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Genome-wide analysis of PTR transporters in *Candida* species and their functional characterization in *Candida auris*

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Abstract

The peptide transport (PTR) or proton-dependent oligopeptide transporter (POT) family exploits the inwardly directed proton motive force to facilitate the cellular uptake of di/tripeptides. Interestingly, some representatives are also shown to import peptide-based antifungals in certain *Candida* species. Thus, the identification and characterization of PTR transporters serve as an essential first step for their potential usage as antifungal peptide uptake systems. Herein, we present a genome-wide inventory of the PTR transporters in five prominent *Candida* species. Our study identifies 2 PTR transporters each in *C. albicans* and *C. dubliniensis*, 1 in *C. glabrata*, 4 in *C. parapsilosis*, and 3 in *C. auris*. Notably, despite all representatives retaining the conserved features seen in the PTR family, there exist two distinct classes of PTR transporters that differ in terms of their sequence identities and lengths of certain extracellular and intracellular segments. Further, we also evaluated the contribution of each PTR protein of the newly emerged multi-drug-resistant *C. auris* in di/tripeptide uptake. Notably, deletion of two *PTR* genes BNJ08_003830 and BNJ08_005124 led to a marked reduction in the transport capabilities of several tested di/tripeptides. However, all three genes could complement the role of native *PTR2* gene of *Saccharomyces cerevisiae*, albeit to varied levels. Besides, BNJ08_005124 deletion also resulted in increased resistance toward the peptide-nucleoside drug Nikkomycin Z as well as the glucosamine-6-phosphate synthase inhibitor, L-norvalyl-N3-(4-methoxyfumaroyl)-L-2,3-diaminopropionoic acid (Nva-FMDP), pointing toward its predominant role in their uptake mechanism. Altogether, the study provides an important template for future structure–function investigations of PTR transporters in *Candida* species.

Key points

- *Candida genome encodes for two distinct classes of PTR transporters.*
- *Candida auris encodes for 3 PTR transporters with different specificities.*
- *BNJ08_005124 in C. auris is involved in the uptake of Nikkomycin Z and Nva-FMDP.*

Keywords PTR/POT transporters · *Candida* species · *Candida auris* · Antifungal peptides · Nikkomycin Z · Nva-FMDP

Introduction

Pathogenic yeasts including *Candida albicans* and non-*albicans Candida* (NAC) species are successful human commensals and assume pathogenic status when exposed to compromised immunity (Sanches et al. 2019). The superficial infections caused by *C. albicans* and NAC species can also extend to disseminated bloodstream and deep-tissue infections (Sanches et al. 2019). While *C. albicans* and few NAC species (e.g., *C. glabrata, C. tropicalis, C. parapsilosis, C. dubliniensis, C. guilliermondii, C. krusei, and C. kefyr*) are the most common species affecting immunocompromised patients, more recently *C. auris* has established itself as a global health threat (https://www.cdc.gov/fungal/candida-auris/index.html) (Chowdhary et al. 2017; Taei et al. 2019). The biggest problem faced with this particular species is its ability to rapidly spread in hospital settings and the high order of resistance against the frontline antifungals (Lockhart et al. 2016; Chowdhary et al. 2017). An increase in the levels of antifungal resistance is also witnessed in

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C. albicans and several other NAC species (Whaley et al. 2017). As a result, fungal research across the globe is currently directed toward the identification of alternative drug targets and therapeutics. Peptide transport serves as one of the primary routes for the acquisition of nitrogen and amino acids by cells, and peptide uptake systems are ubiquitously present in all organisms from bacteria to higher eukaryotes (Newstead 2015). Two distinct classes of proton-coupled peptide transport systems are recognized in fungi: the peptide transporters (PTR/POT), which transport di/tripeptides, and the oligopeptide transporters (OPT), which mediate the uptake of longer peptides (Dunkel et al. 2013; Becerra-Rodríguez et al. 2020). In other words, their specificity depends on the peptide length. Both these families belong to the larger major facilitator superfamily (MFS) of membrane-bound pumps (Garai et al. 2017). It was Paulsen and Skurray (1994) who first noted sequence similarities between different non-ABC-type, proton dependent transporters, like *Arabidopsis thaliana* nitrate transporter Chl1, mammalian PepT1, YhiP/dtpB transport system of *Escherichia coli*, oligopeptide transporter DtpT from *Lactococcus lactis,* and the *Saccharomyces cerevisiae* peptide transporter Ptr2 and hence designated the group as Proton-dependent Oligopeptide Transport (POT) family. The authors also predicted a shared topological domain consisting of 12 putative α-helical membrane-spanning segments, with two unique conserved motifs not known to be present in any other class of proteins at that time. Later, some research groups did not consider the POT family designation completely correct for the set of proteins and proposed the peptide transport family (PTR) to refer to a group of non-ABC type, proton-dependent oligopeptide transporters (Steiner et al. 1995). However, currently, the PTR or POT designations are interchangeably used in the literature and refer to the proton-dependent oligopeptide transporter family with di/tripeptides as the substrates (Newstead 2015). Interestingly, this group of transporters has been identified in both eukaryotes and prokaryotes, except archaea, which can be partly ascribed to the fact that peptides are usually not utilized as nutrient sources in extreme environments in which the archaeal members thrive (Newstead 2017). In *S. cerevisiae*, the PTR transport system comprises of a single transporter ScPtr2 which is the best-known member of the PTR family with homologs reported in *C. albicans* (Perry et al. 1994). An analysis of peptide specificity of ScPtr2 revealed its higher affinity for di/tripeptides with aromatic, branched, or basic amino acids and a relatively lower affinity for negatively charged amino acids, glycine, and proline (Ito et al. 2013). In *C. albicans* two dipeptide/tripeptide transporters of the PTR family have been identified (Dunkel et al. 2013). One encoded by orf19.2583 is designated as CaPtr2 (Dunkel et al. 2013). The second protein encoded by orf19.6937 is more similar to ScPtr2 in terms of its primary structure and is designated as CaPtr22 (Dunkel et al. 2013). It was also noticed by Dunkel and colleagues (Dunkel et al. 2013) that CaPtr22 has a broader substrate spectrum with an increased diversity of tripeptides that can be transported by it. Their counterparts in other *Candida* species have not been identified and/or investigated in detail and form the basis of this study. The primary reason why this class of protein requires investigation is that this class of proton-coupled transporters is also known for its pharmacological importance since they aid in the uptake of drugs that have steric resemblance with substrate peptides (Newstead 2015). Interestingly, in *C. albicans,* the di/tripeptide transporters belonging to the PTR/POT family have demonstrated the ability to import antifungal peptides and thus open avenues for exploiting these membrane-bound pumps as antifungal delivery systems (Schielmann et al. 2017; Skwarecki et al. 2018). Furthermore, the PTR transporters in humans, PepT1 and PepT2, besides catalyzing the uptake of dietary peptides, also recognize and transport many drug molecules with steric resemblance toward peptides like β-lactam antibiotics cefadroxil and cefalexin (Terada et al. 1997). Since peptide-based therapeutics are known to be relatively refractory to resistance development (Pathirana et al. 2018), these systems can be constituent of novel treatment regimens. However, a deeper understanding of the structure and function of these proteins is required to fully utilize their potential. With this background, the current study is aimed at the identification of PTR/POT transporters in predominant *Candida* species to pave the way for exploiting these proteins as efficient antifungal delivery systems. We also attempted to functionally characterize these systems in the newly emerged *C. auris* to encourage further structure–function studies.

Materials and methods

BLAST analysis

To identify the PTR transporters in *Candida* species, the BLAST + feature within the *Candida* Genome Database (CGD) (Inglis et al. 2012) was utilized using the ScPtr2 protein sequence as the query and *C. albicans, C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, and *C. auris* as the target genomes. BLAST was run using the default settings, and results were analyzed based on the E-value and normalized alignment scores (bits).

Identification of protein domains

Protein sequences of all the predicted PTR candidates were retrieved from CGD, and a combined FASTA file was submitted as an input to the DomainViz webserver (Schläpfer et al. 2021). Default parameters were utilized for the job.

Multiple sequence alignment and clustering sequence identity matrix construction

Multiple sequence alignment (MSA) of the identified protein sequences was constructed using PSI/TM-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do:tmcoffee). Within the homology search options, sequence type was set to "transmembrane" and UniRef100 was selected for homology extension. The alignment output was downloaded in the FASTA format and visualized in the Jalview alignment editor, version 2.11.2.0 (Waterhouse et al. 2009) for the identification of conserved motifs. The same MSA was also used to generate the percent identity matrix using BioEdit sequence alignment editor v7. The resultant sequence identities were used to generate the clustering sequence identity matrix with the help of Seaborn data visualization library (https://seaborn.pydata.org/generated/seaborn.clustermap.html) in Python.

Phylogenetic tree construction

The MSA generated above was utilized to generate a phylogenetic tree in MEGA-X (Kumar et al. 2018) using the maximum likelihood method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The generated tree data was exported in Newick format and submitted to iTOL v6 (https://itol.embl.de/) (Letunic and Bork 2021) to generate the final tree figure.

Membrane topology analysis

To analyze the membrane topology, the FASTA file containing protein sequences was submitted to TOPCONS (https://topcons.cbr.su.se) (Tsirigos et al. 2015). The sequence file along with the membrane topology data from TOPCONS was then submitted to Protter (https://wlab.ethz.ch/protter/start/) (Omasits et al. 2014) to generate the membrane topology diagrams for the identified proteins.

Materials

All routine chemicals used in the study were purchased from SRL Pvt. Ltd., Mumbai, India. The dipeptide, tripeptides, Fluconazole, Ketoconazole, Miconazole, Voriconazole, Amphotericin B, and Nikkomycin Z were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). Nourseothricin sulfate was purchased from Gold biotechnology Inc. (St. Louis, MO, USA). Oligonucleotides were purchased from Bioserve Biotechnologies India Pvt. Limited, Hyderabad, India.

Strains and culture conditions

The *C. auris* strain CBS10913T was obtained from the CBS-KNAW fungal culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands, and reported in our previous study (Wasi et al. 2019). The yeast strains were maintained and cultured in the YEPD medium (yeast extract, peptone, and dextrose) from HiMedia Laboratories, Mumbai, India. For the peptide uptake assays, Yeast Carbon base (YCB) media (HiMedia Laboratories, Mumbai, India) was used along with the dipeptides or tripeptides as the nitrogen source. The PTR overexpression transformants in *S. cerevisiae* were selected on a Synthetic Defined medium without uracil. The medium comprised of 0.67% YNB (Yeast nitrogen base) without amino acids (Difco, Becton, Dickinson and Company, MD, USA), 0.2% dropout mix without uracil (Sigma-Aldrich Co. St. Louis, MO, USA), and 2% glucose (HiMedia Laboratories, Mumbai, India). Plasmids were maintained in the *Escherichia coli* DH5α strain (Thermo Fisher Scientific, Waltham, MA, USA) cultured in Luria–Bertani medium (HiMedia Laboratories, Mumbai, India) with 100 µg/ml ampicillin (Amresco, Solon, USA). All the yeast strains used in the study are listed in Supplemental Table S1.

Construction of PTR deletion mutants in CBS10913T

For the construction of deletion mutants, we used the fusion PCR-based deletion strategy (Supplemental Fig. S1). Firstly, 5′- and 3′-UTR regions (nearly 600–700 bps) of the genes (BNJ08_004188, BNJ08_003830, and BNJ08_005124) were amplified from genomic DNA of *C. auris* CBS10913T. The selection marker *NAT1* gene with flippase recognition target (FRT) sites was also PCR amplified in two halves (sharing a 300–350 bp complimentary region with each other) from plasmid pRK625 (kind gift of Dr. Rupinder Kaur, CDFD, Hyderabad, India) (Borah et al. 2011; Kumari et al. 2020). Both 5′- and 3′-UTR amplified products were fused to one-half each of the *NAT1* gene fragments to generate the fusion product. Of note, for amplification of the 5′- and 3′-UTR of the targeted genes and the 5′ and 3′ *NAT1* halves, primers were designed in such a way that the reverse primer for 5′- UTR and forward primer for 3′-UTR share a small complementary region with forward primer for the 5′ *NAT1* fragment and reverse primer for the 3′ *NAT1* fragment amplification, respectively (Supplemental Fig. S1). The fused PCR products were then co-transformed into the CBS10913T strain using electroporation strategy and positive transformants were selected on YEPD agar medium containing 200 µg/ml nourseothricin. The gene deletions were confirmed by gene-specific PCRs from the genomic DNA of transformants. The same was also finally confirmed through DNA sequencing. All the oligonucleotides used for gene

deletions and their confirmations are enlisted in Supplemental Table S2.

Construction of AD-Ura$^-$Ptr2$^-$ strain

For the deletion of *PTR2* encoding gene in *S. cerevisiae* AD1-8u$^-$ strain (Lamping et al. 2007), we used the same fusion PCR-based gene deletion employed with CBS10913T as elaborated in the previous section. However, in this case, the fused PCR products were co-transformed into the host AD1-8u$^-$ strain using the lithium acetate (LiAc) based chemical transformation protocol and positive transformants were selected on YEPD agar medium containing 100 µg/ml nourseothricin. The derivatized host strain was denoted as AD-Ura$^-$Ptr2$^-$.

Molecular cloning of PTR genes in pABC3-His and overexpression in AD-Ura$^-$Ptr2$^-$

Each respective *PTR* encoding gene, viz., BNJ08_004188, BNJ08_003830, and BNJ08_005124 was amplified from the genomic DNA of *C. auris* CBS10913T strain using forward and reverse primers containing *Pac*I and *Not*I restriction sites, respectively (Supplemental Table S2). The PCR product was purified using the Monarch PCR clean-up kit (New England Biolabs, Ipswich, MA, USA). The purified product and the pABC3-His vector (Lamping et al. 2007) were digested with *Pac*I and *Not*I restriction enzymes (New England Biolabs, Ipswich, MA, USA). The resultant fragments were then gel-purified using the Monarch DNA Gel extraction kit (New England Biolabs, Ipswich, MA, USA) and ligated with the help of T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). Positive clones (pABC3-PTR_A-His/ pABC3-PTR_B-His/ pABC3-PTR_C-His) were confirmed by restriction digestion, followed by DNA sequencing. To introduce the *PTR* genes into the AD-Ura$^-$Ptr2$^-$ strain, each respective plasmid was digested with *Asc*I to liberate the transformation cassette, which was then gel-purified using the Monarch DNA Gel extraction kit (New England Biolabs, Ipswich, MA, USA). *S. cerevisiae* AD-Ura$^-$Ptr2$^-$ cells were then transformed with the purified cassette using the LiAc method. The transformants were selected on SD-Ura$^-$ media. Positive colonies were identified using gene-specific PCR.

Peptide uptake assay

For the peptide uptake assay, first overnight primary cultures of the wild type (WT) strain CBS10913T and mutants grown in YEPD media were centrifuged and washed with sterile Milli-Q water. Next, for each strain, $OD_{600} = 0.2$ was set in 1 ml of yeast carbon base (YCB) containing 1 mg of each di/tripeptide. The culture tubes were then incubated at 30 °C for 48 h with shaking. Cell turbidity was determined at 600 nm wavelength using a spectrophotometer. In the case of overexpression strains of *S. cerevisiae*, the assay was performed in a similar manner, except that uracil was also included in the YCB media at a concentration of 20 mg/L (to take care of the uracil auxotrophy of the AD-Ura$^-$Ptr2$^-$ host strain).

Broth microdilution assay (BMD)

Minimum inhibitory concentration (MIC_{80}) for the tested antifungal substrates was determined according to the CLSI broth microdilution method using YEPD medium instead of RPMI as reported previously (Banerjee et al. 2020).

Nikkomycin Z and Nva-FMDP (L-norvalyl-N3-(4-methoxyfumaroyl)-L-2,3-diaminopropionoic acid) sensitivity assay

Nikkomycin Z sensitivity in liquid medium was determined in 96-well microplate format. Herein, two-fold serial dilution of the compound was first prepared in YNB medium containing amino acids (YNB + AA). This medium comprised 0.67% YNB without amino acids (Difco, Becton, Dickinson and Company, Sparks, MD, USA), 0.2% dropout mix without uracil (Sigma-Aldrich Co. St. Louis, MO, USA), 0.002% uracil (SRL Pvt. Ltd., Mumbai, India), and 2% glucose (HiMedia laboratories, Mumbai, India). The medium was then incubated with logarithmic phase cells (~ 10^4 cells/ml) at 30 °C for two days. Cell turbidity was determined at 595 nm wavelength using a microplate reader (Bio-Rad laboratories, Hercules, California, USA).

For serial dilution spot assay, yeast suspensions were prepared in 0.9% NaCl solution and serially diluted fivefold. A 4 µl aliquot from each dilution was spotted onto YNB + AA agar plates with or without Nikkomycin Z (2 µg/ml) and incubated for 48 h at 30 °C. The Nva-FMDP spot assay was also performed similarly, except that YEPD media were used in this case.

Results

Identification of PTR/POT transporters in *Candida* species

To identify the PTR transporters in *Candida* species, we utilized the *Candida* Genome Database (CGD) Multi-genome search using BLAST + feature, wherein we supplied the ScPtr2 sequence as the query and *C. albicans, C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, and *C. auris* as the target genomes. The analysis revealed 2 PTR transporters each in *C. albicans* and *C. dubliniensis*, 1 in *C. glabrata*, 4 in *C. parapsilosis*, and 3 in *C. auris*. Table 1 provides the details

of the proteins and the strain IDs, and Supplemental File S1 summarizes the BLAST results. The *C. glabrata* PTR transporter CAGL0G02453g showed the maximum percent identity to the query (~71%) followed by CPAR2_503540 of *C. parapsilosis* and Cd36_83780 of *C. dubliniensis,* both having around 59% identity. The other 3 candidate PTR proteins of *C. parapsilosis* (CPAR2_800760, CPAR2_804430, and CPAR2_804220) showed relatively lower identities (in the range of 31–34%) to ScPtr2. The second *C. dubliniensis* candidate, Cd36_26850 also showed a relatively lower identity of ~28% to ScPtr2. For *C. albicans*, we observed that CaPtr22 is much more identical to ScPtr2 (~58% identity) than CaPtr2 (~28% identity), as reported earlier too (Dunkel et al. 2013). Interestingly, all the three PTR candidate proteins in *C. auris* showed around 58–62% identity to ScPtr2. From these results, one point emerged that in certain *Candida* species, namely *C. albicans*, *C. parapsilosis*, *C. dubliniensis*, there exists one PTR transporter which is very similar to ScPtr2, while the other candidate protein(s) show low sequence identity to ScPtr2.

To further confirm the status of the identified proteins as PTR/POT family transporters, we exploited the DomainViz web server that uses PFAM (Finn et al. 2016) and Prosite databases (Sigrist et al. 2013) for domain searching and displays the extent of domain conservation along two dimensions: positionality and frequency of occurrence in the input protein sequences (Schläpfer et al. 2021). As evident from Supplemental Fig. S2, PTR2 (PF00854) is the most represented PFAM domain, with all the submitted sequences containing it. One protein also shows MFS_1 PFAM domain, however, HMMSCAN based search of the query (CAGL0G02453g) against PFAM domains results in its exclusion by the PFAM post-processing. Further, all submitted sequences contain either PTR2_1 or PTR2_2 PROSITE signatures (Supplemental Fig. S2). Overall, it could be concluded that all the selected proteins from our BLAST analysis belong to the proton-dependent oligopeptide transporters (POT/PTR family).

Table 1 Putative PTR transporter encoding genes identified in the genome of *Candida* species

S. No	Organism	Strain ID	PTR protein/gene ID
1	*S. cerevisiae*	S288c	ScPtr2
2	*C. albicans*	SC5314	CaPtr2 (CR_01830C_A) CaPtr22 (C3_03800W_A)
3	*C. glabrata*	CBS138	CAGL0G02453g
4	*C. dubliniensis*	CD36	Cd36_83780 Cd36_26850
5	*C. parapsilosis*	CDC317	CPAR2_800760 CPAR2_804430 CPAR2_804220 CPAR2_503540
6	*C. auris*	B8441	BNJ08_004188 BNJ08_005124 BNJ08_003830

Multiple sequence alignment (MSA) to reveal the presence of conserved motifs characteristic of the PTR/POT family

To gain further sequence-related insights, we performed multiple sequence alignment of the identified proteins using PSI/TM-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do:tmcoffee) (Floden et al. 2016) which allows efficient alignment of transmembrane proteins using homology extension on reduced databases. The tool outperforms many other available tools available for the purpose (Floden et al. 2016). We also verified the constructed MSA using another newly developed tool for membrane protein alignment, TM-Aligner (Bhat et al. 2017). Both methods produced comparable results. We visualized the generated MSA in Jalview alignment viewer (Waterhouse et al. 2009) and performed further analysis within this application only. One interesting observation made with the MSA was that two of the *C. parapsilosis* transporters, CPAR2_804430 and CPAR2_804220 are 99.8% identical with a difference of only a single amino acid, whereby isoleucine in the former is replaced by threonine at 223 position (data not shown).

Contrary, to other membrane transporter families, the PTR family representatives show high order of sequence similarity at the level of transmembrane helices (TMHs) (Steiner et al. 1995; Newstead 2015). The constructed MSA also revealed many conserved blocks of amino acids (data not shown). A total of three typical sequence motifs have been reported in the PTR family. One motif is referred to as the ExxERFxYY motif on TMH1 (Newstead 2015). The other two relatively less well-conserved motifs are denoted as the PTR2-1 and PTR2-2 (Newstead 2015). Mutational studies have also revealed their relevance in the transport mechanism of PTR/POT transporters (Hauser et al. 2005; Solcan et al. 2012; Newstead 2015). Figure 1 shows a color-coded MSA (based on identity) of the PTR candidates in *Candida* species as well as *S. cerevisiae* highlighting the regions of the three conserved motifs. It also depicts the conservation scores for each alignment column and consensus motifs. As evident from the sequence logo, all three motifs are well conserved across all the representatives.

Construction of a clustering identity matrix to identify the sequence relatedness between different members

As described previously, several *Candida* species contained PTR protein(s) which show poor sequence identity to ScPtr2. To better understand this heterogeneity amongst the different representatives, we constructed a clustering

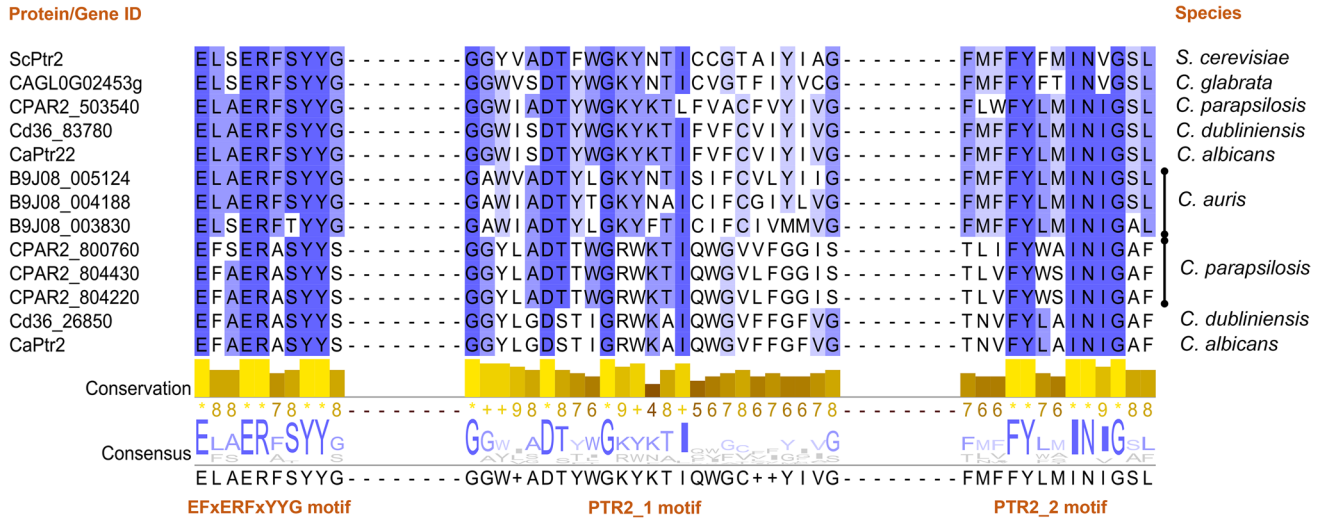

Fig. 1 Snapshot of multiple sequence alignment of all the putative PTR transporter protein sequences constructed using PSI/TM-Coffee and visualized in Jalview highlighting the conservation of the three known motifs present in the PTR family

sequence identity matrix based on the MSA generated above. As shown in Fig. 2A, two major clusters of proteins emerge from the data, while one comprises of ScPtr2, CaPtr22, all three PTR transporters of *C. auris* (BNJ08_004188, BNJ08_005124, and BNJ08_003830). CAGL0G02453g transporter of *C. glabrata*, Cd36_83780 of *C. dubliniensis* and CPAR2_503540 of *C. parapsilosis*. The second cluster comprised of the remaining proteins: CaPtr2 of *C. albicans*; CPAR2_800760, CPAR2_804430, and CPAR2_804220 of *C. parapsilosis*; and Cd36_26850 of *C. dubliniensis*. This observation suggests that the low sequence identity that exists between the *C. albicans* PTR transporters (~26%) extends to many other *Candida* species like *C. parapsilosis* and *C. dubliniensis*, wherein some protein(s) share low similarity amongst themselves. Overall, it could be said that within the PTR family of *Candida* species, two distinct classes of proteins exist, which will be referred to from here onward as ScPtr2-like and ScPtr2-unlike groups. As mentioned before, the functional analysis of CaPtr2 and CaPtr22 also suggested a difference in the substrate repertoire of these two proteins (Dunkel et al. 2013). It is plausible that such a difference in substrate specificity also occurs among

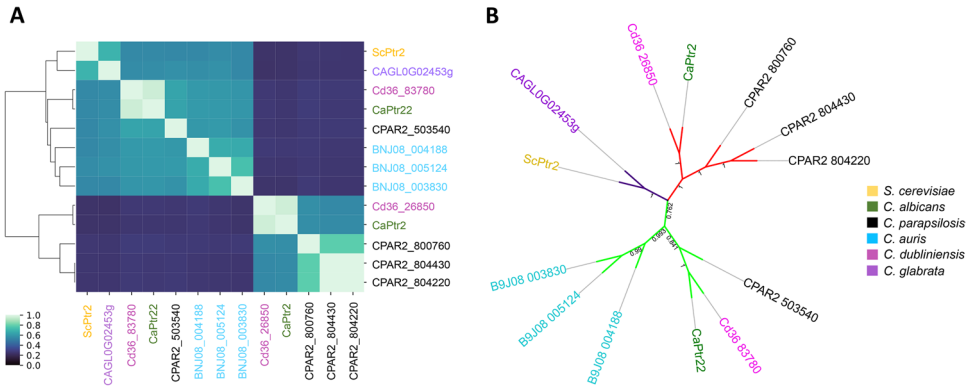

Fig. 2 **(A)** Clustering sequence identity matrix of the PTR protein sequences prepared using Seaborn library in Python. The input identity values for the plot were calculated from MSA generated using BioEdit tool. **(B)** Phylogenetic tree of the *Candida* PTR transporters constructed using the maximum likelihood method and Jones et al. w/freq. model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA X and further modified and visualized in iTOL

the PTR transporters in other *Candida* species. The question however is the need of having two distinct classes of PTR proteins.

Phylogenetic analysis of the PTR representatives from *Candida* species

To obtain an evolutionary perspective of the results obtained so far, we performed phylogenetic analysis of the PTR candidates using the Maximum likelihood method Bootstrap values were calculated using 1,000 reiterations. The topology of the phylogenetic tree showed that the PTR transporters fall into three major groups (Fig. 2B). ScPtr2 and CAGL0G02453g are clustered together and form group 1. Group 2 comprises of CaPtr22, Cd36_83780, CPAR2_503540, BNJ08_004188, BNJ08_005124 and BNJ08_003830. Group 3 comprises the same set of proteins revealed in the sequence identity matrix to be showing remote sequence identity to ScPtr2, viz., CaPtr2, CPAR2_800760, CPAR2_804430, CPAR2_804220, and Cd36_26850. Overall, the grouping resembled the clustering obtained in the hierarchical sequence identity matrix. The obtained topology supports the results obtained from the abovementioned sequence analysis that suggested a distinct class of transporters within the PTR family which shows poor homology to ScPtr2.

Membrane topology analysis of the PTR transporters

Given the results obtained before, we next chose to analyze the topology of the transporters to see if there is any distinction in the way the membrane, intracellular and extracellular segments are arranged within the PTR transporters. We utilized the TOPCONS tool for membrane topology prediction which is a widely used program for consensus prediction of membrane protein topology. TOPCONS combines an arbitrary number of topology predictions into one consensus prediction (Tsirigos et al. 2015) and thus plausibly identifies the number of TMHs and their arrangement accurately. As shown in Fig. 3, all the proteins contain 12 TMHs with both the N- and C-termini toward the intracellular region. The membrane topology also suggested that ScPtr2-like group has some notable differences when compared to the ScPtr2-unlike group as shown in Fig. 3A and 3B, respectively. The length of the central cytoplasmic loop (CCL) and extracellular loop 1 (ECL1) is much longer in the ScPtr2-unlike group compared to the ScPtr2-like group. While the length of CCL in the ScPtr2-like group members is around 53 amino acids, it is around 70 amino acids in the ScPtr2-unlike group members. Similarly, the average length of ECL1 is 28 and 42 amino acids in the former and latter groups, respectively. Therefore, lengths of certain intracellular and extracellular

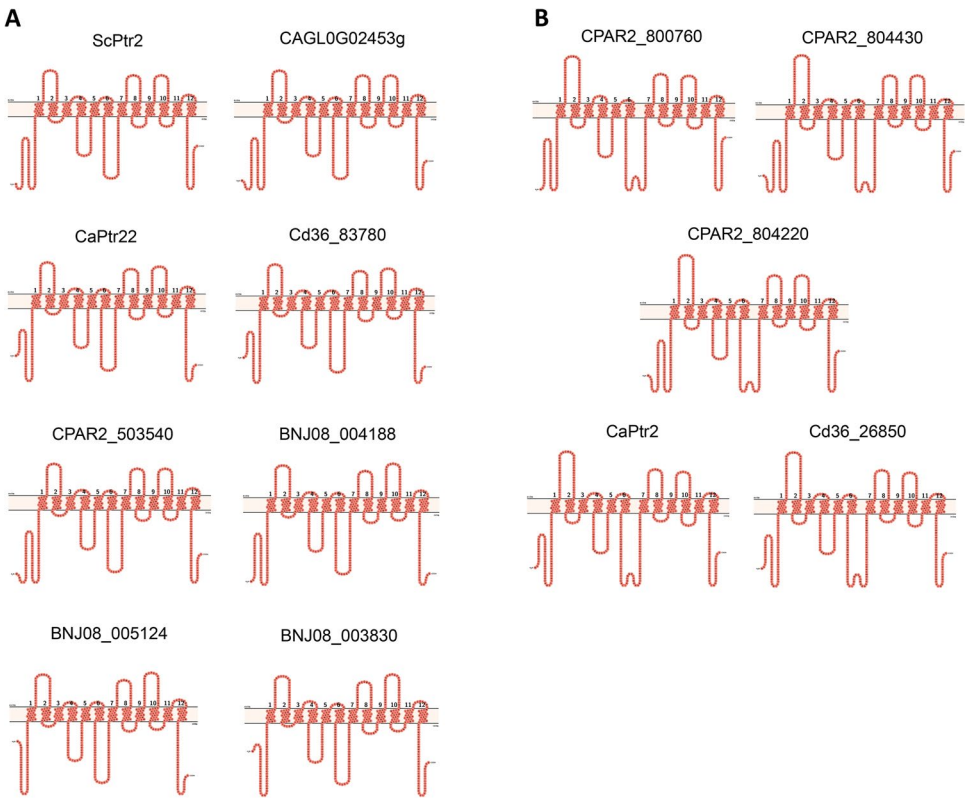

Fig. 3 Membrane topology diagrams of all PTR transporters prepared using Protter. The TM, extracellular and intracellular regions were predicted using TOPCONS and supplied as input to Protter. ScPtr2-like and ScPtr2-unlike groups are shown in panels **(A)** and **(B)**, respectively

segments in the two groups also appear to be distinct, albeit in a subtle manner.

Functional characterization of PTR transporters of *C. auris*

The recent emergence of the multi-drug-resistant pathogen *C. auris* has triggered the search for alternative therapeutics (Černáková et al. 2021). As explained before, PTR systems with their ability to import dipeptide antifungal conjugates are poised to serve as delivery systems for antifungals. Along similar lines, peptide-based antifungal compounds have recently shown promising antifungal activities (Bentz et al. 2021; Shahi et al. 2022). Given that PTR transporters are involved in their uptake (Yadan et al. 1984; Schielmann et al. 2017) and such proteins show increased expression in many clinical isolates (Muñoz et al. 2018; Shahi et al. 2022), we chose to characterize all PTR transporters encoded in the genome of *C. auris*. We constructed deletion mutants for all three identified PTR transporter encoding genes, viz. (BNJ08_004188, BNJ08_003830, and BNJ08_005124) in the CBS10913T (Wild type) strain as described in the methods section. Since all three transporters were highly identical in terms of their primary sequences, we designated BNJ08_004188, BNJ08_003830 and BNJ08_005124 genes as *PTR_A*, *PTR_B* and *PTR_C*, respectively. To assess the contribution of each gene in di/tripeptide transport, we performed a growth assay wherein only the di/tripeptides acted as the nitrogen source for the WT and mutants. Therefore, any reduction in the cellular turbidity points to a role of the gene in peptide uptake. We used 4 dipeptides, namely leucine-glycine (Leu-Gly), histidine-leucine (His-Leu), tyrosine-phenylalanine (Tyr-Phe) and glycine-histidine (Gly-His), and 2 tripeptides, viz., phenylalanine-glycine-glycine (Phe-Gly-Gly) and glycine-glycine-glycine (Gly-Gly-Gly) in our uptake assays. The results are presented in Fig. 4. With the dipeptides, it is evident from the results that barring Gly-His, deletion of *PTR_A* did not result in any significant change in the growth profile compared to the WT. With Gly-His as well, the reduction in cellular turbidity was observed to be rather modest (Fig. 4D). Contrarily, we noticed far more significant consequences on the deletion of *PTR_B* and *PTR_C*, with a major reduction in cellular turbidity observed with the Δ*PTR_C* strain. When Phe-Gly-Gly was used as the substrate, a similar profile was obtained, wherein the reduction in turbidity was of the order Δ*PTR_C* > Δ*PTR_B* > Δ*PTR_A* (Fig. 4E). Interestingly, there was no significant difference in the growth

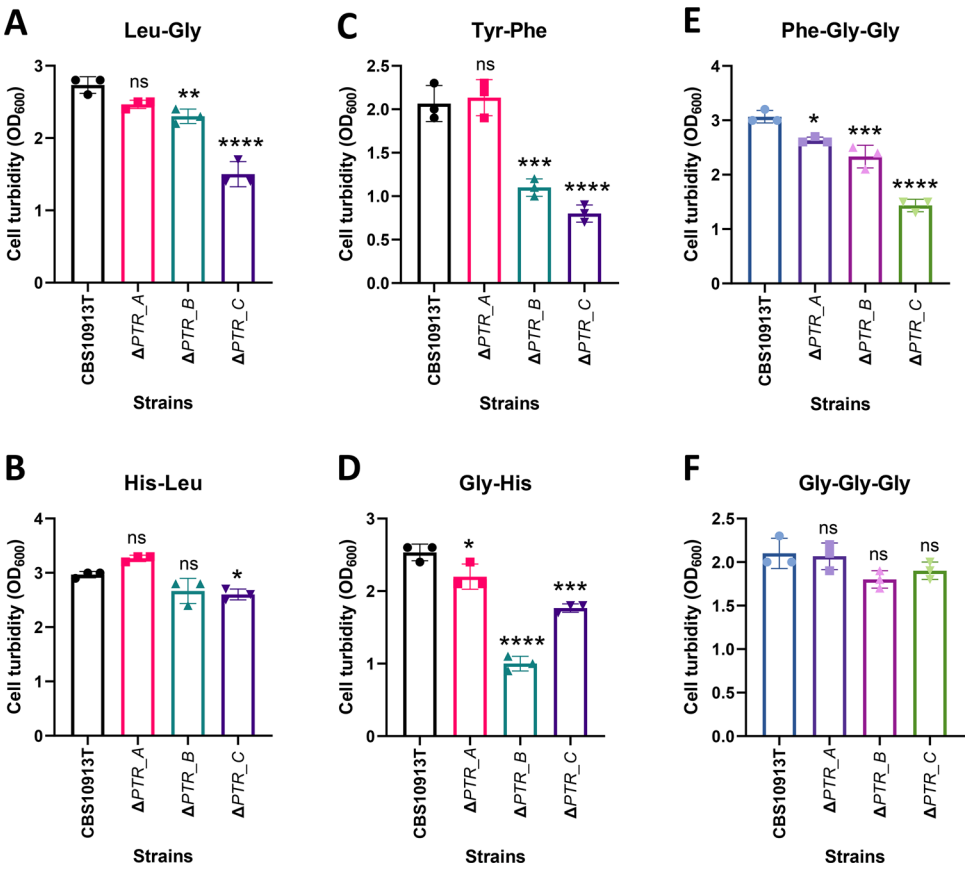

Fig. 4 Di/tripeptide uptake assays in the WT and PTR mutants of *C. auris*. Herein, di/tripeptide utilization capabilities inferred through cellular turbidity served an indicator for their relative contribution in transport as elaborated in the methods. Results are expressed as mean ± SD. One-way ANOVA followed by Dunnett's test has been applied to calculate significance. *, **, ***, and **** indicate p values ≤ 0.05, 0.01, 0.001, and 0.0001, respectively

profile of the WT and PTR mutants with Gly-Gly-Gly as the nitrogen source, plausibly indicating a similar preference for the same by all the transporters (Fig. 4F).

Since recent reports demonstrated increased expression of some of the *C. auris PTR* candidates in drug-resistant clinical isolates (Muñoz et al. 2018; Shahi et al. 2022), we also evaluated the impact of *PTR* deletions on antifungal drug susceptibility. However, when compared to the WT, we did not observe any change in the MIC_{80} values of the mutants against azole drugs and amphotericin B (Supplemental Table S3).

Heterologous overexpression of *C. auris* PTR transporters in *S. cerevisiae* and their functional characterization

We next tried to heterologously express each *PTR* gene of *C. auris* in the conventional yeast model, *S. cerevisiae* and study the strain phenotypes. To do that, we chose the AD1-8u$^-$ system which has been demonstrated to be an excellent system to express membrane transporters (Lamping et al. 2007). The strain contains a gain of function (GOF) mutation in the transcription factor *PDR1* that allows hyperexpression of target genes under the *PDR5* promoter. However, since the PTR transporter encoding gene of *S. cerevisiae* (*PTR2*) was intact in the AD1-8u$^-$ strain, we had to first derivatize the strain by deleting the gene as described in the methods section. The AD-Ura$^-$Ptr2$^-$ strain served as the host for overexpression of each of the *PTR_A*, *PTR_B*, and *PTR_C* genes and resultant strains were denoted as AD-Ptr_A, AD-Ptr_B, and AD-Ptr_C, respectively. We compared the growth patterns of the AD-Ura$^-$Ptr2$^-$ and the PTR transporter overexpression strains in the presence of various di/tripeptides as studied for the PTR mutants of *C. auris* (Fig. 5). In the case of Leu-Gly, Tyr-Phe, and His-Leu dipeptides, all three overexpression strains, viz. AD-Ptr_A, AD-Ptr_B, and AD-Ptr_C, showed highly elevated growth compared to the host strain AD-Ura$^-$Ptr2$^-$. Contrarily, the effect was very marginal in the case of Gly-His dipeptide. Herein, it is important to mention that Gly-His is not a N-end rule dipeptide (dipeptides comprising of bulky hydrophobic or basic amino acids at the N-terminal end) which are preferentially imported by ScPtr2 (Houjian et al. 2007; Ito et al. 2013). We also noted similar trends when tripeptides were used in the assay. All three transporters when overexpressed in the host strain, could increase growth several fold in presence of Phe-Gly-Gly tripeptide. However, with Gly-Gly-Gly, while AD-Ptr_A and AD-Ptr_C showed increased cellular growth, AD-Ptr_B showed similar growth as that of the AD-Ura$^-$Ptr2$^-$ strain. Nonetheless, it could be concluded that all three PTR transporters of *C. auris* complement role of ScPtr2 in dipeptide and tripeptide uptake, albeit differentially. The results are also in line with the high sequence identities between *C. auris* PTR transporters and ScPtr2.

Assessment of Nikkomycin Z and L-norvalyl-N3-(4-methoxyfumaroyl)-L-2,3-diaminopropionoic acid (Nva-FMDP) sensitivity in the PTR deletion mutants

Nikkomycin Z is a peptidyl nucleoside (Fig. 6A) that targets the chitin synthase and has shown promising antifungal activities against many fungal species, including *C. albicans* (Larwood 2020). Of note, in a human phase I trial for treatment of coccidioidomycosis, it has shown promising results (Larwood 2020). A recent in vitro assessment of Nikkomycin Z efficacy against *C. auris* demonstrated an overall mode of 2 mg/L as the MIC (Bentz et al. 2021). However, the compound was not found to be effective against all the tested isolates, the majority being the Clade III strains with MICs ranging between 16 to > 64. Nonetheless, since it is quite active against the maximally drug-resistant clade I isolates, the therapeutic potential should be addressed in detail. Given the previous reports suggesting the role of PTR transporters in their uptake (Yadan et al. 1984), we chose to assess the relative contribution of the three PTR transporters encoded in the *C. auris* genome. Growth assay in liquid medium demonstrated significantly increased resistance toward Nikkomycin Z in the case of Δ*PTR_C* strain only when compared to the WT (Fig. 6B). Notably, serial dilution spot assays on solid media also corroborated the findings from liquid media, wherein the Δ*PTR_C* strain showed enhanced resistance compared to the WT and other PTR mutants (Fig. 6C). However, it is pertinent to mention that all the strains showed much better growth in solid compared to the liquid medium. Besides Nikkomycin Z, the synthetic dipeptide glutamine analogue, Nva-FMDP (Fig. 6D) is another inhibitor of chitin synthesis and previously proposed to be imported by the PTR transporters in *C. albicans* (Schielmann et al. 2017). Recently, clinical isolates of *C. auris* were also reported to be susceptible toward Nva-FMDP (Shahi et al. 2022). To assess the contribution of the PTR genes in the uptake mechanism of Nva-FMDP, we also exposed the three *PTR* mutants toward Nva-FMDP. In alignment with the results obtained using Nikkomycin Z, we observed that the Δ*PTR_C* strain showed significantly increased resistance toward Nva-FMDP compared to the WT and other PTR mutants (Fig. 6E). Hence, these susceptibility assays strongly point to the predominant contribution of transporter encoded by *PTR_C* in the uptake of antifungal peptides.

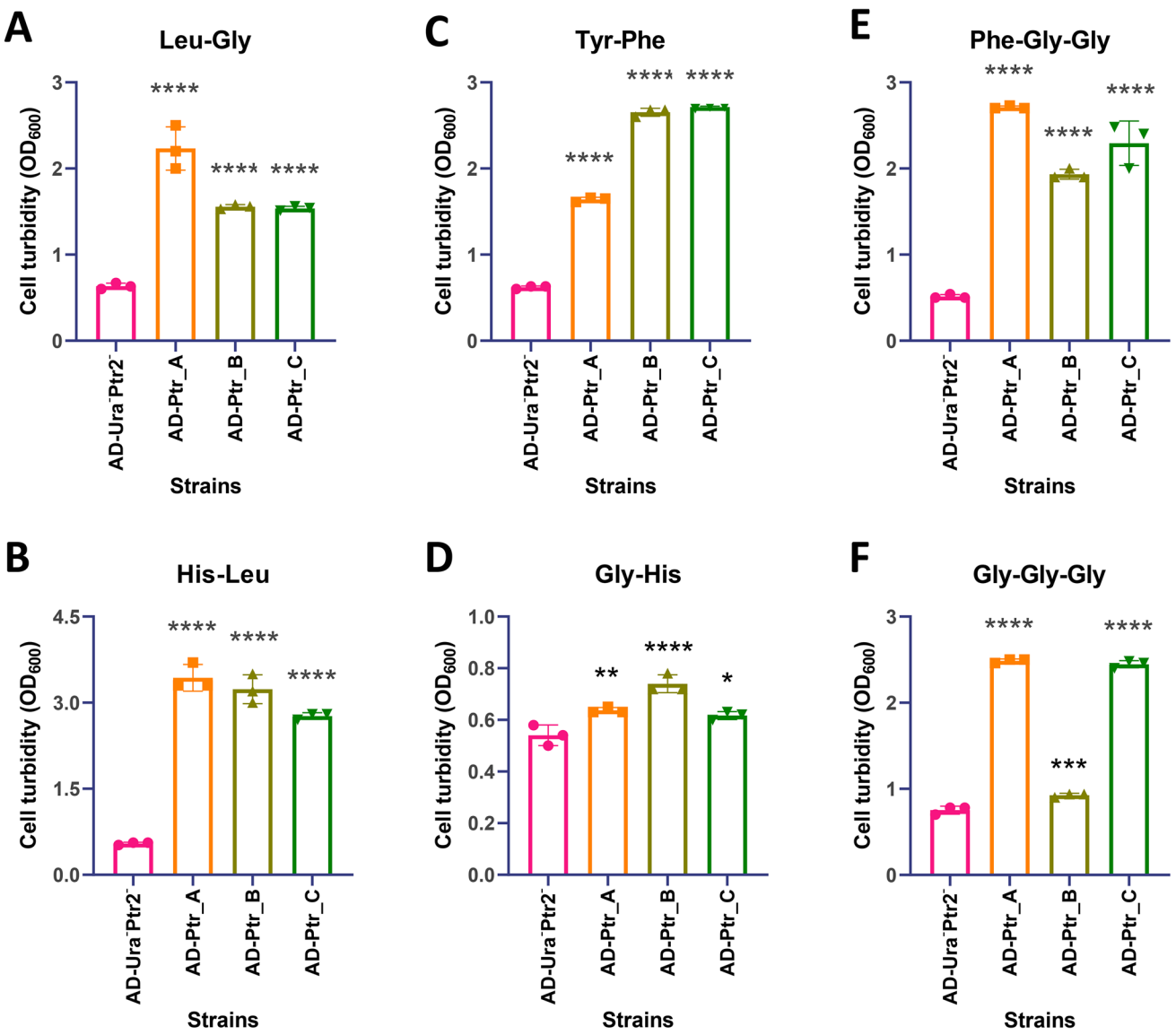

Fig. 5 Di/tripeptide uptake assays in the AD-Ura^{-}Ptr2^{-} host strain and PTR overexpression strains. Results are expressed as mean ± SD. One-way ANOVA followed by Dunnett's test has been applied to calculate significance. *, **, ***, and **** indicate p values ≤ 0.05, 0.01, 0.001, and 0.0001, respectively

Discussion

In this study, we have identified all the putative PTR transporters encoded in the genome of five prominent *Candida* species, including the recently emerged *C. auris*. With a significant increase in the burden of fungal infections due to *Candida* species, effective alternatives to conventional antifungal therapy are urgently needed. With demonstrated efficacy of peptide transporters in transporting peptide drug conjugates, these systems can be exploited as antifungal delivery systems. However, that would require a complete understanding of their structure and function. The present study provides a template for all such future investigations because it reports all the essential primary sequence and structure-related information for the proteins. It is clear from the study that within the PTR/POT family of *Candida* species, there are two distinct classes of transporters, where only one is significantly like ScPtr2 in terms of sequence. Moreover, there are few *Candida* species, namely *C. glabrata* and *C. auris* which harbor only proteins similar to ScPtr2. Given that, both CaPtr2 and CaPtr22 have earlier been shown to be different in terms of their substrate specificity, the reason and implications for such differences need to be understood for future exploitation of these proteins as drug delivery systems.

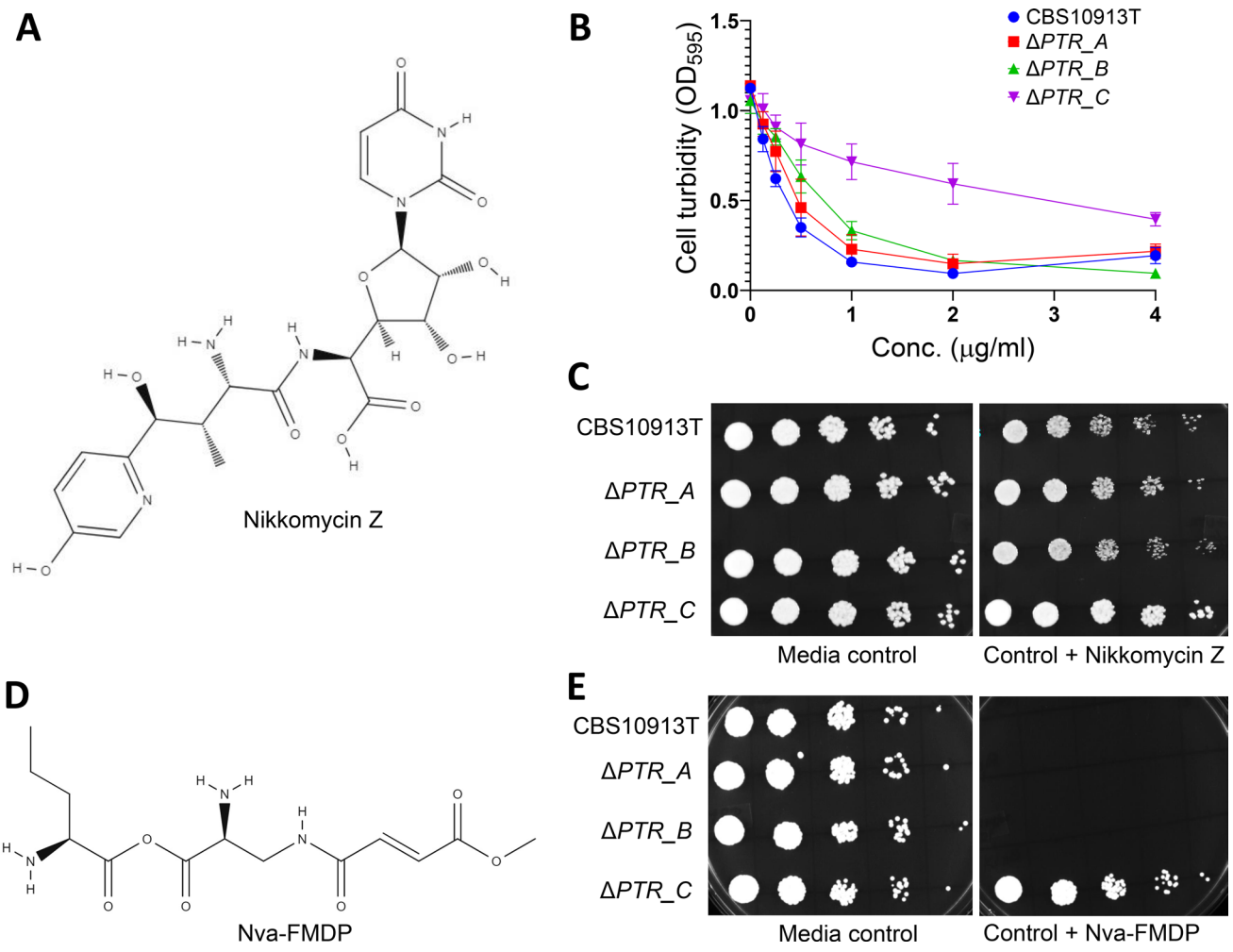

Fig. 6 Nikkomycin Z and Nva-FMDP sensitivity assays. **(A)** Chemical structure of Nikkomycin Z. **(B)** Dose-dependent inhibitory curves of Nikkomycin Z against CBS10913T and the PTR mutants. **(C)** Serial dilution spot assay of CBS10913T and the PTR mutants on solid yeast nitrogen base + amino acids (YNB + AA) medium without (*left panel*) or with 2 μg/ml Nikkomycin Z (*right panel*). **(D)** Chemical structure of Nva-FMDP. **(E)** Serial dilution spot assay of CBS10913T and the PTR mutants on YEPD medium without (*left panel*) or with 72 μg/ml Nva-FMDP (*right panel*)

With the recent emergence of the multi-drug-resistant *C. auris*, alternative therapeutics are in much demand. Under such a scenario, promising activities of peptide-based antifungal compounds like Nva-FMDP and Nikkomycin Z are quite encouraging (Bentz et al. 2021; Shahi et al. 2022). Since previous studies pointed toward a PTR transporter dependent uptake for these compounds (Yadan et al. 1984; Schielmann et al. 2017), and there are three highly identical transporters encoded in *C. auris*, we investigated the relative contribution of each transporter in di/tripeptide as well as Nikkomycin Z and Nva-FMDP uptake. We constructed deletion mutants for all three genes and subjected them to di/tripeptide uptake assay, and Nikkomycin Z and Nva-FMDP sensitivity assays along with the WT. Overall, it could be said that compared to the other two PTR transporters in *C. auris*, *PTR_C* (BNJ08_005124) seems to be the more dominant player responsible for uptake of di/tripeptides and peptide-based therapeutics like Nikkomycin Z and Nva-FMDP. However, the role played by the other two genes *PTR_A* (BNJ08_004188) and *PTR_B* (BNJ08_003830) could not be ignored, more so for *PTR_B* which also showed significant differences in the di/tripeptide uptake assays when compared to the WT. Since these proteins are highly similar to each other and ScPtr2 in terms of their primary sequence, the peptide specificities were required to be studied through unbiased systems lacking native PTR transporters. Therefore, we also overexpressed all three PTR transporters in a *S. cerevisiae* strain lacking the native *PTR2* gene. These assays suggested that all three transporters could efficiently take up the function of di/tripeptide uptake by ScPtr2 in its absence, which was also expected due to their high sequence identity with ScPtr2. However, despite high sequence relatedness,

the subtle differences in peptide specificities between the three PTR transporters of *C. auris* needs to be investigated in future. This becomes further important when we consider the predominant involvement of the transporter encoded by *PTR_C* in uptake of antifungal peptides. Altogether, the study serves as a useful reference for guiding future structure–function studies on the highly relevant PTR/POT membrane transporter family and highlights a novel target gene that is amenable for pharmaceutical exploitation as an antifungal delivery system.

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Author contributions AB was involved in the conceptualization; AB, RK, and SS contributed to the methodology; RK, SS, AML, RP, AP, and AB helped in the formal analysis and investigation; AB and RK were involved in writing—original draft preparation; RK, AML, AP, and AB were involved in writing—review and editing; AB acquired the funding; AB contributed to the resources and supervision.

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Data availability Data are available on request from the authors.

Declarations

Conflicts of interest RK, SS, RP, AML, AP, and AB declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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