the Kennedy pathway due to expression of AtSDC. The addition of AtSDC led to full restoration of β(1,3)-glucan unmasking in the *cho1*ΔΔ mutant similar to that of the wild type (Fig. 7). Thus, production of PE from the Kennedy pathway can bypass the *cho1*ΔΔ mutation regarding β(1,3)-glucan exposure.

This left the possibility that PE production by AtSDC was allowing the regeneration of PS. There is no other PS synthase in *C. albicans*. However, if sufficient product is present, an enzyme can be made to run backward, and the *cho1*ΔΔ mutant possesses the two PS decarboxylase enzymes Psd1 and Psd2 that could potentially generate PS from PE and $CO_2$. Thus, we hypothesized that PS synthesis is restored in the *cho1*ΔΔ mutant when *AtSDC* is expressed. Therefore, we ran thin-layer chromatography to determine if the *cho1*ΔΔ AtSDC mutant synthesizes PS, and we found that it did (Fig. 8A). Furthermore, strains that contain PS are susceptible to the PS-binding toxin papuamide A (PapA) at 10 and 5 μg/ml, while

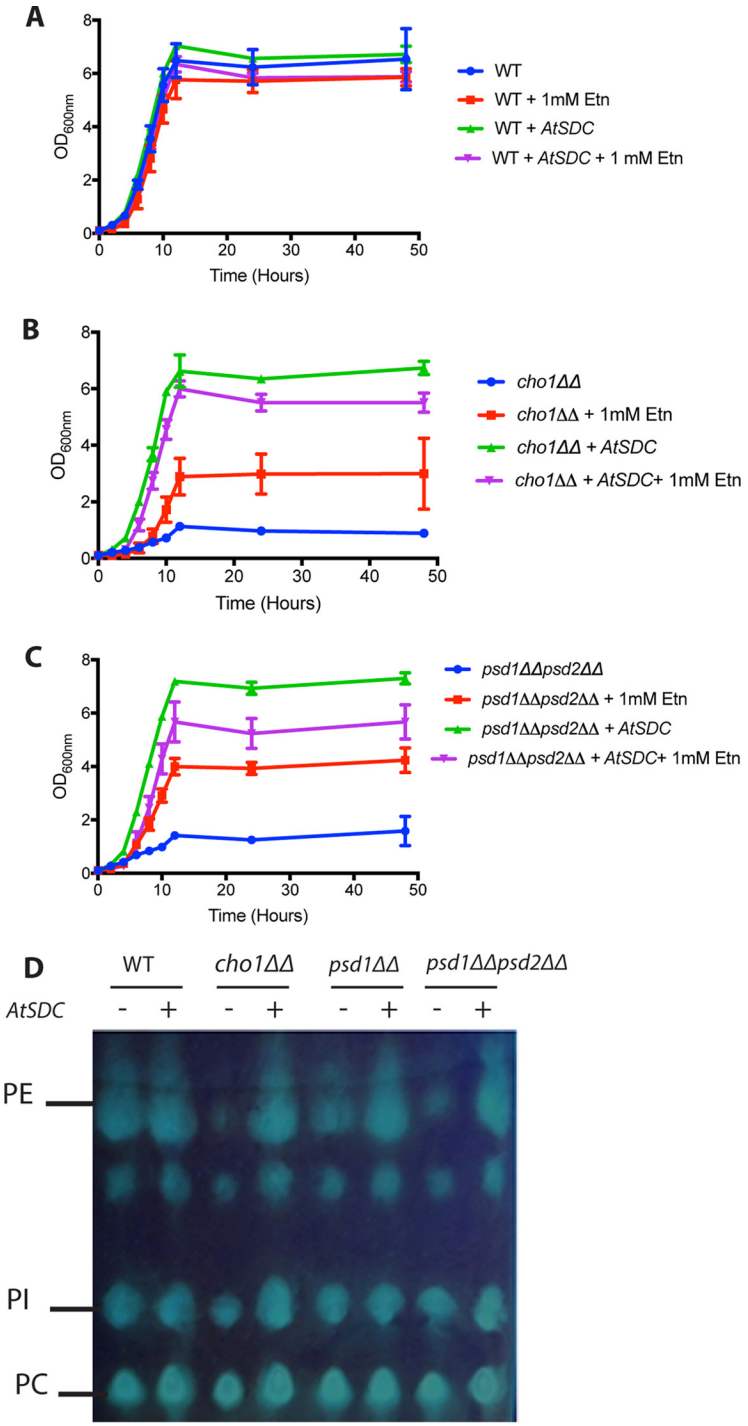


**FIG 4** Expression of *AtSDC* restores ethanolamine prototrophy in *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants. (A to C) Mutant and wild-type (WT) strains with or without *AtSDC* were grown in minimal medium with or without ethanolamine (Etn) at 37°C and a growth curve was plotted. (D) Phospholipids were isolated from each strain grown in minimal medium lacking ethanolamine and then separated by TLC. Lipid spots were visualized with primuline stain under UV light. *cho1*ΔΔ (cho1), *psd1*ΔΔ (psd1), and *psd1*ΔΔ *psd2*ΔΔ (psd1 psd2) strains were included, all with or without AtSDC.

the *cho1*ΔΔ mutant (lacking PS) is resistant, as reported previously (26). In contrast to the *cho1*ΔΔ mutant, the *cho1*ΔΔ AtSDC strain is susceptible, indicating that it contains PS (Fig. 8B). This would explain why *AtSDC* restores $\beta$(1,3)-glucan masking to the *cho1*ΔΔ mutant.

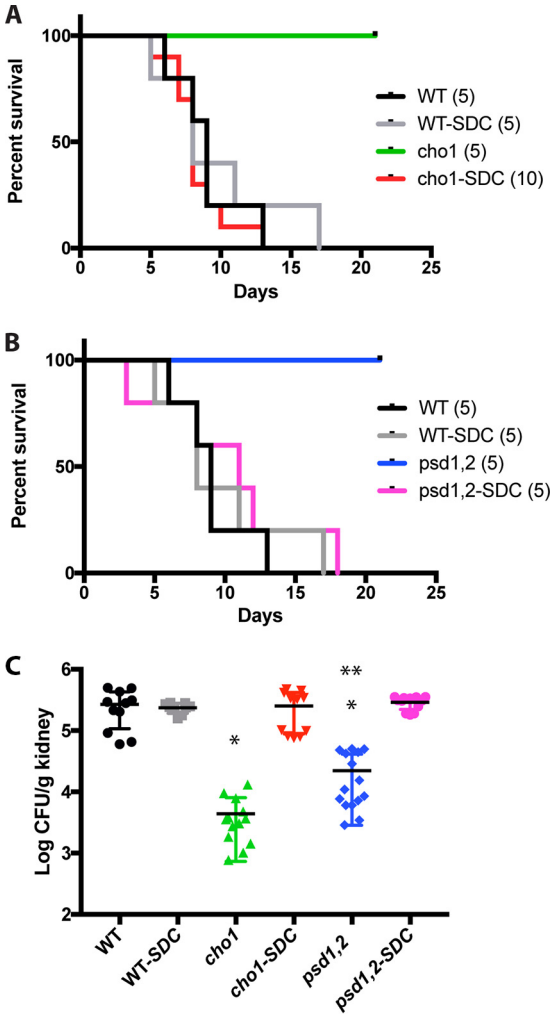


**FIG 5** Expression of *AtSDC* restores virulence in *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ strains in a mouse model of systemic candidiasis. (A and B) Mice were infected by tail vein with $5 \times 10^5$ cells from each strain, and survival was graphed over time. The numbers of mice per strain are shown beside the strain name in parentheses. The *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutant survival curves were significantly different from that of the wild type (*, $P = 0.0004$). (C) Mice were infected with $5 \times 10^5$ cells by tail vein, and after 5 days they were euthanized, kidneys were removed, and CFU/gram of kidney were measured by plating. *, $P < 0.0001$ compared to WT or strains expressing AtSDC; **, $P = 0.0031$ compared to the *cho1*ΔΔ mutant. cho1 (*cho1*ΔΔ), psd1 (*psd1*ΔΔ), psd1,2 (*psd1*ΔΔ *psd2*ΔΔ), and SDC (*AtSDC*) mutant strains were studied.

## DISCUSSION

The data presented in this communication address the main question of whether the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants' losses in virulence are due to a shared loss of PE synthesis from one specific pathway (*de novo*) or if it was due to an overall loss of PE synthesis. Based on the results with AtSDC strains (Fig. 5 and 6), PE synthesized by the Kennedy pathway can compensate for loss of *de novo*-synthesized PE. However, the Kennedy pathway does not compensate for loss of virulence in the *cho1*ΔΔ or *psd1*ΔΔ *psd2*ΔΔ mutant lacking *AtSDC*, and this is likely because the mutants are unable to import sufficient ethanolamine to support virulence in the host. In culture, these mutants require >100 $\mu$M (Fig. 3), and serum ethanolamine is estimated to be 30 $\mu$M (23). In addition, even with 1 mM ethanolamine, neither the *cho1*ΔΔ nor *psd1*ΔΔ *psd2*ΔΔ mutants recover wild-type levels of growth, but they do so with *AtSDC* (Fig. 3), thus one possible explanation is that *C. albicans* is unable to transport ethanolamine into the cell at an efficiency that allows it to restore virulence.

The inability of ethanolamine auxotrophs like the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ

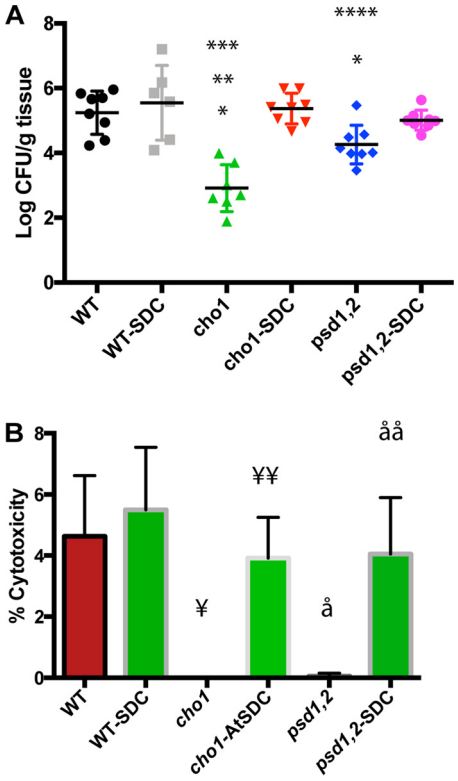


**FIG 6** Expression of *AtSDC* restores virulence to the *cho1*ΔΔ or *psd1*ΔΔ *psd2*ΔΔ strain for *in vivo* and *in vitro* models of OPC. (A) An OPC infection model was performed with mice, and after 5 days they were sacrificed, tongues were removed, and CFU/gram of tongue were measured. *, $P < 0.01$ compared to the WT. **, $P < 0.01$ compared to the *cho1*ΔΔ-*AtSDC* strain. ***, $P < 0.01$ compared to the *psd1*ΔΔ *psd2*ΔΔ strain. ****, $P < 0.01$ compared to the *psd1*ΔΔ *psd2*ΔΔ-*AtSDC* strain. (B) The wild-type (WT), *cho1*ΔΔ, and *psd1*ΔΔ *psd2*ΔΔ strains were compared to their respective *AtSDC* transformants for their abilities to damage FaDu cells based on release of lactase dehydrogenase (LDH). $P = 0.0157$ (¥) and $P = 0.0163$ (å), both compared to the WT; ¥¥, $P = 0.0071$ compared to the *cho1*ΔΔ strain; åå, $P = 0.0201$ compared to the *psd1*ΔΔ *psd2*ΔΔ strains. cho1 (*cho1*ΔΔ), psd1 (*psd1*ΔΔ), psd1,2 (*psd1*ΔΔ *psd2*ΔΔ), and SDC (*AtSDC*) strains were used.

mutants to cause disease is interesting given that auxotrophic mutants in *Candida* spp. are only sometimes associated with avirulence. For instance, an auxotroph for inositol (*ino1*ΔΔ), a metabolite used to synthesize the phospholipid phosphatidylinositol, is not compromised for virulence (27). In addition, auxotrophs for the amino acids lysine, histidine, leucine, and arginine are fully virulent (28, 29). In addition, the related fungal pathogen *Candida glabrata* is a natural niacin auxotroph (30) and *C. albicans* is a biotin auxotroph (31, 32), yet they are both virulent. In contrast, mutants that are auxotrophs for the nucleotide uridine or adenine are avirulent (33, 34), as is a mutant for fatty acid synthesis (*fas2*ΔΔ strain) (35). Thus, it is not a forgone conclusion that auxotrophic mutants are going to be comprised for virulence, but this is clearly the case with the ethanolamine auxotrophs in this study.

This study also has an unusual innovation in that we tested for auxotrophy *in vivo* during infection, which is rarely done because of technical difficulties. Often auxotrophs are assumed to be avirulent due to insufficient nutrient acquisition in the host based on correlations with *in vitro* phenotypes on plates or in culture media. Thus, auxotrophies often cannot be easily tested *in vivo* during infection. However, in this study, with the *AtSDC* gene we were able to demonstrate that provision of ethanolamine from a source other than the host restored virulence *in vivo*. This confirms that ethanolamine auxotrophy is indeed a major contributor to the loss of virulence in the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants.

One potential related explanation for the virulence loss of *cho1*ΔΔ and *psd1*ΔΔ

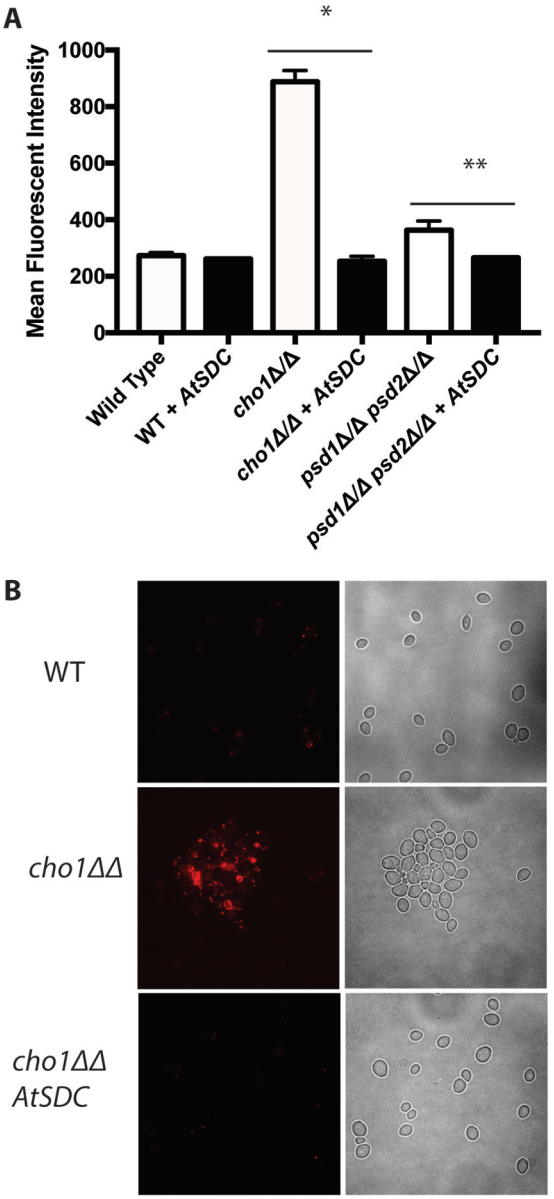


**FIG 7** Expression of *AtSDC* suppresses β(1,3)-glucan unmasking in the *cho1*ΔΔ strain. (A) Cells were stained with anti-β(1,3)-glucan primary and phycoerythrin-conjugated secondary antibodies, and 100,000 cells were measured by flow cytometry for each replicate sample. Experiments were performed with three replicates per strain. (B) Cells were stained with anti-β(1,3)-glucan primary and Cy3-conjugated secondary antibodies and viewed by immunofluorescence microscopy. *, $P < 0.0001$; **, $P = 0.0067$.

*psd2*ΔΔ mutants besides auxotrophy is that they grow more slowly than the wild type, even with ethanolamine supplementation (Fig. 4). In contrast, expression of *AtSDC* restores both mutants to wild-type growth. However, as reported in Chen et al. (13), the *cho1*ΔΔ and *psd1*ΔΔ *psd2*ΔΔ mutants' growth in medium supplemented with ethanolamine was similar to that of the *psd1*ΔΔ single mutant which is fully virulent in the tail vein infection model. Thus, there is not necessarily a strong correlation between *in vitro* growth in culture and virulence, an observation that was also made by Noble et al. in their study of the virulence of ~700 homozygous *Candida albicans* mutants (36).

Finally, it was noted, based on fungal burden levels, that the *psd1*ΔΔ *psd2*ΔΔ mutant had a modest but significantly higher fungal burden in both the systemic model of infection and the OPC model. These differences in fungal burdens do correlate with the *cho1*ΔΔ mutant's β(1,3)-glucan unmasking compared to that of the *psd1*ΔΔ *psd2*ΔΔ

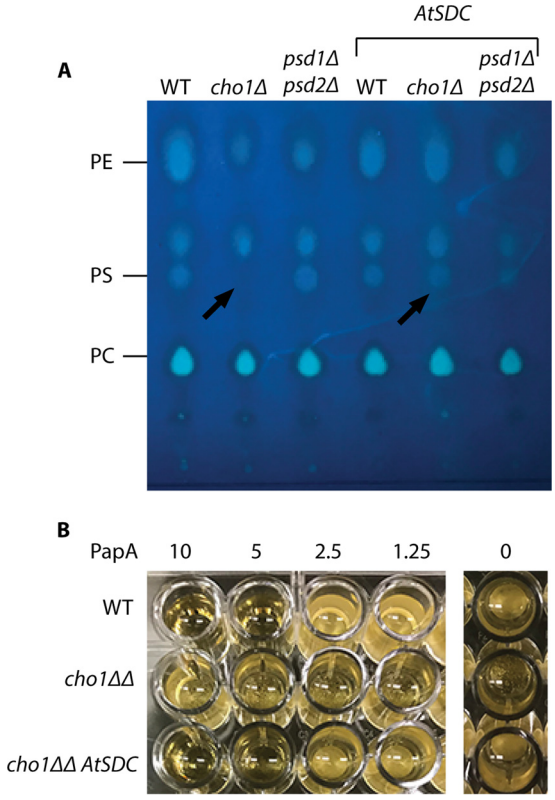


**FIG 8** Expression of *AtSDC* in the *cho1*ΔΔ mutant leads to production of PS. (A) Strains were grown overnight in YPD, and phospholipids were extracted and run on TLC to determine if PS was being synthesized. Lipid spots were compared to purified standards, which are marked on the TLC. The PS spot is marked with a black arrow in the *cho1*ΔΔ and *cho1*ΔΔ *AtSDC* lanes. (B) Cells were grown overnight in YPD and diluted to $10^5$/ml in fresh YPD, and 100 $\mu$l of the culture was added to wells containing YPD and PapA or the methanol-water solvent control at the concentrations shown and then incubated overnight without shaking at 30°C.

mutant (21); however, there may be other explanations, as these mutants are both pleiotropic.

**The role of Kennedy pathway PE in $\beta$-glucan exposure.** Regarding unmasking, another observation made in this study is that supplying sufficient PE via the AtSDC can restore masking in the *cho1*ΔΔ mutant. Although initially we thought it might be acting to restore unmasking in the absence of PS, we have found that AtSDC can cause PS synthesis in the absence of the PS synthase gene. The most likely explanation is that the excess PE synthesized by the Kennedy pathway in the *cho1*ΔΔ strain expressing *AtSDC* causes the Psd1 and/or Psd2 PS decarboxylase enzymes to run in reverse and restores PS and, therefore, $\beta$(1,3)-glucan masking. This is a surprising result and is the first time, to our knowledge, that this has been suggested *in vivo* for a PS decarboxylase enzyme.

## MATERIALS AND METHODS

**Strains and media.** The *C. albicans* strains used in this study are listed in Table 1. The medium used for growth and maintenance of the strains was YPD (yeast extract, peptone, dextrose) broth and plates (37). For growth curves, we used minimal medium (yeast nitrogen base, dextrose) with or without ethanolamine (37). The *AtSDC* strains were generated as follows. First, the *AtSDC* gene was codon optimized by site-directed mutagenesis to change its single CTG codon at amino acid position 447 to TTG in the yeast expression vector pVT103-U (25) and then was PCR amplified with SDO17 (AAAACCATGG ATGGTTGGATCTTTGGAATCTGA) and SDO18 (AAAAAGATCTCTAGACCATGCTCCTACCTAGA), which introduced NcoI and BglII sites that were used to replace the *dTomato* open reading frame found between these sites in the pENO-dTomato-NATr plasmid (38), creating the plasmid pENO1-AtSDC. This plasmid was linearized with EcoRV, transformed by electroporation as previously described (13), and selected on YPD plates containing 200 $\mu$g/ml nourseothricin.

**Papuamide A toxicity assay.** The PapA assay was performed as described in reference 26.

**TABLE 1** *C. albicans* strains used in this study

| Strain | Parent | Genotype | Source or reference |
|---|---|---|---|
| SC5314 | Clinical isolate | Prototrophic wild type | 41 |
| YLC337 | SC5314 | *cho1*ΔΔ | 13 |
| YLC344 | YLC337 | *cho1*ΔΔ::*CHO1-SAT1* | 13 |
| YLC280 | SC5314 | *psd1*ΔΔ | 13 |
| YLC294 | YLC280 | *psd1*ΔΔ::*PSD1-SAT1* | 13 |
| YLC375 | SC5314 | *psd2*ΔΔ *psd1*ΔΔ | 13 |
| SED029 | YLC375 | *psd1*ΔΔ *psd2*ΔΔ::*PSD1-SAT1* | This study |
| SED030 | SC5314 | $P_{ENO1}$-*AtSDC-SAT1* | This study |
| SED031 | YLC337 | *cho1*ΔΔ $P_{ENO1}$-*AtSDC-SAT1* | This study |
| SED032 | YLC280 | *psd1*ΔΔ $P_{ENO1}$-*AtSDC-SAT1* | This study |
| SED033 | YLC375 | *psd1*ΔΔ *psd2*ΔΔ $P_{ENO1}$-*AtSDC-SAT1* | This study |

**Assay of LDH release from FaDu cells during *C. albicans* infection in tissue culture.** For measurement of LDH, cytotoxicity assays were performed using the protocol and materials found in the CytoTox 96 nonradioactive cytotoxicity assay kit (G1780; Promega). Briefly, $5 \times 10^5$ FaDu cells/ml were plated in Earle's balanced salt solution (EBSS) medium with 10% fetal bovine serum (FBS) in a 24-well plate with a total volume of 1 ml/well. *C. albicans* strains were grown overnight in 5 ml YPD. The following day *C. albicans* cultures were washed with water and resuspended in EBSS with 2% human serum. Medium was aspirated from FaDu cells, and 1 ml/well *C. albicans* was added at a multiplicity of infection of 5 and centrifuged at 200 × *g* for 5 min. Wells containing FaDu or *C. albicans* cells were included as controls, and all samples were tested in triplicate by incubating for 4 h at 37°C and 5% $CO_2$. Following incubation, the plate was again centrifuged at 200 × *g* for 5 min, and 50 μl of each supernatant was transferred to a 96-well plate. An equal volume of reconstituted substrate mix was added to each well and incubated for 30 min protected from light. Fifty μl of stop solution was added to each well, and absorbance was measured at 490 nm. Percent cytotoxicity was calculated as described in the manufacturer's protocol by comparing samples to epithelial cells treated with lysis solution (24).

**Hot ethanol extraction of total phospholipids and TLC.** Sample extraction and TLC for Fig. 8 was performed as previously described (13) using plates from GE Healthcare that can separate PS very well, but they are no longer available for sale and are therefore a limited resource. Due to this limitation, for the TLC shown in Fig. 4, which focused on PE, the following exceptions were made to conserve the GE plates. Before running with samples, TLC silica gel plates (M1057290001; Fisher) were prepared by baking them in a drying oven at 100°C for 15 min. After baking, these plates were run with 50 mM EDTA. Plates were then run with a chloroform-methanol (1:1) migration solvent to remove EDTA, dried, and baked in the oven again for 15 min. The samples were then loaded and run as described previously (13). Lipids were visualized on the dried plate by spraying with primuline dye (5 mg of primuline; 206865; Sigma) in 100 ml of acetone and illuminating with UV light.

**Microscopy and flow cytometry.** For flow cytometry, cell staining was performed with an anti-β(1,3)-glucan primary antibody (Biosupplies Australia) and a goat anti-mouse-phycoerythrin secondary antibody (Jackson ImmunoResearch). Three biological replicates for all strains were used in each experiment, and all were analyzed on the same day. In brief, 5-ml cultures of *C. albicans* strains were grown with shaking overnight at 30°C and diluted the following morning to an optical density at 600 nm ($OD_{600}$) of 0.5 in 1 ml of 1× phosphate-buffered saline (PBS). Cells were subsequently washed twice with PBS and blocked for 1 h in PBS plus 3% bovine serum albumin (BSA). Following blocking, cells were rocked at 4°C for 90 min in a 1:800 dilution of anti-β(1,3)-glucan primary antibody in PBS plus 3% BSA. Cells were then washed four times with PBS and rocked once more at 4°C for 20 min in a 1:300 dilution of goat anti-mouse-phycoerythrin secondary antibody in PBS plus 3% BSA. Cells were washed 4 additional times with PBS and finally resuspended in 500 μl of FACS buffer for flow cytometry analysis. Samples were run on a FACSCalibur LSRII flow cytometer (Becton Dickinson), and data for each sample were collected for 100,000 gated events. Data were analyzed with the use of FlowJo 10.11 (FlowJo LLC, OR, USA), and all statistics were run using a Student *t* test.

For immunofluorescence, this procedure was done as described in reference 21. *C. albicans* cells were grown overnight in YPD medium at 30°C. An anti-β(1,3)-glucan antibody (Biosupplies Australia, Pty Ltd., Australia) at a 1:800 dilution was used as the primary antibody, and a goat anti-mouse antibody conjugated to Cy3 (Jackson ImmunoResearch Inc., USA) at a 1:300 dilution was used as the secondary antibody. For imaging, *Candida* cells were resuspended in 100 μl of PBS and visualized with a Leica DM5500B epifluorescence microscope with a Hamamatsu Orca-ER charge-coupled-device digital camera. The pictures were taken through Leica Application Suite AF (advanced fluorescence) software.

**OPC model.** The OPC experiment was performed as previously described (39, 40). Briefly, eight mice per strain of *C. albicans* were immunosuppressed with cortisone acetate, which was administered subcutaneously at 225 mg/kg of body weight on days −1, 1, and 3 relative to infection. To induce infection, the mice were anesthetized with ketamine and xylazine, after which a calcium alginate swab saturated with $10^6$ *C. albicans* cells was placed sublingually for 75 min. After recovery from anesthesia, the mice were provided with food and water *ad libitum*. They were sacrificed after 5 days of infection, and then their tongues were harvested and divided lengthwise. One-half was weighed, homogenized, and quantitatively cultured.

**Mouse systemic infection model.** To prepare strains for injection, 1.0 $OD_{600}$ of cells from overnight test tube cultures were washed 5 times with 1 ml double-distilled water ($ddH_2O$), resuspended, and

counted by hemocytometer. These washed cultures were then diluted to $5 \times 10^6$ cells/ml, and 100 $\mu$l of cell suspension was injected into ICR (Harlan) mice for a total of $5 \times 10^5$ yeast/mouse ($n = 5$ mice for each *Candida* strain/experiment). For fungal burdens, cultures were prepared and injected the same way as they were for the mouse mortality assay described above. Five days after injection, mice were humanely euthanized and then both kidneys were removed. One kidney was weighed and homogenized with a Dounce homogenizer in 5 ml $ddH_2O$. This homogenate was diluted 1:4, and 200 $\mu$l was plated on YPD plus 1 M sorbitol plates for CFU counts.

**Ethics statement.** The mouse studies carried out in this report followed ethical guidelines set forth by the National Institutes of Health for the ethical treatment of animals. For the systemic infection model, these experiments were carried out at the University of Tennessee under the UT-Animal Care and Use Committee (UT-ACUC) approved protocol number 0016-0714. The OPC model was carried out under the approved animal welfare assurance number A3330-01 from the Institutional Animal Care and Use Committee (IACUC) of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center.

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